The Prokaryotes

Third Edition

The Prokaryotes

A Handbook on the Biology of Bacteria

Third Edition

Volume 7: Proteobacteria: Delta and Epsilon Subclasses. Deeply Rooting Bacteria

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Preface

Each of the first two editions of *The Prokaryotes* took a bold step. The first edition, published in 1981, set out to be an encyclopedic, synoptic account of the world of the prokaryotes—a collection of monographic descriptions of the genera of bacteria. The Archaea had not yet been formalized as a group. For the second edition in 1992, the editors made the decision to organize the chapters on the basis of the molecular phylogeny championed by Carl Woese, which increasingly provided a rational, evolutionary basis for the taxonomy of the prokaryotes. In addition, the archaea had by then been recognized as a phylogenetically separate and distinguishable group of the prokaryotes. The two volumes of the first edition had by then expanded to four. The third edition was arguably the boldest step of all. We decided that the material would only be presented electronically. The advantages were obvious and persuasive. There would be essentially unlimited space. There would be no restrictions on the use of color illustrations. Film and animated descriptions could be made available. The text would be hyperlinked to external sources. Publication of chapters would be seriati—the edition would no longer have to delay publication until the last tardy author had submitted his or her chapter. Updates and modifications could be made continuously. And, most attractively, a library could place its subscribed copy on its server and make it available easily and cheaply to all in its community. One hundred and seventy chapters have thus far been presented in 16 releases over a six-year period. The virtues and advantages of the online edition have been borne out. But we failed to predict the affection that many have for holding a bound, print version of a book in their hands. Thus, this print version of the third edition shall accompany the online version.

We are now four years into the 21st century. Indulge us then while we comment on the challenges, problems and opportunities for microbiology that confront us.

Moselio Schaechter has referred to the present era of microbiology as its third golden age—the era of "integrative microbiology." Essentially all microbiologists now speak a common language. So that the boundaries that previously separated subdisciplines from each other have faded: physiology has become indistinguishable from pathogenesis; ecologists and molecular geneticists speak to each other; biochemistry is spoken by all; and—mirabile dictu!—molecular biologists are collaborating with taxonomists.

But before these molecular dissections of complex processes can be effective there must be a clear view of the organism being studied. And it is our goal that these chapters in *The Prokaryotes* provide that opportunity.

There is also yet a larger issue. Microbiology is now confronted with the need to understand increasingly complex processes. And the modus operandi that has served us so successfully for 150 years—that of the pure culture studied under standard laboratory conditions—is inadequate. We are now challenged to solve problems of multimembered populations interacting with each other and with their environment under constantly variable conditions. Carl Woese has pointed out a useful and important distinction between empirical, methodological reductionism and fundamentalist reductionism. The former has served us well; the latter stands in the way of our further understanding of complex, interacting systems. But no matter what kind of synoptic systems analysis emerges as our way of understanding host–parasite relations, ecology, or multicellular behavior, the understanding of the organism as such is sine qua non. And in that context, we are pleased to present to you the third edition of *The Prokaryotes*.

Martin Dworkin
Editor-in-Chief

Foreword

The purpose of this brief foreword is unchanged from the first edition; it is simply to make you, the reader, hungry for the scientific feast that follows. These four volumes on the prokaryotes offer an expanded scientific menu that displays the biochemical depth and remarkable physiological and morphological diversity of prokaryote life. The size of the volumes might initially discourage the unprepared mind from being attracted to the study of prokaryote life, for this landmark assemblage thoroughly documents the wealth of present knowledge. But in confronting the reader with the state of the art, the Handbook also defines where more work needs to be done on well-studied bacteria as well as on unusual or poorly studied organisms.

This edition of *The Prokaryotes* recognizes the almost unbelievable impact that the work of Carl Woese has had in defining a phylogenetic basis for the microbial world. The concept that the ribosome is a highly conserved structure in all cells and that its nucleic acid components may serve as a convenient reference point for relating all living things is now generally accepted. At last, the phylogeny of prokaryotes has a scientific basis, and this is the first serious attempt to present a comprehensive treatise on prokaryotes along recently defined phylogenetic lines. Although evidence is incomplete for many microbial groups, these volumes make a statement that clearly illuminates the path to follow.

There are basically two ways of doing research with microbes. A classical approach is first to define the phenomenon to be studied and then to select the organism accordingly. Another way is to choose a specific organism and go where it leads. The pursuit of an unusual microbe brings out the latent hunter in all of us. The intellectual challenges of the chase frequently test our ingenuity to the limit. Sometimes the quarry repeatedly escapes, but the final capture is indeed a wonderful experience. For many of us, these simple rewards are sufficiently gratifying so that we have chosen to spend our scientific lives studying these unusual creatures. In these endeavors many of the strategies and tools as well as much of the philosophy may be traced to the Delft School, passed on to us by our teachers, Martinus Beijerinck, A. J. Kluyver, and C. B. van Niel, and in turn passed on by us to our students.

In this school, the principles of the selective, enrichment culture technique have been developed and diversified; they have been a major force in designing and applying new principles for the capture and isolation of microbes from nature. For me, the "organism approach" has provided rewarding adventures. The organism continually challenges and literally drags the investigator into new areas where unfamiliar tools may be needed. I believe that organism-oriented research is an important alternative to problem-oriented research, for new concepts of the future very likely lie in a study of the breadth of microbial life. The physiology, biochemistry, and ecology of the microbe remain the most powerful attractions. Studies based on classical methods as well as modern genetic techniques will result in new insights and concepts.

To some readers, this edition of the *The Prokaryotes* may indicate that the field is now mature, that from here on it is a matter of filling in details. I suspect that this is not the case. Perhaps we have assumed prematurely that we fully understand microbial life. Van Niel pointed out to his students that—after a lifetime of study—it was a very humbling experience to view in the microscope a sample of microbes from nature and recognize only a few. Recent evidence suggests that microbes have been evolving for nearly 4 billion years. Most certainly those microbes now domesticated and kept in captivity in culture collections represent only a minor portion of the species that have evolved in this time span. Sometimes we must remind ourselves that evolution is actively taking place at the present moment. That the eukaryote cell evolved as a chimera of certain prokaryote parts is a generally accepted concept today. Higher as well as lower eukaryotes evolved in contact with prokaryotes, and evidence surrounds us of the complex interactions between eukaryotes and

prokaryotes as well as among prokaryotes. We have so far only scratched the surface of these biochemical interrelationships. Perhaps the legume nodule is a pertinent example of nature caught in the act of evolving the "nitrosome," a unique nitrogen-fixing organelle. Study of prokaryotes is proceeding at such a fast pace that major advances are occurring yearly. The increase of this edition to four volumes documents the exciting pace of discoveries.

To prepare a treatise such as *The Prokaryotes* requires dedicated editors and authors; the task has been enormous. I predict that the scientific community of microbiologists will again show its appreciation through use of these volumes—such that the pages will become "dog-eared" and worn as students seek basic information for the hunt. These volumes belong in the laboratory, not in the library. I believe that a most effective way to introduce students to microbiology is for them to isolate microbes from nature, i.e., from their habitats in soil, water, clinical specimens, or plants. *The Prokaryotes* enormously simplifies this process and should encourage the construction of courses that contain a wide spectrum of diverse topics. For the student as well as the advanced investigator these volumes should generate excitement.

Happy hunting!

Ralph S. Wolfe
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Contents

Preface **v**
Foreword by Ralph S. Wolfe **vii**
Contributors **xxix**

Volume 1

1. ***Essays in Prokaryotic Biology***

1.1 How We Do, Don't and Should Look at Bacteria and Bacteriology 3
CARL R. WOESE

1.2 Databases 24
WOLFGANG LUDWIG, KARL-HEINZ SCHLEIFER and ERKO STACKEBRANDT

1.3 Defining Taxonomic Ranks 29
ERKO STACKEBRANDT

1.4 Prokaryote Characterization and Identification 58
HANS G. TRÜPER and KARL-HEINZ SCHLEIFER

1.5 Principles of Enrichment, Isolation, Cultivation, and Preservation of Prokaryotes 80
JÖRG OVERMANN

1.6 Prokaryotes and Their Habitats 137
HANS G. SCHLEGEL and HOLGER W. JANNASCH

1.7 Morphological and Physiological Diversity 185
STEPHEN H. ZINDER and MARTIN DWORKIN

1.8 Cell-Cell Interactions 221
DALE KAISER

1.9 Prokaryotic Genomics 246
B. W. WREN

1.10 Genomics and Metabolism in *Escherichia coli* 261
MARGRETHE HAUGGE SERRES and MONICA RILEY

1.11 Origin of Life: RNA World versus Autocatalytic Anabolism 275
GÜNTER WÄCHTERSHÄUSER

1.12 Biotechnology and Applied Microbiology 284
EUGENE ROSENBERG

1.13 The Structure and Function of Microbial Communities 299
DAVID A. STAHL, MEREDITH HULLAR and SEANA DAVIDSON

2. Symbiotic Associations

2.1 Cyanobacterial-Plant Symbioses 331
DAVID G. ADAMS, BIRGITTA BERGMAN, S. A. NIERZWICKI-BAUER, A. N. RAI and ARTHUR SCHÜßLER

2.2 Symbiotic Associations Between Ciliates and Prokaryotes 364
HANS-DIETER GÖRTZ

2.3 Bacteriocyte-Associated Endosymbionts of Insects 403
PAUL BAUMANN, NANCY A. MORAN and LINDA BAUMANN

2.4 Symbiotic Associations Between Termites and Prokaryotes 439
ANDREAS BRUNE

2.5 Marine Chemosynthetic Symbioses 475
COLLEEN M. CAVANAUGH, ZOE P. MCKINESS, IRENE L.G. NEWTON and FRANK J. STEWART

3. Biotechnology and Applied Microbiology

3.1 Organic Acid and Solvent Production 511
PALMER ROGERS, JIANN-SHIN CHEN and MARY JO ZIDWICK

3.2 Amino Acid Production 756
HIDEHIKO KUMAGAI

3.3 Microbial Exopolysaccharides 766
TIMOTHY HARRAH, BRUCE PANILAITIS and DAVID KAPLAN

3.4 Bacterial Enzymes 777
WIM J. QUAX

3.5 Bacteria in Food and Beverage Production 797
MICHAEL P. DOYLE and JIANGHONG MENG

3.6 Bacterial Pharmaceutical Products 812
ARNOLD L. DEMAIN and GIANCARLO LANCINI

3.7 Biosurfactants 834
EUGENE ROSENBERG

3.8 Bioremediation 850
RONALD L. CRAWFORD

3.9 Biodeterioration 864
JI-DONG GU and RALPH MITCHELL

3.10 Microbial Biofilms 904
DIRK DE BEER and PAUL STOODLEY

Index 939

Volume 2

1. Ecophysiological and Biochemical Aspects

1.1 Planktonic Versus Sessile Life of Prokaryotes 3
KEVIN C. MARSHALL

1.2 Bacterial Adhesion 16
ITZHAK OFEK, NATHAN SHARON and SOMAN N. ABRAHAM

1.3 The Phototrophic Way of Life 32
JÖRG OVERMANN and FERRAN GARCIA-PICHEL

1.4 The Anaerobic Way of Life 86
RUTH A. SCHMITZ, ROLF DANIEL, UWE DEPPENMEIER and GERHARD GOTTSCHALK

1.5 Bacterial Behavior 102
JUDITH ARMITAGE

1.6 Prokaryotic Life Cycles 140
MARTIN DWORKIN

1.7 Life at High Temperatures 167
RAINER JAENICKE and REINHARD STERNER

1.8 Life at Low Temperatures 210
SIEGFRIED SCHERER and KLAUS NEUHAUS

1.9 Life at High Salt Concentrations 263
AHARON OREN

1.10 Alkaliphilic Prokaryotes 283
TERRY ANN KRULWICH

1.11 Syntrophism among Prokaryotes 309
BERNHARD SCHINK and ALFONS J.M. STAMS

1.12 Quorum Sensing 336
BONNIE L. BASSLER and MELISSA B. MILLER

1.13 Acetogenic Prokaryotes 354
HAROLD L. DRAKE, KIRSTEN KÜSEL and CAROLA MATTHIES

1.14 Virulence Strategies of Plant Pathogenic Bacteria 421
BARBARA N. KUNKEL and ZHONGYING CHEN

1.15 The Chemolithotrophic Prokaryotes 441
DONOVAN P. KELLY and ANNE P. WOOD

1.16 Oxidation of Inorganic Nitrogen Compounds as an Energy Source 457
EBERHARD BOCK and MICHAEL WAGNER

1.17 The H_2-Metabolizing Prokaryotes 496
EDWARD SCHWARTZ and BÄRBEL FRIEDRICH

1.18 Hydrocarbon-Oxidizing Bacteria 564
EUGENE ROSENBERG

1.19 Cellulose-Decomposing Bacteria and Their Enzyme Systems 578
EDWARD A. BAYER, YUVAL SHOHAM and RAPHAEL LAMED

1.20 Aerobic Methylotrophic Prokaryotes 618
MARY E. LIDSTROM

1.21 Dissimilatory Fe(III)- and Mn(IV)-Reducing Prokaryotes 635
DEREK LOVLEY

1.22 Dissimilatory Sulfate- and Sulfur-Reducing Prokaryotes 659
RALF RABUS, THEO A. HANSEN and FRIEDRICH WIDDEL

1.23 The Denitrifying Prokaryotes 769
JAMES P. SHAPLEIGH

1.24 Dinitrogen-Fixing Prokaryotes 793
ESPERANZA MARTINEZ-ROMERO

1.25 Root and Stem Nodule Bacteria of Legumes 818
MICHAEL J. SADOWSKY and P. H. GRAHAM

1.26 Magnetotactic Bacteria 842
STEFAN SPRING and DENNIS A. BAZYLINSKI

1.27 Luminous Bacteria 863
PAUL V. DUNLAP and KUMIKO KITA-TSUKAMOTO

1.28 Bacterial Toxins 893
VEGA MASIGNANI, MARIAGRAZIA PIZZA and RINO RAPPUOLI

1.29 The Metabolic Pathways of Biodegradation 956
LAWRENCE P. WACKETT

1.30 Haloalkaliphilic Sulfur-Oxidizing Bacteria 969
DIMITRY YU. SOROKIN, HORIA BANCIU, LESLEY A. ROBERTSON and J. GIJS KUENEN

1.31 The Colorless Sulfur Bacteria 985
LESLEY A. ROBERTSON and J. GIJS KUENEN

1.32 Bacterial Stress Response 1012
ELIORA Z. RON

1.33 Anaerobic Biodegradation of Hydrocarbons Including Methane 1028
FRIEDRICH WIDDEL, ANTJE BOETIUS and RALF RABUS

1.34 Physiology and Biochemistry of the Methane-Producing Archaea 1050
REINER HEDDERICH and WILLIAM B. WHITMAN

Index 1081

Volume 3

A: Archaea

1. The Archaea: A Personal Overview of the Formative Years 3
RALPH S. WOLFE

2. Thermoproteales 10
HARALD HUBER, ROBERT HUBER and KARL O. STETTER

3. Sulfolobales 23
HARALD HUBER and DAVID PRANGISHVILI

4. Desulfurococcales 52
HARALD HUBER and KARL O. STETTER

5. The Order Thermococcales 69
COSTANZO BERTOLDO and GARABED ANTRANIKIAN

6. The Genus *Archaeoglobus* 82
PATRICIA HARTZELL and DAVID W. REED

7. Thermoplasmatales 101
HARALD HUBER and KARL O. STETTER

8. The Order Halobacteriales 113
AHARON OREN

9. The Methanogenic Bacteria 165
WILLIAM B. WHITMAN, TIMOTHY L. BOWEN and DAVID R. BOONE

10. The Order Methanomicrobiales 208
JEAN-LOUIS GARCIA, BERNARD OLLIVIER and WILLIAM B. WHITMAN

11. The Order Methanobacteriales 231
ADAM S. BONIN and DAVID R. BOONE

12. The Order Methanosarcinales 244
MELISSA M. KENDALL and DAVID R. BOONE

13. Methanococcales 257
WILLIAM B. WHITMAN and CHRISTIAN JEANTHON

14. Nanoarchaeota 274
HARALD HUBER, MICHAEL J. HOHN, REINHARD RACHEL and KARL O. STETTER

15. Phylogenetic and Ecological Perspectives on Uncultured Crenarchaeota and Korarchaeota 281
SCOTT C. DAWSON, EDWARD F. DELONG and NORMAN R. PACE

B: Bacteria

1. Firmicutes (Gram-Positive Bacteria)

1.1. Firmicutes with High GC Content of DNA

1.1.1 Introduction to the Taxonomy of Actinobacteria 297
ERKO STACKEBRANDT and PETER SCHUMANN

1.1.2 The Family Bifidobacteriaceae 322
BRUNO BIAVATI and PAOLA MATTARELLI

1.1.3 The Family Propionibacteriaceae: The Genera *Friedmanniella*, *Luteococcus*, *Microlunatus*, *Micropruina*, *Propioniferax*, *Propionimicrobium* and *Tessarococcus* 383
ERKO STACKEBRANDT and KLAUS P. SCHAAL

1.1.4 Family Propionibacteriaceae: The Genus *Propionibacterium* 400
ERKO STACKEBRANDT, CECIL S. CUMMINS and JOHN L. JOHNSON

1.1.5 The Family Succinivibrionaceae 419
ERKO STACKEBRANDT and ROBERT B. HESPELL

1.1.6 The Family Actinomycetaceae: The Genera *Actinomyces*, *Actinobaculum*, *Arcanobacterium*, *Varibaculum* and *Mobiluncus* 430
KLAUS P. SCHAAL, ATTEYET F. YASSIN and ERKO STACKEBRANDT

1.1.7 The Family Streptomycetaceae, Part I: Taxonomy 538
PETER KÄMPFER

1.1.8 The Family Streptomycetaceae, Part II: Molecular Biology 605
HILDGUND SCHREMPF

1.1.9 The Genus *Actinoplanes* and Related Genera 623
GERNOT VOBIS

1.1.10 The Family Actinosynnemataceae 654
DAVID P. LABEDA

1.1.11 The Families Frankiaceae, Geodermatophilaceae, Acidothermaceae and Sporichthyaceae 669
PHILIPPE NORMAND

1.1.12 The Family Thermomonosporaceae: *Actinocorallia*, *Actinomadura*, *Spirillospora* and *Thermomonospora* 682
REINER MICHAEL KROPPENSTEDT and MICHAEL GOODFELLOW

1.1.13 The Family Streptosporangiaceae 725
MICHAEL GOODFELLOW and ERIKA TERESA QUINTANA

1.1.14 The Family Nocardiopsaceae 754
REINER MICHAEL KROPPENSTEDT and LYUDMILA I. EVTUSHENKO

1.1.15 *Corynebacterium*—Nonmedical 796
WOLFGANG LIEBL

1.1.16 The Genus *Corynebacterium*—Medical 819
ALEXANDER VON GRAEVENITZ and KATHRYN BERNARD

1.1.17 The Families Dietziaceae, Gordoniaceae, Nocardiaceae and Tsukamurellaceae 843
MICHAEL GOODFELLOW and LUIS ANGEL MALDONADO

1.1.18 The Genus *Mycobacterium*—Nonmedical 889
SYBE HARTMANS, JAN A.M. DE BONT and ERKO STACKEBRANDT

1.1.19 The Genus *Mycobacterium*—Medical 919
BEATRICE SAVIOLA and WILLIAM BISHAI

1.1.20 *Mycobacterium leprae* 934
THOMAS M. SHINNICK

1.1.21 The Genus *Arthrobacter* 945
DOROTHY JONES and Ronald M. KEDDIE

1.1.22 The Genus *Micrococcus* 961
MILOSLAV KOCUR, WESLEY E. KLOOS and KARL-HEINZ SCHLEIFER

1.1.23 *Renibacterium* 972
HANS-JÜRGEN BUSSE

1.1.24 The Genus *Stomatococcus*: *Rothia mucilaginosa*, basonym *Stomatococcus mucilaginosus* 975
ERKO STACKEBRANDT

1.1.25 The Family Cellulomonadaceae 983
ERKO STACKEBRANDT, PETER SCHUMANN and HELMUT PRAUSER

1.1.26 The Family Dermatophilaceae 1002
ERKO STACKEBRANDT

1.1.27 The Genus *Brevibacterium* 1013
MATTHEW D. COLLINS

1.1.28 The Family Microbacteriaceae 1020
LYUDMILA I. EVTUSHENKO and MARIKO TAKEUCHI

1.1.29 The Genus *Nocardioides* 1099
JUNG-HOON YOON and YONG-HA PARK

Index 1115

Volume 4

1. Firmicutes (Gram-Positive Bacteria)

1.2 Firmicutes with Low GC Content of DNA

1.2.1 The Genera *Staphylococcus* and *Macrococcus* 5
FRIEDRICH GÖTZ, TAMMY BANNERMAN and KARL-HEINZ SCHLEIFER

1.2.2 The Genus *Streptococcus*—Oral 76
JEREMY M. HARDIE and ROBERT A. WHILEY

1.2.3 Medically Important Beta-Hemolytic Streptococci 108
P. PATRICK CLEARY and QI CHENG

1.2.4 *Streptococcus pneumoniae* 149
ELAINE TUOMANEN

1.2.5 The Genus Enterococcus: Taxonomy 163
LUC DEVRIESE, MARGO BAELE and PATRICK BUTAYE

1.2.6 Enterococcus 175
DONALD J. LEBLANC

1.2.7 The Genus *Lactococcus* 205
MICHAEL TEUBER and ARNOLD GEIS

1.2.8 The Genera *Pediococcus* and *Tetragenococcus* 229
WILHELM H. HOLZAPFEL, CHARLES M. A. P. FRANZ, WOLFGANG LUDWIG, WERNER BACK and LEON M. T. DICKS

1.2.9 Genera *Leuconostoc*, *Oenococcus* and *Weissella* 267
JOHANNA BJÖRKROTH and WILHELM H. HOLZAPFEL

1.2.10 The Genera *Lactobacillus* and *Carnobacterium* 320
WALTER P. HAMMES and CHRISTIAN HERTEL

1.2.11 *Listeria monocytogenes* and the Genus *Listeria* 404
NADIA KHELEF, MARC LECUIT, CARMEN BUCHRIESER, DIDIER CABANES, OLIVIER DUSSURGET and PASCALE COSSART

1.2.12 The Genus *Brochothrix* 477
ERKO STACKEBRANDT and DOROTHY JONES

1.2.13 The Genus *Erysipelothrix* 492
ERKO STACKEBRANDT, ANNETTE C. REBOLI and W. EDMUND FARRAR

1.2.14 The Genus *Gemella* 511
MATTHEW D. COLLINS

1.2.15 The Genus *Kurthia* 519
ERKO STACKEBRANDT, RONALD M. KEDDIE and DOROTHY JONES

1.2.16 The Genus *Bacillus*—Nonmedical 530
RALPH A. SLEPECKY and H. ERNEST HEMPHILL

1.2.17 The Genus *Bacillus*—Insect Pathogens 563
DONALD P. STAHLY, ROBERT E. ANDREWS and ALLAN A. YOUSTEN

1.2.18 The Genus *Bacillus*—Medical 609
W. EDMUND FARRAR and ANNETTE C. REBOLI

1.2.19 Genera Related to the Genus *Bacillus*—*Sporolactobacillus*, *Sporosarcina*, *Planococcus*, *Filibacter* and *Caryophanon* 631
DIETER CLAUS, DAGMAR FRITZE and MILOSLAV KOCUR

1.2.20 An Introduction to the Family Clostridiaceae 654
JÜRGEN WIEGEL, RALPH TANNER and FRED A. RAINEY

1.2.21 Neurotoxigenic Clostridia 679
CESARE MONTECUCCO, ORNELLA ROSSETTO and MICHEL R. POPOFF

1.2.22 The Enterotoxic Clostridia 698
BRUCE A. MCCLANE, FRANCISCO A. UZAI,
MARIANO E. FERNANDEZ MIYAKAWA, DAVID LYERLY and
TRACY WILKINS

1.2.23 *Clostridium perfringens* and Histotoxic Disease 753
JULIAN I. ROOD

1.2.24 The Genera *Desulfitobacterium* and *Desulfosporosinus*: Taxonomy 771
STEFAN SPRING and FRANK ROSENZWEIG

1.2.25 The Genus *Desulfotomaculum* 787
FRIEDRICH WIDDEL

1.2.26 The Anaerobic Gram-Positive Cocci 795
TAKAYUKI EZAKI, NA (MICHAEL) LI and YOSHIAKI KAWAMURA

1.2.27 The Order Haloanaerobiales 809
AHARON OREN

1.2.28 The Genus *Eubacterium* and Related Genera 823
WILLIAM G. WADE

1.2.29 The Genus *Mycoplasma* and Related Genera (Class Mollicutes) 836
SHMUEL RAZIN

1.2.30 The Phytopathogenic Spiroplasmas 905
JACQUELINE FLETCHER, ULRICH MELCHER and ASTRI WAYADANDE

1.3 Firmicutes with Atypical Cell Walls

1.3.1 The Family Heliobacteriaceae 951
MICHAEL T. MADIGAN

1.3.2 *Pectinatus*, *Megasphaera* and *Zymophilus* 965
AULI HAIKARA and ILKKA HELANDER

1.3.3 The Genus *Selenomonas* 982
ROBERT B. HESPELL, BRUCE J. PASTER and FLOYD E. DEWHIRST

1.3.4 The Genus *Sporomusa* 991
JOHN A. BREZNAK

1.3.5 The Family Lachnospiraceae, Including the Genera *Butyrivibrio*, *Lachnospira* and *Roseburia* 1002
MICHAEL COTTA and ROBERT FORSTER

1.3.6 The Genus *Veillonella* 1022
PAUL KOLENBRANDER

1.3.7 Syntrophomonadaceae 1041
MARTIN SOBIERJ and DAVID R. BOONE

2. Cyanobacteria

2.1 The Cyanobacteria—Isolation, Purification and Identification 1053
JOHN B. WATERBURY

2.2 The Cyanobacteria—Ecology, Physiology and Molecular Genetics 1074
YEHUDA COHEN and MICHAEL GUREVITZ

2.3 The Genus *Prochlorococcus* 1099
ANTON F. POST

Index 1111

Volume 5

3. Proteobacteria

Introduction to the Proteobacteria 3
KAREL KERSTERS, PAUL DE VOS, MONIQUE GILLIS, JEAN SWINGS, PETER VAN DAMME and ERKO STACKEBRANDT

3.1. Alpha Subclass

3.1.1 The Phototrophic Alpha-Proteobacteria 41
JOHANNES F. IMHOFF

3.1.2 The Genera *Prosthecomicrobium* and *Ancalomicrobium* 65
GARY E. OERTLI, CHERYL JENKINS, NAOMI WARD, FREDERICK A. RAINEY, ERKO STACKEBRANDT and JAMES T. STALEY

3.1.3 Dimorphic Prosthecate Bacteria: The Genera *Caulobacter*, *Asticcacaulis*, *Hyphomicrobium*, *Pedomicrobium*, *Hyphomonas* and *Thiodendron* 72
JEANNE S. POINDEXTER

3.1.4 The Genus *Agrobacterium* 91
ANN G. MATTHYSSE

3.1.5 The Genus *Azospirillum* 115
ANTON HARTMANN and JOSE IVO BALDANI

3.1.6 The Genus *Herbaspirillum* 141
MICHAEL SCHMID, JOSE IVO BALDANI and ANTON HARTMANN

3.1.7 The Genus *Beijerinckia* 151
JAN HENDRICK BECKING

3.1.8 The Family Acetobacteraceae: The Genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia* 163
KAREL KERSTERS, PUSPITA LISDIYANTI, KAZUO KOMAGATA and JEAN SWINGS

3.1.9 The Genus *Zymomonas* 201
HERMANN SAHM, STEPHANIE BRINGER-MEYER and GEORG A. SPRENGER

3.1.10 The Manganese-Oxidizing Bacteria 222
KENNETH H. NEALSON

3.1.11 The Genus *Paracoccus* 232
DONOVAN P. KELLY, FREDERICK A. RAINEY and ANN P. WOOD

3.1.12 The Genus *Phenylobacterium* 250
JÜRGEN EBERSPÄCHER and FRANZ LINGENS

3.1.13 *Methylobacterium* 257
PETER N. GREEN

3.1.14 The Methanotrophs—The Families Methylococcaceae and Methylocystaceae 266
JOHN P. BOWMAN

3.1.15 The Genus *Xanthobacter* 290
JÜRGEN WIEGEL

3.1.16 The Genus *Brucella* 315
EDGARDO MORENO and IGNACIO MORIYÓN

3.1.17 Introduction to the Rickettsiales and Other Intracellular Prokaryotes 457
DAVID N. FREDRICKS

3.1.18 The Genus *Bartonella* 467
MICHAEL F. MINNICK and BURT E. ANDERSON

3.1.19 The Order Rickettsiales 493
XUE-JIE YU and DAVID H. WALKER

3.1.20 The Genus *Coxiella* 529
ROBERT A. HEINZEN and JAMES E. SAMUEL

3.1.21 The Genus *Wolbachia* 547
MARKUS RIEGLER and SCOTT L. O'NEILL

3.1.22 Aerobic Phototrophic Proteobacteria 562
VLADIMIR V. YURKOV

3.1.23 The Genus *Seliberia* 585
JEAN M. SCHMIDT and JAMES R. SWAFFORD

3.2. Beta Subclass

3.2.1 The Phototrophic Betaproteobacteria 593
JOHANNES F. IMHOFF

3.2.2 The *Neisseria* 602
DANIEL C. STEIN

3.2.3 The Genus *Bordetella* 648
ALISON WEISS

3.2.4 *Achromobacter*, *Alcaligenes* and Related Genera 675
HANS-JÜRGEN BUSSE and ANDREAS STOLZ

3.2.5 The Genus *Spirillum* 701
NOEL R. KRIEG

3.2.6 The Genus *Aquaspirillum* 710
BRUNO POT, MONIQUE GILLIS and JOZEF DE LEY

3.2.7 *Comamonas* 723
ANNE WILLEMS and PAUL DE VOS

3.2.8 The Genera *Chromobacterium* and *Janthinobacterium* 737
MONIQUE GILLIS and JOZEF DE LEY

3.2.9 The Genera *Phyllobacterium* and *Ochrobactrum* 747
JEAN SWINGS, BART LAMBERT, KAREL KERSTERS and BARRY HOLMES

3.2.10 The Genus *Derxia* 751
JAN HENDRICK BECKING

3.2.11 The Genera *Leptothrix* and *Sphaerotilus* 758
STEFAN SPRING

3.2.12 The Lithoautotrophic Ammonia-Oxidizing Bacteria 778
HANS-PETER KOOPS, ULRIKE PURKHOLD, ANDREAS POMMERENING-RÖSER, GABRIELE TIMMERMANN and MICHAEL WAGNER

3.2.13 The Genus *Thiobacillus* 812
LESLEY A. ROBERTSON and J. GIJS KUENEN

3.2.14 The Genera *Simonsiella* and *Alysiella* 828
BRIAN P. HEDLUND and DAISY A. KUHN

3.2.15 *Eikenella corrodens* and Closely Related Bacteria 840
EDWARD J. BOTTONE and PAUL A. GRANATO

3.2.16 The Genus *Burkholderia* 848
DONALD E. WOODS and PAMELA A. SOKOL

3.2.17 The Nitrite-Oxidizing Bacteria 861
AHARON ABELIOVICH

3.2.18 The Genera *Azoarcus*, *Azovibrio*, *Azospira* and *Azonexus* 873
BARBARA REINHOLD-HUREK and THOMAS HUREK

Index 893

Volume 6

3. Proteobacteria

3.3. Gamma Subclass

3.3.1 New Members of the Family Enterobacteriaceae 5
J. MICHAEL JANDA

3.3.2 Phylogenetic Relationships of Bacteria with Special Reference to Endosymbionts and Enteric Species 41
M. PILAR FRANCINO, SCOTT R. SANTOS and HOWARD OCHMAN

3.3.3 The Genus *Escherichia* 60
RODNEY A. WELCH

3.3.4 The Genus *Edwardsiella* 72
SHARON L. ABBOTT and J. MICHAEL JANDA

3.3.5 The Genus *Citrobacter* 90
DIANA BORENSHTEIN and DAVID B. SCHAUER

3.3.6 The Genus *Shigella* 99
YVES GERMANI and PHILIPPE J. SANSONETTI

3.3.7 The Genus *Salmonella* 123
CRAIG D. ELLERMEIER and JAMES M. SLAUCH

3.3.8 The Genus *Klebsiella* 159
SYLVAIN BRISSE, FRANCINE GRIMONT and PATRICK A. D. GRIMONT

3.3.9 The Genus *Enterobacter* 197
FRANCINE GRIMONT and PATRICK A. D. GRIMONT

3.3.10 The Genus *Hafnia* 215
MEGAN E. MCBEE and DAVID B. SCHAUER

3.3.11 The Genus *Serratia* 219
FRANCINE GRIMONT and PATRICK A. D. GRIMONT

3.3.12 The Genera *Proteus*, *Providencia*, and *Morganella* 245
JIM MANOS and ROBERT BELAS

3.3.13 *Y. enterocolitica* and *Y. pseudotuberculosis* 270
ELISABETH CARNIEL, INGO AUTENRIETH, GUY CORNELIS, HIROSHI FUKUSHIMA, FRANÇOISE GUINET, RALPH ISBERG, JEANNETTE PHAM, MICHAEL PRENTICE, MICHEL SIMONET, MIKAEL SKURNIK and GEORGES WAUTERS

3.3.14 *Yersinia pestis* and Bubonic Plague 399
ROBERT BRUBAKER

3.3.15 *Erwinia* and Related Genera 443
CLARENCE I. KADO

3.3.16 The Genera *Photorhabdus* and *Xenorhabdus* 451
NOEL BOEMARE and RAYMOND AKHURST

3.3.17 The Family Vibrionaceae 495
J. J. FARMER, III

3.3.18 The Genera *Vibrio* and *Photobacterium* 508
J. J. FARMER, III and F. W. HICKMAN-BRENNER

3.3.19 The Genera *Aeromonas* and *Plesiomonas* 564
J. J. FARMER, III, M. J. ARDUINO and F. W. HICKMAN-BRENNER

3.3.20 The Genus *Alteromonas* and Related Proteobacteria 597
VALERY V. MIKHAILOV, LYUDMILA A. ROMANENKO and ELENA P. IVANOVA

3.3.21 Nonmedical: *Pseudomonas* 646
EDWARD R. B. MOORE, BRIAN J. TINDALL, VITOR A. P. MARTINS DOS SANTOS, DIETMAR H. PIEPER, JUAN-LUIS RAMOS and NORBERTO J. PALLERONI

3.3.22 *Pseudomonas aeruginosa* 704
TIMOTHY L. YAHR and MATTHEW R. PARSEK

3.3.23 Phytopathogenic Pseudomonads and Related Plant-Associated Pseudomonads 714
MILTON N. SCHROTH, DONALD C. HILDEBRAND and NICKOLAS PANOPOULOS

3.3.24 *Xylophilus* 741
ANNE WILLEMS and MONIQUE GILLIS

3.3.25 The Genus *Acinetobacter* 746
KEVIN TOWNER

3.3.26 The Family Azotobacteraceae 759
JAN HENDRICK BECKING

3.3.27 The Genera *Beggiatoa* and *Thioploca* 784
ANDREAS TESKE and DOUGLAS C. NELSON

3.3.28 The Family Halomonadaceae 811
DAVID R. ARAHAL and ANTONIO VENTOSA

3.3.29 The Genus *Deleya* 836
KAREL KERSTERS

3.3.30 The Genus *Frateuria* 844
JEAN SWINGS

3.3.31 The Chromatiaceae 846
JOHANNES F. IMHOFF

3.3.32 The Family Ectothiorhodospiraceae 874
JOHANNES F. IMHOFF

3.3.33 *Oceanospirillum* and Related Genera 887
JOSÉ M. GONZÁLEZ and WILLIAM B. WHITMAN

3.3.34 *Serpens flexibilis*: An Unusually Flexible Bacterium 916
ROBERT B. HESPELL

3.3.35 The Genus *Psychrobacter* 920
JOHN P. BOWMAN

3.3.36 The Genus *Leucothrix* 931
THOMAS D. BROCK

3.3.37 The Genus *Lysobacter* 939
HANS REICHENBACH

3.3.38 The Genus *Moraxella* 958
JOHN P. HAYS

3.3.39 *Legionella* Species and Legionnaire's Disease 988
PAUL H. EDELSTEIN and NICHOLAS P. CIANCIOTTO

3.3.40 The Genus *Haemophilus* 1034
DORAN L. FINK and JOSEPH W. ST. GEME, III

3.3.41 The Genus *Pasteurella* 1062
HENRIK CHRISTENSEN and MAGNE BISGAARD

3.3.42 The Genus *Cardiobacterium* 1091
SYDNEY M. HARVEY and JAMES R. GREENWOOD

3.3.43 The Genus *Actinobacillus* 1094
JANET I. MACINNES and EDWARD T. LALLY

3.3.44 The Genus *Francisella* 1119
FRANCIS NANO and KAREN ELKINS

3.3.45 Ecophysiology of the Genus *Shewanella* 1133
KENNETH H. NEALSON and JAMES SCOTT

3.3.46 The Genus *Nevskia* 1152
HERIBERT CYPIONKA, HANS-DIETRICH BABENZIEN, FRANK OLIVER GLÖCKNER and RUDOLF AMANN

3.3.47 The Genus *Thiomargarita* 1156
HEIDE N. SCHULZ

Index 1165

Volume 7

3. Proteobacteria

3.4 Delta Subclass

3.4.1 The Genus *Pelobacter* 5
BERNHARD SCHINK

3.4.2 The Genus *Bdellovibrio* 12
EDOUARD JURKEVITCH

3.4.3 The Myxobacteria 31
LAWRENCE J. SHIMKETS, MARTIN DWORKIN and HANS REICHENBACH

3.5. Epsilon Subclass

3.5.1 The Genus *Campylobacter* 119
TRUDY M. WASSENAAR and DIANE G. NEWELL

3.5.2 The Genus *Helicobacter* 139
JAY V. SOLNICK, JANI L. O'ROURKE, PETER VAN DAMME and ADRIAN LEE

3.5.3 The Genus *Wolinella* 178
JÖRG SIMON, ROLAND GROSS, OLIVER KLIMMEK and ACHIM KRÖGER

4. Spirochetes

4.1 Free-Living Saccharolytic Spirochetes: The Genus *Spirochaeta* 195
SUSAN LESCHINE, BRUCE J. PASTER and ERCOLE CANALE-PAROLA

4.2 The Genus *Treponema* 211
STEVEN J. NORRIS, BRUCE J. PASTER, ANNETTE MOTER and ULF B. GÖBEL

4.3 The Genus *Borrelia* 235
MELISSA J. CAIMANO

4.4 The Genus *Leptospira* 294
BEN ADLER and SOLLY FAINE

4.5 Termite Gut Spirochetes 318
JOHN A. BREZNAK and JARED R. LEADBETTER

4.6 The Genus *Brachyspira* 330
THADDEUS B. STANTON

5. Chlorobiaceae

5.1 The Family Chlorobiaceae 359
JÖRG OVERMANN

6. Bacteroides and Cytophaga Group

6.1 The Medically Important *Bacteroides* spp. in Health and Disease 381
C. JEFFREY SMITH, EDSON R. ROCHA and BRUCE J. PASTER

6.2 The Genus *Porphyromonas* 428
FRANK C. GIBSON and CAROLINE ATTARDO GENCO

6.3 An Introduction to the Family Flavobacteriaceae 455
JEAN-FRANÇOIS BERNARDET and YASUYOSHI NAKAGAWA

6.4 The Genus *Flavobacterium* 481
JEAN-FRANÇOIS BERNARDET and JOHN P. BOWMAN

6.5 The Genera *Bergeyella* and *Weeksella* 532
CELIA J. HUGO, BRITA BRUUN and PIET J. JOOSTE

6.6 The Genera *Flavobacterium*, *Sphingobacterium* and *Weeksella* 539
BARRY HOLMES

6.7 The Order Cytophagales 549
HANS REICHENBACH

6.8 The Genus *Saprospira* 591
HANS REICHENBACH

6.9 The Genus *Haliscomenobacter* 602
EPPE GERKE MULDER and MARIA H. DEINEMA

6.10 *Sphingomonas* and Related Genera 605
DAVID L. BALKWILL, J. K. FREDRICKSON and M. F. ROMINE

6.11 The Genera *Empedobacter* and Myroides 630
CELIA J. HUGO, BRITA BRUUN and PIET J. JOOSTE

6.12 The Genera *Chryseobacterium* and *Elizabethkingia* 638
JEAN-FRANÇOIS BERNARDET, CELIA J. HUGO and BRITA BRUUN

6.13 The Marine Clade of the Family Flavobacteriaceae: The Genera Aequorivita, Arenibacter, Cellulophaga, Croceibacter, Formosa, Gelidibacter, Gillisia, Maribacter, Mesonia, Muricauda, Polaribacter, Psychroflexus, Psychroserpens, Robiginitalea, Salegentibacter, Tenacibaculum, Ulvibacter, Vitellibacter and Zobellia 677
JOHN P. BOWMAN

6.14 Capnophilic Bird Pathogens in the Family Flavobacteriaceae: Riemerella, Ornithobacterium and Coenonia 695
PETER VAN DAMME, H. M. HAFEZ and K. H. HINZ

6.15 The Genus *Capnocytophaga* 709
E. R. LEADBETTER

6.16 The Genera *Rhodothermus*, *Thermonema*, *Hymenobacter* and *Salinibacter* 712
AHARON OREN

7. Chlamydia

7.1 The Genus *Chlamydia*—Medical 741
MURAT V. KALAYOGLU and GERALD I. BYRNE

8. Planctomyces and Related Bacteria

8.1 The Order Planctomycetales, Including the Genera *Planctomyces*, *Pirellula*, *Gemmata and Isosphaera* and the Candidatus Genera *Brocadia*, *Kuenenia* and *Scalindua* 757
NAOMI WARD, JAMES T. STALEY, JOHN A. FUERST, STEPHEN GIOVANNONI, HEINZ SCHLESNER and ERKO STACKEBRANDT

9. Thermus

9.1 The Genus *Thermus* and Relatives 797
MILTON S. DA COSTA, FREDERICK A. RAINEY and M. FERNANDA NOBRE

10. Chloroflexaceae and Related Bacteria

10.1 The Family Chloroflexaceae 815
SATOSHI HANADA and BEVERLY K. PIERSON

10.2 The Genus *Thermoleophilum* 843
JEROME J. PERRY

10.3 The Genus *Thermomicrobium* 849
JEROME J. PERRY

10.4 The Genus *Herpetosiphon* 854
NATUSCHKA LEE and HANS REICHENBACH

11. Verrucomicrobium

11.1 The Phylum Verrucomicrobia: A Phylogenetically Heterogeneous Bacterial Group 881
HEINZ SCHLESNER, CHERYL JENKINS and JAMES T. STALEY

12. Thermotogales

12.1 Thermotogales 899
ROBERT HUBER and MICHAEL HANNIG

13. Aquificales

13.1 Aquificales 925
ROBERT HUBER and WOLFGANG EDER

14. Phylogenetically Unaffiliated Bacteria

14.1 Morphologically Conspicuous Sulfur-Oxidizing Eubacteria 941
JAN W. M. LA RIVIÈRE and KARIN SCHMIDT

14.2 The Genus *Propionigenium* 955
BERNHARD SCHINK

14.3 The Genus *Zoogloea* 960
PATRICK R. DUGAN, DAPHNE L. STONER and HARVEY M. PICKRUM

14.4 Large Symbiotic Spirochetes: *Clevelandina*, *Cristispira*, *Diplocalyx*, *Hollandina* and *Pillotina* 971
LYNN MARGULIS and GREGORY HINKLE

14.5 *Streptobacillus moniliformis* 983
JAMES R. GREENWOOD and SYDNEY M. HARVEY

14.6 The Genus *Toxothrix* 986
PETER HIRSCH

14.7 The Genus *Gallionella* 990
HANS H. HANERT

14.8 The Genera *Caulococcus* and *Kusnezovia* 996
JEAN M. SCHMIDT and GEORGI A. ZAVARZIN

14.9 The Genus *Brachyarcus* 998
PETER HIRSCH

14.10 The Genus *Pelosigma* 1001
PETER HIRSCH

14.11 The Genus *Siderocapsa* (and Other Iron- and Maganese-Oxidizing Eubacteria) 1005
HANS H. HANERT

14.12 The Genus *Fusobacterium* 1016
TOR HOFSTAD

14.13 Prokaryotic Symbionts of Amoebae and Flagellates 1028
KWANG W. JEON

Index 1039

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PART 3

Proteobacteria

SECTION 3.4

Delta Subclass

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CHAPTER 3.4.1

The Genus *Pelobacter*

BERNHARD SCHINK

The genus *Pelobacter* was proposed as a taxonomic entity consisting of strictly anaerobic, Gram-negative, nonspore-forming, rod-shaped bacteria that use only a very limited number of substrates. The members of the genus are all unable to ferment sugars and therefore cannot be grouped with any other genus in the family Bacteroidaceae (Krieg and Holt, 1984). The genus comprises five different species, *P. acidigallici* (Schink and Pfennig, 1982), *P. venetianus* (Schink and Stieb, 1983), *P. carbinolicus* (Schink, 1984), *P. propionicus* (Schink, 1984), and *P. acetylenicus* (Schink, 1985), which all are based on 3–5 described strains.

Comparisons of the various *Pelobacter* species by DNA-DNA hybridization experiments revealed that the genus is rather inhomogenous; therefore, a reorganization may perhaps be necessary in the future (J. P. Touzel and B. Schink, unpublished observations). Whereas the species *P. venetianus*, *P. carbinolicus*, and *P. acetylenicus* form a homogenous cluster, *P. acidigallici* and *P. propionicus* appear to be only distantly related to the others. These findings are consistent to some extent with the fermentation patterns of these species (see below). Comparison of three *Pelobacter* species with other anaerobes on the basis of 16S rRNA structure analysis supports this view. Whereas *P. venetianus* and *P. carbinolicus* exhibit a rather high similarity, with an S_{AB} of 0.70, *P. acidigallici* is related to both at an S_{AB} of only 0.53 (Stackebrandt et al., 1989). It is interesting to note that these three Pelobacter strains did not show any resemblance to other fermenting Gram-negative strict anaerobes; instead, they appeared to be highly related to several strains of sulfur-reducing anaerobes, namely, *Desulfuromonas succinoxidans*, *D. acetexigens*, and *D. acetoxidans*, to which they are even more closely related than *P. acidigallici* is to the other two *Pelobacter* species. Since *Pelobacter* species and the obligately sulfur-respiring bacteria are quite diverse metabolically and the latter are supposed to have derived directly from phototrophic ancestors, it has been suggested that the genus *Pelobacter* represents a group of fermenting bacteria that developed a fermentative metabolism as a "secondary" evolutionary event and that they are separate from the first fermentative bacteria (Stackebrandt et al., 1989).

Habitat

All *Pelobacter* strains have been isolated so far from marine or freshwater sediments. The name *Pelobacter* was based on this origin (Greek *pelos* meaning mud, sediment). Enrichments from sewage sludges led to similar isolates as well. Viable counts using the characteristic substrates gallic acid, acetoin, polyethylene glycol, and acetylene showed that there were approximately 100 cells/ml of each of the *Pelobacter* species in sediment and up to 2,500 cells/ml in sewage sludge. Since their substrate ranges are comparably small, their ecological niche in such sediments can be understood rather well in most cases. *P. acidigallici* is restricted to the utilization of trihydroxybenzenoids, which are probably its only energy source in its natural habitat. *P. venetianus*, *P. carbinolicus*, *P. propionicus*, and *P. acetylenicus* were enriched and isolated with polyethylene glycol, 2,3-butanediol, and acetylene, respectively, but the ecological importance of these substrates in the respective environments is questionable. Since all these species can also ferment ethanol, either in syntrophic cooperation with hydrogen scavengers or in pure culture, it appears more probable that degradation of this important fermentation intermediate is their predominant function in these environments. *P. carbinolicus* has been identified as the dominant ethanol-degrading bacterium in digesting industrial sewage sludge (Dubourguier et al., 1986), and high numbers (10^6–10^7 cells/ml) of syntrophically ethanol-oxidizing anaerobes were detected also in other sewage sludges (Schink et al., 1985) and in freshwater creek sediments (Eichler and Schink, 1985). *Pelobacter* has to compete for ethanol with certain homoacetogenic bacteria, e.g., *Clostridium aceticum* (Wieringa, 1940) or *Acetobacterium carbinolicum* (Eichler and Schink, 1984), which appear to be at least as successful in freshwater sediments (Schink et al., 1985), especially if the sediment is slightly acidic (Schink et al., 1985) or the temperature is low (Conrad et al., 1989). The ecological importance of ethanol fermentation to propionate by *P. propionicus* has been elucidated by enumerations and by tracer experiments (Schink et al., 1985). These studies revealed that up to 20% of the total ethanol turnover can go through propionate and that bacteria forming propionate from ethanol contribute significantly to the total ethanol-metabolizing microbial community.

It has to be concluded that bacteria of the metabolic types represented by the various *Pelo-*

This chapter was taken unchanged from the second edition.

bacter species make up a significant part of the anaerobic microbial population in sediments and sewage sludge. No *Pelobacter*-like bacteria have so far been isolated from the rumen. The numerically predominant, syntrophically ethanol-oxidizing *Pelobacter* species represent new isolates of the metabolic type of the S-strain in the mixed culture "*Methanobacillus omelianskii*" (Bryant et al., 1967). These *Pelobocter* species have become accessible to pure culture growth in our laboratory by the use of unusual substrates that all can be converted easily into acetaldehyde, the key intermediate in the energy metabolism of these bacteria (see next section).

Isolation

Growth Media

All *Pelobacter* strains have been enriched and isolated in a carbonate-buffered, sulfide-reduced mineral medium that contained only one organic energy and carbon source. Since they grow with substrates that yield 2-carbon intermediates exclusively, they have to form pyruvate and sugars via reductive carboxylation of acetyl coenzyme A and need carbon dioxide for this reaction. Use of a bicarbonate-buffered medium is therefore recommended for enrichment, isolation, and maintenance. Three different versions of this medium are described below for the isolation of *Pelobacter* from freshwater, estuarine, and marine sediment, respectively (after Widdel and Pfennig, 1981; Schink and Pfennig, 1982):

Pelobacter Growth Media

Dissolve in 1 liter of distilled water:

Autoclave the complete mineral medium in a vessel equipped with 1) a filter inlet to allow flushing of the headspace with sterile oxygen-free gas; 2) screw-cap inlets for addition of thermally unstable additives after autoclaving; 3) a silicon tubing connection from the bottom of the vessel out to a dispensing tap (if possible with a protecting bell) for sterile dispensing of the medium (do not use latex tubing; it releases compounds which are highly toxic to many anaerobes); and 4) a stirring bar.

After autoclaving, connect the vessel with the still-hot medium to a line of oxygen-free nitrogen/carbon dioxide mixture (90%: 10%) at low pressure (<100 mbar), flush the headspace and cool it under this atmosphere to room temperature, perhaps with the help of a cooling water bath.

The mineral medium is amended with the following additions from stock solutions that have been sterilized separately (amounts/l of medium): a) 30 ml of 1 M $NaHCO_3$ solution (autoclaved in a *tightly closed* screw-cap bottle with about 30% headspace; the bottle should be autoclaved inside another protective vessel, e.g., a polypropylene beaker, to avoid spills of carbonates if the bottle breaks in the autoclave); b) 2 ml of 0.5 M $Na_2S9{\cdot}H_2O$ solution (autoclaved separately under oxygen-free gas atmosphere as above); c) 1 ml of trace element solution, e.g., SL 10 (Widdel et al., 1983); d) 0.5 ml of 10-fold concentrated, filter-sterilized vitamin solution (Pfennig, 1978); and e) adequate amounts of sterile 1 M HC1 or 1 M $Na_2\ CO_3$ to adjust the pH to 7.1–7.3.

The complete medium is dispensed into either screw-cap bottles or screw-cap tubes which are filled completely to the top, leaving a lentil-sized air bubble for pressure equilibration. Enrichment cultures usually produce gas in the first enrichment stages and are better cultivated in half-filled serum bottles (50–100 ml volume) under a headspace of nitrogen: carbon dioxide mixture (90%:10%).

This mineral medium is amended with the respective organic substrates for enrichment and cultivation of pure cultures. The vitamin mixture is not really needed by all strains.

Selective Enrichment

All enrichment cultures were set up in our laboratory at 27–30°C in 50-ml fluid cultures inoculated with about 5 ml of sediment or sludge. Smaller inocula may also lead to isolation of the same bacteria, but this has not been evaluated in our lab. *P. acidigallici* can be selectively enriched with either one of its growth substrates (see Table 1) at 5–10 mM concentration. For enrichment of *P. venetianus*, either polyethylene glycol (mol wt 106–20,000; 0.1% w/v) or methoxyethanol (10 mM; Tanaka and Pfennig, 1988) is recommended. In this medium, 10 mM 2,3-butanediol enriches for *P. carbinolicus* from marine sediments and for *P. propionicus* from freshwater sediments. *P. acetylenicus* is successfully enriched with mineral medium under the above-mentioned nitrogen: carbon dioxide atmosphere containing 5–10% acetylene in addition.

Isolation

After 3–4 transfers in liquid medium, a homogeneous microbial population should have become established in liquid enrichment cultures. Purification of *Pelobacter* species is most easily done by serial dilution in agar deep cultures ("agar shakes"; Pfennig, 1978). Roll tubes have not been used and are not necessary since these bacteria neither consume nor produce insoluble gaseous compounds. *P. acetylenicus* can be easily purified with 10 mM acetoin as substrate. Other procedures (streaking on agar plates in an anoxic glove box or on agar surfaces in flat agar bottles) have not been tried yet, but there is no reason why such methods would not be successful as well, if the solid media are incubated under a nitrogen/carbon dioxide atmosphere.

Preservation

Liquid cultures were maintained in our laboratory in 50-ml bottles at 4°C for 4–12 weeks

Table 1. Properties of the five *Pelobacter* species.

	P. acidigallici	*P. venetianus*	*P. carbinolicus*	*P. acetylenicus*	*P. propionicus*
Width (μm)	0.5–0.8	0.5–1.0	0.5–0.7	0.6–0.8	0.5–0.7
Length (μm)	1.5–3.5	2.5	1.2–3.0	1.5–4.0	1.2–6.0
GC content (mol%)	51.8	52.2	52.3	57.1	57.4
Substrate metabolized					
Gallic acid	+	–	–	–	–
Pyrogallol	+	–	–	–	–
Phloroglucinol	+	–	–	–	–
Phloroglucinolcarboxylate	+	–	–	–	–
Acetoin	–	+	+	+	+
2,3-Butanediol	–	+	+	+	+
Ethylene glycol	–	+[a]	+	–	–
Polyethylene glycols	–	+	–	–	–
Ethanol	–	+[b]	+[b]	+[b]	+
n-Propanol	–	+[b, c]	+[b, c]	+[b, c]	+[c]
n-Butanol	–	+[b, c]	+[b, c]	+[b, c]	+[c]
1,2-Propanediol	–	+[c]	–	+[c]	–
Acetylene	–	–	–	+	–
Lactate	–	–	–	–	+
Pyruvate	–	–	–	–	+
Glycerol	–	+[c]	–	+[c]	–
Typical Products	Acetate (CO_2)	Acetate, ethanol	Acetate, ethanol	Acetate, ethanol	Acetate, propionate

[a]Growth is possible only at very low concentration (<1 mM) or in continuous culture.
[b]Growth is possible only in the presence of a hydrogen-scavenging anaerobe, e.g., a methanogenic bacterium.
[c]Growth is possible only in the presence of small amounts of acetate for cell carbon synthesis.
Symbols: +, growth; –, no growth.

between transfers. Longer storage intervals may be possible as well. Long-term preservation is easily accomplished by storage of dense cell suspensions in glass capillaries kept in liquid nitrogen.

Identification

Morphological and Cytological Properties

Cells of all *Pelobacter* species are Gram-negative, short rods that do not form spores (Fig. 1). The cell ends are usually rounded; however, *P. acetylenicus* cells can be slightly pointed (Fig. 1e). The cell sizes vary from 0.5 to 1.0 μm in width and from 1.2 to 6.0 μm in length. More exact cell sizes of the different type strains are listed in Table 1. The temperature optima for growth are in the range of 28–35°C; the pH optima are 6.5–7.5. On the basis of the GC content of the DNA, two clusters can be defined; the one (*P. acidigallici*, *P. venetianus*, *P. carbinolicus)* has a GC value at 50–53 mol%, the other one *(P. acetylenicus*, *P. propionicus)* at 57–58 mol%. It should be noted that this clustering does not agree with the subgroupings based on DNA and RNA homologies (see "Introduction," this chapter) or with physiological similarities.

Cytochromes have been found only in *P. propionicus*. A *b*-type cytochrome was detected at a very low level (Schink et al., 1987), but there is no evidence that this cytochrome plays any role in ATP-generating electron transport (see below).

Physiological Properties and Biochemistry

Table 1 presents a listing of all substrates used by the various *Pelobacter* species described so far. It is obvious from this table that *Pelobacter acidigallici* differs in its substrate spectrum from all other *Pelobacter* species. It utilizes only trihydroxybenzenoids for growth, and it ferments them to acetate as sole fermentation product. With this, it resembles *Eubacterium oxidoreducens* (Krumholz and Bryant, 1986) to some extent, but does not depend on formate as an external co-substrate for degradation of these compounds. Growth experiments with increased substrate concentrations have revealed that the pathway of gallic acid fermentation leads via pyrogallol and phloroglucinol (Samain et al., 1986) and has nothing in common with the pathway of anaerobic benzoate degradation (Evans, 1977). Obviously, three hydroxyl groups in alternating position at the ring polarize the π-electron system sufficiently to permit selective reduction to dihydrophloroglucinol and ring opening by a thiolytic or hydrolytic mechanism. The isomerization of pyrogallol to phloroglucinol has recently been characterized as a unique transhydroxylase reaction involving a tetrahydroxybenzene as co-substrate (Brune and Schink, 1990). The third trihydroxybenzene isomer, hydroxyhy-

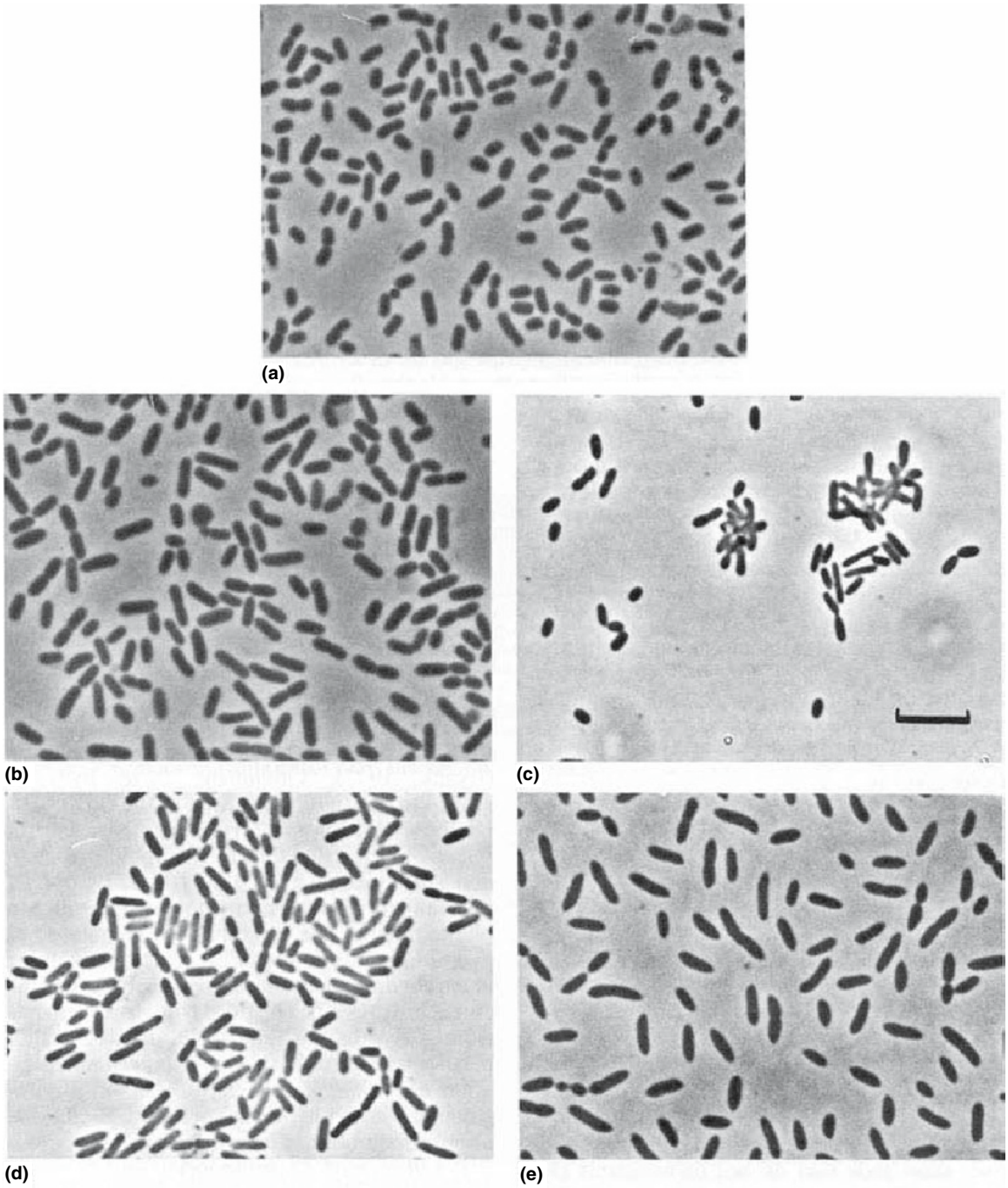

Fig. 1. Phase contrast photomicrographs of cells of *Pelobacter* species. Bar = 5 µm for all figures. (a) *P. acidigallici*; (b) *P. venetianus*; (c) *P. carbinolicus*; (d) *P. acetylenicus*; (e) *P. propionicus*.

droquinone, is not metabolized by these bacteria, but it is fermented by other new anaerobic bacteria, also via phloroglucinol (A. Brune, S. Schnell and B. Schink, unpublished observations).

P. venetianus, *P. carbinolicus*, and *P. acetylenicus* have been enriched and isolated with polyethylene glycol, 2,3-butanediol, or acetylene, respectively. They can all grow with acetoin, some also with ethylene glycol or ethanolamine. All these substrates are converted to acetaldehyde, which is further dismutated to acetate and ethanol as final products. ATP is formed exclusively by substrate level phosphorylation via the acetate kinase reaction. The higher homologs of

ethylene glycol, 1,2-propanediol and 1,2-butanediol, are dismutated to propanol and propionate or butanol and butyrate, respectively, provided that the medium contains some acetate (2–3 mM) for synthesis of cell material. Glycerol undergoes a similar dismutation to 3-hydroxypropionate and 1,3-propanediol. It is interesting to note that glycerol fermentation also requires the presence of acetate and that this substrate cannot itself be assimilated.

In the presence of hydrogen-scavenging anaerobes, e.g., homoacetogens or methanogens, *Pelobacter* oxidizes primary aliphatic alcohols to the corresponding acids; with propanol and butanol, acetate is again required for cell matter synthesis. This syntrophic oxidation of ethanol via "interspecies hydrogen transfer" was first observed with the S-strain isolated from the syntrophic mixed culture "*Methanobacillus omelianskii*" (Bryant et al., 1967), which was lost many years ago. *Pelobacter* strains are the only representatives of this type of metabolism that are available today in pure cultures. Pure and mixed culture experiments have been carried out recently to understand the energetics and kinetics of interspecies hydrogen transfer in model cultures of *P. acetylenicus* and hydrogen-scavenging homoacetogenic and methanogenic partners (Seitz et al., 1988; Seitz et al., in preparation).

The biochemistry of polyethylene glycol degradation is not yet understood, neither in *P. venetianus* nor in other polyethylene glycol-degrading anaerobes (Dwyer and Tiedje, 1986; Wagener and Schink, 1988). *P. venetianus* degrades all polymers from the dimer up to a molecular weight of 40,000. Growth experiments in batch and continuous culture have shown that ethylene glycol can also support growth if it is provided at limiting amounts and that acetaldehyde is the first free intermediate in both polymer and monomer degradation (Strass and Schink, 1986). Perhaps the polymer is attacked by a diol dehydratase-like reaction that transforms the terminal ether linkage into an unstable half-acetal linkage yielding acetaldehyde as product. Unfortunately, the cleavage reaction is very difficult to demonstrate in cell-free extracts, and a B_{12} compound of atypical structure appears to be involved (E. Schramm and B. Schink, unpublished observations). This cleavage reaction occurs inside the cells, and it is not clear how high-molecular-weight polyethylene glycols are able to cross the cytoplasmic membrane at sufficiently high transport rates.

Anaerobic degradation of polyethylene glycols is of major ecological concern because many industrially produced nonionic surfactants contain polyethylene glycols as hydrophilic moieties that may be subject to anaerobic degradation in anoxic sediments and sludge (Wagener and Schink, 1987, 1988).

P. carbinolicus is related to *P. venetianus* and has basically the same biochemistry and physiology. It degrades ethylene glycol rather than polyethylene glycols, and it was originally enriched and isolated with acetoin or 2,3-butanediol as substrate. 2,3-Butanediol is oxidized to acetoin, which undergoes oxidative cleavage to acetyl CoA and acetaldehyde by a dichlorophenol indophenol-dependent enzyme analogous to pyruvate dehydrogenase (Oppermann et al., 1988). The physiological electron acceptor of this enzyme is not yet known. Acetaldehyde is either oxidized by a benzyl viologen-dependent enzyme to acetyl CoA or, depending on the electron balance, reduced to ethanol.

P. acetylenicus is the first strict anaerobe known to ferment an unsaturated hydrocarbon in pure culture. Again, the first intermediate of acetylene fermentation is acetaldehyde, which is further dismutated to acetate and ethanol. The enzyme that hydrates acetylene to acetaldehyde could not be demonstrated in a cell-free assay system; perhaps unusual cofactors are also involved in this reaction. A report on an acetylene-hydratase enzyme in cell-free extracts of an aerobic *Rhodococcus* species (deBont and Peck, 1980) could not be reproduced in our hands either. The function of such an acetylene-hydrating enzyme in an anoxic environment is hard to understand since acetylene is probably not an important substrate in such environments. Perhaps its main role is hydration of other possibly toxic compounds, such as nitriles or cyanides, but there is no experimental evidence so far for such activities.

P. propionicus differs from the other *Pelobacter* species by producing propionate as one of its main fermentation products. Degradation of acetoin, 2,3-butanediol, and ethanol probably follows the same routes via acetaldehyde and acetyl CoA, as outlined above for the other species. The biochemistry of propionate formation from these C-2 compounds has been studied in detail (Schink et al., 1987). The key reaction is catalyzed by pyruvate synthase (pyruvate ferredoxin oxidoreductase), which operates here in the opposite direction to that predicted by its chemical equilibrium. The equilibrium is shifted by the exergonic propionate-forming reaction chain via methylmalonyl CoA; this chain is not coupled to ATP-yielding electron transport phosphorylation in these bacteria. This reaction in *P. propionicus* and other bacteria with similar biochemical capacities (Stams et al., 1984; Samain et al., 1982) is probably responsible for the formation of C-3 compounds from C-2 compounds in significant amounts in anoxic sediments and sludges (Goldberg and Cooney, 1981; Schink et al., 1985).

Applications

All *Pelobacter* strains discussed in this chapter may act as important syntrophic oxidants of primary aliphatic alcohols in sediments and sludges, where they have been found to be predominant (Dubourgier et al., 1986). Of technological interest may be the capacity of *P. venetianus* and similar isolates to degrade polyethylene glycols and to attack nonionic surfactants based on these compounds. These surfactants are of growing interest in the industrial manufacture of detergents, soaps, emulsifiers, etc. Aerobic degradation of polyethylene glycol-containing surfactant wastes creates enormous problems of foam formation in conventional activated-sludge sewage treatment and in natural waters. Anaerobic degradation of most of these compounds to methane can easily be achieved in packed column reactors, in which *P. venetianus*-like anaerobes ferment the polyethylene glycol moieties to substrates for the methanogenic microbial community (Wagener and Schink, 1987).

P. propionicus produces acetate and propionate from the substrates listed in Table 1. Propanol is fermented together with acetate exclusively to propionate. This fermentation may be of interest for biological production of propionate at high purity.

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Literature Cited

Brune, A., B. Schink. 1990. Conversion of pyrogallol to phloroglucinol, and other hydroxyl transfer reactions catalyzed by cell-free extracts of *Pelobacter acidigallici*. J. Bacteriol. 172:1070–1076.

Bryant, M. P., E. A. Wolin, M. J. Wolin, R. S. Wolfe. 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Arch. Mikrobiol. 59:20–31.

Conrad, R., F. Bak, H. J. Seitz, B. Thebrath, H. P. Mayer, H. Schütz. 1989. Hydrogen turnover by psychrotrophic homoacetogenic and mesophilic methanogenic bacteria in anoxic paddy soil and lake sediment. FEMS Microbiol. Ecol. 62:285–294.

deBont, J. A. M., M. W. Peck. 1980. Metabolism of acetylene by *Rhodococcus* A 1. Arch. Microbiol. 127:99–104.

Dubourguier, H. C., E. Samain, G. Prensier, G. Albagnac. 1986. Characterization of two strains of *Pelobacter carbinolicus* isolated from anaerobic digestors. Arch. Microbiol. 145:248–253.

Dwyer, D. F., J. M. Tiedje. 1986. Metabolism of polyethylene glycol by two anaerobic bacteria, *Desulfovibrio desulfuricans* and a *Bacteroides* sp. Appl. Environ. Microbiol. 52:852–856.

Eichler, B., B. Schink. 1984. Oxidation of primary aliphatic alcohols by *Acetobacterium carbinolicum* sp. nov., a homoacetogenic anaerobe. Arch. Microbiol. 140:147–152.

Eichler, B., B. Schink. 1985. Fermentation of primary alcohols and diols and pure culture of syntrophically alcohol-oxidizing anaerobes. Arch. Microbiol. 143:60–66.

Evans, W. C. 1977. Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature 270:17–22.

Goldberg, J., C. L. Cooney. 1981. Formation of short-chain fatty acids from H_2 and CO_2 by a mixed culture of bacteria. Appl. Environ. Microbiol. 41:148–154.

Krieg, N. R., J. G. Holt. 1984. Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins. Baltimore.

Krumholz, L. R., M. P. Bryant. 1986. *Eubacterium oxidoreducens* sp. nov. requiring H_2 or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. Arch. Microbiol. 144:8–14.

Oppermann, F. B., A. Steinbüchel, H. G. Schlegel. 1988. Utilization of methylacetoin by the strict anaerobe *Pelobacter carbinolicus* and consequences for the catabolism of acetoin. FEMS Microbiol. Lett. 55:47–52.

Pfennig, N. 1978. *Rhodocyclus purpureus* gen. nov. and sp. nov., a ring-shaped, vitamin B_{12}-requiring member of the family Rhodospirillaceae. Int. J. Syst. Bacteriol. 23:283–288.

Samain, E., G. Albagnac, H. C. Dubourguier, J. P. Touzel. 1982. Characterization of a new propionic acid bacterium that ferments ethanol and displays a growth factor dependent association with a Gram-negative homoacetogen. FEMS Microbiol. Lett. 15:69–74.

Samain, E., G. Albagnac, H. C. Dubourguier. 1986. Initial steps of catabolism of trihydroxybenzenes in *Pelobacter acidigallici*. Arch. Microbiol. 144:242–244.

Schink, B. 1984. Fermentation of 2,3-butanediol by *Pelobacter carbinolicus* sp. nov. and *Pelobacter propionicus* sp. nov., and evidence for propionate formation from C_2 compounds. Arch. Microbiol. 137:33–41.

Schink, B. 1985. Fermentation of acetylene by an obligate *anaerobe, Pelobacter acetylenicus* sp. nov. Arch. Microbiol. 142:295–301.

Schink, B., N. Pfenning. 1982. Fermentation of trihydroxybenzenes by *Pelobacter acidigallici* gen. nov., sp. nov., a new strictly anaerobic, nonsporeforming bacterium. Arch. Microbiol. 133:195–201.

Schink, B., M. Stieb. 1983. Fermentative degradation of polyethylene glycol by a strictly anaerobic, Gram-negative, nonsporeforming bacterium, *Pelobacter venetianus* sp. nov. Appl. Environ. Microbiol. 45:1905–1913.

Schink, B., D. R. Kremer, T. A. Hansen. 1987. Pathway of propionate formation from ethanol in *Pelobacter propionicus*. Arch. Microbiol. 147:321–327.

Schink, B., T. J. Phelps, B. Eichler, J. G. Zeikus. 1985. Comparison of ethanol degradation pathways in anoxic freshwater environments. J. Gen. Microbiol. 131:651–660.

Seitz H.-J., B. Schink, R. Conrad. 1988. Thermodynamics of hydrogen metabolism in methanogenic cocultures degrading ethanol or lactate. FEMS Microbiol. Lett. 55:119–124.

Stackebrandt, E., U. Wehmeyer, B. Schink. 1989. The phylogenetic status of *Pelobacter acidigallici*, *Pelobacter vene-*

tianus, and *Pelobacter carbinolicus*. 1989. System. Appl. Microbiol. 11:257–260.

Stams, A. J. M., D. R. Kremer, K. Nicolay, G. H. Wenk, T. A. Hansen. 1984. Pathway of propionate formation in *Desulfobulbus propionicus*. Arch. Microbiol. 139:167–173.

Strass, A., B. Schink. 1986. Fermentation of polyethylene glycol via acetaldehyde in *Pelobacter venetianus*. Appl. Microbiol. Biotechnol. 35:37–42.

Tanaka, K., N. Pfenning. 1988. Fermentation of 2-methoxyethanol by *Acetobacterium malicum* sp. nov. and *Pelobacter venetianus*. Arch. Microbiol. 149:181–187.

Wagener, S., B. Schink. 1987. Anaerobic degradation of nonionic and anionic surfactants in enrichment cultures and fixed-bed reactors. Wat. Res. 21:615–622.

Wagener, S., B. Schink. 1988. Fermentative degradation of nonionic surfactants and polyethylene glycol by enrichment cultures and by pure cultures of homoacetogenic and propionate-forming bacteria. Appl. Environ. Microbiol. 54:561–565.

Widdel, F., N. Pfenning. 1981. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov. sp. nov. Arch. Microbiol. 129:395–400.

Widdel, F., G.-W. Kohring, F. Mayer. 1983. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov. Arch. Microbiol. 134:286–294.

Wieringa, K. T. 1940. The formation of acetic acid from CO_2 and H_2 by anaerobic bacteria. Antonie van Leeuwenhoek 6:251–262.

Prokaryotes (2006) 7:12–30
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CHAPTER 3.4.2

The Genus *Bdellovibrio*

EDOUARD JURKEVITCH

Introduction

Bdellovibrio spp. are Gram negative, motile, and uniflagellated bacteria. What characterizes this bacterial genus as unique is its predatory behavior: Bdellovibrios attack other Gram negative cells, penetrate their periplasm, multiply in their cytoplasm, and finally burst their cell envelopes to start anew (Figure 1). Although many predator bacteria may lyse other bacteria, and many "micropredators" have been described, these *Bdellovibrio*-like organisms can grow without bacterial prey and do not divide inside the periplasm (Varon and Shilo, 1978).

Bdellovibrio research began with their serendipitous discovery by Stolp and Petzold (1962). The physiology, ecology, taxonomy, interactions with prey, and cell cycle of *Bdellovibrio* were established in the 1960's and 70's mainly by the groups of Shilo and Varon, Stolp and Starr, Rittenberg, Hespell, Diedrich, Ruby, Tudor, Thomashow, and more recently Williams.

Bdellovibrios can be described as predators, or parasites, or symbionts (Starr, 1975). In this review, the terms predator-prey and parasite-host will be used interchangeably.

Bdellovibrios are found in wet, aerobic environments. As described in other sections, their importance in shaping or influencing bacterial community dynamics and structure is still unknown. Similarly, the way these predators interact with potential prey, their survival mechanisms, and their preferred habitats in natural settings or man-made biotops are poorly understood.

Particularly fascinating is the two-phased cell cycle of bdellovibrios, which are dimorphic. During the "attack phase," small cells (0.5 to 1.5 μm long, 0.5 μm in diameter, Figure 2) swim at speeds up to one hundred body lengths·sec^{-1} (Stolp, 1967) to find prey. Prey-encounter is apparently by random collision, as evidence of chemotaxis has not been detected. After encountering and attaching to a substrate cell, a predatory cell penetrates the periplasm, shedding its long-sheathed flagellum in the process. The "growth phase" can then be initiated. The invaded bacterium usually rounds into the ensemble predator-prey, called a bdelloplast (Figure 3). Within this confined space *Bdellovibrio* engages in restricted development, turning the short cell into an unseptated filament at the expense of the host's cytoplasm (Figure 4). After a lag phase of about 45 min, DNA begins to replicate within the bdelloplast and proceeds for a couple of hours along with cell growth, and then the filament divides by multiple fission into progeny attack cells, the number determined by the size of the host. Progeny bursts from the ghost cell, ready for another attack cycle. Within the bdelloplast, the bdellovibrio cell is protected from photooxidation (Friedberg, 1977) and phage attack (Varon and Seijffers, 1975) and shows increased resistance to pollutants (Varon and Shilo, 1981). Host-independent mutants growing axenically can be isolated. The cell cycle can be extended to include the development of a cyst-like stage called a bdellocyst, which, however, has been seen in only two strains.

Basic biological questions such as predator-prey interactions, host recognition, regulation of a simple developmental program, and cell-cell signaling can be addressed by using this two-membered model. We shall deal with each of these issues in the linked sections.

Taxonomy

The taxomony of *Bdellovibrio* is still fragmentary. Historically, microorganisms that have been assigned to the genus *Bdellovibrio* exhibit characteristic features: flagellum-driven high motility, attachment and penetration through the outer membrane of Gram-negative hosts, intraperiplasmic growth, cell multiplication at the expense of host cytoplasm, lysis of prey, and finally release of *progeny*. Their wide habitat and heterogeneity can be interpreted in terms of development of a common lifestyle by convergent evolution or increased heterogeneity by divergent evolution from a common very ancient ancestor (Varon and Shilo, 1978).

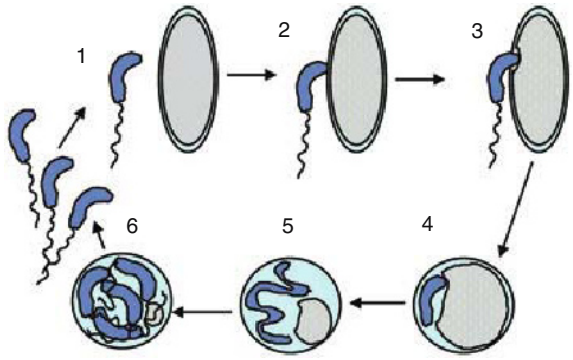

Fig. 1. The life cycle of *Bdellovibrio*. 1. Attack-phase cells; 2. Attachment to the prey's outer membrane; 3. Penetration; 4. Initiation of growth; 5. Intraperiplasmic growth; 6. Differentiation into attack cells and lysis of the prey envelope.

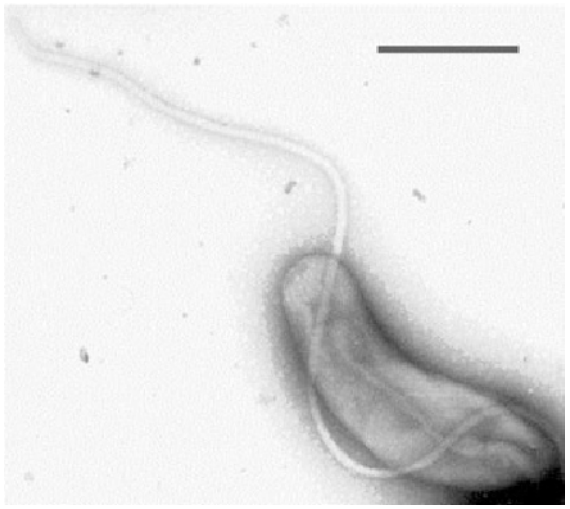

Fig. 2. A *Bdellovibrio* cell in the attack phase. The flagellum is wrapped around the cell. Bar: 0.5 µm.

The genus has been placed within the Proteobacteria (Ribosomal Database Project), myxobacteria and sulfate-reducing bacteria being their closest relatives. Based on G+C content, DNA/DNA (Table 1) and DNA/rRNA homology, and enzyme migration patterns, the genus was divided into three species: *B. bacteriovorus*, *B. stolpii* and *B. starrii* (Siedler et al., 1972). *Bdellovibrio* strain W, one of the two bdellocyst-forming isolates (Hoeniger et al., 1972), and the marine isolates were left undefined. The ion requirements of these halophilic isolates have been described (Taylor et al., 1974; Marbach and Shilo, 1978). Based on the similarities in host ranges, Taylor et al. (1974) subdivided the 13 analyzed isolates into three groups. Mole %G+C of three isolates from one group was similar (38.5–38.8) and much lower than that of any of the terrestrial isolates, whereas G+C content of

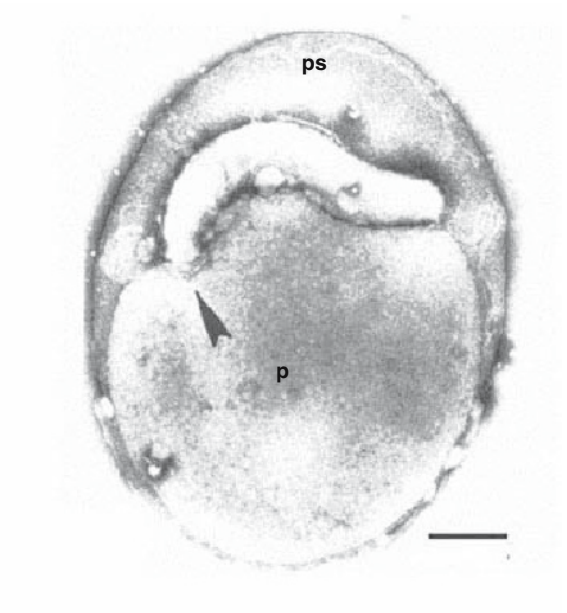

Fig. 3. An *E. coli* cell with a *B. bacteriovorus* 109J parasite in the intraperiplasmic space. The parasite at its entry pole is associated with the host cytoplasmic membrane (arrow). ps: periplasm. p: protoplasm. Bar: 0.2 µm. (From Abram et al., 1974.)

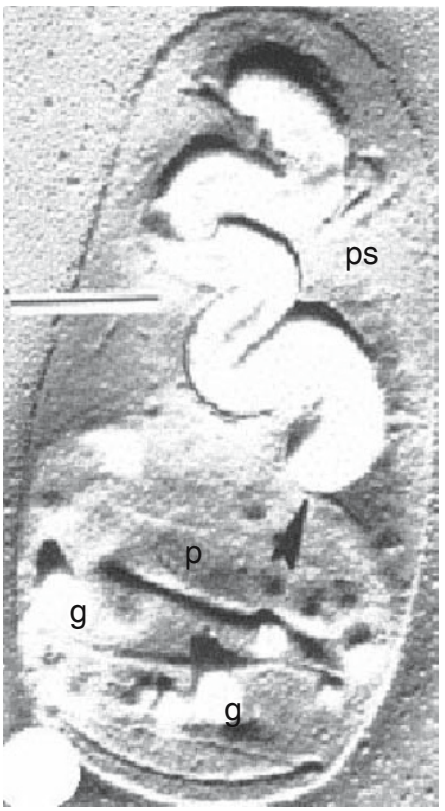

Fig. 4. Intraperiplasmic growth of *B. bacteriovorus* 109J in *Spirillum serpens*. p: protoplast. ps: periplasmic space. g: polymetaphosphate granules. Bar: 1 µm. (From Abram et al., 1974.)

Table 1. Genetic characteristics of *Bdellovibrio*.

Species	Genome size (kbp)	Mol% G+C	% DNA/DNA relative reassociation					
			109D	100	A3.12	UKi2	W	N6804
B. bacteriovorus 109D	2060	51	100	103				
B. bacteriovorus 100	2170	49.5		100	1	0		
B. starrii A3.12	2610	43.5			100	16	23	0
B. stolpii UKi2	2290	41.8				100	28.5	37
Strain W	2030	43.7					100	32
N 6804 (ICPB 3294)	2040	37.4						100

one isolate from another group was 43.5%. Marbach et al. (1976) separated halophilic bdellovibrios into 10 groups, based on prey range. However, no such grouping was found by Torrella et al. (1978) or by Schoeffield et al. (1991), and host range was shown to depend on the experimental conditions, thereby raising questions about the usefulness of this parameter as a taxonomic criterion. Bacteriophages of bdellovibrios (bdellophages) have been isolated (Sagi and Levisohn, 1976; Althauser et al., 1972; Varon and Levisohn, 1972; Hashimoto et al., 1970) and in some instances used to define taxomic groups of bdellovibrios (Althauser et al., 1972; Varon, 1974).

16S rRNA

Resolution of the phylogeny of *Bdellovibrio* has been increased by the analysis of the 16S rRNA molecule. Donze et al. (1991) analyzed 11 terrestrial strains and one marine isolate using partial sequences of the *16S rDNA* gene. Their results confirmed the placement of Bdellovibrio within the Proteobacteria and showed *Bdellovibrio* strains were related more closely to each other than to other bacteria, supporting the claim of common ancestry. The heterogeneity of the genus *Bdellovibrio* could be divided into two groups, *B. bacteriovorus* strains forming one and *B. stolpii*, *B. starrii* and the marine isolate the other (Figure 5). However, the large phenotypic and molecular (%G+C content, DNA/DNA reassociation) differences between the marine and terrestrial isolates, the scant molecular information available on the diversity of marine strains, and the heterogeneity observed within the species *B. bacteriovorus* call for further studies.

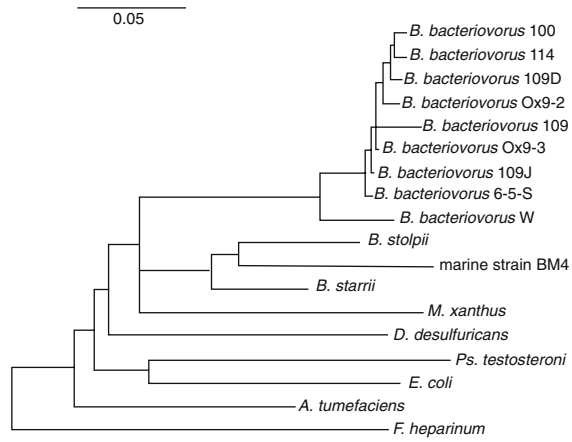

Fig. 5. Phylogenetic tree for 12 strains of *Bdellovibrio* based on the analysis of the 16S rRNA gene. Bar: fractional difference in nucleotide sequence. (From Donze et al., 1991.)

Serological Classification

Immunological approaches have been shown to offer a level of sensitivity high enough to resolve taxonomic groups of bdellovibrios (Schoeffield et al., 1991; Schelling et al., 1983; Kramer et al., 1977). Using techniques such as immunodiffusion, immunoelectrophoresis, agglutination, and indirect immunofluorescence, halophilic and nonhalophilic bdellovibrios can be separated into several serologically distinct subgroups: the terrestrial subgroups, sharing a common antigen (Schelling et al., 1983; Kramer et al., 1977); the halophilic isolates (Schoeffield et al., 1991), each identified by at least one common antigen; and the aquatic, salt-loving strains, sharing a common antigen with terrestrial strain W (Schoeffield et al., 1991). Thus, such immunology-based tools can help determine taxonomy.

Habitat and Ecology

Bdellovibrios have been isolated from a wide range of water systems: estuaries (brackish water), sea coasts and oceans, rivers, sewage, fish ponds, runoff (irrigation water), and man-made water supplies. Also, organisms have been found in the gills of crabs, in sediments, on submerged surfaces, in soils, in rice paddies, and in the rhizosphere of plants. Table 2 presents a summary of biotops in which *Bdellovibrio* were detected. Interestingly, the concentration of recovered *Bdellovibrio* in rivers correlated with water pollution. Whereas no predators could be detected

Table 2. *Bdellovibrio* in natural and man-made biotops.

Origin	Isolation steps	Prey	*Bdellovibrio* PFU·g^{-1}, ml^{-1} or cm^{-2}	Reference
Oyster shells in brackish water	Differential centrifugation	*Vibrio parahaemolyticus*	Up to 9.4×10^2	Kelley et al. 1997
Coastal sea water and aquaculture farms	Filtration	Various Gram-negatives	0 to 3.8×10^2	Pan et al. 1997
Soil and sewage	Filtration	*Escherichia coli* O157:H7; Salmonella spp.	N.D.	Fratamico and Cooke 1996
Rhizosphere of Chinese cabbage	Filtration	*Pseudomonas fluorescens*	Up to 10^5	Elsherif and Grossmann 1996
Aquatic plant surfaces	Differential centrifugation	*Vibrio parahaemolyticus*	Up to 1.2×10^2	Williams et al. 1995
Gills of blue crab (Callinecus sapidus)	Differential centrifugation	*Vibrio parahaemolyticus*	2×10^3	Kelley and Williams 1992
Brackish tidal pond*, saltwater aquarium**	Direct plating	*Vibrio parahaemolyticus*	40*, 10^2**	Schoeffield and Williams 1990
Man-made water supplies[a]	Concentrated samples	*Legionella* spp.	Positive, N.S.	Richardson 1990
Rice paddies, Japan	1.2µm filtration	*Xanthomonas oryzae*	0 to 10^3	Uematsu 1980
River water, Great Britain	Dilution to extinction	*Escherichia coli*	0–3×10^3;[b] 2×10^4 to 5×10^4;[c]	Fry and Staples 1976
Osaka Bay, Japan	N.S.	*Vibrio parahaemolyticus*	2×10^4 to 1.5×10^6	Miyamoto and Kuroda 1975
Oahu, Hawaii, USA	Filtration	Various Gram-negatives	1 to 2×10^2	Taylor et al. 1974
Sewage, Great Britain	Homogenization	Achromobacter spp.	9×10^2	Staples and Fry 1973
Soil, Australia	Filtration	*Rhizobium* spp.; *Agrobacterium* spp.	2 to 1.2×10^3	Parker and Grove 1970
Soil, USA	Direct plating	*Escherichia coli*	4.5×10^4	Klein and Casida 1967
Soil, USA	Filtration	Various Gram-negatives	4×10^1 to 2×10^2	Stolp and Starr 1963

N.S.: not specified; [a]: hospital water, shower unit, industry, etc; [b]: unpolluted water; [c]: polluted water.

in pure well waters (Varon and Shilo, 1978), *Bdellovibrio* counts increased from 10 to 3500 PFU·mL^{-1} (plaque-forming units) at the source and near an urbanized section of the river, respectively (Lambina et al., 1974). Although a higher total bacterial load in the sample may promote *Bdellovibrio* growth, the predator has been shown sensitive to environmental pollutants such as heavy metals, detergents, and pesticides (Varon and Shilo, 1978). Bdellovibrios are aerobic, multiplying under oxic conditions, but they are able to survive anoxic periods as attack-phase cells or as bdelloplasts (Schoeffield et al., 1991). Furthermore, halo-tolerant predators have been shown to grow under microaerobic conditions. Spells of low oxygen tension occur in soils and in water, and the parasite seems to be adapted to these conditions. The range of possible niches for bdellovibrios to survive and be active, therefore, may not be restricted to permanently aerobic biotas.

Another environment in which bdellovibrios have been found is biofilms on surfaces. In the continuous space between the solid phases of biofilms, dissolved chemicals, suspended particles, and cells move freely (Wanner, 1989). It is therefore plausible that bdellovibrios due to their small size, high motility, and mode of multiplication may strongly influence the bacterial composition of their associated biofilms. Recently, an isolate that effectively reduces the level of biofilm *E. coli* cells on stainless steel has been reported (Fratamico and Cooke, 1996). In some instances, biofilms seem to offer better conditions for the multiplication and survival of bdellovibrios as they have been found in the fabric of natural marine biofilms but not always recovered from the surrounding water (Kelley et al., 1997; Kelley et al., 1992; Williams et al., 1995). In these specific econiches, bdellovibrios might become entrapped in the gel-matrix of biofilms, benefiting from higher concentrations of prey cells that ensure survival, a greater multiplication rate, and also physical protection. Surface-associated bdellovibrios were shown to survive various environmental insults but free-living cells died rapidly (Markelova et al., 1998).

A high density of prey has often been shown to be necessary for *Bdellovibrio* survival. Various authors have reported that prey concentrations of at least 10^5 to 10^6 CFU·g^{-1} soil or mL^{-1} are required for *Bdellovibrio* survival (Keya and Alexander, 1975; Uematsu, 1980). Calculations based on the Lotka-Volterra predator-prey model (Varon and Zeigler, 1978) indicate that at least 3×10^6 prey cells are needed to give bdellovibrios a 50% chance of survival. From this type of calculation, the general conclusion was that *Bdellovibrio* can survive only in special ecological niches. However, low-level, steady-state bdellovibrio populations were sustained for long periods in continuous cultures at low host density (10^4 cells·mL^{-1}).

As seen above, biofilms can potentially provide a richer habitat for bdellovibrios. Biofilms may provide an environment more conducive to bdellovibrio development in low microbial density biotas, the predator expanding beyond that realm during bacterial population surges. Notably, the calculated minimal prey levels usually relate to bdellovibrio predation upon a defined bacterial species in pure culture. Because bdellovibrios are usually not stringently host-range specific, the concentration of substrate cells in natural settings may well be high enough to sustain bdellovibrio populations. Moreover, only a fraction (>1 to a few percent) of the bacterial cells contained in environmental samples are typically amenable to cultivation. This fact has to be considered (Amann et al., 1995), as did Rice et al. (1998), who quantified the number of bdellovibrio-susceptible bacteria in an estuarine environment. Seventy to 85% of recovered bacteria were found susceptible to bdellovibrios isolated from the same sampling sites. Assuming that almost 10% of the bacteria in samples were retrieved on culture, the authors calculated that the susceptible populations were sufficient to ensure survival of the predators. Therefore, *Bdellovibrio* spp. may have a modulatory role in nature (Rice et al., 1998), eradication being prevented by natural oscillations in a predator-prey system (Varon, 1979).

Survival of bdellovibrios in nature has been the subject of conjecture because cell composition in bdellovibrios is known to change rapidly in starving conditions, and bacterial suspensions quickly lose viability (Marbach et al., 1976; Hespell et al., 1974), signifying that a free swimming cell must find a prey rapidly. However, bdellovibrios were shown to survive longer periods in nutrient-poor environments (Fry and Staples, 1976; Daniel, 1969), possibly due to population heterogeneity (Varon and Shilo, 1978) or better resilience of bdelloplasts (certainly as bdellocysts, although the number of strains able to develop this morphology seems to be small). The molecular responses to starvation are unknown.

Isolation

The peculiar cell cycle of *Bdellovibrio* dictates its method of isolation, whether from sewage, plant parts, submerged surfaces, or water samples. Basically, bdellovibrios and phages are isolated in a similar manner. The sample, or serial dilutions of it, is mixed with a potential prey bacterium in melted soft agar and poured onto an agar

plate containing a diluted growth medium. The bacterial predator forms plaques that have to be differentiated from those formed by protozoa or bacteriophages.

Relatively low levels of bdellovibrios are usually retrieved from environmental sources that contain much higher levels of unsusceptible bacteria and "blurring" through overgrowth results, which can interfere with the detection of bdellovibrio plaques. Moreover, bdellovibrio growth can be hindered by bacteria-excreted compounds as well as by bacteriophages. A number of protocols have been developed based on the physical separation of *Bdellovibrio* cells from the rest of the bacterial population, with the purpose of obtaining enriched fractions of the predator while reducing contaminating protozoa, bacteria, or viruses.

The number of bdellovibrio plaques detected in any environmental sample depends on the host bacterium chosen, the processing of the sample, and the isolation protocol. *Bdellovibrio* shows variation in host range, and no single bacterial species can potentially support the growth of all isolates. This problem is compounded by the lack of information on the structure and diversity of *Bdellovibrio* populations. However, *Vibrio parahaemolyticus* was shown to be an effective host for the retrieval of *Bdellovibrio* from estuarine environments (Schoeffield and Williams, 1990). *Pseudomonas* spp., *Aquaspirillum serpens*, a number of phytopathogens such as *Erwinia carotovora* sspp. *carotovora*, *E. amylovora*, *Xanthomonas oryzae* and *P. syringae* have been used to isolate soil and water bdellovibrios (Stolp and Starr, 1963; Scherff, 1973; Uematsu, 1980; Epton et al., 1989; Elsherif and Grosman, 1996).

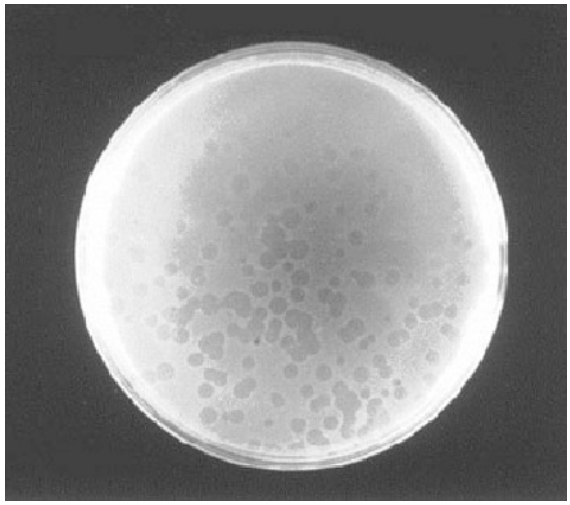

Fig. 6. Lytic plaques of *Bdellovibrio* on *Pseudomonas corrugata*.

Direct Isolation of *Bdellovibrio* from Environmental Samples

The most common approach is based on the use of one or more filtration steps with or without differential centrifugation of the sample analyzed. The compositions of the agar and soft agar can also vary. *Bdellovibrio* plaque development is favored in low-nutrient media, resulting in a higher plaque yield (Staples and Fry, 1973), but growth of contaminating bacteria is inhibited in plain agar medium (Uematsu, 1980).

Based on Stolp (1981): A water sample or 50-g soil in 500 mL of sterile buffer is shaken vigorously for 1 h, then centrifuged for 5 min at 2000g to remove gross particles. The supernatant is passed through a series of membrane filters of decreasing pore size (3.0, 1.2, 0.8 and 0.45 μm). Filtrates from the last two steps are serially diluted, and 100-μL aliquots are mixed with approximately 109 cells of the prey bacterium in molten soft top agar. The mixture is poured onto an agar plate and incubated at 28–32°C. Rapidly developing lytic regions visible after 24 h are the result of bacteriophage multiplication. These plaques are usually small and do not grow further. They should be marked to differentiate them from the slower growing bdellovibrios. Plaques becoming visible within two to three days and showing further expansion for a few more days are potentially bdellovibrios (Figure 6). Small and highly motile *Bdellovibrio* cells can be detected by examination of crushed plaque material in wet-mounts with a phase-contrast microscope.

Because only a fraction of the bdellovibrios is detected using this method, a number of variations have been proposed that increase the efficiency of recovery, thereby enabling more accurate quantification. From Uematsu (1980): Samples are collected, filtered through a 1.2-μm membrane filter, mixed with a phage-resistant host in molten top plain agar and plated on bottom plain agar. From Varon and Shilo (1970): The sample is centrifuged at 800 g for 5 min or passed through filter paper to remove coarse particles, then the bacteria are concentrated by centrifugation at 27,000 g for 20 min. The supernatant, which contains free bacteriophages, is discarded. The pellet is resuspended in a few mL and passed through a 1.2-μm membrane filter. Serial dilutions of the filtrate are then mixed with the prey in molten agar and plated on solid DN.

Sixty to 70% of bdellovibrios may be recovered with less than 1% of prey cells passing through the filter, as compared with 0.1% recovery with sequential filtration.

A second method, using a Ficoll gradient for purifying bdellovibrios from mixed samples, was also proposed by Varon and Shilo (1970): Samples are prepared as above but instead of being filtered, the resuspended pellet is layered on top of a 14-mL linear Ficoll gradient of 1 to 15% (Mw = 400,000) in DN, in 1.5 × 9.5 cm centrifugation tubes. The tubes are centrifuged horizontally in the cold at 1,620 g. The upper 3 mL contains 80 to 90% of the *Bdellovibrio* with less than 2% contamination, whereas the other bacteria concentrate lower along the gradient.

Low-nutrient media:

DN: (Diluted Nutrient, Seidler and Starr, 1969a) 0.08% Difco Nutrient Broth is supplemented with $CaCl_2 \cdot 2H_2O$, 2 mM, and $MgCl_2 \cdot 6H_2O$, 3 mM, after autoclaving, and pH is adjusted to 7.2 with 0.1 N NaOH.

YP (Yeast Extract Peptone, Stolp and Starr, 1963) contains 0.3% yeast extract, 0.06% peptone in 0.05 M Tris buffer, pH 7.5.

Plain agar (Uematsu, 1980): $MgCl_2 \cdot 6H_2O$, 20 mM; $CaCl_2 \cdot 2H_2O$, 30 mM, pH 6.5–8. Bottom agar: 1.2 to 1.5%. Top agar: 0.6%.

Plating: Aliquots of 4-mL molten top agar are kept at 42°C in a water bath prior to mixing with prey and sample suspensions.

Isolation of Marine Bdellovibrios

Halophilic or halotolerant bdellovibrios have been isolated from marine environments such as estuaries, sediments, coastal waters, open sea and submerged surfaces (Williams et al., 1995; Williams and Falkler, 1984; Williams, 1988; Taylor et al., 1974; Marbach and Shilo, 1978). The chemical composition of sea water has to be considered for isolating marine bdellovibrios. These organisms have a different G+C content than the terrestrial strains (Taylor et al., 1974) and their requirements for Na^+, K^+, and Ca^{2+} dictate the use of adapted media for their isolation (Marbach and Shilo, 1978). Marine bacteria such as *Vibrio parahaemolyticus* P-5 can be used as prospective hosts. Moreover, the relative low abundance of *Bdellovibrio* in these biotas may require concentration of large sample volumes.

From Marbach et al. (1976): Both direct and enrichment approaches can be used. If needed, the water samples are concentrated by centrifugation (10,000 g, 30 min) or collected on a 0.1-μm membrane. The concentrate is then mixed with a potential host bacterium in soft agar and poured on bottom agar. Alternatively, 5 mL of the sample is added to 3.3 mL of molten top agar having 0.7 mL of the prey suspension for a final agar concentration of 0.65%. The mixture is then poured onto large Petri dishes and incubated at 25°C (Schoeffield and Williams, 1990).

The medium used for isolating marine strains of *Bdellovibrio* should contain at least 25% sea water or appropriate salts.

Polypeptone (Pp 20) medium (Williams et al., 1982): Filtered ocean water, 1 L; Polypeptone, 1 g; agar, 15 g for bottom agar and 6.5 g for top agar; pH 7.7–7.8.

Synthetic marine salt solution (Marbach and Shilo, 1978): NaCl, 500 mM; KCl, 10 mM; $MgSO_4$, 25 mM; $MgCl_2$, 25 mM; $CaCl_2$, 10 mM.

Specific Enrichment for *Bdellovibrio*

Stolp (1968) devised a method yielding large numbers of *Bdellovibrio* plaques, thereby greatly facilitating the isolation of the predator on a specific host bacterium whenever quantification is not needed. This approach has been applied to obtain bdellovibrios lytic to *Rhizobium* and *Agrobacterium* (Parker and Grove, 1970), *Legionella* (Richardson, 1990) and *Azospirillum brasilense* from 2-years-stored air-dried soils (Germida, 1987).

Based on Stolp, modified by Ruby (1991): One-hundred milliliter aliquots of a dense suspension of the prospective prey bacterium (10^{10} cells·mL^{-1}) are prepared in DN medium or HM buffer in Erlenmeyer flasks. The sample (soil, 100 mg; sewage, 0.5 mL; river water, 1 mL) is added and the flasks are incubated on a rotary shaker. The suspension is examined daily over 2 to 4 days for lysis (reduction in optical density) and for the presence of small, highly motile presumptive bdellovibrio cells or bdelloplasts by phase contrast microscopy. If no bdellovibrios are apparent, the incubation can be prolonged or a 1-mL aliquot can be transferred into a fresh suspension of substrate bacterium. When bdellovibrios are detected, the enrichment culture is centrifuged for 5 min at 2000g (250g; Germida, 1987) and the supernatant filtered through a 0.45-μm membrane. Serial dilutions are plated on the prospective prey bacterium. Developing plaques are checked microscopically for bdellovibrios.

HM buffer: Hydroxyethyl piperazine-N′-2-ethanesulfonic acid, 25 mM, adjusted to pH 7.6 with NaOH and supplemented with $CaCl_2 \cdot 2H_2O$, 1 mM, and $MgCl_2 \cdot 6H_2O$, 1 mM.

Isolation of Host-Independent-Saprophytic *Bdellovibrio* Mutants

Wild-type bdellovibrios isolated from natural habitats are obligate parasites. However, host-independent (H-I) mutants can be recovered from all wild-type strains at a low frequency of 10^{-5} to 10^{-8} (Cotter and Thomashow, 1992a; Varon and Seijffers, 1975; Seidler and Starr,

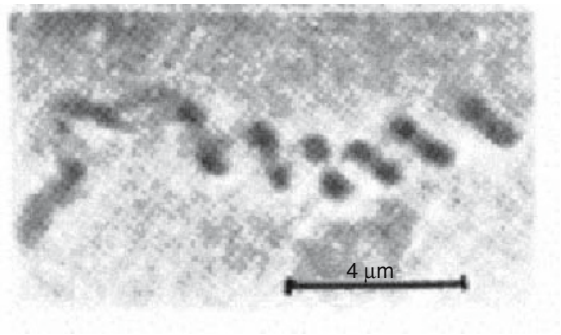

Fig. 7. Growth phase of a host-independent mutant of *B. bacteriovorus* 10^9D growing in enriched nutrient broth (From Eksztejn and Varon, 1977.)

1969b; Stolp and Starr, 1963). This suggests that the H-I phenotype results from a single mutational event. Although these axenic mutants are able to lyse substrate cells, they do so less efficiently than their wild-type counterparts (Cotter and Thomashow, 1992b; Varon and Seijffers, 1975; Stolp and Starr, 1963), and they tend to lose this ability completely after numerous transfers, seemingly the result of a second mutational event (Cotter and Thomashow, 1992b; Gray and Ruby, 1991). Host-independence may have pleiotropic effects as only partial phenotypic complementation can be achieved (Cotter and Thomashow, 1992b) and the mutants, contrary to the colorless wild-type cells, produce pigments protective against photooxidative damage (Friedberg, 1977).

The axenic mutants show strong proteolytic activities and are oxidase positive; most are catalase positive and possess a cytochrome *a* and *c* component (Seidler and Starr, 1969b; Diedrich et al., 1970; Stolp and Starr, 1963). Strikingly, the cell cycle of H-I mutants still shows the dimorphic pattern of filamentous growth, division, and differentiation (Figure 7; Seidler and Starr, 1969a). This observation has triggered many investigations of the possible involvement of prey-derived signals in the initiation or completion of the specific growth phases of the parasite.

From Shilo and Bruff (1965): A *Bdellovibrio* suspension (10^9 cells·mL^{-1}) is filtered through a 0.45-µm membrane. The filtrate is transferred to nutrient broth (NB) or DN broth (containing 10^8 heat-killed host cells) and incubated at 30°C with vigorous shaking. For growth on plates, solid NB or PY (peptone, 10 g; yeast extract, 3 g, pH 6.8) is supplemented with 1% autoclaved culture supernatant fluid of the same bdellovibrio strain grown in NB. In that case, a large (10^4 cells per plate) inoculum is required.

Although more exacting, the method described by Seidler and Starr (1969b) was successful with all isolates tested, contrary to the protocol of Shilo and Bruff (1965), which did not always yield axenic mutants:

1. Selection of Sm^r *Bdellovibrio*. The host-dependent (H-D) strain is grown in DN broth on a Sm^r host. After 24 h, streptomycin is added (50 µg·mL^{-1}) and the culture is incubated for another 12 h. Next, the H-D strain is heavily (15%, v/v) inoculated in DN broth with the Sm^r host in the presence of streptomycin. When a lysate is obtained, it is washed from the antibiotic by centrifugation, resuspended in DN broth, and filtered through a 0.45-µm membrane.

2. A Sm^s host suspension is inoculated with the filtrate. Five to 10 mL of the resulting lysate are transferred to the selection medium (DN or PY broth amended with 10^9 cells·mL^{-1} heat-killed prey cells and with streptomycin) and incubated for 3 to 6 days. Cultures are transferred twice in PY broth with a heavy inoculum (15 to 25%) and the H-I isolates can then be plated on solid PY.

Preparation of Pure Cultures

Pure cultures of *Bdellovibrio* are obtained in the manner of bacteriophages. A single plaque of the original isolation event is lifted, resuspended in HM buffer or in dilute medium, passed through a 0.45-µm filter, and serially diluted. Plating of the dilutions on the original host results in growth of plaques. The procedure is performed at least three times.

Identification

Bdellovibrios are best identified by morphological features along with the particular growth requirements of the bacteria, i.e. a cell cycle featuring an obligate, intraperiplasmic, developmentally separated stage inside the substrate bacterium, leading to the generation of progeny cells. The physiology of both host and parasite is intimately linked to the interaction between the two and is dealt with separately. No molecular markers have been developed to date that enable the detection and identification of bdellovibrios in samples without relying on isolation, cultivation, and morphological identification.

Morphology and Ultrastructure

After isolation, plaques have to be detected before filtrates can be examined under phase contrast for small, highly motile cells, swimming at a speed of up to 100 body-lengths per second (Stolp, 1967). Electron microscopy of subsequent lysates reveals small, vibrioid or rod-shaped bacteria. The size of bdellovibrio can

vary between 0.25 and 0.40 μm in width and 1 and 2 μm in length (Ruby, 1991), but smaller cells (0.2 μm, width; 0.8 μm, length) have been reported (Pan et al., 1997). The cell is propelled by a long (up to 30 μm), thick-sheathed polar flagellum. The sheath is an extension of the outer membrane but is of different biochemical composition (Thomashow and Rittenberg, 1985). To date and under the proper conditions, only two isolates have been shown to enter the bdellocyst stage.

The *Bdellovibrio* Cell Cycle

Chemotaxis Attack-phase bdellovibrios in liquid culture have been shown to lose viability rapidly (Hespell et al., 1973). Therefore, the steps leading to attachment and penetration within the host cell are crucial. No chemotactic responses towards potential hosts, their exudates, or their lysates have been detected (Straley and Conti, 1977; LaMarre et al., 1977). However, positive and negative chemotaxis was measured for numerous compounds including organic and amino acids, sugars and inorganic ions, but very different responses were obtained with different *Bdellovibrio* strains (Straley et al., 1979). All the tested strains showed aerotaxis. Because nonhalophilic and halophilic bdellovibrios have been shown not to grow or to grow less, respectively, in microaerobic conditions (Schoeffield et al., 1996), aerotaxis lets the cells find optimal niches. Similarly, the chemotactic responses may drive the predator towards ecological niches rich in prey (LaMarre et al., 1977) or may help the cells find compounds that alleviate starvation in the extracellular environment (Varon and Shilo, 1978).

Because there is no active "marauding," the process of finding prey appears to be based on random collisions and therefore on the concentration of both predator and prey cells. At low prey density (10^4 cells·mL^{-1}), a high initial predator : prey ratio (1 : 250) is necessary to obtain significant predation (Varon and Zeigler, 1978).

In pelagic habitats, many bacteria are attached to particles, and clusters of bacteria continuously form and disperse around patches centered on the lysis of a large cell or protozoal excretion (Caldwell, 1977; Blackburn et al, 1998). Chemotaxis towards organic compounds may indicate bacteria-rich niches and therefore be helpful in bringing bdellovibrios in proximity to potential prey.

Attachment Attachment to substrate cells is affected by many factors such as the composition and the pH of the medium, oxygen tension, and temperature (Varon and Shilo, 1968). It is reversible within the first minutes of an encounter, but agitation is unable to separate attached bdellovibrios after 20 to 30 min (Varon and Shilo, 1968). Reversible attachment to non-hosts as well as to inorganic surfaces has been reported (Varon and Shilo, 1978). Although completely different in their mechanisms, two-stage attachment processes (the first reversible, the second irreversible) are required in many bacteria-host systems: *Agrobacterium tumefaciens* for DNA transfer into a plant cell; *Rhizobium* for cell invasion (Vande Broek and Vanderleyden, 1995) and colonization of plant surfaces (Romantschuk, 1992); and pathogenic *E. coli* for penetration into epithelial cells (Mulvey et al., 1998). Recognition sites on the surface of the bacterial prey of bdellovibrios are still uncharacterized. *B. bacteriovorus* 109D and *B. stolpii* UKi2 interacted differently with various components of the host cell's outer membrane. Whereas strain 109D specifically required core sugar of the host's lipopolysaccharide for irreversible attachment to occur, *B. stolpii* UKi2 seemed to require specific proteins to interact with its prey (Schelling and Conti, 1986).

Corroborating the involvement of specific moieties in the recognition process, detailed electron microscopy revealed that, upon attachment, the nonflagellated pole has strands believed to mediate the attachment of parasite to host protoplast, even before a pore is created. The parasite is subsequently anchored to the host protoplast (Figure 8; Abram et al., 1974).

Two extracellular components of bacterial cells have been shown to exert opposite effects on the attachment of bdellovibrios. Bacterial capsules did not hinder *B. bacteriovorus* 109J to infect *E. coli* cells (Koval and Bayer, 1997), but the proteinaceous S-layer of *Aquaspirillum ser-*

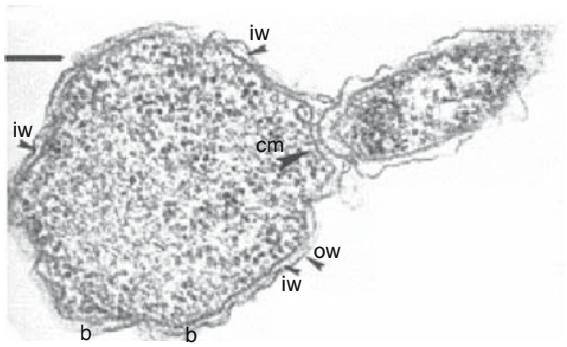

Fig. 8. Penetration of *B. bacteriovorus* 10^9 into an *E. coli* cell. The penetration pole is flattened against the host cytoplasmic membrane. b: bulges in the host membranes. cm: cytoplasmic membrane. iw: peptidoglycan layer. ow: outer membrane. The penetrating parasite shows constriction at the entry pore. (From Abram et al., 1974.)

pens, *A. sinuosum*, and *Aeromonas salmonicida* played a protective role, rendering the bacteria resistant to penetration by bdellovibrios (Koval and Hynes, 1991).

Penetration Penetration is completed within 5 to 20 min after attachment. It may involve mechanical "drilling" through the prey's membranes (Stolp, 1981), although enzymatic action is essential (Physiology). The inhibition of invasion by streptomycin (Varon and Shilo, 1968) supports the involvement of a tightly regulated response to initial attachment and production of enzymes. On the other hand, penicillin has no effect on invasion but inhibits intraperiplasmic multiplication (Varon et al., 1969). The driving force for entry may be generated by fluxes of water entering the infected host and causing a differential expansion of the host protoplast and cell wall, leading to their separation. The bdellovibrio cell, which is bound to the cytoplasmic membrane, is then passively pulled into the periplasm, shedding its flagellum in the process. During attack and penetration, the prey-cell's membrane-derived oligosaccharides are lost to the extracellular medium and are not used by the predator (Ruby and McCabe, 1988).

Intraperiplasmic Growth The invaded periplasm offers a protected environment for the predator to enter a new developmental stage characterized by elongation into a spiraling cell, DNA replication, and finally fragmentation of the filament by the formation of septa and the biosynthesis of one flagellum per progeny cell. The ghost host cell is then lysed by the action of lytic enzymes, and the progeny is released. The filamentous cell developing at the expense of the host's cytoplasmic content grows in proportion to the size of the prey (Kessel and Shilo, 1976) and exhibits a very efficient energy metabolism. Therefore, the number of progeny varies, from 5.7 per *E. coli* (Seidler and Starr, 1969b) to 20 to 30 cells in *Aquaspirillum serpens* (Stolp, 1967). In liquid culture, the initial cell cycle is completed within 2 to 4 h, depending on the age of the bdellovibrios (Varon et al., 1969). The signals leading to initiation of growth within the periplasm, cell elongation, division, and differentiation into attack phase cells have been sought, but a consistent picture of their mode of action has not been obtained.

Bdellocysts Under special growth conditions, bdellovibrios enter a cyst-like stage. The formation of bdellocysts has been reported in two strains, one of which (strain W) is the subject of a number of investigations (Burger et al., 1968; Hoeniger et al., 1972; Tudor and Conti, 1977; Tudor and Conti, 1978). Bdellocysts of strain W developing on *Rhodospirilum rubrum* mature within 4 h. The predator enlarges into a kidney-shaped cell enclosed by a structureless, amorphous outer layer. A finely particulate inner layer surrounds the more particulate plasma membrane of the predator cell. Structures resembling storage granules are present (Figure 9). Bdellocysts are more resistant than vegetative cells to dessication, high temperatures, and sonication (Tudor and Conti, 1977), and their germination is favored by L-glutamate, K^+, and NH_4^+ (Tudor and Conti, 1978).

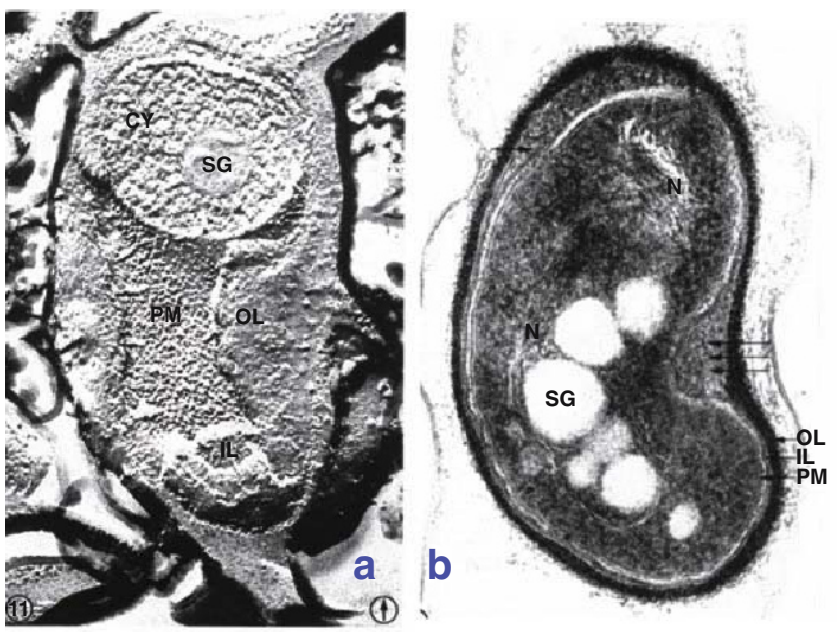

Fig. 9. Mature resting body of *Bdellovibrio* sp. strain W in *Rhodospirillum rubrum*. Panel a: Freeze-etching, × 173,000. Panel b: Negative staining, × 150,000. OL: outer layer. IL: inner layer. PM: particulate plasma membrane. CY: granular cytoplasm. SG: storage granule. Arrows: infolding of the inner layer. N: fibrillar nucleoplasm. (From Hoeniger et al., 1972.)

Cultivation

The cultivation of bdellovibrios in a diluted broth or buffer suspension of host cells routinely yields high concentrations of the predator (10^9 cells·ml^{-1}). However, these suspensions rapidly lose their viability because bdellovibrios have very high endogenous respiration rates (Hespell et al., 1973). Therefore, aerating cultures on a rotary shaker and filling flasks only to one third of their volume is strongly recommended. Viability of the predators can be augmented by adding 5 mM glutamate to the fresh lysates.

Propagation of *Bdellovibrio*

One single plaque with a small piece of its surrounding unlysed prey cell lawn is excised from a Petri dish, resuspended in 10 to 15 mL of a diluted medium, such as DN broth or YP, and shaken at 30°C for 24 to 48 h. The prey cells first multiply, creating a suspension dense enough to enable a high yield of bdellovibrios. This first lysate can then be subcultured.

Routine Culture

HM buffer or DN broth (30–40 ml) is inoculated with freshly grown, stationary phase host suspension to a final concentration of 10^8 to 10^9 cells·ml^{-1} and 100 to 200 µl of lysate. Overnight incubation on a rotary shaker usually lyses all the prey cells. The resulting bdellovibrio suspension can then be supplemented with 5 mM glutamate and stored at 4°C.

Large Scale Culture

According to Ruby (1991): In a 500-mL Erlenmeyer flask, 50 to 100 µl of a fresh *B. bacteriovorus* 109J lysate are added to 200 ml of DN broth amended with 40 ml of an *Escherichia coli* ML35 stationary phase culture, totalling 1.6×10^{11} cells. The flask is shaken overnight and total lysis is observed under a phase-contrast microscope. The culture is cooled on ice, centrifuged at 700g for 15 min, and the pellet discarded. The supernatant, containing most of the attack-phase bdellovibrios, is concentrated at 10,000g for 15 min. The pellet is resuspended in HM buffer, harvested again by centrifugation, and resuspended in HM buffer. The yield should be about 6×10^{11} cells.

Single-Cycle Growth of Synchronous Cultures

Synchronous cultures are tools essential for investigating developmentally regulated events in the cell cycle of bdellovibrios. A 2 : 1 attack-phase predator-to-prey ratio enables concomitant attack of all host cells within minutes. By separating the bdelloplasts from excess attack phase cells, a uniform synchronous single-cycle suspension is obtained. The developing intraperiplasmic bdellovibrio cells can be prematurely released from the bdelloplast for further analysis. This technique can be used to study the cell cycle of wild type cells, which are known to differ from their host-independent variants.

From Thomashow and Rittenberg (1978a) and McCann et al. (1998): *E. coli* cells are grown into stationary phase in Luria or nutrient broth, centrifuged at 5,000g for 10 min, washed once with cold HM buffer, and then resuspended in the same buffer. A fresh lysate of attack phase *B. bacteriovorus* 109J is harvested in the cold by centrifugation at 10,000g for 20 min and treated as the host suspension. Combining 10^{10} predator with 5×10^9 prey cells·ml^{-1} results in a synchronous, single-cycle culture in which host penetration occurs in less than 30 min and the cycle is complete in 3.5 h. Because the culture is very dense, proper aeration has to be ensured by vigorous shaking of the flask.

Premature Release from Bdelloplasts

From Ruby (1991): Bdelloplasts of *B. bacteriovorus* 109J can be obtained from a synchronous culture at any time in its development by cooling the culture on ice, removing the unattached, attack-phase cells by centrifugation at 1,100g for 5 min at 0°C, and resuspending the pellet by vigorous vortexing in cold HM buffer. This step is repeated twice to yield a virtually attack-phase bdellovibrio-free bdelloplast pellet as observed by phase-contrast microscopy. The pellet is warmed to room temperature, resuspended in 2 volumes of 10 mM EDTA in 120 mM Tris-hydrochloride (pH 7.6), and incubated at 30°C for 3 min to permeabilize the outer membrane. The bdelloplasts become transparent and the envelopes are lysed when 10 volumes of lytic enzyme concentrate are added and then incubated at 30°C for 15 to 20 min. The suspension is cooled to 0°C, centrifuged at 10,000g for 20 min, and resuspended in 1 ml HM buffer. The released bdellovibrios can then be purified on a Ficoll gradient in HM buffer. The band is removed from the tube and washed three times in HM buffer at 0°C (Ruby and Rittenberg, 1983). The use of a diaminopimelic acid (DAP)-deficient mutant host results in higher yields of released bdellovibrios. Culture of this mutant host in DAP-deficient medium for one doubling before harvest will weaken its envelope.

Preparation of a Lytic Enzyme Concentrate

From Ruby and Rittenberg (1983): Progeny bdellovibrios from 3 to 4 liters of a synchronous culture (300 ml in a 2-liter flask) are separated from the dithiothreitol (50 $\mu g \cdot ml^{-1}$) amended lytic solution by two successive centrifugations at 10,000g for 20 min. The supernatant is concentrated 60 times by pressure filtration using a membrane with a molecular cut-off of 30,000 daltons. The concentrate is centrifuged at 10,000g for 20 min and 0.5% polyethylene glycol is added. Aliquots are frozen in liquid nitrogen and stored at –70°C.

Preservation

Bdellovibrios can be kept as plaques on solid medium at 4°C for 1 to 2 weeks. Lysates amended with 5 mM glutamate and conserved at 4°C should be transferred every few weeks. For long-term conservation, aliquots of a fresh lysate are concentrated to half their original volume in freeze-resistant tubes and glycerol is added to a final concentration of 25%. The tubes are quickly frozen in liquid nitrogen and kept at –80°C. To revive the culture, thawed aliquots are serially diluted and plated on double agar seeded with the appropriate host. Alternatively, storage as bdelloplasts also maintains viability for long periods (Stolp, 1981): one ml of host bacteria is added to 5 ml of fresh lysate; the final concentrations of bdellovibrio and host bacteria are equal. The mixture is incubated for 30 min on a shaker to produce a high density of invaded prey cells. Glycerol is added to a final concentration of 10% and the vials are conserved in a deep freezer at –70°C.

Physiology

Upon attachment of attack phase bdellovibrios, the host cell is rapidly immobilized (Stolp and Starr, 1963). Major physiological features change quickly in the host, following an orchestrated sequence, even before the initiation of intraperiplasmic growth by the predator: the prey cell stops respiring, its peptidoglycan changes (Thomashow and Rittenberg, 1978a; 1978b; 1978d), the cell loses control over uptake, secretion, and synthesis (Varon et al., 1969; Rittenberg and Shilo, 1970), and its nucleic acids are degraded (Matin and Rittenberg, 1972; Hespell and Odelson, 1978; Hespell et al., 1976). Bdellovibrios, in contrast to viruses and most intracellular parasites, do not depend on the metabolism of their host: they normally proliferate in ultraviolet irradiated- or heat-killed prey cells (Varon et al., 1969). However, the use of prey is highly organized: degradation of the substrate cell leads to precursors that are used directly as building blocks, which drives an efficient coupling between anabolic and catabolic processes. The result is a highly efficient energy metabolism.

Lytic-Enzymatic Activities

Host morphology changes dramatically upon penetration by bdellovibrio: the host becomes a bdelloplast, which is usually round. Penetration of noncovalently bound outer-membrane components can be explained by the action of mechanical forces. However, for pore formation and passage through the peptidoglycan to occur, covalent bonds must be ruptured by enzymes (Fackrell and Robinson, 1973; Huang and Starr, 1973). The lytic activities must permit penetration on the one hand and prevent premature lysis of the host on the other. Rapid solubilization of peptidoglycan, which is the result of differentially regulated glycanase and peptidase activities, has been demonstrated during penetration, the free diaminopimelic acid (DAP) and the amino sugar being released to the extracellular medium (Thomashow and Rittenberg, 1978a). Free DAP is then rapidly incorporated into the peptidoglycan of the bdelloplast by a soluble enzyme activity only present during intraperiplasmic growth (Araki and Ruby, 1988). A lipopolysaccharidase is also active during invasion. The glycanase is inactivated rapidly by peptidoglycan deacetylation, while peptidase activity continues at a reduced pace throughout the bdelloplast stage (Thomashow and Rittenberg, 1978a; 1978c). Prevention of bdelloplast superinfection by other bdellovibrios has been attributed to deacetylation of the peptidoglycan backbone (Thomashow and Rittenberg, 1978d). Acylation of the host's peptidoglycan with long-chain fatty acids may stabilize the outer membrane of the bdelloplast, although the peptidoglycan is not essential for its osmotic stability (Thomashow and Rittenberg, 1978b; 1978c). Many hosts do not round up. Because strain W infected cells and heat-killed substrate cells, which do not round, have no detectable glycanase, Tudor et al. (1990) concluded that glycanase activity is responsible for the formation of a round bdelloplast, but deacetylation is not required for exclusion. A second burst of lytic activity takes place at the end of the intraperiplasmic growth phase, the newly synthesized enzymes degrading the remaining peptidoglycan (Thomashow and Rittenberg, 1978a; Fackrell and Robinson, 1973; Huang and Starr, 1973).

Energy Metabolism

At least some strains of bdellovibrios have a very high endogenous respiration rate, seven times the rate of *E. coli*, that increases little during the growth phase (Rittenberg and Shilo, 1970; Hespell et al., 1973).

Unlike most bacteria, free swimming and intraperiplasmically growing bdellovibrios have their endogenous respiration partially or totally shut-off by acetate and glutamate, respectively, and by mixtures of amino acids, and without increase in respiration rate (Hespell et al., 1973). This may be due to saturation of the tricarboxylic acid cycle or oxidative phosphorylation capacities (Hespell, 1976: Hespell et al., 1973). These findings and the inability of bdellovibrios to utilize (and possibly transport) sugars (Ruby, 1989) suggest substrate-level phosphorylation is not a major energy source (Rittenberg and Hespell, 1975a). Notably, bdellovibrios can use nucleoside monophosphates and ATP from the substrate cell or an exogenous source directly, and they may also use prey-derived phospholipids (Ruby and McCabe, 1986; Ruby et al., 1985; Rittenberg and Langley, 1975b). During the intraperiplasmic stage, the parasite DNases degrade the substrate cell's DNA in a timely regulated manner, providing the parasite with most of the nucleosides needed for growth (Rosson and Rittenberg, 1979; Matin and Rittenberg, 1972). The other nucleosides come from RNA breakdown and ribonucleotide and deoxyribonucleotide conversions (Rosson and Rittenberg, 1981), inasmuch as bdellovibrios seem unable to synthesize nucleotide denovo (Engelking and Seidler, 1974).

Rittenberg and Hespell (1975a) calculated that 16% of the total energy requirement for growth could be saved if the predator used intact prey fatty acids instead of denovo synthesizing fatty acids from acetate. Indeed, bdellovibrios growing intraperiplasmically are capable of totally or partially conserving substrate cell fatty acids and possibly phospholipids, thereby greatly reducing their energy need (Kuenen and Rittenberg, 1975).

Similarly, fatty acids and hexosamines from the lipopolysaccharide (LPS) of the prey's outer membrane were retrieved in large proportions from the LPS of intracellularly growing bdellovibrios (Nelson and Rittenberg, 1981a). Thin layer chromatography resolved lipid A isolated from the predator into two fractions shown to contain an unequal distribution of fatty acids originating from both substrate and bdellovibrio cells (Nelson and Rittenberg, 1981b).

Another peculiar feature of the bdellovibrio physiology is its unbalanced growth. During the growth phase, the DNA concentration increases faster than the protein concentration, the original ratio being restored by protein synthesis, which continues after DNA replication stops (Gray and Ruby, 1989). This property may permit the generation of the largest number of progeny with a minimal cell mass (Gray and Ruby, 1989).

This extraordinarily efficient metabolism, combining both scavenging and a finely regulated degradation of the substrate cell, can explain the outstanding energy efficiency of intraperiplasmically growing bdellovibrios, which were shown to achieve a YATP (biomass yield per ATP molecule expended) of 26 when compared with 10.5 for bacteria grown on rich medium (Rittenberg and Hespell, 1975a).

Transport

Outer Membrane Proteins Little is known about the transport capacities of *Bdellovibrio*, including its export pathways that involve breakdown of prey cytoplasm by predator proteases and nucleases and its import pathways that use the breakdown products and other solutes. Secretion of bdellovibrio products may involve export pathways designated as types I, II and III (Genin and Boucher, 1994; Schatz and Dobberstein, 1996) or other mechanisms. Interestingly, type III secretion pathways are found in plant and animal pathogens and are involved in the secretion of peptides directly into the host's cytoplasm (Finlay and Cossart, 1997; Baker et al., 1997).

More data are available on uptake routes. As described in energy metabolism, bdellovibrios use phosphate-ester containing molecules obtained from the prey. The molecules are transferred to the parasite, and in the case of phosphate nucleosides, transport seems to involve two different systems, one specific for the growth and the other for the free-living stage (Ruby and McCabe, 1986; Ruby et al., 1985). Inorganic phosphorus transport is low and reflects the lack of need for this element, which is obtained in organic form directly from the prey (Ruby and McCabe, 1986). Sugar transport pathways are similarly lacking (Ruby and McCabe, 1988). Mechanisms of fatty acid and lipopolysaccharide incorporation into bdellovibrio molecules are not known.

The very efficient metabolism of bdellovibrios is epitomized by the transfer of molecules that move solute: outer membrane proteins (OMPs) are routed to the outer membrane of the predator and cytoplasmic membrane of the prey (Diedrich et al., 1983; 1984; Talley et al., 1987; Tudor and Karp, 1994). The structure of the relocated proteins seems to be conserved in that

OmpF protein of *E. coli* transferred to the outer membrane of *B. bacteriovorus* 109D during intraperiplasmic growth had unaltered function in colicin A bdellovibrio-killing experiments (Diedrich et al., 1983). The process of acquiring outer membrane porin-like molecules from the substrate cell has limited adaptability. In OmpF mutants, the OmpC protein was transferred to the predator, although a clear preference for OmpF was detected when wild-type strains were the prey (Diedrich et al., 1984). However, other porin-like proteins were not found in the bdellovibrio outer membrane. Furthermore, it could be demonstrated that the protein relocalization of bdellovibrios is influenced by both the prey (smooth versus rough strains produce different results) and the history of the cultured predator (Talley et al., 1987).

The acquired proteins may play a role in the passive uptake of nutrients in the free-living phase of *Bdellovibrio* (Diedrich et al., 1983). Alternatively, proteins relocated to the host's cytoplasmic membrane may be responsible for the increased permeability of this membrane to small hydrophilic molecules and ions (Rittenberg and Shilo, 1970; Cover et al., 1984a). Thus, breakdown products from the cytoplasm might diffuse to the periplasmic space to be taken up by the predator (Tudor and Karp, 1994).

Properties of Bdelloplast and of Predator Membranes Change in the cell wall structure of the prey upon attack by *Bdellovibrio* is not limited to its peptidoglycan. The cell surface becomes more hydrophobic (Cover and Rittenberg, 1984b), maybe as a result of peptidoglycan modification with long-chain fatty acids (Thomashow and Rittenberg, 1978b) or cleavage of glucosamine residues from the substrate cell lipopolysaccharide (Thomashow and Rittenberg, 1978a). The cytoplasmic membrane of the substrate cell becomes permeable to lactose and small hydrophilic molecules (Rittenberg and Shilo, 1970; Cover et al., 1984a). However, the permeability of the outer membrane layer to small hydrophilic molecules is not altered, and most periplasmic proteins are retained (Odelson et al., 1982; Cover et al., 1984b).

Signaling The dimorphic cell cycle of bdellovibrios, which include an obligatory intracellular cell multiplication stage, is highly ordered, and physiology involved in attachment, penetration, and intraperiplasmic growth is highly regulated. The transition between the phases of the cell cycle seems to involve signals, whose function and nature are still elusive. It is obvious that during the cell cycle important changes occur in gene expression and protein synthesis. It was recently demonstrated by two-dimensional gel analysis of synchronous cultures that protein synthesis is affected by the growth stage of the cell: more than 30 polypeptides falling into nine kinetic groups were detected (McCann et al., 1998).

Wild-type bdellovibrios cultured on complex media amended with extracts obtained from prey cells have a developmental cycle similar to the one occurring intraperiplasmically (Friedberg, 1978; Horowitz et al., 1974; Reiner and Shilo, 1969; Introduction) and in host-independent (H-I) mutants. Bdellovibrios, obtained by premature release from bdelloplasts before the onset of DNA replication, differentiate into attack cells but complete their DNA synthesis round only if DNA replication has been initiated. The addition of host cell extracts enables initiation of DNA replication (Ruby and Rittenberg, 1983; Gray and Ruby, 1990). In an extensive review on the subject, Gray and Ruby (1991) proposed a two-signal model, the first triggering the differentiation of attack-phase bdellovibrios into growth-phase cells, the second initiating and sustaining subsequent rounds of DNA replication. Attempts at purifying the early and late putative signal molecules from prey cells were not conclusive, although these molecules were shown to be nondialyzable, heat stable, DNAse resistant, and possibly proteinaceous (Gray and Ruby, 1990; Friedberg, 1978; Horowitz et al., 1974; Reiner and Shilo, 1969). Interestingly, axenic growth of prey-dependent bdellovibrios in a rich medium without prey extracts can be triggered by heat shock, eliminating the need for a growth inducing signal. Heat shock may trigger changes in gene transcription (Gordon et al., 1993).

In contrast to prey-derived signals, Eksztejn and Varon (1977) detected a cell-density dependent, division-promoting activity identified as an apparent cyclic peptide produced by bdellovibrios during the middle to late growth stages.

The influence of cell density was already noted by Shilo and Bruff (1965), who reported colony growth of H-I mutants of *B. starrii* only with a high density inoculum. More recently, Cotter and Thomashow (1992a) and (1992b) reported that H-I mutants could be differentiated into two classes, defined as type I and type II: type I develop small colonies growing close one to the other, and type II exhibit large, density-independent growth. Type I mutants arise at a frequency of 10^{-6} to 10^{-7} and type II at a frequency of 10^{-8} to 10^{-9}. The latter may result from a double mutation, lending support to the two-signal hypothesis (Thomashow and Cotter, 1992). Type II mutants could be partially com-

plemented by a 1 kilobase fragment (the hit locus) containing four putative open reading frames of unknown function and not homologous to any sequence in the databases (Cotter and Thomashow, 1992b).

Cell-cell communication may therefore exist in bdellovibrios but its nature, whether peptidic like in *Myxococcus* (Dworkin, 1996), N-acyl-L-homoserine lactone based, quorum-sensing (Dunlap, 1997), or other, is unknown.

Applications

The characteristic life-style of bdellovibrios makes them attractive candidates for a number of applications concerned with reducing or modulating bacterial populations, i.e. biological control of pathogens, water purification, and biofilm control.

Surprisingly little emphasis has been put on studying the possible role of *Bdellovibrio* in agricultural applications: Bdellovibrios efficiently eradicated *Xanthomonas oryzae* from rice paddy field water and caused rapid decline in populations of *E. carotovora* sspp. *carotovora* in soil (Uematsu, 1980). *Bdellovibrio* isolates from the rhizosphere of soybean were used to control bacterial blight caused by *Pseudomonas glycinea* (Scherff, 1973). Reduction in disease severity and systemic symptoms were significant.

An attempt was made to control soft rot and black leg of potato but mixed results were obtained (Epton et al., 1989).

That bdellovibrios might be deleterious to plant growth-promoting rhizobacteria was suggested by Germida (1987), who isolated bdellovibrios parasitic to *Azospirillum brasilense* from soils and by Elsherif and Grossman (1996), who could show an increase in bdellovibrio counts from the rhizosphere of plants inoculated with a beneficial strain of *Pseudomonas fluorescens*.

A role for *Bdellovibrio* in the self-purification of natural water systems has been suggested (Daniel, 1969; Fry and Staples, 1976). Because bdellovibrios are highly sensitive to certain chemicals and metals, they can be used as indicators for those compounds in water (Varon and Shilo, 1981).

A few attempts have been made to use bdellovibrios in animal models: Nakamura (1972) effectively treated *Shigella flexneri*-induced keratoconjunctivitis in rabbit with bdellovibrio, but bdellovibrio used as predator against pathogens in the intestinal tract of rabbits was unsuccessful (Westergaard and Kramer, 1977). It has been proposed that because of its unique physiology *Bdellovibrio* may be a source of novel chemicals and biochemicals (Ruby, 1991), although the evidence is unpublished. Undeniably, this intriguing bacterial group is a great model for the study of a myriad of subjects central to biology, e.g. predator-prey interactions, host recognition, development of prokaryotes, and cell-cell signaling.

Conclusion

Although much has been done to understand the ecology and the biology of bdellovibrios, one can still ask whether bdellovibrios play a role in determining the structure of bacterial populations. How do they survive? How is their cell cycle regulated, and what special functions are needed to complete it? Those are few of the very basic questions still left unanswered.

The advent of genomics should greatly improve our understanding of bdellovibrio biology and thereby its relation with the environment. Looking at the metabolic properties of this particular microbial predator, a few comparisons with other organisms can already be drawn: transport of exogenous phosphorylated nucleosides, sometimes accompanied by a marked reduction in de novo nucleotide biosynthesis capabilities, has been shown in intracellular parasites of eucaryotic cells such as *Rickettsia*, *Mycoplasma* and, *Chlamydia* (Stephens et al., 1998; Fraser et al., 1995; Moulder, 1991). This type of adaptation points to converging phenotypic evolution between intracellular parasites, although the biochemical mechanisms widely differ. Similarly, it will be possible to assess the dynamics of genome evolution and reduction by looking for "missing" genes and for the ones pointing to the particular lifestyle of this organism when the *Bdellovibrio* genome becomes available.

Literature Cited

Abram, D., Castro e Melo, J., and D. Chou. 1974. Penetration of Bdellovibrio bacteriovorus into host cells. J. Bacteriol. 118:663–680.

Althauser, M., Samsonoff, W.A., Anderson, C., and S.F. Conti. 1972. Isolation and preliminary characterization of bacteriophages for Bdellovibrio bacteriovorus. J. Virol. 10:516–524.

Amann, R., Ludwig, W., and K.H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual cells without cultivation. Microbiol. Rev. 59:143–161.

Araki, Y., and E.G. Ruby. 1988. A soluble enzyme activity that attaches free diaminopimelic acid bdelloplast peptidoglycan. Biochem. 27:2624–2629.

Baker, B., P. Zambrisky, B. Staskawicz, S.P. Dinesh-Kumar. 1997. Signaling in plant-microbe interactions. Science 276:726–733.

Burger, A., Drews, G., and R. Ladwig. 1968. Wirtskreis und infecktionscyclus eines neu isolierten Bdellovibrio bacteriovorus-stammes. Arch. Mikrobiol. 61:261–279.

Blackburn, N., Fenchel, T., and J. Mitchell. 1998. Microscale nutrient patches in planktonic habitats shown by chemotactic bacteria. Science 282:254–256.

Caldwell, D.E. 1977. The planktonic microflora of lakes. CRC Crit. Rev. Microbiol. 5:305–370.

Cotter, T.W. and M.F. Thomashow. 1992a. A conjugation procedure for Bdellovibrio bacteriovorus and its use to identify DNA sequences that enhance the plaque-forming ability of a spontaneous host-independent mutant. J. Bacteriol. 174:6011–6017.

Cotter, T.W. and M.F. Thomashow. 1992b. Identification of a Bdellovibrio bacteriovorus genetic locus, hit, associated with the host-independent phenotype. J. Bacteriol. 174:6018–6024.

Cover, W.H., Martinez, R.J., and S.C. Rittenberg. 1984a. Permeability of the boundary layers of Bdellovibrio bacteriovorus 109J and its bdelloplasts to small hydrophobic molecules. J. Bacteriol. 157:385–390.

Cover, W.H., and S.C. Rittenberg. 1984b. Change in the surface hydrophobicity of substrate cells during bdelloplast formation by Bdellovibrio bacteriovorus 109J. J. Bacteriol. 157:391–397.

Daniel, S. 1969. Etude de l'influence de Bdellovibrio bacteriovorus dans l'auto-epuration marine. Rev. Intern. Ocean. Med. 15–16:61–102.

Diedrich, D.L., Portnay Duran, C., and S.F. Conti. 1984. Acquisition of Escherichia coli outer membrane proteins by Bdellovibrio sp. strain 109D. J. Bacteriol. 159:329–334.

Diedrich, D.L., Portnoy, C.A., and S.F. Conti. 1983. Bdellovibrio possesses a prey-derived OmpF protein in its outer membrane. Curr. Microbiol. 8:51–56.

Diedrich, D.L., Denny, C.F., Hashimoto, T., and S.F. Conti. 1970. Facultatively parasitic strain of Bdellovibrio bacteriovorus. J. Bacteriol. 101:989–996.

Donze, D., Mayo, J.A., and D.L. Diedrich. 1991. Relationships among the bdellovibrios revealed by partial sequences of 16S ribosomal RNA. Curr. Microbiol. 23:115–119.

Dunlap, P.V. 1997. N-acyl-L-homoserine lactone inducers in bacteria: unity and diversity. Shapiro, J.A., and M. Dworkin. Bacteria as multicellular organisms. Oxford University Press. Oxford, 69–106.

Dworkin, M. 1996. Recent advances in the social and developmental biology of the myxobacteria. Microbiol. rev. 60:70–102.

Eksztejn, M., and M. Varon. 1977. Elongation and cell division in Bdellovibrio bacteriovorus. Arch. Microbiol. 114:175–181.

Elsherif, M., and F. Grossmann. 1996. Role of biotic factors in the control of soil-borne fungi by fluorescent pseudomonads. Microbiol. Res. 151:351–357.

Engelking, H.M., and R.J. Seidler. 1974. The involvement of extracellular enzymes in the metabolism of Bdellovibrio. Arch. Microbiol. 95:293–304.

Epton, H.A.S., Walker, N.M., and D.C. Sigee. 1989. Bdellovibrio: a potential control agent for soft rot and black leg of potato. Klement, Z.. Plant pathogenic bacteria – Part A. Akademia Kiado. Budapest, 207–212.

Fackrell, H.B., and J. Robinson. 1973. Purification and characterization of a lytic peptidase produced by Bdellovibrio bacteriovorus 6–5-S. Can. J. Microbiol. 19:659–666.

Finlay, B.B., and P. Cossart. 1997. Exploitation of mammalian host cell functions by bacterial pathogens. Science 276:718–725.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., Bult, C.J., Kervalage, A.R., Sutton, G., Kelley, J.M., Fritchman, J.L., Weidman, J.F., Small, K.V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T.R., Saudek, D.M., Phillips, C.A., Merrck, J.M., Tomb, J.F., Dougherty, B.A., Bott, K.F. Hu, P.-C., Lucier, T.S., Peterson, S.N., Smith, H.O., Hutchinson III, C.A. and J.C. Venter. 1995. The minimal gene complement of Mycoplasma genitalium. Science 270:397–403.

Fratamico, P.M., and P.H. Cooke. 1996. Isolation of bdellovibrios that prey on Escherichia coli 0157:H7 and Salmonella species and application for removal of prey from stainless steel surfaces. J. Food Saf. 16:161–173.

Friedberg, D. 1978. Growth of host-dependent Bdellovibrio in host-free system. Arch. Microbiol. 116:185–190.

Friedberg, D. 1977. Effect of light on Bdellovibrio bacteriovorus. J. Bacteriol. 131:399–404.

Fry, J.C., and D.G. Staples. 1976. Distribution of Bdellovibrio bacteriovorus in sewage works, river water, and sediments. Appl. Environ. Microbiol. 31:469–474.

Genin, S., and C.A. Boucher. 1994. A superfamily of proteins involved in different secretion pathways in gram-negative bacteria: modular structure and specificity of the N-terminal domain. Mol. Gen.Genet. 243:112–118.

Germida, J.J. 1987. Isolation of Bdellovibrio spp. that prey on Azospirillum brasilense in soil. Can. J. Microbiol. 33:459–461.

Gordon, R.F., Stein, M.A., and D.N. Diedrich. 1993. Heat shock-induced growth of Bdellovibrio bacteriovorus. J. Bacteriol. 175:2157–2161.

Gray, K.M., and E.G. Ruby. 1991. Intercellular signalling in the bdellovibrio developmental growth cycle. Dworkin, M. Microbial Cell-Cell Interactions. ASM. Washington DC, 333–366.

Gray, K.M., and E.G. Ruby. 1990. Prey-derived signals regulating duration of the developmental growth phase of Bdellovibrio bacteriovorus. J. Bacteriol. 172:4002–4007.

Gray, K.M., and E.G. Ruby. 1989. Unbalanced growth as a normal feature of development of Bdellovibrio bacteriovorus. Arch. Microbiol. 152:420–424.

Hashimoto, T., Diedrich, D.L., and S.F. Conti. 1970. Isolation of a bacteriophage for Bdellovibrio bacteriovorus. J. Virol. 5:97–98.

Hespell, R.B. 1976. Glycolytic and tricarboxylic acid enzymes activities during intraperplasmic growth of Bdellovibrio bacteriovorus . J. Bacteriol. 128:677–680.

Hespell, R.B., and D.A. Odelson. 1978. Metabolism of RNA-ribose by Bdellovibrio bacteriovorus during intraperiplasmic growth. J. Bacteriol. 136:936–946.

Hespell, R.B., Thomashow, M.F., and S. C. Rittenberg. 1974. Changes in cell composition and viability of Bdellovibrio bacteriovorus during starvation. Arch. Microbiol. 97:313–327.

Hespell, R.B., Rosson, R.A., Thomashow, M.F., and S.C. Rittenberg. 1973. Respiration of Bdellovibrio bacteriovorus strain 109J and its energy substrates for intraperiplasmic growth. J. Bacteriol. 113:1280–1288.

Hoeniger, J., Ladwig, R., and H. Moor. 1972. The fine structure of "resting bodies" of Bdellovibrio sp. strain W developed in Rhodospirillum rubrum. Can. J. Microbiol. 18:87–92.

Horowitz, A.T., Kessel, M. and M. Shilo. 1974. Growth cycle of predaceous bdellovibrios in a host-free extract system and some properties of the host extract. J. Bacteriol. 117:270–282.

Huang, J.C.C., and M.P. Starr. 1973. Possible enzymatic basis of bacteriolysis by bdellovibrios. Arch. Microbiol. 89:147–167.

Kelley, J.I., Turng, B.F., Williams, H.N., and M.L. Baer. 1997. Effects of temperature, salinity, and substrate on the colonization of surfaces in situ by aquatic bdellovibrios. Appl. Environ. Microbiol. 63:84–90.

Kelley, J.I., and H.N. Williams. 1992. Bdellovibrios in Callinectus sapidus, the blue crab. Appl. Environ. Microbiol. 58:1408–1410.

Kessel., M., and M. Shilo. 1976. Relationship of Bdellovibrio elongation and fission to host cell size. J. Bacteriol. 128:477–480.

Keya, S.O., and M. Alexander. 1975. Regulation of parasitism by host density: the Bdellovibrio-Rhizobium interrelationship. Soil Biol. Biochem. 7:231–237.

Klein, D.A., and L.E. Casida, Jr. 1967. Occurence and enumeration of bdellovibrio bacteriovorus in soil capable of parasitizing Escherichia coli and indigenous soil bacteria. Can. J. Microbiol. 13:1235–1241.

Koval, S.F., and M. E. Bayer. 1997. Bacterial capsules: no barrier against Bdellovibrio. Microbiol. 143:749–753.

Koval, S.F., and S.H. Hynes. 1991. Effect of paracrystalline protein surfaces layers on predation by Bdellovibrio bacteriovorus. J. Bacteriol. 173:2244–2249.

Kramer, T.T., and J.M. Westergaard. 1977. Antigenicity of bdellovibrios. Appl. Environ. Microbiol. 33:967–970.

Kuenen, J.G., and S.C. Rittenberg. 1975. Incorporation of long-chain fatty acids of the substrate organism by Bdellovibrio bacteriovorus during intraperiplasmic growth. J. Bacteriol. 121:1145–1157.

LaMarre, A.G., Straley, S.C., and S.F. Conti. 1977. Chemotaxis toward amino acids by Bdellovibrio bacteriovorus. J. Bacteriol. 131:201–207.

Lambina, V.A., Chuvilskaya, N.A., Ledova, L.A., Afinogenova, A.V. and I.V. Averburg. 1974. Quantitative characteristics of distribution of Bdellovibrio bacteriovorus in river waters. Microbiologia 42:715–720.

Miyamoto, S., and K. Kuroda. 1975. Lethal effect of fresh sea water on Vibrio parahaemolyticus and isolation of Bdellovibrio parasitic against the organism. J. Microbiol. 19:309–317.

Marbach, A., and M. Shilo. 1978. Dependence of marine bdellovibrios on potassium, calcium, and magnesium ions. Appl. Environ. Microbiol. 36:169–177.

Marbach, A., Varoon, M., and M. Shilo. 1976. Properties of marine bdellovibrio. Microb. Ecol. 2:284–295.

Matin, A., and S.C. Rittenberg. 1972. Kinetics of deoxyribonucleic acid destruction and synthesis during growth of Bdellovibrio bacteriovorus strain 109D on Pseudomonas putida and Escherichia coli. J. Bacteriol. 111:664–673.

Markelova, N.Y., Shahamat, M., Levin, M.A., and R.R. Colwell. 1998. Effect of various pollutants on Bdellovibrio's contribution to the self-purifiaction processes. 8th Inter. l Symp. Microb. Ecol. 227.

McCann, M.P., Solimeo, H.T., Cusick, Jr., F., Panunti, B., and C. McCullen. 1998. Developmentally regulated protein synthesis during intraperiplasmic growth of Bdellovibrio bacteriovorus 109J. Can. J. Microbiol. 44:50–55.

Moulder, J.W. 1991. Interaction of Chlamydiae and host cells in vitro. Microbiol. Rev. 55:143–190.

Mulvey, M.A., Lopez-Boado, Y.S., Wilson, C.L., Roth, R., Parks, W.C., Heuser, S.J., and S.J. Hultgren. 1998. Induction and evasion of host defenses by type 1-piliated uropathogenic Escherichia coli. Science 282:1494–1497.

Nakamura, M. 1972. Alteration of Salmonella pathogenicity by other bacteria. Am. J. Clin. Nutr. 25:1441–1451.

Nelson, D.R., and S.C. Rittenberg. 1981a. Incorporation of substrate cell lipid A components into the lipopolysaccharide of intraperiplasmically grown Bdellovibrio bacteriovorus. J. Bacteriol. 147:860–868.

Nelson, D.R., and S.C. Rittenberg. 1981b. Partial characterization of lipid A of intraperiplasmically grown Bdellovibrio bacteriovorus. J. Bacteriol. 147:869–874.

Odelson, D.A., Patterson, M.A., and R.B. Hespell. 1982. Periplasmic enzymes in Bdellovibrio bacteriovorus and Bdellovibrio stolpii. J. Bacteriol. 151:756–763.

Pan, C-L., Hsu, Y-L., Tsai, G-J., Kuo, H-J, Chang, C-M., Wang, F-J., and C-S. Wu. 1997. Isolation and identification of Bdellovibrio from coastal areas of Taiwan. Fish. Sci. 63:52–59.

Parker, C.A., and P.L. Grove. 1970. Bdellovibrio bacteriovorus parasitizing Rhizobium in western Australia. J. Appl. Bacteriol. 33:253–255.

Reiner, A.M., and M. Shilo. 1969. Host-independent growth of Bdellovibrio bacteriovorus in microbial extracts. J. Gen. Microbiol. 59:401–410.

Rice, T.D. Williams, H.N. and B.F. Turng. 1998. Susceptibility of bacteria in estuarine environments to autochtonous bdellovibrios. Microb. Ecol. 35:256–264.

Richardson, I.R. 1990. The incidence of Bdellovibrio spp., in man-made water systems: coexistence with legionellas. J. Appl. Bacteriol. 53:134–140.

Rittenberg, S.C., and M. Shilo. 1970. Early host damage in the infection cycle of Bdellovibrio bacteriovorus. J. Bacteriol. 102:149–160.

Rittenberg, S.C. and R.B. Hespell. 1975a. Energy efficiency of intraperiplasmic growth of Bdellovibrio bacteriovorus. J. Bacteriol. 121:1158–1165.

Rittenberg, S.C., and D. Langley. 1975b. Utilization of nucleoside monophosphates per se for intraperiplasmic growth of Bdellovibrio bacteriovorus. J. Bacteriol. 121:1137–1144.

Romantschuk, M. 1992. Attachment of plant pathogenic bacteria to plant surfaces. Annu. Rev. Phytopathol. 30:225–243.

Rosson, R.A., and S.C. Rittenberg. 1979. Regulated breakdown of Escherichia coli deoxyribonucleic acid during intraperiplasmic growth of Bdellovibrio bacteriovorus 109J. J. Bacteriol. 140:620–633.

Rosson, R.A., and S.C. Rittenberg. 1981. Pyrimidine metabolism of Bdellovibrio bacteriovorus grown intraperiplasmically and anexically. J. Bacteriol. 146:108–116.

Ruby, E.G. 1991. The genus Bdellovibrio. A. Balows, H.G. Truper, M. Dworkin, W. Harder, and K.H. Schleifer. The Prokaryotes. Springer-Verlag. New York, NY. 3400–3415.

Ruby, E.G. 1989. Cell-envelope modifications accompaying intracellular growth of Bdellovibrio bacteriovorus. Moulder, J.W. Intracellular parasitism. CRC Press. Boca Raton, 17–34.

Ruby, E.G., and J. McCabe. 1988. Metabolism of periplasmic membrane-derived oligosaccharides by the predatory bacterium Bdellovibrio bacteriovorus 109J. J. Bacteriol. 170:646–652.

Ruby, E.G., and J.B. McCabe. 1986. An ATP transport system in the intracellular bacterium Bdellovibrio bacteriovorus 109J. J. Bacteriol. 167:1066–1070.

Ruby, E.G., McCabe, J.B., and J.I. Barke. 1985. Uptake of intact nucleoside monophosphate by Bdellovibrio bacteriovorus 109J. J. Bacteriol. 163:1087–1094.

Ruby, E.G., and S.C. Rittenberg. 1983. Differentiation after premature release of intraperiplasmically growing Bdellovibrio bacteriovorus. J. Bacteriol. 154:32–40.

Sagi, B., and R. Levisohn. 1976. Isolation of temperate bacteriophages for Bdellovibrio bacteriovorus. Isr. J. Med. Sci. 12:709–710.

Schatz, G., and B. Dobberstein. 1996. Common principles of protein translocation across membranes. Science 271:1516–1526.

Schelling, M.E., and S.F. Conti. 1983. Serotyping of bdellovibrios by agglutination and indirect immunofluorescence. J. Syst. Bacteriol. 33:816–821.

Schelling, M., and S. Conti. 1976. 1986. Host receptor sites in the attachment of Bdellovibrio bacteriovorus and Bdellovibrio stolpii. FEMS Microbiol. Let. 36:319–323.

Scherff, R.H. 1973. Control of bacterial blight of soybean by Bdellovibrio bacteriovorus. Phytopathol. 63:400–402.

Schoeffield, A.J., Williams, H.N., Turng, B.F., and W.A. Falkler, Jr. 1996. A comparison of the survival of intraperiplasmic and attack phase bdellovibrios with reduced oxygen. Microb. Ecol. 32:35–46.

Schoeffield, A.J., and H.N. Williams. 1990. Efficiencies of recovery of bdellovibrios from brackish-water environments by using various bacterial species as prey. Appl. Environ. Microbiol. 56:230–236.

Schoeffield, A.J., Falkler, Jr., W.A., Desai, D., and H.N. Williams. 1991. Serogrouping of halophilic bdellovibrios from Chesapeake Bay and environs by immunodiffusion and immunoelectrophoresis. Appl. Environ. Microbiol. 57:3470–3475.

Siedler, R.J., Mendel, M., and J.N. Baptist. 1972. Molecular heterogeneity of the bdellovibrios: evidence for two new species. J. Bacteriol. 109:209–217.

Seidler, R.J., and M.P. Starr. 1969a. Isolation and characterization of host-independent bdellovibrios. J. Bacteriol. 100:769–785.

Seidler, R.J., and M.P. Starr. 1969b. Factors affecting the intracellular parasitic growth of Bdellovibrio bacteriovorus developing within Escherichia coli. J. Bacteriol. 97:912–923.

Shilo, M., and B. Bruff. 1965. Lysis of Gram negative bacteria by host-independent ectoparasitic Bdellovibrio bacteriovorus isolates. J. Gen. Microbiol. 40:317–328.

Staples, D.G., and J.C. Fry. 1973. Factors which influence the enumeration of Bdellovibrio bacteriovorus in sewage and river water. J. Appl. Bacteriol. 36:1–11.

Starr, M.P. 1975. Bdellovibrio as a symbiont: The association of bdellovibrios with other bacteria interpreted in terms of a generalized scheme for classifying organismic associations. Symposia of the Society of Experimental Biology 29:93–124.

Stephens, R.S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., Mitchell, W., Olinger, L., Tatusov, R.L., Zhao, Q., Koonin, E.V., and R.W. Davis. 1998. Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. Science 283:754–759.

Stolp, H. 1981. The genus Bdellovibrio. Starr, M.P., Stolp, H., Troper, H.G., Balows, A., and Schegel. The Prokaryotes. Springer-Verlag. Berlin, 618–629.

Stolp, H. 1968. Ein rauberischer backterienparasit. Naturwiss. 55:57–63.

Stolp, H. 1967. Lysis von bacterien durch den parasiten Bdellovibrio bacteriovorus. I.W.F., Gottingen Film C972.

Stolp, H., and M.P. Starr. 1963. Bdellovibrio bacteriovorus gen. et sp., a predatory, ectoparasitic, and bacteriolytic microorganism. Anton. van Leeuw. 29:217–248.

Stolp, H., and H. Petzold. 1962. Untersuchungenuber einen obligat parasitischen mikroorganismus mit lytischer aktivat fur Pseudomonas-bakterien. Phytopatho. Zeit. 45:364–390.

Straley, S.C., LaMarre, A.G., Lawrence, L.J., and S.F. Conti. 1979. Chemotaxis of Bdellovibrio bacteriovorus toward pure compounds. J. Bacteriol. 140:634–642.

Straley, S.C. and S.F. Conti. 1977. Chemotaxis of Bdellovibrio bacteriovorus toward prey. J. Bacteriol. 132:628–640.

Talley, B.G., and McDade, R.L. and D.L. Diedrich. 1987. Verification of the protein in the outer membrane of Bdellovibrio bacteriovorus as the OmpF protein of its Escherichia coli prey. J. Bacteriol. 169:694–698.

Taylor, V.I., Baumann, P., Reichelt, J.L., and R.D. Allen. 1974. Isolation, enumeration, and host range of marine bdellovibrios. Arch. Microbiol. 98:101–114.

Thomashow, M.F. and T.W. Cotter. 1992. Bdellovibrio host-dependence: the search for signal molecules and genes that regulate the intrapreriplasmic growth cycle. J. Bacteriol. 174:5767–5771.

Thomashow, L.S. and S.C. Rittenberg. 1985. Isolation and composition of sheathed flagella from Bdellovibrio bacteriovorus. J. Bacteriol. 161:1047–1054.

Thomashow, M.F., and S.C. Rittenberg. 1978a. Intraperiplasmic growth of Bdellovibrio bacteriovorus 109J: solubilization of Escherichia coli peptidoglycan. J. Bacteriol. 135:998–1007.

Thomashow, M.F., and S.C. Rittenberg. 1978b. Intraperiplasmic growth of Bdellovibrio bacteriovorus 109J: attachment of long-chain fatty acids to Escherichia coli peptidoglycan. J. Bacteriol. 135:1015–1023.

Thomashow, M.F., and S.C. Rittenberg. 1978c. Penicillin-induced formation of osmotically stable spheroplasts in nongrowing Bdellovibrio bacteriovorus. J. Bacteriol. 133:1484–1491.

Thomashow, M.F., and S.C. Rittenberg. 1978d. Intraperiplasmic growth of Bdellovibrio bacteriovorus 109J: N-deacetylation of Escherichia coli peptidoglycan amino sugars. J. Bacteriol. 135:1008–1014.

Torrella, F., Guerrero, R., and R.J. Seidler. 1978. Further taxonomic characterization of the genus Bdellovibrio. Can. J. Microbiol. 24:1387–1394.

Tudor, J.J., and M.A. Karp. 1994. Translocation of an outer membrane protein into prey cytoplasmic membranes by bdellovibrios. J. Bacteriol. 176:948–952.

Tudor, J.J., McCann, M., and I.A. Acrich. 1990. A new model for the penetration of prey cells by bdellovibrios. J. Bacteriol. 172:2421–2426.

Tudor, J.J., and S.F. Conti. 1978. Characterization of germination and activation of Bdellovibrio bdellocysts. J. Bacteriol. 133:130–138.

Tudor, J.J., and S.F. Conti. 1977. Characterization of bdellocysts of Bdellovibrio sp. J. Bacteriol. 131:314–322.

Uematsu, T. 1980. Ecology of Bdellovibrio parasitic to rice bacterial leaf blight pathogen, Xanthomonas oryzae. Rev. Plant Protec. Res. 13:12–26.

Vande Broek, A., and J. Vanderleyden. 1995. The role of bacterial motility, chemotaxis, and attachment in bacteria-plant interactions. Mol. Plant Microb. Inter. 8:800–810.

Varon, M. 1979. Selection of predation-resistant bacteria in continuous culture. Nature 277:386–388.

Varon, M. 1974. The bdellophage parasitic three-membered system. CRC Crit. Rev. Microbiol. 221–241.

Varon, M., and M. Shilo. 1981. Inhibition of the predatory activity of Bdellovibrio by various environmental pollutants. Microb. Ecol. 7:107–111.

Varon, M., and M. Shilo. 1978. Ecology of aquatic bdellovibrios. Droop, M.R., and H.W. Jannash. Advances in microbial ecology. Academic Press. London, 1–48.

Varon, M., and B. Zeigler. 1978. Bacterial predator-prey interaction at low prey density. Appl. Environ. Microbiol. 36:11–17.

Varon, M., and J. Seijffers. 1975. Symbiosis-independent and symbiosis-incompetent mutants of Bdellovibrio bacteriovorus 109J. J. Bacteriol. 124:1191–1197.

Varon, M., and M. Shilo. 1974. Isolation of host-dependent and nonparasitic mutants of the facultative parasitic Bdellovibrio UKi2. J. Bacteriol. 119:635–637.

Varon, M., and R. Levisohn. 1972. Three-membered parasitic systems: A bacteriophage, Bdellovibrio bacteriovorus, and Escherichia coli. J. Virol. 9:519–525.

Varon, M., and M. Shilo. 1970. Method for separation of bdellovibrio from mixed bacterial population by filtration through millipore or by gradient differential centrifugation. Rev. Intern. Oceanogr. 18–19:145–151.

Varon, M., Dickbuch, S., and M. Shilo. 1969. Interaction of Bdellovibrio bacteriovorus and host bacteria. II. Intracellular growth and development of Bdellovibrio bacteriovorus in liquid cultures. J. Bacteriol. 99:136–141.

Varon, M., and M. Shilo. 1968. Interaction of Bdellovibrio bacteriovorus and host bacteria: I. Kinetic studies of attachment and invasion of Escherichia coli B by Bdellovibrio bacteriovorus. J. Bacteriol. 95:744–753.

Wanner, O. 1989. Modeling of population dynamics. Characklis, W.G. and P.A. Wilderer. Structure and function of biofilms. Wiley & Sons. New York, NY. 91–110.

Westergaard, J.M., and T.T. Kramer. 1977. Bdellovibrio in the intestinal flora of vertebrates. Appl. Environ. Microbiol. 34:506–511.

Williams, H.N., Schoeffied, H.N., Guether, D., Kelley, J., Shah, D., and W.A. Falkler. 1995. Recovery of bdellovibrios from submerged surfaces and other aquatic habitats. Microb. Ecol. 29:39–48.

Williams, H.N., and W.A. Falkler. 1984. Distribution of bdellovibrios in the water column of an estuary. Can. J. Microbiol. 30:971–974.

Williams, H.N., Falkler, W.A., and D.E. Shay. 1982. Seasonal distribution of bdellovibrios at the mouth of the Patuxent River in the Chesapeake Bay. Can. J. Microbiol. 28:111–116.

Williams, H.N. 1988. A study of the distribution of bdellovibrios in estuarine sediment over an annual cycle. Microb. Ecol. 15:9–20.

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CHAPTER 3.4.3

The Myxobacteria

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The myxobacteria are Gram-negative, unicellular bacteria with rod-shaped vegetative cells (Fig. 1). Because of their gliding movement, colonies develop as thin, spreading swarms, particularly on media low in organic constituents (Fig. 2). Under starvation conditions, the myxobacteria undergo an impressive process of cooperative morphogenesis: the vegetative cells aggregate into large mounds and then form a fruiting body by directed cell movement (Fig. 3). Myxobacterial fruiting bodies show various degrees of complexity. They typically measure between 50 and 500 μm and can be seen with the naked eye. Within the maturing fruiting body the vegetative cells convert into short, optically refractile myxospores (Figs. 1 and 3). The myxospores are desiccation resistant and allow the organism to survive unfavorable environmental conditions. Many features of the life cycle are richly illustrated in a series of film clips created by Hans Reichenbach and assembled by Martin Dworkin (see the online version of *The Prokaryotes*). The clips are presented with the permission of the Institut für den Wissenschaftlichen Film.

Commentary and References for Time-lapse Film.

This time lapse film is from a series of films created by Dr. Hans Reichenbach. It was assembled by Dr. Martin Dworkin and is presented with the permission of the Institut fur den Wissenschaftlichen Film, Göttingen, Germany.*

1. Movement of cells of *Archangium violaceum* on agar. Note that movement ceases while the cells are dividing. [2 frames/min. over approximately 8 hr.; Kühlwein, H. and H. Reichenbach, 1968a]

2. Movement of cells of *Myxococcus xanthus* on agar. [4 frames/min over approximately 1.5 hr.; Kühlwein, H. and H. Reichenbach, 1968b]

3. Movement of cells of *Myxococcus virescens* over agar. [8 frames/min. over approximately 1 hr.; Kühlwein, H. and H. Reichenbach, 1968c]

4. Spiral movement characteristic of masses of cells of *Chondromyces apiculatus* on an agar surface. [1 frame/min. over approximately 16 hr.; Kühlwein, H. and H. Reichenbach, 1968d]

5. Aggregation of *Chondromyces apiculatus* as a prelude to fruiting body formation. Note the streams of cells moving into the aggregation center. [1 frame/min. over approximately 13 hr.; Kühlwein, H. and H. Reichenbach, 1968e]

6–8. Side views of the development of the fruiting body of *Chondromyces apiculatus* on a cellophane membrane overlaid on agar. The aggregated spherical mass of cells excretes a slime stalk, which propels the mass of cells upward. The cells within the knob at the top of the stalk differentiate into individual sporangioles containing the myxospores. [4, 8, and 4 frames/min. over 3, 3, and 5 hr.; Kühlwein, H. and H. Reichenbach, 1968f]

9–10. Germination of sporangioles of *Chondromyces apiculatus*. The myxospores of *C. apiculatus* are contained in sporangioles arrayed around the head of the fruiting body. Prior to germination the sporangioles fall off the fruiting body. The sporangiole swells and movement of the cells within the sporangiole becomes discernable. The sporangiole lyses at one end and then at the other, whereupon the cells emerge and constitute a small swarm, leaving the empty hull behind. Note that as the swarm moves, individual cells, which can move faster than the entire swarm, never escape from the swarm. Opposing swarms move toward each other and eventually fuse. The attractive mechanism is unknown. [2 frames/min. over 23.5 hr.; Kühlwein, H. and H. Reichenbach, 1968g]

11. Germination of myxospores of *Myxococcus xanthus*. Unlike the resting cells of *Chondromyces*, which are contained in sporangioles, the myxospores of *Myxococcus* germinate as individual cells. The cells lose their optical refractility and break out of their spore coat, leaving the empty hull behind. The cells elongate, reacquire their characteristic gliding motility and move off.

* The real time of each segment can be calculated from the fact that the film was projected at a speed of 24 frames/second and that the rate at which the frames were taken will be presented for each segment.

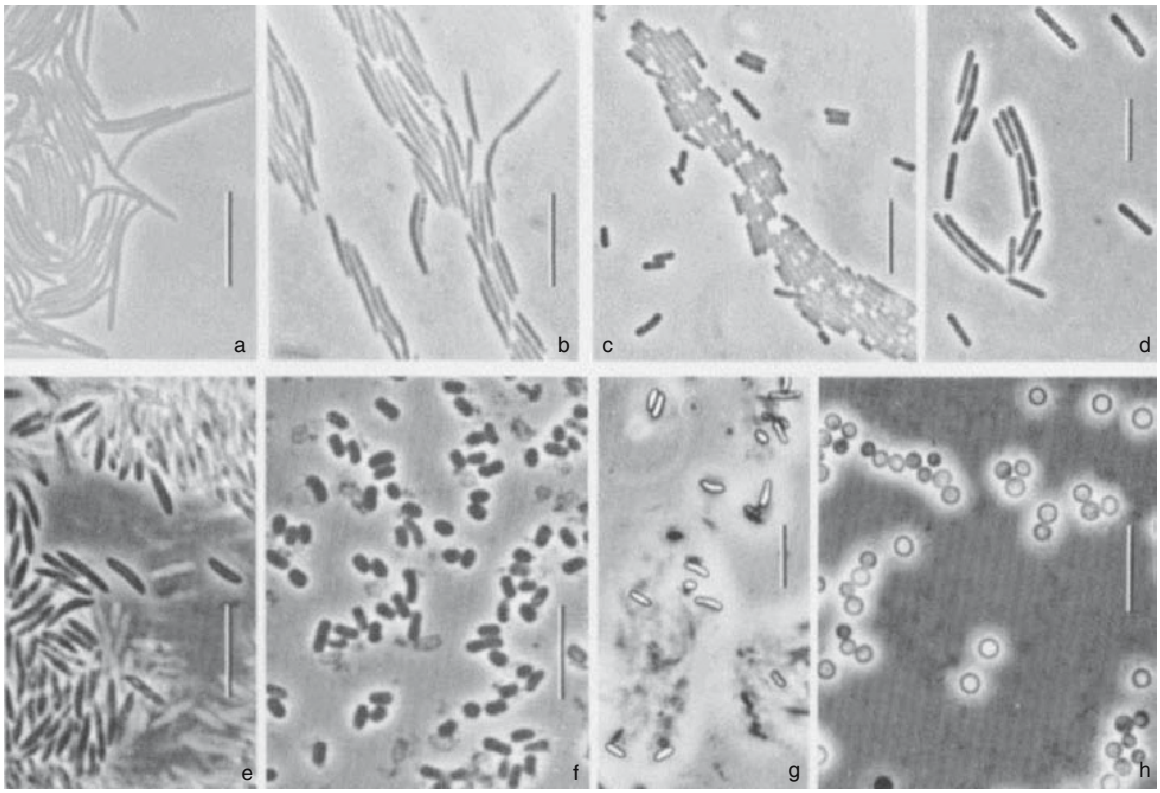


Fig. 1. Various types of myxobacterial cells. (a–d) Vegetative cells. (a and b) The Cystobacterineae type. a) *Cystobacter ferrugineus*, cells from a liquid culture. b) *Stigmatella aurantiaca*, cells in situ on agar surface in a chamber culture. (c and d) The Sorangineae type. c) *Chondromyces crocatus* in a chamber culture. d) *Sorangium compositum* in a chamber culture. Cells of the Nannocystineae resemble those of the Sorangineae. (e–h) Myxospores of members of the Cystobacterineae. e) Myxospores of *Cystobacter ferrugineus* from a crushed, degenerated, *Archangium*-like fruiting body; under oil immersion, the high optical refractility of the myxospores is not apparent. f) Myxospores of *Cystobacter velatus* from a crushed fruiting body sporangiole; oil immersion. g) Experimentally induced myxospores of *Stigmatella aurantiaca* on the agar surface in a chamber culture; under the 40X dry objective, the high optical refractility of the myxospores becomes very conspicuous. And h) myxospores from a *Myxococcus xanthus* fruiting body; the optical refractility of these spherical myxospores is so high that it is recognizable even under oil immersion. All photographs are in phase contrast. Bars = 10 μm.

Several cells can be seen dividing. [2 frames/min. over an approximately 9 hr.; Reichenbach, H. 1966]

12–13. Slime trails of *Chondromyces apiculatus*. The gliding cells leave a visible trail on the agar surface. Cells move slowly when laying the trail, but more rapidly if they move onto a previously laid trail. Cells may join on a trail and move together as a group. [5 frames/min. over a 1 and 2 hr period for *C. apiculatus*; Kühlwein, H. and H. Reichenbach, 1968h]

14–15. Rippling. Cells of the Myxococcaceae characteristically undergo what Reichenbach originally referred to as "rhythmic oscillations." Starting at the center of a swarm of *Chondrococcus coralloides* (now *Corallococcus coralloides*), rhythmically pulsating ripples travel outward. The oscillations periodically stop and start. The phenomenon is shown at a lower and higher magnification. [1 frame/min. for approximately 3 hr and, at a two-fold higher magnification, 8 frames/min. over about 0.5 hr.; Kühlwein, H. and H. Reichenbach, 1968i]

16. Edge of a swarm of *Archangium violaceum*. The cells move in a pack. If a cell occasionally moves away from the pack, it quickly returns. [8 frames/min. over 1.5 hr.; Kühlwein, H. and H. Reichenbach, 1968j]

17. Lysis of bacteria. Living cells of *Sarcina flava* are lysed by *Archangium violaceum*. Note that the cells are lysed at a distance, as evidenced by their loss of phase darkness. Note also that the cells of Archangium seem to head directly for the Sarcina, as if they can detect them at a distance. [4 frames/min. over 3.5 hr.; Reichenbach, H., 1968]

The phylogenetic position of the myxobacteria has been elucidated by a comparison of 16S rRNA (Ludwig et al., 1983; Oyaizu and Woese, 1985) and 16S rRNA gene sequences (Shimkets and Woese, 1992; Sproer et al., 1999). According

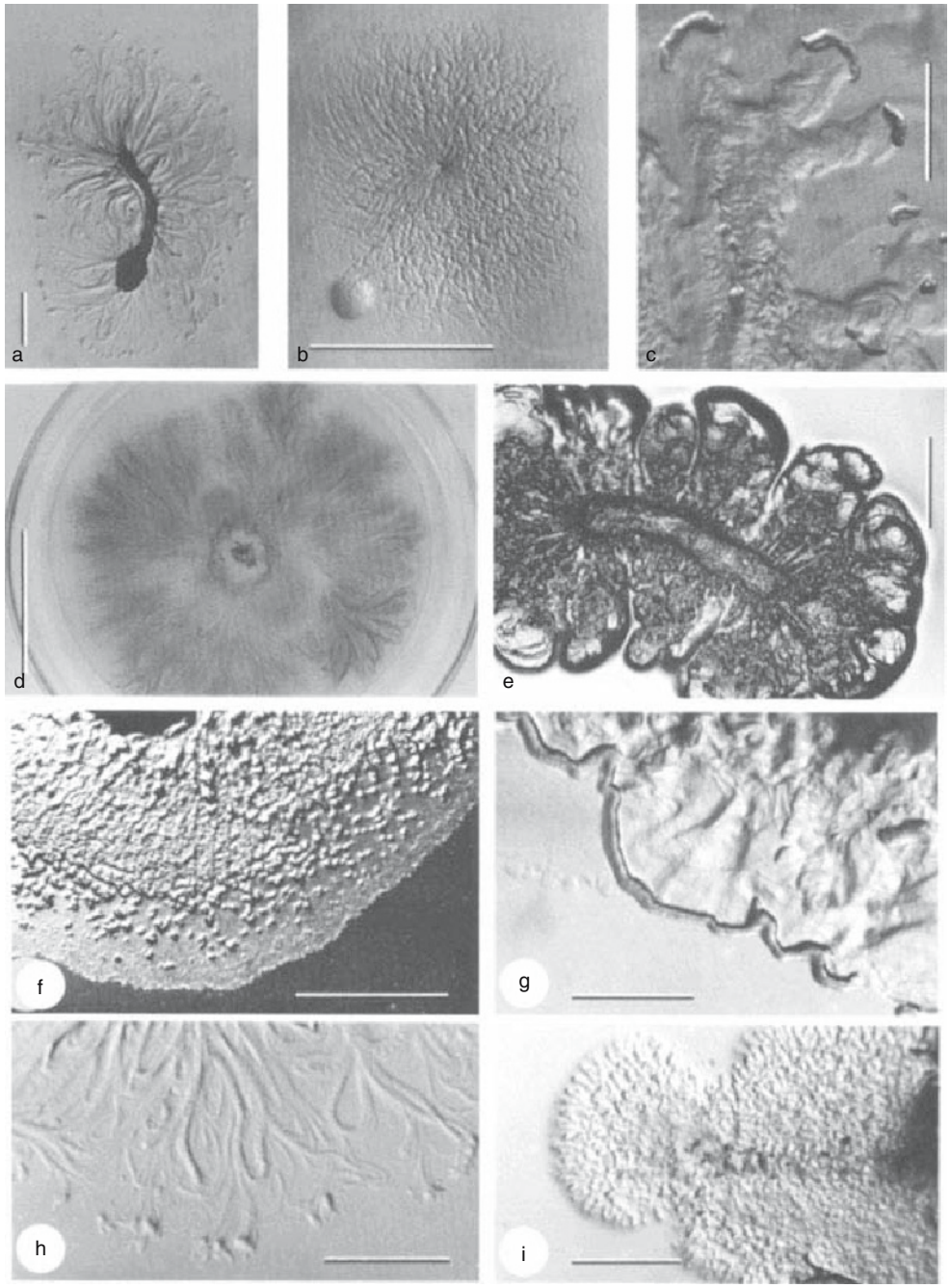


Fig. 2. Swarm colonies of various myxobacteria. (a, b, d, f and h) Swarms of members of the Cystobacterineae. (c, e and g) Swarms of members of the Sorangineae. i) Swarms of members of the Nannocystineae. a) *Stigmatella erecta* on CY agar; dissecting microscope, oblique illumination. Bar = 1 mm. b) *Myxococcus xanthus*, very young colony on Casitone agar; dissecting microscope, oblique illumination. Bar = 0.5 mm. c) *Polyangium* sp. on water agar, swarm edge; the spreading colony falls apart into cell clumps, which separate, thereby etching trenches into the agar surface; dissecting microscope, oblique illumination. Bar = 1 mm. d) *Cystobacter violaceus* (formerly *Archangium violaceum*) on VY/2 agar; from the inoculum in the center, the swarm has spread over the whole agar plate. Bar = 30 mm. e) *Nannocystis exedens* on Casitone agar; the swarm developed from the ridge in the center, and the agar plate has become deeply corroded; Leitz Aristophot. Bar = 5 mm. f) *Corallococcus coralloides* (Reichenbach, 2005) on Casitone agar; the central part of the swarm is covered with rudimentary fruiting bodies; Leitz Aristophot; Bar = 5 mm. g) *Polyangium* sp. on a streak of *Escherichia coli* (which is still recognizable at the left) on water agar; the swarm edge is a compact, ridge-shaped mass of vegetative cells, behind which the agar surface is deeply corroded; dissecting microscope, oblique illumination. Bar = 1 mm. h) *Stigmatella erecta* on Casitone agar, swarm edge with flame-like protrusions; dissecting microscope, oblique illumination. Bar = 1 mm. And i) *Nannocystis exedens* on a streak of *Micrococcus luteus* on water agar; the entire swarm has sunk into the agar, which is deeply corroded; dissecting microscope, oblique illumination. Bar = 2 μm.

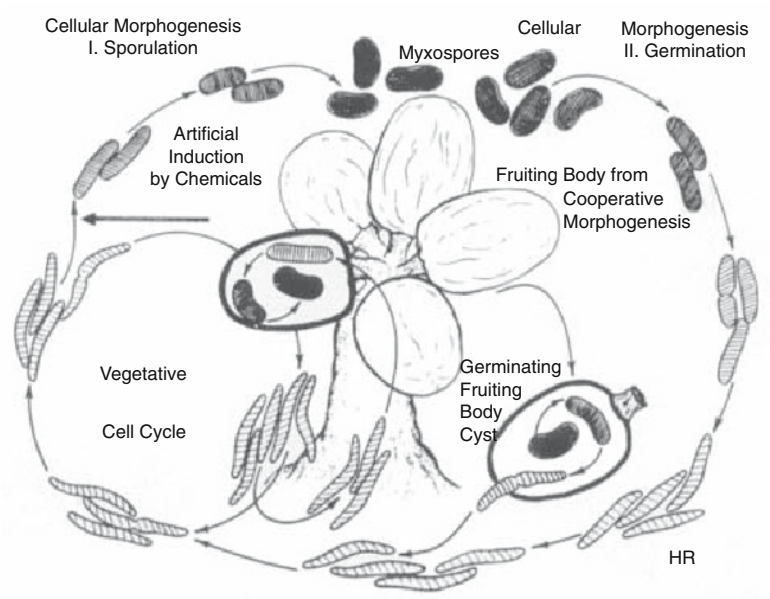


Fig. 3. Cellular morphogenesis in myxobacteria shown with *Stigmatella aurantiaca* as an example. Fruiting body and cells are not drawn to scale. From Gerth and Reichenbach (1978).

to these data, the myxobacteria belong to the Deltaproteobacteria, which also contains the genera *Bdellovibrio*, *Geobacter*, *Pelobacter* (Lonergan et al., 1996), *Anaeromyxobacter* (Sanford et al., 2002) and certain sulfate reducers such as *Desulfovibrio* and *Desulfonema* (Stackebrandt et al., 1988). About 50 species of myxobacteria have been assigned to seventeen different genera (Reichenbach, 2005; see the section Taxonomy of Myxobacteria in this Chapter).

While myxobacterial fruiting bodies had been observed by botanists early in the nineteenth century, they continued to be mistaken for fungi for almost a century. The first report in the scientific literature appears to be by Heinrich Friedrich Link (Link, 1809), who described a little "gasteromycete," *Polyangium vitellinum*, a taxon that is still valid. The description was accompanied by an exact and beautiful illustration (Link, 1809; Ditmar, 1814; see Fig. 14 below). Two more species, *Stigmatella aurantiaca* and *Chondromyces crocatus*, were defined by the British mycologist, M. J. Berkeley, who classified them as hyphomycetes (fungi imperfecti) (Berkeley, 1857). Again, both taxa are still valid. *Stigmatella aurantiaca* was described two more times as a new fungus under different names and with different systematic positions before it was finally recognized as a myxobacterium by the United States botanist Roland Thaxter (1892). Thaxter was the first to elucidate the astonishing and unexpected life cycle of the myxobacteria, which he published, together with descriptions of a substantial number of new species, in a series of brilliant articles (Thaxter, 1892; Thaxter, 1893; T. Thaxter, 1897; Thaxter, 1904). However, the reception by the scientific community was less than enthusiastic, and it took at least 20 years before Thaxter's work became widely accepted. In the first decade of the twentieth century, however, four voluminous studies on myxobacteria were published (Baur, 1905; Quehl, 1906; Vahle, 1910; Kofler, 1913) which deal with many problems that later became important topics of research. One of those four authors later became a very influential geneticist involved in breeding research (Erwin Baur). In the following decades, research was confined mainly to taxonomic, ecological and morphological questions (Jahn, 1911; Jahn, 1924; Krzemieniewska and Krzemieniewski, 1926; 1927a, 1927b; 1930). During that period, new species were discovered and the wide geographical distribution of myxobacteria was recognized. Furthermore, two large subgroups that could be distinguished by the shape of their vegetative cells were recognized (Krzemieniewska and Krzemieniewski, 1928), and some myxobacteria degrade cellulose and could be cultivated on a medium containing only cellulose and a few mineral salts (Imshenetski and Solntseva, 1936; Krzemieniewska and Krzemieniewski, 1937a, 1937b). For some time, cellulose decomposition by myxobacteria was a principal focus of interest, mainly in Russia, where it was studied in the laboratory of A. A. Imschenezki (for a review, see Imshenetski, 1959).

Footnote: In the remainder of the chapter the following abbreviations are used for genera of myxobacteria: *Ar*., *Archangium*; *Cb*., *Cystobacter*; *Cm*., *Chondromyces*; *Cl*., *Corallococcus*; *Ha*., *Haploangium*; *Ko*., *Kofleria*; *Me*., *Melittangium*; *Mx*., *Myxococcus*; *Na*., *Nannocystis*; *Pl*., *Polyangium*; *Sg*., *Stigmatella*; and *So*., *Sorangium*.

Studies utilizing pure cultures of myxobacteria did not become common practice until the middle of the twentieth century because myxobacteria were generally believed to be incapable of growth suspended in liquid media. Until then, physiological studies were tedious and only moderately conclusive, because the myxobacteria grew only as a film along the glass wall of the container and the surface of the liquid. Even under these experimental restrictions some insights were gained into the nutritional requirements of myxobacteria. Norén (1955), Loebeck and Klein (1956), and Mason and Powelson (1958) reported that at least some strains of *Myxococcus* grew well as suspension cultures. Subsequently, many more examples of myxobacteria growing in a dispersed manner have been described. Ironically, it later turned out (Dworkin, 1984) that dispersed growth of *Myxococcus* (*Mx.*) *xanthus* strains had been observed as early as 1948, but this information had never been published (Woods, 1948).

Beginning in the 1950s, the emphasis of myxobacterial research shifted to problems in developmental biology, with *Mx. xanthus* as the preferred subject. A major breakthrough was the discovery by Dworkin and Gibson (1964a) that vegetative cells of *Mx. xanthus* in suspension cultures could be induced to convert into myxospores by the addition of certain chemicals, such as 0.5 M glycerol. This was the first clearly defined experimental system for the investigation of a morphogenetic process in a myxobacterium, and it led to a long series of studies on the morphological, physiological and biochemical events that take place during induced myxospore formation.

Genetic analysis began with the development of systems for the transfer of plasmids, transposons and genes into and between strains of *Mx. xanthus*, based on the use of coliphage P1 (Kaiser and Dworkin, 1975; O'Connor and Zusman, 1983; Shimkets et al., 1983) and *Mx. xanthus* phages Mx4 and Mx8 (Campos et al., 1978; Martin et al., 1978). The availability of these genetic techniques made *Mx. xanthus* accessible as a model system for the study of morphogenetic processes and gliding motility. While these methods are not easily adaptable to other myxobacteria, a more versatile system that uses conjugation with *Escherichia coli* for the transfer of plasmids and transposons has been successfully applied to other myxobacterial genera (Breton et al., 1985). Progress in myxobacterial research has gained considerable momentum. The discovery that many myxobacterial strains produce secondary metabolites, most of which are novel compounds, has stimulated an even broader interest in myxobacterial biochemistry, physiology and taxonomy (for reviews, see Reichenbach and Höfle [1993] and Reichenbach [2001]).

Our knowledge of the myxobacteria has been summarized repeatedly in recent years. Apart from the articles mentioned above, the following reviews should be consulted for further details: 1) for books, Rosenberg (1984), Dworkin and Kaiser (1993), and Brun and Shimkets (1999); 2) for taxonomy, Reichenbach (2004); 3) for developmental biology, Shimkets (1990b), Shimkets (1992), Shimkets (1999), Zusman (1991), Kaiser (1993), Kaiser (1996), Kaiser (1998), Kaiser (1999), Kaiser (2001), Dworkin (1996), Crawford and Shimkets (1999), and Bonner and Shimkets (2001); 4) for genomics, Shimkets (1997a), Shimkets (1997b), and Ryding and Shimkets (2000); 5) for motility, Shi and Zusman (1993a), Hartzell and Youderian (1995), Youderian (1998), Ward and Zusman (1999), Kaiser (2000a), and Kearns and Shimkets (2001a). In addition, several movies are available from the Institut für den Wissenschaftlichen Film, Göttingen, Germany, that illustrate cell behavior, colony formation, and developmental processes in various species of myxobacteria (Reichenbach et al., 1965d; Reichenbach et al., 1965e; Reichenbach et al., 1965f; Reichenbach et al., 1965g; Reichenbach et al., 1980; Grimm et al., 1971; Kühlwein et al., 1971a; Kühlwein et al., 1971b).

Myxobacterial Ecology

The essential questions with regard to the ecology of any particular group of organisms are: Where are they? What is there? What do they do? How do their characteristic biochemical, physiological and developmental properties relate to their behavior in nature? With what do they interact?

Habitats

With regard to the first two questions—Where are they? and What is there?, a great deal of anecdotal and subjective information has been compiled on the habitats of the myxobacteria (e.g., Dawid, 2000; Reichenbach and Dworkin, 1992) since their recognition as bacteria by Roland Thaxter in 1892. Until recently (Dawid, 2000), however, these data have not been subjected to any sort of systematic analysis.

In addition, a number of other factors have made it difficult to draw useful quantitative descriptions. In view of the multicellular nature of the fruiting body, which may contain 10^4–10^6 resting cells, it is difficult, if not impossible, to determine the number of myxobacteria in a natural sample. Does a sample that contains one fruiting body represent 1 or 10^4–10^6 organisms?

Table 1. Convenient and preferential sources for the isolation of different myxobacteria.[a]

Substrate	Myxobacterial species typically found[b]
Soil	*Nannocystis exedens*,[5] *Sorangium cellulosum*,[4] *Archangium serpens*,[4] *Corallococcus coralloides*,[4] *Polyangium* spp.,[3] *Cystobacter* spp.,[3] *Melittangium* spp.,[3] *Myxococcus fulvus*,[2] *Mx. virescens*,[2] and *Mx. stipitatus*[2]
Dung of herbivores	*Myxococcus fulvus*,[5] *Corallococcus coralloides*,[5] *Mx. virescens*,[4] *Cystobacter fuscus*,[4] *Cb. ferrugineus*,[4] *Archangium serpens*,[4] *Nannocystis exedens*,[3] *Cb. violaceus*,[3] *Polyangium* spp.,[3] *Stigmatella erecta*,[2] *Mx. xanthus*,[2] *Melittangium* spp.,[2] and *Cb. velatus*[1]
Bark and rotting wood	*Stigmatella aurantiaca*,[4] *Chondromyces apiculatus*,[4] *Sorangium cellulosum*,[4] *Corallococcus coralloides*,[4] *Myxococcus fulvus*,[3] *Cm. pediculatus*,[2] and *Haploangium* spp.[2]

[a]The frequency of myxobacteria on a specific substrate may vary substantially in different environments. Furthermore, some species may be underestimated from a particular source because the isolation technique usually applied may not result in the isolation of a particular organism.

[b]Frequency of the encountered species: [5], ubiquitous; [4], very frequent; [3], moderately frequent; [2], relatively rare; and [1], rare.

The principal habitats of myxobacteria are soil, dung, decaying plant material, and the bark of living and dead trees. Nevertheless, myxobacteria have been isolated from marine environments as well as from a variety of climatic zones (Dawid, 2000). However, since the myxospores may tolerate considerable environmental extremes, and since most isolation techniques involve the cultivation of samples that have been extensively dried, it has not been possible to determine, in many cases, whether the myxobacteria were there adventitiously and simply survived as spores or were growing and thriving in that environment (Reichenbach, 1999). In a few cases, the isolated organisms were tested for their ability to grow under conditions similar to those in which they were isolated. Occasionally, they were able to (Dawid et al., 1988) but more often were not (Rückert, 1983). Iizuka et al. (1998) have isolated two myxobacteria from marine environments, which have been subsequently characterized and assigned to the new genus *Haliangium* (Fudou et al., 2002). The organisms were reported to require 2% NaCl for their growth. However, since this measurement was made on the basis of colony swarm expansion rather than growth per se, the claim must be viewed with caution.

A survey of common sources for different myxobacteria is given in Table 1. Most myxobacteria appear to be primarily soil organisms. However, almost all are "micropredators" (Singh, 1947) and are attracted by habitats with rich microbial communities, which they may colonize as secondary substrates. Even if certain myxobacteria can be isolated more easily from sources other than soil, their presence in the soil of the same environment can often be demonstrated. The preferred substrate of a particular species may vary under different climatic and edaphic conditions. Thus, in the central United States, *Chondromyces* species are common inhabitants of bark and rotting wood but are rare in soil (Nellis and Garner, 1964; H. Reichenbach, unpublished observations). In India, they are found regularly in soil, in the rhizosphere of plants, and on rotting wood, bark, and dung (Agnihothrudu et al., 1959; Singh and Singh, 1971). In Europe, in spite of apparently similar climatic conditions to those found in the United States, they appear to be extremely rare organisms and have been found almost exclusively on dung, rotting wood, and bark (Zukal, 1896; Quehl, 1906; Jahn, 1924; Krzemieniewska and Krzemieniewski, 1946). Dawid (1979) tested several thousand samples from the Siebengebirge, a mountain ridge near Bonn, Germany, and obtained only one isolate of *Chondromyces*, a strain of *Cm. apiculatus*, from a piece of rotting wood. Similar results have been obtained by Krzemieniewska and Krzemieniewski (1946) and by one of us (H. Reichenbach) who isolated myxobacteria over decades from all kinds of samples collected at many different sites in Europe.

Myxobacteria are very common in soils of neutral or slightly alkaline pH (Krzemieniewska and Krzemieniewski, 1927b; Norén, 1950; Norén, 1952; Sabados-Saric, 1957; Kühlwein, 1960; Brockman and Boyd, 1963; Peterson, 1965; Peterson and Norén, 1967; Michoustine, 1968; McCurdy, 1969b; Rückert, 1975b; Rückert, 1976; Rückert, 1979; Brockman, 1976; Hook, 1977; Rückert and Heym, 1977; Dawid, 1978; Dawid, 1979). They have been isolated from samples collected in tropical rain forests and in the arctic tundra, in steppes, deserts, and in bogs, at sea level as well as at high altitudes. Warm and seasonally dry areas, such as the southwestern United States, Mexico, northern India, the Mediterranean countries, and the Canary Islands, have been found to be especially rich in different myxobacteria. The same is true for areas with underground limestone in temperate climates. Still, a myxobacterial species may show an unequal distribution even within the same climatic zone. The case of *Chondromyces* has just been mentioned. Another example is *Sg. auran-*

tiaca, which is common in the middle of the North American continent (Nellis and Garner, 1964; McCurdy, 1969b; Reichenbach and Dworkin, 1969a), but very rare in Europe (Krzemieniewska and Krzemieniewski [1946] isolated myxobacteria in Europe 15 years before they found their first strain of *Sg. aurantiaca*). The decisive factor that controls the distribution may be the warm, humid American summers, which are usually in contrast to those in Europe. For unknown reasons, *Sg. aurantiaca* seems to be restricted to rotting wood and bark on both continents. Locations with acid soils and raw humus underground, such as the *Rhododendron* forests of West Virginia (H. Reichenbach, unpublished observations), can be totally devoid of myxobacteria. But under otherwise favorable conditions such as sufficiently high humidity and temperature, myxobacteria have also been isolated from soils with a bulk pH of below 5 (Rückert, 1975b; Rückert, 1979). In cool, humid environments, such as in Finland, northern Minnesota, and mountain ranges, myxobacteria may be abundant, albeit limited to a few species. Tropical rain forests are not necessarily rich in myxobacteria, perhaps owing to the nature of the underground material. Myxobacteria have also been isolated from Antarctic soil samples but at least in one case, the organisms had a temperature optimum around 30°C, which raises some doubts as to whether they were really indigenous (Rückert, 1985). In another instance, however, the isolated bacteria were true psychrophiles that developed, very slowly indeed, at temperatures between 4 and 9°C but did not grow at 18°C (Dawid et al., 1988). While the vegetative stages of the latter organisms closely resembled those of myxobacteria of the *Polyangium* and *Nannocystis* type, their identity could not be established with certainty since they did not produce fruiting bodies. No mesophilic myxobacteria were found in the Antarctic samples examined by Dawid et al. (1988).

Dung of various animals, especially of herbivores such as rabbits, hares, deer, moose, sheep and goats, is an excellent substrate for myxobacteria. Rabbit dung was the preferred source of the early myxobacteria investigators and appears to serve as natural bait. Apparently, however, myxobacteria can also pass unscathed through the digestive tract of animals (Kühlwein, 1950). Also, the observation of myxobacteria on dung pellets collected on the surface of deep snow layers suggests that, occasionally, they were deposited with the dung (Rückert, 1975b; H. Reichenbach, unpublished observations). However, the organisms primarily appear to arise in the surrounding soil after the dung has been dropped. This conclusion is based on the fact that aged dung is a better source of myxobacteria than is fresh dung, that dung collected on soil rich in myxobacteria results in a greater yield of strains than one from poor soil, and that the same species found on dung can also be found in the surrounding soil. A number of myxobacteria are regularly found on dung, such as *Mx. fulvus*, *Mx. virescens*, *Cl. coralloides*, *Cb. fuscus* or *Sg. erecta*, but no myxobacterium depends on dung-derived growth factors, as was thought for some time, and all strains isolated from dung can be cultivated on relatively simple media. Of course, occasional stimulation by substances such as vitamins found in dung extracts is conceivable.

Bark and rotting wood are good sources of certain myxobacteria. This includes the cellulose degraders of the genus *Sorangium*, but several noncellulolytic species are also regularly found in those habitats. Results vary with different species of trees: In Europe, relatively good yields have been reported with bark from living elder (*Sambus racemosa*), beech (*Fagus silvatia*), and black locust (*Robinia pseudacacia*; Dawid, 1979). In Minnesota and other locations in the United States, bark and rotting wood are reliable sources for *Stigmatella aurantiaca* and *Chondromyces apiculatus*. The latter has also been found regularly on wood samples from southeast Asia and on dry, rotten "leaves" of *Opuntia* cacti from the Canary Islands (H. Reichenbach, unpublished observations). The difference from the situation in Europe has already been pointed out. Clearly, other less obvious factors, perhaps climatic ones, also play a role in the distribution of myxobacteria. However, probably no myxobacteria have an absolute dependence on bark and wood. The cellulose degraders are also common in soil and dung, and because the other species are bacteriolytic, they often grow well on dung and in culture on media that contain peptone or protein. In fact, why in nature they grow preferentially on wood and bark is not understood. Even when they are found on dung, they may be there mainly because the dung of herbivores is always abundant in plant residues. However, *Haploangium* has only been found on bark and wood so far. It has never been cultivated, so its nutritional requirements are unknown.

Although myxobacteria have repeatedly been isolated from freshwater habitats (Geitler, 1925; Jeffers, 1964; Brauss et al., 1967; Brauss et al., 1968; Gräf, 1975; Hook, 1977; Trzilová et al., 1980; Trzilová et al., 1981), they probably are merely washed in from the soil. Myxobacteria have been used to classify rivers for the type of pollution present (Gräf, 1975; Trzilová et al., 1980; Trzilová et al., 1981). While myxobacteria appear not to be typical aquatic organisms, our experience with cultivated strains suggests that they can survive and thrive in suitable aquatic

niches. A clear indication of this is the case of a myxobacterium that parasitizes the green alga *Cladophora*, with fruiting body formation inside the emptied-out algal cells (Geitler, 1925). Another example may be the occurrence of structures resembling myxobacterial fruiting bodies on eggs of the water beetle *Dytiscus marginalis* (Jackson, 1959).

Dawid (2000) has compiled data on myxobacteria isolated from 1398 samples collected over an 18-year period from all continents. Representatives of 30 species and 11 of the 12 known genera of myxobacteria were isolated (only *Haploangium* was not). Approximately 20,000 strains representing 17 species were isolated from a limited area in Germany alone. Among the 55 nations and 9 states of the United States, myxobacteria were ubiquitous. The genera most frequently encountered were *Myxococcus*, *Corallococcus* (Reichenbach, 2005) and *Archangium*. Whether this reflects their true predominance or the bias of the isolation procedure is unclear.

Myxobacteria were found most commonly in environments with pH 6–8.

The search for new organisms able to produce novel and useful bioactive compounds has stimulated attempts to isolate bona fide marine myxobacteria. While isolation of myxobacteria from marine environments has been reported (Brockman, 1967; Rückert, 1975c; Dawid, 1979), in no case have these authors claimed that the organisms require or even can grow in the presence of the concentration of NaCl found in seawater. Several recent reports show strains with moderate halotolerance (Iizuka et al., 1998; Iizuka et al., 2003a; Iizuka et al., 2003b; Li et al., 2002).

A most unusual myxobacterium, *Anaeromyxobacter dehalogenans*, which is outside the bounds of what is usually defined as a myxobacterium, has recently been described (Sanford et al., 2002). This organism is an anaerobe that uses acetate as an electron donor and a variety of chlorinated phenols as electron acceptors. While it has not been shown to produce fruiting bodies, it is a characteristically pigmented, Gram-negative rod that moves by means of gliding motility. The result of 16S rRNA/DNA analysis places it within the Myxococcales as a separate genus. Clearly, the taxonomic and phylogenetic boundaries of the myxobacteria have not yet been reached or even defined.

Ecologically Relevant Characteristics of Myxobacteria

The features that are characteristic of the myxobacteria are gliding motility, a complex life cycle culminating in the formation of fruiting bodies and myxospores, the ability to degrade insoluble macromolecules, and the production of an extensive battery of bioactive compounds. Each of these properties plays an important role in establishing the particular ecological function of the myxobacteria.

Degradation of Insoluble Macromolecules

In the grand scheme of molecular turnover in nature, bacteria play the major role. And myxobacteria have long been known to play an important part in the degradation of such insoluble macromolecules as protein, cellulose, peptidoglycan, lipids and nucleic acids as well as intact cells, both eukaryotic and prokaryotic, and their subcellular fragments (see the section Lysis of Other Bacteria in this Chapter). They do so by excreting a powerful battery of hydrolytic enzymes. As such, however, they are at the mercy of the diluting effect of diffusion of their enzymes away from the cell and the effect of hydrolyzed products diffusing back toward the cell. Thus, the suggestion on theoretical grounds that *Mx. xanthus* when feeding on macromolecules behaves as a bacterial "wolf pack" (Dworkin, 1973a), seeking at all times to optimize its feeding by maintaining a high cell density, has been experimentally demonstrated: *Mx. xanthus* can feed on hydrolyzed protein quite efficiently at a low cell density but can only utilize the equivalent high molecular weight protein when at a high cell density (Rosenberg et al., 1977). The characteristic swarms of migrating cells accomplish this, and the fruiting body may be a mechanism for maintaining the resting myxospores at a high cell density in preparation for future germination and feeding.

The Function of Gliding Motility

Myxobacteria exist in nature on surfaces—soil particles, leaf surfaces, bark and other detritus. Thus, flagellar motility through an aqueous milieu is likely to be of little value. The ability to crawl over a surface, on the other hand, seems well suited for the myxobacterial life style. Thus, their gliding motility is consistent with their ecological niche.

Developmental Aspects of Myxobacterial Ecology

The myxobacteria, with the exception of the taxonomically problematic *Anaeromyxobacter dehalogenans*, are aerobes and are found on surfaces or close to the surface of soil. As such, they are exposed to the vicissitudes of desiccation, irradiation and temperature extremes. Myxospores

while do not possess the extraordinary resistance of endospores, but they are sufficiently resistant (Sudo and Dworkin, 1969) to withstand the kinds of extremes they are likely to encounter in normal environmental situations. The fruiting body can be assumed to maintain the resting cells packed closely enough together so that upon germination they can begin feeding at the optimally high cell density. The sporangioles, whether multiple on a stalk (as in *Stigmatella* or *Chondromyces*), or single on a stalk (as in *Haploangium* or *Melittangium*), or lying on the substrate (as in *Cystobacter*, *Polyangium*, *Sorangium* or *Nannocystis*), may be simply an optimal packaging mechanism of the resting myxospores.

Myxobacterial Bioactive Compounds

The myxobacteria produce an astounding variety of bioactive compounds (Reichenbach, 1989; Reichenbach, 1993; Reichenbach, 2001; Reichenbach and Höfle, 1998). Among over 2000 bacteriolytic strains of myxobacteria, 55% produce bioactive compounds; among over 700 cellulolytic myxobacteria, 95% produce bioactive compounds (Dawid, 2000). These include inhibitors of protein synthesis as well as compounds interfering with electron transport, nucleic acid synthesis, and actin and tubulin synthesis. Their structures include polyketides, linear and cyclic peptides and heterocyclic molecules. Clearly, when the group is fully characterized, the spectrum and number of bioactive compounds produced will rival those produced by the Actinomycetes.

The biological function of antibiotics is equivocal. While antibiotics might assist a soil organism in its struggle for nutritional "lebensraum," the matter has not been settled. How then do the myxobacteria interact with other myxobacteria and other bacteria in nature?

Lytic Activities

The ability of the myxobacteria to kill and lyse a wide variety of Gram-positive and Gram-negative bacteria, yeast, fungi, algae and protozoa was noticed early and often and came to be considered as a characteristic property of the group (see the section Lysis of Other Bacteria in this Chapter). Thus they might reasonably play some sort of role in nature, either to control other microbial populations or to clear the soil of the carcasses of other dead or dying cells. In fact, Callao et al. (1966) demonstrated that after *Mx. xanthus* and *Azotobacter* were simultaneously inoculated into soil, the number of *Azotobacter* decreased substantially.

Symbiosis

Myxobacteria have been shown to exist in a close symbiotic association with other bacteria. Early on, Pinoy (1913) demonstrated that a culture of *Cm. crocatus*, which constructs the most complex of all the myxobacterial fruiting bodies, existed in a close association with a non-myxobacterial companion. This matter was more carefully examined and defined by Jacobi et al. (1997), who showed that the growth of almost all strains of *Cm. crocatus* examined depended on a small rod-shaped bacterium tentatively identified as belonging to the genus *Sphingobacterium* present in the sporangioles of the fruiting body.

Myxobacterial Interactions and Territoriality

When observing the multicellular behavior of the myxobacteria, an obvious question is how able or inclined they are to interact with other myxobacteria. Will related species form mixed fruiting bodies? Will their migrating swarms fuse? And if not, what is the basis upon which the myxobacterial cells distinguish between self and non-self? Do the myxobacteria establish territories? These questions were examined by Smith and Dworkin, who observed the interactions between feeding and developing swarms of *Mx. xanthus* and *Mx. virescens* (Smith and Dworkin, 1994).

A common observation in natural samples of myxobacterial fruiting bodies is that the localized areas in which they occur contain only one species of fruiting body, i.e., the myxobacteria do indeed seem to establish an exclusive territory. When cultures of *Mx. xanthus* and *Mx. virescens* were mixed and placed under fruiting conditions, the cells sorted themselves out and established separate fruiting body territories. And the contents of the individual fruiting bodies were limited to a single species. Furthermore, *Mx. virescens* was able to produce an extracellular material that, when applied to *Mx. xanthus*, caused spheroplasting and loss of viability. The toxic material had no effect on other bacteria and its properties were consistent with those of bacteriocins, which have previously been described for the myxobacteria (McCurdy, 1974). Thus, the myxobacteria do establish territoriality, the cells of different species do not mix, and one mechanism of exclusion may be myxobacterial bacteriocins (Smith and Dworkin, 1994).

Isolation

Although myxobacteria are common in many types of soil (a teaspoonful of soil is usually sufficient to isolate four or five species), they are

rarely mentioned in articles on soil microbiology. The reason is that the usual dilution and plating techniques used for the isolation of soil microorganisms are unlikely to reveal the presence of myxobacteria. Owing to the slime matrix they produce, myxobacterial cells do not disperse easily when the soil sample is shaken in water, so that the organisms are highly underrepresented in number when the suspension is plated. Even when other, more suitable methods are used, the estimated numbers probably are only approximate ones; depending on the type of soil, they are in the range of 10^3 to 4.5×10^5/g and thus under favorable conditions seem to be a rather substantial component (Singh, 1947; Sabados-Saric, 1957; McCurdy, 1969b). Another problem is that on lean media the myxobacterial colonies develop as easily overlooked delicate, spreading swarms, while on rich media they remain compact and are not recognized as myxobacteria. In addition, they grow relatively slowly so they are often overgrown by other soil microorganisms, particularly fungi. The fruiting bodies, however, are conspicuous and have probably been frequently observed by soil biologists. The use of available nucleotide probes and fluorescent in situ hybridization techniques should make it possible now to arrive at a more realistic appraisal of myxobacterial numbers.

The isolation of myxobacteria can start from fruiting bodies that have developed on natural substrates or from swarms growing in agar cultures. These isolations can also be preceded by enrichment and baiting techniques. The methods have frequently been reviewed (Kühlwein and Reichenbach, 1965; McCurdy, 1969b; Peterson, 1969).

Collection and Treatment of Samples

Soil to be used for the isolation of myxobacteria should be taken from the upper few centimeters of the soil profile. Soils rich in higher organisms, and samples collected from between plant roots and near the base of stems, give the best yields. If the material cannot be processed soon after collection, it should be air-dried as quickly as possible because otherwise the sample may become moldy, creating problems later during isolation. A few cubic centimeters of soil are sufficient for most isolation techniques. In general, it is better to have several small samples from different localities than one large batch from a single spot. Dry material can be stored for long periods at room temperature. Apparently, in the natural substrate, myxobacteria are much more stable than, for example, fruiting bodies dried on filter paper. Thus, we have found essentially the same species over a 12-year period of sampling of stored soil samples, and we are regularly able to isolate myxobacteria from samples stored 10–15 years (H. Reichenbach and M. Dworkin, unpublished observations).

With dung, better results are usually obtained if the samples are neither very fresh nor very old, but are completely free of low-molecular weight components. It is very important to dry the samples if they have to be stored for more than one day, and such dry dung will yield myxobacteria even after months and years of storage. Rabbit dung to be used for baiting (see below) should be taken from wild animals; pellets from laboratory or domestic rabbits are usually unsuitable, perhaps because they become soaked with urine in the cage.

Bark and wood for the isolation of myxobacteria must be taken from tree species that are low in resins and tannin compounds, and specimens have to be quickly dried if they are not to be processed immediately. Successful isolations are often obtained with wood that has already been decomposed to a rather soft state and with bark from the base of the tree and from fallen trees.

Direct Isolation from Natural Substrates

Isolation can often be achieved directly from the natural substrate. Bark of living or dead trees, rotting wood, or dung pellets are kept in large Petri dishes lined with two to three layers of filter paper. The samples are soaked in distilled water containing cycloheximide (up to 0.08 mg/ml) for a few hours to suppress the growth of molds. The water is then decanted and appropriate amounts of water are added at intervals during cultivation. If the culture is kept too dry, it may soon be covered with molds; if the substrates are inundated, no myxobacterial fruiting bodies will develop. The air in the incubator should be kept sufficiently humid so that the crude cultures do not dry out too quickly. The initially saturated system is allowed to dry out gradually over 14–21 days; in this way it will eventually pass through the optimum for the development of myxobacterial fruiting bodies. Such crude cultures are likely to contain a variety of fungi, mites, springtails, nematodes, and other organisms that may contaminate other cultures. The danger of a contamination with mites is especially serious and can quickly spoil a culture collection. The cultures should therefore be kept in tightly closed plastic bags or, more conveniently, in a separate incubator, which should frequently be sterilized.

The cultures can be incubated at room temperature (around 20°C) or at 30°C. We have never observed unequivocal differences in the yield of myxobacteria when parallel crude cultures were kept at different temperatures, but we have found that at room temperature, the development

of myxobacteria proceeds more slowly and molds tend to spread more vigorously. Thus, we keep such cultures at 30°C (see also Krzemieniewska and Krzemieniewski, 1927b). However, samples from mountains at high altitudes or from arctic environments may contain myxobacteria that are adapted to lower temperatures (see the section Habitats in this Chapter), and even in forest soils in Central Europe, strains are occasionally found that grow reasonably well at 30°C but produce fruiting bodies only at lower temperatures.

Initially, the crude cultures should be inspected for fruiting bodies every day, beginning with the third or fourth day of incubation, because fruiting bodies often spring up and then rapidly collapse and become very inconspicuous. Also, the fruiting bodies may soon become overgrown by other organisms. Most fruiting bodies will appear during the first 10 days, either on the substrate itself or on the filter paper lining; only rarely will anything of interest develop later than 18–20 days. Sometimes, fruiting bodies appear very early on the substrate, within hours after moistening; probably, in such cases, desiccated structures have simply been rehydrated and thus have become recognizable, while the fruiting bodies that appear later are produced de novo. In the later phases of culture development, spherical or ridge-shaped masses of vegetative cells, which are usually bright yellow, orange, or red, can often be observed on the filter paper and can be used for isolation; in this way one may obtain species whose fruiting bodies are too small and inconspicuous to be recognized directly on the substrate. These organisms are almost always members of the Sorangineae, usually *Polyangium* and *Nannocystis* strains, which produce swarms with a massive ridge at the edge. Sometimes, cellulose decomposers may develop on the filter paper lining itself.

Isolation from Soil by Baiting

A baiting technique can be used to isolate myxobacteria from soil (Krzemieniewska and Krzemieniewski, 1926). A large Petri dish is filled with soil, which is moistened with distilled water up to its water-holding capacity. Autoclaved dung pellets from wild rabbits are partly buried in the soil. The culture is then incubated and examined as described above. Vegetative cells will migrate to the dung pellets, colonize them, and in time develop the characteristic fruiting bodies. Here, as with the technique described above, the discovery of the myxobacterial fruiting bodies is greatly facilitated by their tendency to appear locally in large numbers and by their brightly colored and glistening appearance. With some experience, they can be located quickly with the unaided eye, despite their relatively small size (50–500 µm). For a more careful examination of the culture, a dissecting microscope with incident illumination and a magnification between 10 and 40X is perfectly adequate (see Fig. 2).

Isolation from Swarms

The bacteriolytic properties of myxobacteria can be used for their enrichment from natural substrates, particularly from soil and plant debris. Streaks of living food organisms (e.g., *E. coli*) on water agar (WAT agar, see below), in the form of a cross-streak, three parallel streaks, or a number of circular patches, are inoculated with a small quantity (approximately the size of a lentil) of the material to be analyzed (Singh, 1947). The cross and the patches are inoculated in the center, and the parallel streaks at the ends. Care should be taken not to scatter the inoculum over the plate. To facilitate application of the sample, the soil may first be moistened with sterile water and then applied with sterile swabs.

To restrict the development of fungi, the addition of the antibiotic cycloheximide (25–100 µg/ml) to the enrichment medium has been recommended (WCX agar, see below; Brockman and Boyd, 1963). Results are indeed superior when cycloheximide is included, and the cultures can be maintained for a longer period. The antibiotic does not control the growth of amoebae, a major nuisance with this isolation technique. Also, it does not result in the selection of particular myxobacteria or in the suppression of any other gliding bacteria, such as *Cytophaga*-like bacteria, flexibacters, *Taxeobacter*, or *Herpetosiphon*, which can also be isolated with this method.

WAT Agar and WCX Agar

$CaCl_2 \cdot 2H_2O$	0.1% (w/v)
Agar	1.5% (w/v)
HEPES	20 mM

Prepare media in distilled water and adjust pH to 7.2. Autoclave the medium. Then, add 25 mg of cycloheximide per ml from a filter-sterilized stock solution, yielding WCX Agar. As the pH is very difficult to adjust in this completely unbuffered medium, addition of, e.g., 20 µM *N*-2-(hydroxy-ethyl)piperazine-*N*′-2-ethane-sulfonic acid (HEPES), is advisable.

Many bacteria and yeasts can be used as food organisms by the myxobacteria. Food organisms that are readily recognized and can be easily eliminated later are, of course, preferred. Selection for myxobacteria works better if the prey microbes used are alive rather than dead. In the first study, an *Aerobacter* strain was used as the food organism (Singh, 1947). We have found the following bacteria to be useful: 1) *Micrococcus luteus* is easy to distinguish, is nonmotile, and is relatively large, so that it is not readily spread

around; while it is not well utilized by soil amoebae, not all strains of myxobacteria, specifically *Nannocystis* strains, grow on it. 2) *Escherichia coli* is a favorable substrate for myxobacteria, including *Nannocystis*, which can be subcultured indefinitely on it; but soil amoebae also use it well, and this sometimes makes *E. coli* inconvenient. 3) Autoclaved yeast, *Saccharomyces cerevisiae*, can be cheaply prepared from commercial bakers' yeast; the pH of the suspension must be adjusted to 7.0, since the yeast cake is usually acidic; unfortunately, the autoclaved yeast is also a good substrate for many contaminants. Generally, living *E. coli* is our preferred food organism.

The cultures are incubated at 30°C and checked under a dissecting microscope for the appearance of myxobacterial swarms and fruiting bodies, at first daily, beginning on the second or third day, and later at longer intervals (Fig. 2). After about 3 weeks, no additional myxobacteria are likely to appear, and the cultures can be discarded. Most myxobacteria appear within 8–14 days. The myxobacteria grow first on the lawn of the food organism, but later often spread over the agar surface between the smears. A fast-spreading organism such as *Cystobacter* may reach the end of a streak within 1–2 days. If two streaks are made in a crossed pattern, different myxobacteria may develop on each of the four arms. Different organisms also may follow one another on the same streak. As a rule, species of the Cystobacterineae are seen first, while the Sorangineae develop later. Between two and five different myxobacteria can be expected on one plate. Three parallel plates are usually sufficient to secure most of the myxobacterial types that are readily isolated from a sample, with the exception of the cellulose degraders. Fruiting bodies are often produced within the swarms after a couple of days. Fruiting bodies may also appear on the soil crumbs or other particles of the inoculum. To detect the delicate, film-like, swarms of myxobacteria, one must use a dissecting microscope with a tilting mirror so that light can be applied at a shallow angle to the surface of the plate.

The sooner a swarm is recognized, the more easily a myxobacterium can be isolated, as at the beginning, the contaminants may still be confined to the site of the inoculum. Transfers are then made from the swarm edge, which is usually the purest part of the colony.

A disadvantage of isolating myxobacteria from the swarm stage is that most species cannot be identified by their swarm morphology. With some experience, however, at least certain genera or types can be differentiated (Fig. 2). The ubiquitous *Nannocystis*, for example, produces a network of trails outside the streak of the food organism. These trails are deeply etched into the agar and end with a small cluster of cells. On the streak, there is often a heavy, meandering ridge. *Polyangium* shows a similar pattern, but with coarser structures. Swarms with an edge that consists of a massive, often brightly colored ridge are in general typical for the suborder Sorangineae. *Cystobacter* and *Archangium* swarms are usually tough slime sheets with a pattern of delicate, but conspicuous, branched, radiating veins. The swarms of the Myxococcaceae tend to be smooth, soft-slimy sheets, often with dense fields of tiny waves or ripples. If there are radial veins, they typically are wavy and meandering. Swarms of *Mx. stipitatus* show a bright yellow fluorescence in ultraviolet (UV) light of 366-nm wavelength (Lampky and Brockman, 1977). The shape of the vegetative cells under the microscope may also provide a clue to the identification of the swarm observed.

Isolation by Direct Plating of Heated and Dried Soil on Antibiotic-containing Media

Karwowski et al. (1996) have described a method for the isolation of *Myxococcus* from soil by plating treated soil samples directly on a medium containing antibiotics.

Soil was air-dried at room temperature overnight, diluted in sterile distilled water, heated at 56°C for 10 min and then plated on CY-C10 medium. (CY-C10 medium is CY medium with 1% Casitone instead of 0.3% and with 50 μg of cycloheximide/ml.) Vancomycin, ristocetin or tiacumycin B at 10 μg/ml was also included. The combination of heat, desiccation and antibiotics killed or inhibited the growth of many non-myxobacterial cells, and the higher concentration of Casitone inhibited excessive swarming of the myxobacteria, while still allowing their colonies to be recognized. Colonies were picked and plated on media allowing fruiting body formation, and from these plates the final purification and identification were achieved. This approach was effective for isolating *Myxococcus*.

Isolation of Cellulose Decomposers

For the isolation of cellulose-degrading myxobacteria of the genus *Sorangium*, a mineral agar with cellulose as the only carbon source is used. While NH_4^+ is an excellent nitrogen source for *Sorangium*, much better results are obtained when NO_3^- is used in the isolation medium, as in ST21 agar. The cellulose is applied in the form of sterile filter paper, which is placed on top of the agar surface. Since the cultures must be incubated for a longer time before the cellulose decomposers can be recognized, inclusion of cycloheximide in the medium is essential.

ST21 Agar and ST21CX Agar

Solution A:

K_2HPO_4	0.1% (w/v)
Yeast extract (Difco)	0.002% (w/v)
Agar	1% (w/v)

Make up in about two-thirds of the water volume in distilled water.

Solution B:

KNO_3	0.1% (w/v)
$MgSO_4 \cdot 7H_2O$	0.1% (w/v)
$CaCl_2 \cdot 2H_2O$	0.1% (w/v)
$FeCl_3$	0.02% (w/v)
$MnSO_4 \cdot 7H_2O$	0.01% (w/v)

Make up in the remaining water volume. Autoclave separately. Combine solutions A and B and add 1 ml of trace element solution (see below) per liter of medium. For crude and enrichment cultures, add 25 mg of cycloheximide per ml from a filter-sterilized stock solution, yielding ST21CX agar.

Trace Element Solution (10× Original Formulation; Drews, 1974)

$MnCl_2 \cdot 4H_2O$	100 mg
$CoCl_2$	20 mg
$CuSO_4$	10 mg
$Na_2MoO_4 \cdot 2H_2O$	10 mg
$ZnCl_2$	20 mg
LiCl	5 mg
$SnCl_2 \cdot 2H_2O$	5 mg
H_3BO_3	10 mg
KBr	20 mg
KI	20 mg
EDTA, Na-Fe^{+3} salt (trihydrate)	8 g

Dissolve in 1 liter of distilled water and filter sterilize. This solution is stable for months at room temperature. Use the mixture at 1 ml/liter of medium.

The filter paper is inoculated in the center with a pea-sized amount of soil or plant debris, either in one spot or in the form of a streak. The cultures are incubated at 30°C. Unequivocal cellulose decomposition cannot be expected before 8–10 days of incubation, and most *Sorangium* strains are seen only after 10–20 days. They are recognized as bright, more or less translucent patches, which are yellow, orange, brown, or even black, often several cm across, and consisting of macerated cellulose, slime, and usually masses of densely packed fruiting bodies. The latter are responsible for most of the color, but also the vegetative parts of the swarms may be tinged yellow and orange, and at the edges, dense, orange ridges often develop. Besides the sorangia, other cellulolytic bacteria may also appear. The lysed areas always contain complex mixtures of a variety of bacteria, protozoa, and nematodes. In such cultures, particularly at later stages, noncellulolytic myxobacteria can also develop that live off the cellulose degraders (Brockman and Boyd, 1963). They appear in the form of long, heavy, orange ridges, which are the edges of expanding swarms, and are usually strains of *Nannocystis*, *Polyangium* or *Chondromyces*. Often fruiting bodies are also produced, not only in the macerated areas, but also on the edges of the intact filter paper, on the inoculum, and on the agar just beyond. Of course, these latter myxobacteria, not being cellulolytic, have to be subcultured on streaks of food bacteria and not again on filter paper. As the fruiting bodies observed in the lysis zones are not necessarily those of the cellulose degraders, in case of doubt, subcultures are best started both from fruiting bodies and from the swarm edge, using both types of substrates.

Isolation from Fresh Water

Sediments and plant and animal debris collected in lakes, ponds, rivers, etc. may be processed in the same way as described above. If free-floating myxobacteria are to be isolated, between 10 and 1000 ml of water is passed through a sterile membrane filter of small pore size (0.15–0.45 µm). The filter is then placed right side up on the surface of rabbit dung agar (another lean medium, e.g., VY/2 agar with a reduced yeast content or CY agar with a lowered peptone concentration, would also do). The cultures are incubated at a suitable temperature, e.g., 34°C. After 4–5 days, myxobacterial swarms and fruiting bodies may be observed (Gräf, 1975).

Rabbit Dung Agar

Boil 20 g of dry rabbit dung (wild rabbits) in 1 liter of distilled water for 20 min. Make up the filtrate to 1 liter again, and add 1.5% agar. Adjust the pH to 7.2, and autoclave the medium.

In summary, the various direct isolation techniques all have certain limitations, and each may be more or less appropriate for the isolation of a specific organism. For the cellulose degraders there is little choice. However, with some experience, they may also occasionally be found on dung pellets. Conversely, the cellulose plates are also useful for the isolation of *Chondromyces*, *Stigmatella*, *Polyangium* and *Nannocystis*. While *Myxococcus* is most easily obtained on dung pellets; *Cb. fuscus*, *Sg. erecta*, *Archangium*, *Corallococcus*, *Chondromyces* and *Melittangium* are also often found with this technique. The most generally effective technique is the use of bacterial smears; under these conditions, myxobacteria with small and inconspicuous fruiting bodies as well as strains that do not form fruiting bodies at all under the culture conditions are reliably isolated. Bark and rotting wood incubated in a moist chamber are in some cases excellent sources for fast and reliable isolation of *Stigmatella aurantiaca* and *Chondromyces* species.

Purification Methods

In the simplest case, pure cultures of myxobacteria can be obtained in one step from fruiting bodies, which usually contain an uncontaminated population of myxospores, at least as long as they are young. By carefully transferring material from fruiting bodies to a suitable agar medium, such as CY or VY/2 agar (see below), pure swarms can be obtained. An excellent tool for such delicate work is a glass rod drawn out into a fine tip. More convenient for a swift operation is the sharp point of a disposable, 1-ml, injection syringe. Direct isolation from fruiting bodies is possible with *Myxococcus* species because they produce soft-slimy fruiting bodies, and enough myxospores to start a culture can be obtained just by touching the fruiting body at the top. The fruiting bodies of *Chondromyces* and *Stigmatella* consist of sporangioles that are borne on a relatively long stalk that lifts them above the heavily contaminated substrate surface. The sporangioles can be plucked off and used as an inoculum. With all other myxobacteria, the chance of a selective transfer from fruiting bodies is low because they are too intimately interwoven with the substrate and the slime layer of the swarm.

VY/2 Agar

Bakers' yeast (commercial yeast cake)	0.5% (w/v)
$CaCl_2 \cdot 2H_2O$	0.1% (w/v)
Cyanocobalamin	0.5 mg/µl
Agar	1.5% (w/v)

Adjust pH to 7.2. Autoclave the medium. The yeast may be stored as an autoclaved stock suspension for several weeks. To obtain a uniform suspension of the yeast cells in the agar medium, add the yeast to the molten medium.

CY Agar

Casitone (Difco)	0.3% (w/v)
Yeast extract (Difco)	0.1% (w/v)
$CaCl_2 \cdot 2H_2O$	0.1% (w/v)
Agar	1.5% (w/v)

Adjust the pH to 7.2. Autoclave the medium.

ENRICHMENT TECHNIQUES. If direct isolation is not possible, enrichment techniques must be applied. Fruiting bodies or vegetative cells are transferred to streaks of a food organism on WCX agar. Three parallel streaks can be made on one plate, and each streak can be inoculated at both ends. As the swarm is often firmly attached to the substrate or forms a very tough slime sheet, from which tiny bits cannot easily be removed, or because the myxobacteria migrate within the agar, the inoculum often with a small piece of agar must be cut out using the tools mentioned above. An inoculation loop would be much too coarse and would risk carrying over too many contaminants. The bacterial smears are best inoculated at their edge so that the developing swarm also has a chance to spread over the free agar surface, which sometimes gives a better separation from the contaminants. New transfers can be made from the purest-looking sections of the developing swarms, preferentially from the swarm edge, until swarms are obtained that seem pure enough to justify a transfer to VY/2 or CY agar. A transfer to smears of autoclaved *E. coli* on WCX agar may be necessary as an intermediary step, particularly if the myxobacterium grows only within the streaks of the living *E. coli*. Much time can be saved later if the cultures are initially transferred within short intervals (1–2 days) before the contaminants have multiplied and spread. While it is advisable to make several transfers from the crude culture—by inoculating all three streaks of the enrichment plate, if possible from different sites—to enrich for the organism and to have a choice of contaminants to deal with, transfers can also be made one at a time, so that the three streaks of a plate can be used consecutively.

Sometimes transferred fruiting bodies do not germinate. This may happen, for example, if fruiting bodies found on a dung pellet have erroneously been identified as a bacteriolytic species (which is indeed the case with almost all dung organisms) and therefore were transferred to a bacterial smear, while in reality the fruiting bodies had been produced by a cellulose degrader. The latter will readily develop if inoculated to filter paper on ST21 agar (see the section Isolation of Cellulose Decomposers in this Chapter). However, some bacteriolytic myxobacteria do not always germinate on bacterial streaks, e.g., *Cb. fuscus*. With these strains, transfer of fruiting bodies directly to VY/2 agar sometimes helps. After germination, the swarms should be inoculated as soon as possible on bacterial streaks, because many contaminants can grow vigorously on VY/2 agar. If a sufficient quantity of fruiting bodies can be obtained from the crude culture, some of them should first be heated in water (see the section Purification by Heating in this Chapter) before they are inoculated to the growth medium. Note at least one myxobacterium (*Haploangium*) will not germinate on any known medium and has not been cultivated so far.

If the myxobacteria are left growing on the streaks for a longer time, they usually will produce fruiting bodies. Fruiting bodies can be very useful as starting material for further, more sophisticated purification steps. Also, fruiting bodies are important for the identification of the isolated strains.

The main contaminants in the enrichment cultures are other bacteria. Occasionally, other gliding organisms, more often small Gram-negative rods, become trapped in the slime of the swarm and are carried around by the moving myxobac-

teria. These two types of contaminants are often difficult to eliminate. Nematodes, fungi, and especially soil amoebae may also become a serious problem.

The first step in purification is to eliminate all animals from the culture because they move very actively and thus quickly spread bacterial contaminants over the plate. The transfer of nematodes can usually be avoided if some caution is observed, because they are large enough to be easily recognized and eliminated. Occasionally, however, a culture is heavily infested with nematodes, or the worms cannot be seen because an opaque substrate is used, e.g., cellulose plates. In these cases, the plate can be frozen by simply storing it in an –80°C deep-freeze for one or two days, which seems to kill all nematodes. After thawing the plate, it is important to make a transfer to a fresh plate immediately after the surface of the plate has softened sufficiently, because the thawed agar will be very runny and soft (Reichenbach, 1983). Exposing the plate to ammonia vapors (M. Aschner, personal communication) can also kill the amoebae, which sometimes multiply extremely fast. We use a 5% ammonia solution in a Petri dish and place the culture plate upside down over the ammonia dish for 1–2 min. The plate is then left with a closed lid for another 2–5 min, and then the myxobacteria are transferred to a fresh plate because the old one is now strongly alkaline. The myxobacteria usually survive the treatment very well, even when no fruiting bodies are present. A further transfer should be made as soon as possible from the fresh culture because a few amoebae, probably encysted stages, sometimes survive. Soil amoebae do not seem to be inhibited by cycloheximide, nystatin, or freezing.

Occasionally, cycloheximide-resistant fungi are present, but dusting the inoculum with nystatin powder can almost always inhibit them. The myxobacteria grow between the nystatin particles and can then be transferred to another plate.

The remaining contaminants are bacteria. By transferring pieces from the fast-spreading swarm edge, most of the larger and less motile organisms are quickly eliminated, in particular *Bacillus* species and their spores, but the slime matrix of the swarm may still shelter many small, mainly Gram-negative bacteria. Apart from the slime, the relatively slow growth of myxobacteria compared with that of typical contaminants is a major factor that makes purification time-consuming and difficult. Usually it takes 3–6 weeks before an isolated strain is pure; only rarely can it be accomplished in 1–2 days (by direct transfer of fruiting bodies). Fortunately, those isolations that may take 1–2 years (e.g., of a cellulose degrader) are equally rare.

Often spreading growth under selective nutritional conditions is not sufficient to shake off contaminants, or the procedure becomes too time-consuming. In such cases, a number of more specific purification steps may be tried.

PURIFICATION BY PLATING. Plating of diluted cell suspensions, the technique of choice for the isolation of most soil bacteria, is only partly useful with myxobacteria. The first difficulty is that the slime makes it difficult to suspend the cells homogeneously in water. We have sometimes had success using a small (1 ml) sterilized tissue homogenizer to overcome this problem. If fruiting bodies are available, treatment with ultrasound for 1–2 min may result in a suspension of myxospores. (The use of ultrasound to kill contaminants selectively, as suggested by Sutherland [1976b], has not appeared very promising in our hands.) Cell suspensions can also be produced in a mixer with or without glass beads (McCurdy, 1963). The cell suspensions can be diluted without problems in sterile distilled water, although a special dilution medium (DM) has also been used.

Dilution Medium (DM; McCurdy, 1963)

Soluble starch	0.5% (w/v)
$MgSO_4 \cdot 7H_2O$	0.05% (w/v)
K_2HPO_4	0.025% (w/v)

The second difficulty is that quite a few myxobacteria, particularly members of the suborder Sorangineae, do not readily produce swarms from single cells. Often a long and tedious optimization and adaptation procedure is required before single-cell colonies are obtained, even from pure strains. Nevertheless, with many myxobacteria, a reasonably high proportion of the cell population will grow to form colonies, so that plating may be successful. However, in our experience, plating has a chance only if the myxobacterium is already nearly pure, which may be due to their relatively low plating efficiency and relatively slow growth rate. CY agar is often an appropriate medium for plating; sometimes the yield is improved if the peptone concentration is increased to 0.8–1.2% or if 0.5% glucose is added. ECM agar and SP agar have also been recommended. While VY/2 agar is a good growth medium, its turbidity reduces its utility and, in addition, myxospores sometimes do not germinate on this medium.

ECM Agar (McCurdy, 1963)

> Suspend washed cells of *E. coli* (100 mg of dry mass per 100 ml) in a medium containing: $MgSO_4 \cdot 7H_2O$, 0.05% (w/v); NaCl, 0.6% (w/v); and agar, 1.5% (w/v). Adjust the pH to 7.2 and autoclave the medium. On this medium, myxobacterial colonies are surrounded by lysis zones.

SP Agar (McDonald and Peterson, 1962; McCurdy, 1963)

Raffinose	0.1% (w/v)
Sucrose	0.1% (w/v)
Galactose	0.1% (w/v)
Soluble starch	0.5% (w/v)
Casitone (Difco)	0.25% (w/v)
$MgSO_4 \cdot 7H_2O$	0.05% (w/v)
K_2HPO_4	0.025% (w/v)
Agar	1.5% (w/v)
Vitamin solution	2.5 ml/liter

Vitamin Solution (McDonald and Peterson, 1962)

Thiamine	100 mg
Riboflavin	75 mg
Pyridoxine	75 mg
Ca pantothenate	200 mg
p-Aminobenzoic acid	5 mg
Nicotinamide	75 mg
Choline HCl	200 mg
Folic acid	1 mg
Inositol	1000 mg
Biotin	0.05 mg
Cyanocobalamin	0.05 mg

Dissolve these ingredients in 1 liter of 100% ethanol. Add 2.5 ml per liter of medium.

Whether the diluted cell suspensions are spread on the agar surface or included in pour plates does not seem to make much difference. In the latter case, the agar content of the medium should be reduced to about 1% and temperatures above 40°C of the molten agar should be avoided. Myxobacterial colonies may be recognized by their swarming behavior, which they also tend to show when embedded in agar. On rich media, swarming may be considerably reduced, and the colonies may become compact and look like those of ordinary bacteria. If they are dense enough, however, they may be brightly colored in shades of yellow, orange, red, or purple.

PURIFICATION BY HEATING. This procedure can be used if fruiting bodies with mature myxospores are present. The fruiting bodies are suspended in 1 ml of sterile water and incubated in a water bath. After 10, 20 and 40 min, samples are transferred to CY or VY/2 agar and carefully spread all over the plate. The useful temperature—too high for the contaminant, but still withstood by the myxobacterium—has to be found by trial and error. We usually work at 58°C, a temperature most myxospores can survive for at least 10 min. This temperature is often sufficient to kill contaminating bacteria, fungi, and amoebae. Only rarely do myxobacteria tolerate higher temperatures, and some do not withstand 58°C, in which case 56°C or less may still lead to success. However, this method should be tried in the later part of the purification process, because then the chances are higher that thermoresistant contaminants, e.g., *Bacillus* spores, will no longer be present. It may take a week or longer at 30°C before swarms can be seen on the plates, as a substantial part of the myxospore population may also have been killed. However, often swarms are visible within 1–2 days.

PURIFICATION WITH ANTIBIOTICS. This procedure may also be useful if mature myxospores are present. Fruiting bodies are suspended in 1 ml of a rich growth medium, e.g., EBS medium (see below). To this is added 0.1 ml of a concentrated mixture of potent antibiotics, and the suspension is shaken overnight at 30°C (Reichenbach, 1983). The myxospores do not germinate in the rich medium, but the contaminants will grow and be killed. If a contaminant is resistant to the inhibitors, the culture will be turbid the next morning, and the procedure must be repeated with a different mixture of antibiotics. (Occasionally the liquid becomes turbid owing to suspended dead cells. In this case, the culture fluid can be replaced by fresh medium and the culture incubated a few hours more.) We use three increasingly aggressive combinations of antibiotics, AB-1, -2, and -3 solutions (see below), which we apply consecutively when necessary. If the supernatant is still clear on the next morning, it is replaced by sterile distilled water, and the fruiting bodies are washed by shaking for an additional 4–6 h. They are then transferred to VY/2 or CY agar or to streaks of autoclaved *E. coli* on WCX agar. Usually swarms develop after 1–5 days. Sometimes an individual swarm is still contaminated, although no foreign colonies can be seen on the plate. Therefore, several swarms should always be isolated and tested separately for purity. Again, the procedure is less effective during the early stages of purification, before the majority of the contaminants have been removed. Note that the vegetative growth of myxobacteria is, in general, as sensitive to inhibition by antibiotics as is the growth of other Gram-negative bacteria. However, the metabolically quiescent myxospores within the fruiting bodies are resistant as long as their germination is prevented.

EBS Medium

Peptone from casein (Merck, Darmstadt, Germany) 0.5% (w/v)	
Proteose peptone (Difco)	0.5% (w/v)
Peptone from meat (Merck)	0.1% (w/v)
Yeast extract (Difco)	0.1% (w/v)

The peptone from casein is a tryptic digest. Adjust the pH to 7.0 and autoclave the medium.

AB-1 Solution

Chloramphenicol	20 mg
Streptomycin sulfate	30 mg
Tetracycline HCl	25 mg
Na cephalotin	20 mg

Dissolve the above ingredients in 50 ml of distilled water and filter sterilize. The solution remains stable for several weeks at 4°C.

AB-2 Solution

Chloramphenicol	20 mg
Streptomycin sulfate	30 mg
Tetracycline HCl	25 mg
Kanamycin sulfate	35 mg
Erythromycin	25 mg
Polymyxin B sulfate	20 mg

Dissolve the above in 50 ml of distilled water and filter sterilize. The solution remains stable for several weeks at 4°C. Use only as a last resort since it is often inhibitory to myxobacteria.

AB-3 Solution

Chloramphenicol	20 mg
Tetracycline HCl	30 mg
Neomycin sulfate	20 mg
Gentamycin sulfate	30 mg
Collistin methane sulfonate	30 mg

Dissove the above in 50 ml of distilled water and filter sterilize. The solution remains stable for several weeks at 4°C. This mixture is specifically designed to eliminate pseudomonads. It usually serves that purpose but is less well tolerated by the myxobacteria than is AB-1 solution.

The inclusion of inhibitors, e.g., neomycin and sulfanilamide, directly in the isolation medium has been suggested (McDonald, 1967; McCurdy, 1969b). However, while some myxobacteria can be isolated in this way, we do not recommend this approach. (Cycloheximide is of course an exception, as it does not inhibit prokaryotes.) It is remarkable how many soil bacteria can grow in the presence of high doses of powerful antibiotics, and there is always the danger of a counterselection of some myxobacteria. Addition of antibiotics to purification media is a different story, however, as some groups of myxobacteria are naturally resistant to certain potent antibiotics (see later).

PURIFICATION OF CELLULOSE DECOMPOSERS. The purification of *Sorangium* strains is especially tedious and time-consuming because the organisms only grow slowly on the selective substrate, giving the contaminants ample time to multiply. Furthermore, within the water-soaked filter pad, many contaminants spread rapidly over the whole area, complicating the problem of separating the myxobacteria from the other organisms.

The first transfer from the crude culture should be made again to filter paper on ST21CX agar (see the section Isolation of Cellulose Decomposers in this Chapter) to retain the selective conditions. For the preparation of subcultures, filter paper is cut into small rectangles, about 1.5 × 3 cm, three or four of which are placed at some distance from each other on the agar surface. The filter pads are inoculated from different parts of the original swarm. In this way, an initial reduction of the contaminants may be achieved. The next step is to remove nematodes and amoebae using the techniques recommended above. To eliminate bacterial contaminants, transfers can be made to streaks of autoclaved *E. coli* on WCX agar to which 250 mg of kanamycin sulfate has been added per liter medium (after autoclaving, from a filter-sterilized stock solution: KAN4 agar). All *Sorangium* strains tested so far are completely resistant to kanamycin as well as to neomycin and gentamycin (H. Reichenbach, unpublished observations). Most sorangia grow reasonably well on autoclaved (but not on living) *E. coli*, often forming large, delicate swarms (another advantage of this purification step). Pieces from the swarm edge that appear to be uncontaminated can be transferred to VY/2 agar. The pure strain is reinoculated to filter paper to make sure that the desired organism has been isolated. We have never observed a *Sorangium* strain that lost its ability to degrade cellulose during isolation. Addition of kanamycin initially to the crude culture would prevent the appearance of many other myxobacteria and, at the outset, select for kanamycin-resistant contaminants.

If the method just described fails, heating of the fruiting bodies or treatment with the antibiotic solution may lead to success. If the *Sorangium* isolate is already relatively pure, it may also be transferred to chitin agar (CT7 agar; see below) or to cellulose overlay agar (CEL3 agar, see below). Most but not all sorangia also decompose chitin and grow relatively quickly, producing large swarms on chitin agar with chitin as the only carbon, nitrogen, and energy source. Large swarms also arise on cellulose overlay agar, but development is delayed so that the contaminants have a better chance to predominate. On both media, the sorangia penetrate the agar, so that sometimes a pure inoculum can still be obtained from the deeper layers of the plate even though the surface is contaminated.

CEL3 Agar

Cellulose powder	0.5% (w/v)
KNO_3	0.1% (w/v)
Agar	1.0% (w/v)

Cellulose powder MN 300 from Macherey and Nagel (Düren, Germany) works well. Adjust the pH to 7.2. Autoclave the medium. Autoclave the KNO_3 separately and add when the medium has cooled. Pour as a thin layer on top of ST21 agar plates.

Another strategy often helpful in the purification of sorangia is incubation at 38°C, e.g., on KAN4 agar + autoclaved *E. coli*. Most but not all strains are able to grow at this temperature, sometimes vigorously so, while growth of the contaminants is often prevented.

TESTING FOR PURITY. The final task in pure culture isolation is to check for purity of the culture. Some swarm material may be streaked

on nutrient agar, on which myxobacteria, in contrast to most contaminants, grow only slowly or not at all. Other media useful for purity control are CY and MYX agar. Although most myxobacteria grow on those media, they are usually easily distinguished from nonmyxobacterial colonies.

MYX Agar

Glutamate Na	0.5% (w/v)
Yeast extract (Difco)	0.1% (w/v)
$MgSO_4 \cdot 7H_2O$	0.1% (w/v)
Glucose	0.2% (w/v)
Agar	1.5% (w/v)

Autoclave glucose separately. Adjust the pH to 7.2. Autoclave the medium. Finally, add the autoclaved glucose to the autoclaved medium.

In addition, some material may be inoculated into a liquid medium, e.g., nutrient broth or CAS medium, and incubated with shaking for one day at 30°C. Under such conditions, myxobacteria usually grow in clumps, flakes, or as a ring around the glass wall, if at all. Dispersed growth at that stage is almost always an indication of contamination.

Myxobacteria can often be differentiated easily from other bacteria by their size, cell shape, and their inability to swim actively. Thus microscopic examination will also often quickly reveal contaminants, although this is not always reliable and requires some experience.

Counting Myxobacteria in Soil

The quantitative determination of myxobacteria in soil is not likely to give accurate results because, in the swarm and in the fruiting bodies, the cells are always linked together by slime, and in the fruiting bodies, cells are often surrounded by the tough walls of the sporangioles. Still, estimates are possible and have been obtained in the following way: Small circular smears (about 2 cm in diameter) of a food organism are placed on a water agar or a silica gel plate, and each circle is inoculated with a small quantity of soil which has been carefully homogenized and weighed (Singh, 1947). Alternatively, the weighed and homogenized soil is made into a paste with sterile water, and small drops are applied to the ends and centers of cross-streaks of a food organism on water agar or, for counting cellulose decomposers, to filter paper disks on mineral agar (Brockman, 1976). After incubation, the number of myxobacteria is calculated from the number of swarms and fields of fruiting bodies that have developed. Another approach is to add soil dilutions to antibiotic cell extract (ACE) agar (McCurdy, 1969b). This method has severe limitations because the medium allows a rapid development of contaminants, and only *Myxococcus* and *Corallococcus* cells can be counted. Nevertheless, the numbers that have been obtained are sometimes quite impressive.

ACE Agar (Antibiotic Cell Extract Agar; McCurdy, 1969b)

SP medium	
Yeast extract	0.1% (w/v)
Yeast (or *E. coli*) cells	0.5% (dry w/v)
Neomycin	10 mg/ml
Sulfanilamide	10 mg/ml
Cycloheximide	100 mg/ml

Add the remaining ingredients to the SP medium (see the section Purification by Plating in this Chapter).

The myxobacteria are detected by their swarm morphology and surrounding lysis zones.

Cultivation

Contrary to a belief that still seems widely held, myxobacteria are not particularly fastidious organisms. With the exception of *Haploangium*, all species have been cultivated, and almost all can be grown in axenic, pure culture. Of course, as with all other organisms, myxobacteria have their peculiarities, but no unusual requirements, and not even particularly complex ones, have been discovered to date. Exotic substrates such as the rabbit dung media that were so popular for a long time are not necessary. Although they were not always easy to develop, fully defined, synthetic media have been developed for some myxobacteria. In connection with possible commercial antibiotic production, quite a few myxobacteria have been cultivated in large-scale fermentors (5– 50 m^3 and greater), often on technical media, such as corn steep powder and soy meal.

Plate Cultures

Myxobacterial cultures are often incubated for extended periods (1–4 weeks) when dehydration may occur, so that plates must be poured thicker than for the usual bacterial work. Also, a high humidity in the incubator is helpful.

All myxobacteria, including the cellulose degraders, seem to grow well on yeast agar (VY/2 agar). The yeast cells are usually decomposed, but not always completely and sometimes not at all. Vitamin B_{12} is occasionally required (e.g., by many *Polyangium* strains) so cyanocobalamin is included in this standard medium. Typically, on VY/2 agar, gliding motility is considerably stimulated, and the swarm colonies tend to become very large. Fruiting bodies are often formed even after several transfers on VY/2 agar, although sooner or later most strains cease fruiting on this medium as well as on most other growth media. Cultures on VY/2 agar are usually

rather stable, and most strains need not be transferred more often than every 2–3 weeks (30°C). The reason is probably that VY/2 agar is relatively lean, nutrients are only gradually solubilized, and the pH does not change very much. The medium therefore is also well suited for stock cultures.

Another useful medium is CY agar (see the section Purification Methods in this Chapter). On this medium, growth often is more vigorous, but the swarms spread less and may become somewhat slimy and often the cells die earlier (after 1–2 weeks) because of copious ammonia production. The choice of the peptone for media of this type is critical. In general, the most suitable products appear to be pancreatic and tryptic digests of casein, e.g., Casitone (Difco), tryptone (Difco), or peptic digests (Marcor, Merck). But other peptones such as soybean peptones can also be used. Meat peptones, on the other hand, are usually not useful, perhaps because their phosphate content is too high. In any case, the suitability of a peptone for cultivation of the myxobacterial strain must first be tested. How critical the quality of the peptone can be is seen from the fact that even the specific batch of a particular peptone brand may make a difference. Thus, for example, not every batch of Difco Casitone supported successful induction of myxospore formation with *Stigmatella aurantiaca* (Gerth and Reichenbach, 1978); similar observations have also been made with glycerol induction of myxospores of *Mx. xanthus*. Replacement of peptone by a total protein hydrolysate, e.g., casamino acids (Difco), is often not possible. But casamino acids are a perfect N source for *Cystobacter* and *Sorangium* strains.

Watson and Dworkin (1968) showed that *Mx. xanthus* lacked hexokinase and was unable to take up, metabolize, or grow on sugars. This, however, cannot be generalized for the myxobacteria, despite earlier impressions that this might be the case. Many myxobacteria are known that efficiently metabolize sugars, e.g., *Stigmatella* and *Cystobacter*. In fact, it seems that only the *Myxococcus* species, *Nannocystis*, and perhaps some *Corallococcus* strains are unable to use carbohydrates. In some cases, e.g., certain *Corallococcus* strains, polysaccharides such as starch can be utilized, although no mono- and disaccharides can be used. Apparently the starch is degraded to the trisaccharide, which can be metabolized (Irschik and Reichenbach, 1985a). Thus, it may sometimes be useful to supplement a peptone medium with starch or glucose. Another beneficial effect of this may be that the pH rises more slowly, so that the culture can be maintained for a longer time. We have found the following formulations useful for plate cultivation:

CYG2 Agar

Casitone (Difco)	0.3% (w/v)
Yeast extract (Difco)	0.1% (w/v)
$CaCl_2 \cdot 2H_2O$	0.1% (w/v)
$MgSO_4 \cdot 7H_2O$	0.1% (w/v)
Agar	1.5% (w/v)

Adjust the pH to 7.2. Autoclave, then add 0.3% (w/v) glucose from an autoclaved stock solution.

STK2 Agar

Base agar:

Casitone (Difco)	0.1% (w/v)
Yeast extract (Difco)	0.05% (w/v)
$MgSO_4 \cdot 7H_2O$	0.1% (w/v)
Agar	1.2% (w/v)

Adjust the pH to 7.2. Autoclave the medium and pour into Petri dishes. After the agar has solidified, add the following top agar.

Top Agar

Use the same base agar ingredients, but increase the agar concentration to 1.5% (w/v), and prepare the medium in about 85% of the final total water volume. In the remaining water, dissolve (as a percentage of the total volume of the top agar) soluble starch (0.2% [w/v]; see below) and K_2HPO_4 (0.02% [w/v]). Heat the starch solution in a water bath with stirring and then autoclave to prevent clumping of the starch. Combine the two solutions (the base agar solution and the starch/K_2HPO_4 solution) after autoclaving.

In the form just described, STK2 agar can be used for checking starch degradation. If it is only to be used for cultivation, just the top agar (but with 1.2% agar) is required.

The cellulose decomposers utilize NO_3^- or NH_4^+ as the only nitrogen (N) source and can be cultivated on simple synthetic media like CK6 and CA2 agar (see below). They respond favorably to the addition of an organic N source, such as peptone (0.1–0.2%), but usually they will not grow in the absence of a suitable carbohydrate, like glucose or starch. *Sorangium* strains thus can also be grown on VY/2, CEL3, and STK2 agar, and many strains will grow as well on CT7 agar, but for the reasons just mentioned, relatively few will grow on CY agar.

CK6 Agar (Modified from Couke and Voets, 1967)

Solution A:

$MgSO_4 \cdot 7H_2O$	0.15% (w/v)
Fe^{+3} citrate	0.002% (w/v)
Agar	1.5% (w/v)

Prepare this solution in 80% of the final water volume.

Solution B:

KNO_3	0.2% (w/v)
K_2HPO_4	0.025% (w/v)

Prepare this solution in 10% of the final water volume.

Solution C:

Glucose	0.5% (w/v)
$CaCl_2 \cdot 2H_2O$	0.15% (w/v)

Prepare this solution in 10% of the final water volume. After autoclaving, combine the three solutions.

CA2 Agar (Mullings and Parish, 1984)

Base agar:
Agar 1.5% (w/v)

Stock solution A:

KNO_3 7.5 g
K_2HPO_4 7.5 g

Dissolve these ingredients to give 100 ml.

Stock solution B:

$MgSO_4 \cdot 7H_2O$ 1.5 g

Dissolve to give 100 ml.

Stock solution C:

$CaCl_2 \cdot 2H_2O$ 0.27 g
$FeCl_2$ 0.15 g

Dissolve these to give 100 ml. Autoclave. Supplement the base agar with 1% (v/v) of each of the three stock solutions, 1% (w/v) of glucose (from an autoclaved 20% stock solution) and trace elements.

Since myxobacterial swarms tend to spread quickly, cultures are more conveniently kept in Petri dishes than on agar slants in tubes. In the latter, the swarm edge soon reaches the glass surface and the cells dry out and die; also transfer of sticky, tenacious swarms is easier from plates. The plates are inoculated best at one spot at the side to give the swarm as much space as possible. In large swarms, living cells are often found only in a rather narrow band along the edge. Therefore, the inoculum should always be taken from the edge, at least as long as there are no fruiting bodies in the interior zone. Many myxobacteria of the suborder Sorangineae tend to penetrate deeply into the agar, in which case an agar strip has to be cut out and used as an inoculum.

Viable Counting

Accurate viable counts are possible only with strains that grow in a dispersed fashion in liquid media. Plating efficiency has to be tested for every strain. Given below are some media that allow plating efficiencies close to 100%: CT and CTT agar for *Mx. xanthus* FB; PT agar for *Mx. fulvus* Mx f2; and SG agar for *Sg. aurantiaca* Sg a1. CF (clone-fruiting) agar supports the formation of colonies from single cells of *Mx. xanthus*, with the subsequent production of fruiting bodies (Hagen et al., 1978). If a plating medium is not satisfactory for a specific strain, usually a modification of the peptone and ion concentrations or the addition of some yeast extract will make it suitable.

CT Agar (Dworkin, 1962)

Casitone (Difco) 2% (w/v)
$MgSO_4 \cdot 7H_2O$ 8 mM
Potassium phosphate buffer, pH 7.2 0.01 M
Agar 2% (w/v)

Autoclave $MgSO_4 \cdot 7H_2O$ separately.

A variation of the pH sometimes improves the results; a pH of 7.6 is closer to the optimum than is 7.2 (H. Reichenbach, unpublished observations).

CTT Agar (Bretscher and Kaiser, 1978)

Casitone (Difco) 1% (w/v)
$MgSO_4 \cdot 7H_2O$ 8 mM
Potassium phosphate buffer, pH 7.6 1 mM
Tris buffer, pH 7.6 10 mM
Agar 1.5% (w/v)

For semisolid media, agar is at 0.6% (w/v). Autoclave the medium.

PT Agar (for *Mx. fulvus* Mx f2)

Casitone (Difco) 0.4% (w/v)
Yeast extract (Difco) 0.4% (w/v)
$MgSO_4 \cdot 7H_2O$ 0.2% (w/v)
$CaCl_2 \cdot 2H_2O$ 0.1% (w/v)
Agar 1.5% (w/v)

Adjust the pH to 7.2. Autoclave the medium.

SG Agar (Gerth, 1975)

Casitone (Difco) 1.5% (w/v)
$MgSO_4 \cdot 7H_2O$ 0.1% (w/v)
$CaCl_2 \cdot 2H_2O$ 0.2% (w/v)
Agar 1.5% (w/v)

Adjust the pH to 7.0. Autoclave the medium.

CF Agar (Hagen et al., 1978)

Casitone (Difco) 0.015% (w/v)
Na pyruvate 0.1% (w/v)
Na citrate 0.2% (w/v)
$(NH_4)_2SO_4$ 0.02% (w/v)
$MgSO_4 \cdot 7H_2O$ 8 mM
Potassium phosphate buffer, pH 7.6 1 mM
Tris buffer, pH 7.6 10 mM
Agar 1.5% (w/v)

Autoclave the medium.

Excellent viable counts, e.g., for survival curves after treatment with mutagens, have been obtained by us with *Mx. fulvus* Mx f2 and *Sg. aurantiaca* Sg a1 by adapting a miniaturized counting technique recommended by Sharpe and Kilsby (1971). One of the plating media mentioned above is prepared with a reduced agar concentration of 0.6% (w/v). The bacteria are diluted directly in this soft agar at 40°C, and 0.1-ml drops of appropriate dilutions are put into Petri dishes, covered with a drop of sterile agar of the same composition, and incubated at 30°C. Note that vegetative cells of *Mx. xanthus*, and perhaps other myxobacteria as well, are sensitive to temperatures higher than 43°C. Microcolonies are counted under a dissecting microscope. Up

to 150 colonies per drop can easily be counted (H. Reichenbach and M. Dworkin, unpublished observations). The method has several advantages. Without difficulty, 40 drops can be placed into one Petri dish, using the bottom and the lid. Besides enormous economy in materials and incubator space, results are obtained earlier (after 3–4 days instead of 5–8 days) and with superior accuracy (more replicates, e.g., 5 instead of 3); less divergence between replicates; and a better chance to hit the optimum dilution step, because a wide interval of dilution steps can conveniently be plated. Plating efficiency can be 100%. The peripheral drops often show a reduced colony count, probably because they dry more easily. Therefore a ring of sterile agar drops should be placed around the edge of the plate.

Production of Fruiting Bodies in Culture

Usually, fruiting bodies are not produced on media that allow good growth. High nutrient concentrations seem to repress the fruiting process. As already mentioned, on VY/2 agar, fruiting bodies are often produced during several transfers, but a vegetatively propagated strain normally stops fruiting soon after isolation and often cannot be made to fruit again. The reason for this is not really understood; perhaps there is a selection for nonfruiting variants. In several cases, induction conditions for fruiting have been found for certain individual strains. Such strains reliably form fruiting bodies even after many transfers of purely vegetative growth, but other strains of the same species usually do not respond to the same conditions. With freshly isolated strains, fruiting can often be induced by transferring swarm material from a lean growth medium, like VY/2 agar, to plain water agar (WAT agar) or to sterile filter paper pads on water agar. Also, fruiting may continue for some time on streaks of living *E. coli* cells on water agar. The nutrients carried over from the growth medium and the low nutrient level provided by the lysing *E. coli* cells, respectively, allow reasonable growth for some time, leading to sufficiently high cell densities; the subsequent more-or-less abrupt depletion of nutrients then somehow triggers development. The need for a high population density for fruiting has been established for several myxobacteria, e.g., *Mx. xanthus* (Wireman and Dworkin, 1975) and *Cm. apiculatus* (H. Reichenbach, unpublished observations).

Using some simple tricks, myxobacterial strains can sometimes be maintained in the fruiting state for a long period. We were able to do so with *Sg. aurantiaca* over a period of years by storing the strain in the form of fruiting bodies on WAT agar at room temperature (20°C). After 2–3 months, when the culture began to dry out, the fruiting bodies were inoculated to VY/2 agar and incubated at 30°C. The resulting swarms were immediately reinoculated to WAT agar, incubated for a few days at 30°C, during which time the organism began to fruit again, and then stored at room temperature as before. One or two more transfers to VY/2 agar were possible, but after that the strain failed to produce fruiting bodies. This procedure of cycling the culture between rich media that support growth and lean media that induce fruiting body formation will probably work with many different myxobacteria.

With several *Chondromyces* species (*Cm. apiculatus*, *Cm. crocatus*, *Cm. pediculatus* and *Cm. lanuginosus*), we have obtained fruiting bodies when the organism was kept in a mixed culture with cellulose degrading bacteria (not myxobacteria) on filter pads placed on ST21 agar plates. The cultures are incubated in the light (on the bench top in the laboratory) at room temperature (20–24°C). Transfers are made every 3–4 weeks by inoculating macerated cellulose and *Chondromyces* fruiting bodies together onto fresh filter pads on ST21 agar. Within 4–5 days, new fruiting bodies appear. Using this procedure, several of our strains have continued to produce dense populations of fruiting bodies over a period of years (H. Reichenbach, unpublished observations). The method can also be used with pure cultures of *Chondromyces*. The swarm material is simply inoculated into a developing culture of a cellulose degrader. While *Polyangium* strains often form fruiting bodies with this procedure, myxobacteria of the suborder Cystobacterineae rarely do so. In general, a slightly reduced temperature (24–28°C) seems to favor fruiting. Also, in two cases, a requirement of light for fruiting body differentiation has been demonstrated—with *Cm. apiculatus* (Reichenbach, 1974a, b) and with *Sg. aurantiaca* (Qualls et al., 1978).

Chamber Cultures

For the study of the social behavior of myxobacteria and of morphogenetic events, chamber cultures that can be observed and followed under the microscope are extremely useful. Figure 4 illustrates one procedure for making such cultures.

Liquid Cultures

If a freshly isolated myxobacterial strain is cultured for the first time in a liquid medium in a shake flask, the bacteria almost always grow in the form of flakes and nodules and as a film along the glass wall. In static cultures, they grow as a sheet on the glass in the upper parts of the vessel

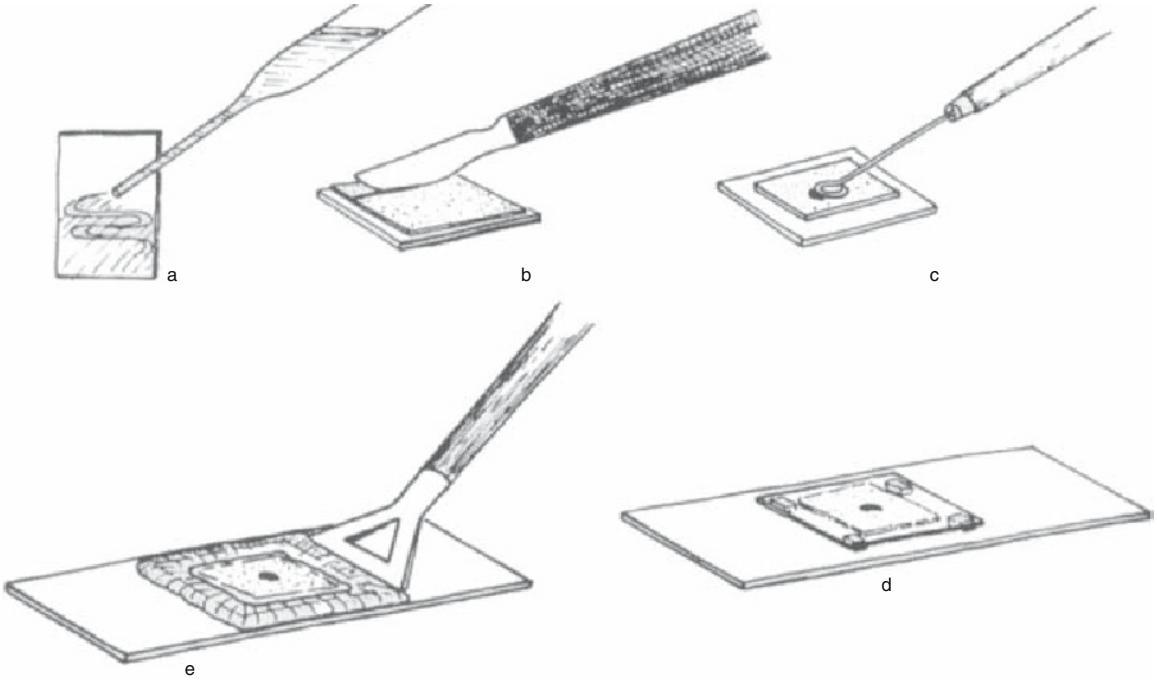


Fig. 4. Method for making a chamber culture: a) Coat a sterile cover slip (e.g., 40 × 22 mm) with a thin agar layer. The molten agar is applied with a Pasteur pipette; the hotter the agar, the thinner the resulting layer. b) Using a sterile scalpel, remove the agar layer along the edges of the cover glass. c) Inoculate the agar film. d) Mount the cover slip with the agar layer downward on a sterile slide; tiny pieces of glass from a broken microscopic slide are used as supports. e) Seal the chamber carefully with a molten mixture of paraffin and Vaseline (2 : 1) and incubate at a suitable temperature. From Heunert (1973).

and as a pellicle along the surface of the liquid. However, if repeated transfers are made from shake cultures, preferentially using the supernatant as an inoculum, strains that grow in a dispersed fashion may be obtained. While with many strains only a few such transfers are required, other strains may resist dispersed growth completely. Thus, for example, while most strains of *Myxococcus* can eventually be grown in dispersed culture, we once transferred a strain of *Mx. fulvus* for months in a variety of liquid media without ever observing the slightest inclination to dispersed growth (H. Reichenbach, unpublished observations). Cells of most members of the suborder Sorangineae show a strong tendency to stick together, and in spite of many attempts, we have never been able to get *Chondromyces*, *Polyangium* and *Nannocystis* to grow homogeneously in liquid cultures. The best that could be achieved was growth as tiny flakes with *Nannocystis* and *Polyangium*. With *Sorangium*, however, we have been able to select many completely dispersed strains. The list of myxobacteria that have been grown in a dispersed fashion is very long by now and, with the exception of the last-mentioned organisms, comprises virtually all taxa. However, even the recalcitrant species, which do not grow in a completely dispersed fashion, can at least be cultivated in suspension in liquid medium.

The main factor that determines whether *Mx. xanthus* cells will grow in clumps is the ability to produce an extracellular matrix composed of fibrils (Arnold and Shimkets, 1988a; Dworkin, 1999). Loss of type IV pili because of a *pilA* mutation, which encodes the major structural pilin subunit, also eliminates clumping (Wu et al., 1997), but this may be due to the fact that pilus mutants are often severely restricted in fibril synthesis. Fibril formation is also required for divalent cation-dependent agglutination (Shimkets, 1986), which is thought to mimic clumping in culture. The Sorangineae, which persistently fail to grow in a dispersed state, produce a slime that is chemically different from that of the Cystobacterineae (as shown by Congo red adsorption to the slime of the latter, but none to the slime of the former; McCurdy, 1969b). Thus, in the case of this group of the myxobacteria, the nature of the excreted slime may play a role in determining their mode of growth.

A homogeneously growing strain may start to clump again if growth conditions are changed, e.g., in a medium of different composition. Thus, cells of *Sg. aurantiaca* Sg a1, a strain that grows in a dispersed fashion, form tiny nodules if $CaCl_2$

is added to the medium. Freshly isolated *Cystobacter fuscus* cultures grow homogeneously when inoculated into a casamino acids medium, but produce nodules and flakes for many transfers in Casitone medium. Cells of *Sorangium cellulosum* strain So ce14 yield a homogeneous suspension when shaken overnight in Casitone medium (in which they cannot grow, however); when reinoculated into AMB growth medium, they produce nodules again. In this connection, although the myxobacteria are the classical "slime bacteria" (*myxo* is a combining form meaning "slime"), they do not produce copious amounts of slime, either on plates or in liquid media, which rarely become recognizably viscous. Only on certain agar media is a larger quantity of slime sometimes seen, e.g., with *So. cellulosum* on CA2 agar.

To facilitate the transition to dispersed growth, the addition of 0.1% agar to the liquid medium has been suggested (Schürmann, 1967). The agar forms tiny flakes that float in the liquid and are colonized by the myxobacteria. However, the selection of truly dispersed strains is not noticeably speeded up by this procedure. Sometimes replacing the usual rotary shaker with a reciprocating shaker is helpful, since the slime sheets are broken up more efficiently on the latter, and the transition to dispersed growth may be faster, although patience is still required (K. Gerth, personal communication).

Liquid media used for cultivation of myxobacteria are of the same general composition as the agar media mentioned above (see the sections Direct Isolation and Purification in this Chapter), but the nutrient requirements stand out more clearly in liquid media, and the organisms respond almost immediately to inadequacies of the medium. The prototype for the bacteriolytic myxobacteria is CT liquid medium, which was designed to provide the most rapid growth (3.5-h doubling time), maximum cell yield ($4–5 \times 10^9$ cells/ml), and uniform cell suspension of *Mx. xanthus* FB (Dworkin, 1962; the medium has the same composition as the CT agar described in the section Viable Counting, only without the agar). However, most other myxobacteria grow better when the peptone concentration is reduced, as in CAS medium and in MD1 medium (see below). The latter medium was originally developed for *Nannocystis*, but it is also often useful for *Archangium*, *Melittangium* and *Cystobacter*. Also, in many cases it is superior for starting liquid cultures with newly isolated strains.

CAS Medium

Casitone (Difco)	1% (w/v)
$MgSO_4 \cdot 7H_2O$	0.1% (w/v)

Do not adjust the pH (which is 6.8) if Difco Casitone is used. Autoclave the medium.

MD1 Medium (Behrens et al., 1976)

Casitone (Difco)	0.3% (w/v)
$CaCl_2 \cdot 2H_2O$	0.07% (w/v)
$MgSO_4 \cdot 7H_2O$	0.2% (w/v)
Cyanocobalamin	0.5 mg/ml
Trace elements	(see the section Isolation of Cellulose Decomposers in this Chapter)

Do not add vitamin solution for most strains. Autoclave the medium.

What has been said about the choice of peptone in agar media holds also for liquid media. For many myxobacteria, the addition of glucose (0.2–0.5%), starch, or another useful sugar to the peptone medium may be favorable. In general, the organisms do not grow faster with the carbohydrate, but they grow for a longer time, and they reach a higher cell density; also, the rise of the pH is delayed, so that the culture becomes more stable.

As already mentioned, some myxobacteria, e.g., *Cystobacter* strains, grow well on casamino acids, e.g., in CAC medium.

CAC Medium

Casamino acids, vitamin-free (Difco)	0.2% (w/v)
Sodium glutamate	0.3% (w/v)
$MgSO_4 \cdot 7H_2O$	0.1% (w/v)
Cyanocobalamin	0.5 mg/ml
Trace elements	

After autoclaving, complement the medium with 0.5% (w/v) glucose and 0.5 mM phosphate buffer (pH 6.5) from autoclaved stocks.

Stigmatella aurantiaca Sg a15 can be cultivated in the following defined medium:

STG Medium (Kunze et al., 1984)

Casamino acids, vitamin-free (Difco)	0.1% (w/v)
$MgSO_4 \cdot 7H_2O$	0.1% (w/v)
$(NH_4)_2SO_4$	0.1% (w/v)
$CaCl_2 \cdot 2H_2O$	0.05% (w/v)
Phosphate buffer, pH 7.0	1 mM
Trace elements	
Vitamins (see earlier)	

Autoclave. Complement the medium with 0.5% (w/v) glucose from an autoclaved stock solution. Alternatively, fructose, maltose, starch, or dextrin 10 could also be used.

The cost of the medium is of considerable importance when large-scale culture is necessary. Many myxobacteria have been cultivated on various technical media, like corn steep powder, maize gluten, soy meal, skim milk, or yeast cells (Gerth et al., 1983; Kunze et al., 1984; Kunze et al., 1985; Kunze et al., 1987). Zein medium is an example of a production medium.

Zein Medium (Kunze et al., 1987)

Zein (maize gluten, Maizena, Hamburg, Germany)	1% (w/v)
Peptone from casein (Merck, Darmstadt, Germany)	0.1% (w/v)
$MgSO_4 \cdot 7H_2O$	0.1% (w/v)

The casein peptone is tryptic digested. Adjust the pH to 7.3. Autoclave the medium.

All myxobacteria of the bacteriolytic type rely totally, or to a large extent, on peptides and amino acids for nitrogen, carbon and energy. As a consequence, relatively large quantities of ammonia are released, which (by regulatory mechanisms and by making the medium alkaline) seriously limits growth and productivity. Thus, in cultures of *Mx. virescens* Mx v48, concentrations of 35–42 mM NH_4^+ and more were measured in the culture supernatant and were 80–140 mM within the cells (Gerth and Reichenbach, 1986). On-line extraction of ammonia from a laboratory fermentor via hydrophobic-membrane hollow fibers maintained the NH_4^+ concentration in the medium between 3 and 7.5 mM with dramatic effects: the generation time was lowered from about 4 to 2 h, the cell density increased by 233%, and the yield of the antibiotic myxovirescin rose from 8 to 115 mg/liter (K. Gerth, personal communication).

For a few strains of bacteriolytic myxobacteria, fully defined, synthetic media have been developed. Examples are given in Table 2. Gen-

Table 2. Composition of five fully defined, synthetic media for bacteriolytic myxobacteria.[a]

Mx. xanthus FB		*Ar. violaceum*		*Cb. fuscus/Cb. ferrugineus*	
Component	S medium[b]	Ml medium[c]	Al medium[d]	medium[e]	Hpl6 medium[f]
L-Ala	50	1000	—	50	—
L-Arg	100	100	—	100	—
L-Asn	500	500	100	100	—
L-Asp·K	—	—	5000	—	—
L-Cys	—	100	—	—	—
L-Glu·Na	—	—	—	1000	—
L-Gln·Na	—	—	—	—	1000
L-Gly	50	100	—	50	—
L-His	50	100	—	50	—
L-Ile	500	1000	100	300	40
L-Leu	1000	2000	50	500	—
L-Lys	250	500	—	150	—
L-Met	50	500	10	50	—
L-Phe	—	1000	100	—	40
L-Pro	500	1000	—	250	—
L-Ser	100	200	—	100	—
L-Thr	100	100	—	100	—
L-Trp	50	1000	—	—	—
L-Tyr	600	400	—	500	—
L-Val	100	200	100	100	—
Djekolic acid	100	—	—	—	—
Glycogen	3000	—	—	—	—
D-Glucose	—	—	—	—	5000
Na pyruvate	—	—	5000	—	—
Spermidine·3HCl	—	—	125	—	—
Cyanocobalamin	—	—	1	—	—
$(NH_4)_2SO_4$	—	—	5000	—	—
$MgSO_4 \cdot 7H_2O$	2000	200	2000	200	1000
$Ca_3(PO_4)_2$	—	—	—	20	—
$CaCl_2$	—	2	1.1	—	—
KCl	—	—	—	20	—
NaCl	—	200	—	—	—
$FeCl_3 \cdot 6H_2O$	—	2	2.7	—	—
KH_2PO_4	—	140	—	—	—
Phosphate buffer	A	—	B	—	C
Tris buffer	—	A	A	D	—
Trace elements	—	—	—	—	1 ml/liter

Abbreviations: —, 0 mg/ml; A, 10 mM, pH 7.6; B, 1 mM, pH 7.6; C, 0.5 mM, pH 6.5; and D, 20 mM, pH 7.5.

[a] In mg/ml.

[b] From Dworkin (1962).

[c] From Witkin and Rosenberg (1970).

[d] Minimal medium: from Bretscher and Kaiser (1978).

[e] From Mayer (1967). The organism is now called "*Cystobacter violaceus*."

[f] Reichenbach (1984a) and H. Reichenbach (unpublished observations). Some strains also require thiamine and/or biotin when grown in this minimal medium.

eration times increase substantially in such media. Thus, with *Mx. xanthus* FB, the doubling times were 3.5 h in CT medium, 8 h in S medium, and 6.5 h in M1 medium, 6.5 h in M1 medium without lysine, 9.5 h in M1 medium without threonine, and 11.5 h in M1 medium without lysine and threonine (Filer et al., 1973).

The cellulose degraders can be cultivated on very simple media, such as CK1 or CK6 medium (see below). The addition of a small quantity of peptone, e.g., 0.05% (w/v) peptone from a tryptic digest of casein (Merck, Darmstadt, Germany), usually improves growth and keeps the cells in the dispersed state, whereas without peptone they sometimes form cell clusters or tiny clumps (Irschik et al., 1987). Media with peptone as the only N source usually also allow good growth, e.g., AMB medium.

CK1 Medium (Kleinig et al., 1971; modified from Couke and Voets, 1967)

$MgSO_4 \cdot 7H_2O$	0.3% (w/v)
$CaCl_2 \cdot 2H_2O$	0.18% (w/v)
Fe^{+3}-citrate	20 mg/liter
KNO_3	0.2% (w/v)
K_2HPO_4	0.025% (w/v)
Glucose	1% (w/v)

Autoclave nitrate and phosphate together and $CaCl_2$ and glucose together and separate from the other ingredients Then add the three solutions when cooled.

CK6 Medium (Irschik et al., 1987; modified from Couke and Voets, 1967)

$MgSO_4 \cdot 7H_2O$	0.15% (w/v)
$CaCl_2 \cdot 2H_2O$	0.15% (w/v)
$FeCl_2 \cdot 7H_2O$	8 mg/liter
KNO_3	0.2% (w/v)
K_2HPO_4	0.025% (w/v)
Glucose	0.5% (w/v)

Autoclave nitrate and phosphate together and $CaCl_2$ and glucose together and separate from the other ingredients. Then add the three solutions when cooled.

AMB Medium (Ringel et al., 1977)

Soluble starch	0.5% (w/v)
Casitone (Difco)	0.25% (w/v)
$MgSO_4 \cdot 7H_2O$	0.05% (w/v)
K_2HPO_4	0.025% (w/v)

Autoclave the medium.

MD1 medium (see above) can also be used for cellulose degraders if it is supplemented with 0.1% (w/v) glucose or soluble starch (Irschik et al., 1987). Some strains can grow in liquid media, like CB medium containing Casitone as the only C, N, and energy source (Sarao et al., 1985), but this must be exceptional because none of the many *Sorangium* strains we have tested would do so.

CB Medium (Casitone Broth; Sarao et al., 1985)

Casitone (Difco)	0.25% (w/v)
$MgSO_4 \cdot 7H_2O$	0.05% (w/v)
K_2HPO_4	0.0025% (w/v)

For liquid cultures of myxobacteria, ordinary Erlenmeyer flasks are perfectly satisfactory as culture vessels. For agitation, a rotary shaker at about 150 rpm is sufficient. As the oxygen demand of myxobacteria is rather moderate, flasks with baffles give little or no improvement in growth and actually are unfavorable with peptone-containing media because they produce too much foam. Several types of antifoam can be used with myxobacteria, but the type of antifoam acceptable in a certain situation and tolerated by a specific strain must always be tested first. We have found that with small culture volumes and limited foaming, a few drops of a 0.5% (w/v) cholesterol solution in acetone works well. In fermentors, as a rule, more efficient antifoams must be applied (Mizrahi et al., 1977; Ringel et al., 1977). We have had good results in many different myxobacterial fermentations with polyoxypropylene-based antifoams at 0.02–0.03% (w/v), for instance, of antifoam agents LB625 and M115 (both from Brenntag, Mülheim/Ruhr, Germany; Gerth et al., 1980; Irschik et al., 1983b), or with silicone antifoam agent at 0.005–0.02% (w/v; Merck, Darmstadt, Germany; these sometimes require further additions later during fermentation; Irschik et al., 1983a; Kunze et al., 1984; Kunze et al., 1985). Many, but not all, myxobacteria can be cultivated in the presence of an Amberlite adsorber resin, e.g., with 1% XAD-1180 (Röhm and Haas, Darmstadt, Germany; e.g., Gerth et al., 1983; Kunze et al., 1985). This strategy often is extremely useful for the production and recovery of secondary metabolites that are excreted by the cells.

Mass cultivation of myxobacteria in fermentors has not presented any serious problems. Mechanical stress seems not to be a limiting factor. If no continuous control and regulation of culture parameters is required, cultivation in large flasks (10–15 liters) also gives excellent results. The flasks are aerated with three air outlets of sintered glass at the ends of silicone tubing, which are lowered to close to the bottom of the vessel. The aeration produces enough turbulence in the liquid so that additional stirring is unnecessary. The appropriate temperature is achieved by placing the flask in an incubation room or by connecting a length of silicone tubing coiling within the culture broth to a thermostat with a pump. With such an inexpensive system, yields on the order of 8–12 g cells/liter (wet weight, corresponds to 2–3 g of dry weight) can be obtained.

Cultivation in commercial fermentors permits the continuous monitoring of various culture parameters and allows them to be balanced and controlled. Although the optimum conditions depend on the type of fermentor used, the strain, and the medium, some generalizations are possi-

ble: In fermentations on the scale of 50–5000 liters, typical aeration rates are between 0.01 and 0.2 liter air per liter of medium per min, which, with stirring rates between 150 and 650 rpm, result in oxygen levels between 90 and 100% saturation (Mizrahi et al., 1977; Gerth et al., 1980; Gerth et al., 1982; Gerth et al., 1983; Irschik et al., 1983b; Irschik et al., 1983a; Irschik et al., 1987; Kunze et al., 1984; Kunze et al., 1985, Kunze et al., 1987). In a special study on myxovirescin production with *Mx. virescens* Mx v48 with fed-batch fermentations in a peptone medium, a cell yield of 0.5 g (dry weight) per g oxygen consumed and of 0.31 g per g peptone was obtained (Nigam et al., 1984). The oxygen consumption was 6.25 g of O_2 per liter of culture in 55 h. Respiration quotients between 0.3 and 1.3 mol of CO_2 per mol of O_2 were calculated under different culture conditions, with values of 0.8–1.0 during logarithmic growth. The maximum dry weight yield was 3.1 g/liter. The specific growth rate, m, varied between 0.05 and 0.12/h during exponential growth, depending on the feeding rate.

Other types of special fermentations have also been performed with myxobacteria; for instance, continuous cultures of *Mx. virescens* Mx v48 have been achieved in a chemostat with on-line extraction of myxovirescin in a vortex chamber (Hecht et al., 1987), and fermentations have been established with alginate-immobilized cells (Younes et al., 1984; Younes et al., 1987; Vuillemard et al., 1988). Cells of *So. cellulosum* So ce12 immobilized in 1-mm alginate beads are extremely stable and could be maintained in fermentors under continuous exchange of the medium for up to 75 days before the cells degenerated (Becker, 1990; K. Becker, personal communication). The system allowed the production conditions for the antibiotic sorangicin to be defined more clearly, and under optimal conditions, volume/time yields are five times higher than those measured with suspended cells in batch cultures.

When fermentations are started with an inoculum of 5–10% (v/v), the results are almost always satisfactory. Sometimes the age of the inoculum is a critical factor, and beyond a certain, relatively early stage but still within the logarithmic phase, the cells will start to grow only after a long and erratic lag period or not at all. This has been observed with *Polyangium* and *Sorangium* strains (H. Reichenbach, unpublished observations).

Under optimal growth conditions the doubling times of myxobacteria are between 3.5 and 15 h, with the members of the suborder Sorangineae comprising the most slowly growing myxobacteria. Cell densities of up to 10^{10}/ml can be reached, for example, with *So. cellulosum*. Cell mass yields are in the range of 4–12 g of wet weight per liter (e.g., Irschik et al., 1983b; Irschik et al., 1983a; Irschik et al., 1985b). The dry mass is 20–25% of the wet weight.

The pH range for growth is rather narrow, approximately 6.5–8.5; in general, there is no good growth below a pH of 6.6 or above pH 8.0. While some of the cellulose decomposers will grow at a pH down to about 5.0 (Krzemieniewska and Krzemieniewski, 1937a; Couke and Voets, 1967), below pH 5.8, growth is marginal (H. Reichenbach, unpublished observations). As with other bacteria, the pH in myxobacterial cultures can be stabilized by adding a buffer. HEPES and 3-[*N*-morpholino]propanesulfonic acid (MOPS) buffers (50 mM) are well tolerated by many myxobacteria, and Tris may also be used. (Note that many of the media formulations do not contain phosphate; the low amount of phosphate contained in the peptones—in a 0.2% [w/v] solution of peptone from casein, 0.5 mM phosphate was present [Becker, 1990]—is usually sufficient to satisfy the phosphate requirement of myxobacteria.)

The temperature optimum for growth is usually between 32 and 36°C, and there is a sharp maximum around 38°C. The minimum is less clear. *Myxococcus xanthus* FB grew exponentially with a generation time of 14 h at 14°C, the lowest temperature tested (Janssen et al., 1977); the lower limit for cellulose decomposition by two *Sorangium* strains was found to be 11°C (Krzemieniewska and Krzemieniewski, 1937a). *Myxococcus fulvus* Mx f2 grows, albeit slowly, at 6°C. However, other myxobacteria obviously have a different temperature range: The psychrophilic myxobacteria from Antarctica have already been mentioned (Dawid et al., 1988), and at the other end of the scale, strains of *Archangium*, *Polyangium* and *Sorangium* that are able to grow at 40°C have been known for some time (McCurdy, 1969b). About 70% of the *Sorangium* strains grow at 40°C and about 80% at 38°C. A variety of other myxobacteria can grow at these somewhat elevated temperatures, e.g., most *Polyangium*, many *Archangium*, *Cystobacter*, *Chondromyces* and some *Mx. virescens* strains, but usually not *Mx. fulvus* and *Corallococcus*.

Most myxobacteria do not require vitamins when growing in the usual complex media, but, as already mentioned, a vitamin dependence, usually for thiamine or biotin or both, may develop when the organism is transferred to a minimal medium. *Stigmatella aurantiaca* Sg a15 needs both thiamine and B_{12} (Kunze et al., 1984); B_{12} dependence is not unusual and has also been observed with many *Polyangium* (but not with *Sorangium*), some *Nannocystis*, and one strain of *Corallococcus macrosporus*.

The mineral requirements of myxobacteria are similar to those of other bacteria. Myxobacteria seem to need relatively high Mg^{+2} concentrations (the optimum is often around 5–10 mM, equivalent to 0.075–0.15% $MgSO_4 \cdot 7H_2O$). *Sorangium* strains require Ca^{+2} as well and grow better on media with Ca^{+2} than with Mg^{+2} (McCurdy, 1969b). The addition of Co^{+2} sometimes has a stimulating effect (Ringel et al., 1977; Gerth et al., 1982). *Myxococcus xanthus* is sensitive to elevated concentrations of monovalent cations (Mason and Powelson, 1958), and the salt tolerance of myxobacteria is generally low. An exception is *Mx. virescens*, which seems to form fruiting bodies better when 0.5% (w/v) NaCl is added to the medium (Rückert, 1978). This organism can still grow on media containing 1% NaCl, although at a clearly reduced rate, and, in fact, it can be selectively isolated on streaks of *E. coli* on WCX agar at that salt concentration.

Usually, myxobacterial cultures are kept in the dark or, in an incubation room, under continuous illumination. As already mentioned, some myxobacteria produce fruiting bodies only when the cultures are illuminated. Also, a phototactic behavior has been reported for some myxobacteria (Aschner and Chorin-Kirsch, 1970). Illuminated cultures often are much more deeply colored than those grown in the dark because carotenoid synthesis is induced by light (Burchard and Dworkin, 1966b). As color is sometimes a taxonomically relevant characteristic, cultures used for taxonomic work should at least be intermittently illuminated.

Isolation of Mutants

Mutant strains of myxobacteria can be isolated without difficulty provided strains capable of dispersed growth and procedures for obtaining a high plating efficiency are available. *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) is often (but not always) an excellent mutagen for myxobacteria. The useful concentration range is 50–150 µg NTG/ml when the cultures are treated for 1 h. Also UV irradiation has successfully been used to obtain mutants. However, in contrast to NTG, UV was ineffective in generating carotenoid mutants in *Mx. fulvus* Mx f2. Further details on the application of different mutagens and a comparison of their effects on a variety of myxobacteria can be found in the literature (e.g., Burchard and Parish, 1975; Hodgkin and Kaiser, 1977; Grimm, 1978).

In recent years, transposon insertion mutagenesis has enjoyed particular favor because of the ease in identifying the gene containing the mutation. Transposon insertions have been generated with Tn*5* (Kuner and Kaiser, 1981), Tn*5 lac* containing a *lacZ* reporter (Kroos and Kaiser, 1984), Tn*V* containing an origin of replication (Furuichi et al., 1985), and magellan-4 (Youderian et al., 2003). Transposon mutagensis typically employs a suicide vector that is introduced by specialized transduction with coliphage P1 (Shimkets et al., 1983), conjugation with RP4 (Breton et al., 1985), or electroporation (Kashefi and Hartzell, 1995).

One typically mutagenizes cells and then screens for a particular phenotype of interest. One can also take the opposite approach and eliminate specific genes whose sequences are known. Mutations can be introduced in a directed way by homologous insertion of a nonreplicating circular DNA molecule containing a small portion of a gene lacking both 5′ and 3′ coding sequences (Shimkets and Asher, 1988). Integration of a truncated gene inactivates that gene and any genes following it in the same transcriptional unit. Such systematic mutagenesis has already been applied to a large cosmid (Lancero et al., 2002). The genome sequences of *Mx. xanthus* and *So. cellulosum* are now complete. This will facilitate the directed mutagenesis approach.

Preservation Methods

Vegetative cultures of myxobacteria on agar plates kept at room temperature or at 30°C will usually remain viable for about 2–3 weeks. Storage of such cultures at 4°C leads to a rapid loss of viability.

If fruiting bodies are produced, the cultures remain viable for a much longer time. One has to be certain, however, that the fruiting bodies do indeed contain myxospores, since under some conditions the fruiting bodies are empty or contain only vegetative cells.

Storage in the Form of Desiccated Fruiting Bodies

Pieces of agar that contain fruiting bodies can be placed on sterile filter paper in an empty Petri dish, dried in an evacuated desiccator, and stored as such for years. This method is especially useful with myxobacteria such as *Nannocystis*, whose fruiting bodies are mainly located within the agar.

Alternatively, vegetative cells can be transferred from a suitable growth medium, preferably VY/2 agar, to small rectangles (about 1.5 × 3 cm) of sterile filter paper on water (WAT) agar, and the cultures are incubated at 28°C. Usually, large numbers of fruiting bodies develop within 2–3 days. These fruiting bodies are allowed to mature for about 8 days. The filter pads are then placed into suitable containers, such as screw-cap

tubes or small medicine bottles, and dried in an evacuated desiccator over silica gel for a few days. The containers are then tightly closed and stored at room temperature or at 6°C (it is not known which temperature is better). The dried fruiting bodies survive for about 5–15 years. This method has the advantage of being easy and cheap, and it conserves the ability of the strain to form fruiting bodies. It works, however, only if the strain has not been transferred too many times since its isolation. The method should therefore be applied as soon as a strain is pure and, if fruiting is a desired feature, even earlier. Sometimes myxospores are still produced on the filter although fruiting bodies are no longer formed. In that case, the filter can still be dried and a viable preparation produced. The growth medium taken from the vegetative cells must not be too rich. Thus, for example, fruiting bodies usually do not appear after transfer from CY agar. Sometimes the organism must be grown directly on the filter paper to obtain fruiting bodies. This may be done on filter pads on water agar after streaking autoclaved yeast or food bacteria onto the filter. In this way, *Chondromyces* strains are sometimes induced to fruit. The cellulose decomposers almost always produce many fruiting bodies when growing on filter paper, which has been placed on ST21 agar. As the fabric of the filter paper is destroyed during growth, the paper can no longer be removed, but the macerated mass may be transferred to a fresh filter pad, which is then treated as described above.

To start a culture from dried fruiting bodies, the filter pad is placed upside down on CY agar, VY/2 agar, or WAT agar with streaks of autoclaved *E. coli*. After 24 h, the filter strip should be shifted to another place on the plate, because reduced oxygen access may prevent germination under the wet paper. When the paper is removed, many of the soaked and softened fruiting bodies will stick to the agar surface.

Alternatively, if a sufficient quantity of fruiting bodies is obtainable, e.g., after growth on VY/2 agar, after having been transferred to water agar or, in the case of the cellulose degraders, on filter paper on ST21 agar, they may be dried in skim milk. The fruiting bodies are suspended in skim milk, and a few drops of the suspension are applied to a freeze-dried milk plug in an ampoule. The ampoules are kept at 4°C for at least 10 min, and the plug is then dried again for about 5 h (this time at room temperature) in a desiccator connected to an efficient pump. After that, the ampoules are filled with nitrogen gas and sealed. Strains thus preserved also retain their ability to form fruiting bodies and may remain viable for a longer time than on filter paper. While drying in skim milk also works well with myxospores, including those that were experimentally induced (Dworkin and Gibson, 1964a), results with vegetative cells are highly erratic. Vegetative cells invariably die during drying, and only those cultures that contain myxospores survive. Experiments with true lyophilization and with protective substances other than skim milk did not give better results.

An excellent and convenient method for the preservation of myxobacteria is freezing at –80°C. Samples of 1 ml are transferred from exponentially growing liquid cultures to screw-cap tubes and placed directly into the deep freeze. Alternatively, the bacteria may be scraped from the surface of a young agar culture, transferred to 1 ml of CAS medium, and frozen. All of the myxobacteria that we have tested so far (26 species) have survived in the deep freeze for 8–10 years, the longest period over which data have been collected. Storage at ultra-low temperature is the best way to preserve strains growing in the dispersed state, nonfruiting, nonspore-forming strains, and mutants. It is also a fast and reliable method for the storage of all kinds of strains of a culture collection. However, if the temperature rises above –30 to –25°C, the cells will die unless they are transferred to a growth medium within 5–10 h.

For safe long-term storage, freezing in liquid nitrogen is the method of choice. We use the same cell suspension as described above, but add 5% dimethyl sulfoxide (DMSO) to the suspension medium, which is usually CAS medium. We fill 0.5-ml amounts of the suspension into glass ampoules, seal them, and immerse them in liquid nitrogen without further precautions. The DMSO may not be required since all strains stored without DMSO have survived thus far for 2 years.

Storage of frozen cell suspensions at –25°C is not recommended because most myxobacteria will die within several days or, at the most, a few weeks.

To reactivate frozen cultures, the tubes or ampoules are immersed in cold water to speed up thawing. The cells must be transferred to fresh medium immediately after they have thawed because if the cells remain in the original medium after thawing, they will lyse within 15–30 min. We generally use VY/2 agar for the reactivation of the culture, usually with good results, but CY agar, CAS medium, and MD1 medium can also be used. If experience is lacking with a certain strain, the thawed suspension should always be inoculated into several different media, including a liquid medium. Sometimes, albeit rarely, recovery will occur only on a specific medium, although the other media normally support growth. For example, we have observed that some strains can be reactivated only in a liquid medium or only on agar plates. If a liquid

medium is used, care should be taken not to dilute the organisms too much, e.g., by inoculating a few drops into 100 ml. In some cases, for example with particularly fragile mutants, reactivating the cells by embedding them in a growth medium containing 0.6% (w/v) agar is useful. The soft agar appears to provide a matrix that protects cells that have been slightly damaged by freezing and thawing (D. Zusman, personal communication).

At least some myxobacteria can be stored in a 1% (w/v) NaCl solution at room temperature. The cell suspension must not be too dense: the liquid should be just visibly turbid; otherwise the cells will lyse overnight. For one strain of *Mx. xanthus*, a suspension of vegetative cells has been kept alive for 5 months in this way. In another case, we suspended vegetative cells and glycerol-induced myxospores of *Mx. xanthus* FB in 1% (w/v) NaCl solution and kept the suspensions in sealed ampoules for 7 years at room temperature. After that time, a culture could be started from the myxospores, but not from the vegetative cells. While the original strain was very sensitive to NaCl, the revived strain grew on CY agar with 1% (w/v) NaCl. This clearly indicates that in such cases the bacteria are not completely dormant and that a selection for certain characteristics may have taken place.

Characterization

Nutrition and Energy Metabolism

All myxobacteria decompose macromolecules, but there are two sharply separated metabolic groups. Group 1 myxobacteria decompose cellulose and are classified in two genera, *Sorangium* and *Byssophaga*. The few strains that have been studied in detail are all able to grow on inorganic nitrogen (NH_4^+ or NO_3^-) as the only nitrogen source, although their growth is stimulated if a small quantity (0.05–0.1% [w/v]) of peptone is added. They also grow on organic nitrogen compounds as the only nitrogen source, provided a carbohydrate is also present. A few strains appear even to grow on peptone alone (Sarao et al., 1985). These organisms utilize cellulose and sugars such as glucose equally well, but cellulase production is repressed in the presence of sugar (Krzemieniewska and Krzemieniewski, 1937a; Krzemieniewska and Krzemieniewski, 1937b; Pronina, 1962; Couke and Voets, 1967).

Group 2 myxobacteria, by far the majority of species, depend on an amino acid-containing medium such as peptone for growth. They obtain oligopeptides by hydrolyzing proteins. Their dependence on peptides may be such that developing defined amino acid media for them is difficult or impossible. In other cases, the organisms grow on remarkably simple media with glucose, glutamate, and two essential amino acids (see the section Cultivation in this Chapter). In nature, these organisms seem to subsist mainly on other organisms, which they degrade by means of a variety of hydrolytic exoenzymes. Proteases, nucleases, lipases, glycanases, and cell wall lytic enzymes have been demonstrated. These myxobacteria are particularly efficient in the destruction of other bacteria and of yeast cells and therefore have been called "micropredators" (Singh, 1947). However, they do not depend on living organisms and could as well be regarded as scavengers. Their association with animal dung is probably due to its rich mixture of many types of living and dead cells and to its content of organic matter at every level of decomposition.

Almost all known myxobacteria (with the possible exception of *Anaeromyxobacter dehalogenans*) are strictly aerobic organotrophs. As a prerequisite for aerobic energy generation, *Myxococcus* possesses *a*-, *b*-, and *c*-type cytochromes as well as reduced β-nicotinamide adenine dinucleotide (NADH)-oxidase (Dworkin and Niederpruem, 1964b; Kleinig, 1972) and a complete tricarboxylic acid cycle (Watson and Dworkin, 1968; McCurdy and Khouw, 1969c; Sarao et al., 1985). The genus *Myxococcus* cannot utilize mono- and disaccharides (Watson and Dworkin, 1968) and depends on amino acids (or pyruvate) as a carbon and energy source (Bretscher and Kaiser, 1978). Recently it was discovered that methyl oleate dramatically increases the growth yield of *Mx. xanthus*, suggesting the presence of a fatty acid β oxidation pathway (Lau et al., 2002). Some myxobacteria can take up and metabolize oligosaccharides, which they produce from polysaccharides such as starch through the action of special hydrolytic enzymes (Irschik and Reichenbach, 1985a). This ability may be more common than presently believed, for virtually all *Myxococcus* and *Corallococcus* strains are able to degrade one or more polysaccharides, e.g., yeast cell wall β-glucan, xylan, and starch.

An overview of the metabolism of myxobacteria has been given by Shimkets (1984). Apparently, myxobacterial metabolism is similar to that of other bacteria, and unique biochemical pathways have not been discovered. Of course, myxobacterial metabolism has often been studied mainly in relation to biochemical reorganization during morphogenesis, and many enzymes have been described that are turned on or off at certain stages of development. These data will be found among the reviews cited earlier.

The glycolytic pathway seems to play a major role in the glucose metabolism of *So. cellulosum* (Sarao et al., 1985; D. Hofmann, personal com-

munication). In *Mx. xanthus*, glucose is not metabolized, presumably because hexokinase (ATP-dependent) and pyruvate kinase are absent (Watson and Dworkin, 1968). Nevertheless, an intact phosphofructokinase and a fructose-1,6-diphosphatase are present (Watson and Dworkin, 1968), implying that glycolysis and gluconeogenesis are both possible. The explanation for this apparent contradiction is not obvious. In *Cl. coralloides* strain Cc c127, fructose-1,6-diphosphate aldolase activity is very weak, and intracellular hexose seems to be metabolized via the pentose phosphate pathway (Irschik and Reichenbach, 1985a). The latter pathway is present in other myxobacteria, e.g., *So. cellulosum* (D. Hofmann, unpublished observations). In fact, this pathway must be present in all myxobacteria because all are able to synthesize the pentose required in anabolic reactions. In *Mx. xanthus*, the initial enzyme, phosphoenolpyruvate carboxylase, has been shown to be present (Watson and Dworkin, 1968). The conversion of fructose-6-phosphate into cell wall precursors has also been demonstrated (Filer et al., 1977). In *So. cellulosum*, gluconeogenesis appears to be blocked, which may explain the dependence of this organism on external carbohydrates (D. Hofmann, unpublished observations).

Morphology and Fine Structure

The vegetative cells of the myxobacteria are rod-shaped, typically 3–6 μm long and 0.7–1.0 μm wide. They are found in two morphological types: 1) slender, flexible rods with more-or-less tapering ends and 2) cylindrical, somewhat rigid rods with bluntly rounded ends (Krzemieniewska and Krzemieniewski, 1928; Fig. 1a–d). These different cell types correlate with a number of other basic characteristics and represent suborders of the Myxobacterales, the Cystobacterineae and the Sorangineae/Nannocystineae. The cell shape makes it possible to assign a strain at once to one of the two groups.

Most electron microscopic studies on myxobacteria are rather old now, and a thorough reinvestigation would be desirable. In addition to *Mx. xanthus*, several other myxobacteria have been studied, including *Mx. fulvus*, *Mx. virescens*, *Cystobacter*, *Stigmatella*, *Sorangium* and *Chondromyces* species (Voelz and Dworkin, 1962; Voelz, 1965; Voelz, 1966a; Voelz, 1966b; Voelz, 1967; Abadie, 1967; Abadie, 1968; Abadie, 1971a; Abadie, 1971b; Schmidt-Lorenz and Kühlwein, 1968; McCurdy, 1969a; Reichenbach et al., 1969b; MacRae and McCurdy, 1975; Lampky, 1976; Galván et al., 1986). The myxobacteria are typical Gram-negative bacteria. Both the tapered and the cylindrical cell types appear to be identical in fine structure, with ultrathin sections revealing a typical triple-layered outer membrane and a thin peptidoglycan sheet. The cells divide by septum formation (Abadie, 1971b; MacRae and McCurdy, 1975). Within the cells, mesosomes and mesosome-like membrane bodies are often seen (Voelz, 1965; Abadie, 1967; Abadie, 1968; Abadie, 1971b; MacRae and McCurdy, 1975; Lampky, 1976; Galván et al., 1986). The visualization of ribosomes was initially a problem, because they were difficult to fix properly (Voelz, 1967), but it later turned out that they are of the usual type. At low phosphate levels, the formation of polysomes seems to be induced in *Mx. xanthus* (Voelz, 1966a). Polysomes were also observed in cells of *Cm. crocatus*, and they were often attached to the cytoplasmic membrane and arranged in a helical fashion (Abadie, 1971b). Unusual disk-shaped, membrane-associated structures have been described from *Cm. crocatus* (MacRae and McCurdy, 1975). In cross sections they appear as two double tracks, each 7.5–11 nm wide and between 55 and 130 nm long, connected to the interior of the cytoplasmic membrane by 11- to 15-nm-long, fibrillar extensions. Several classes of intracellular granules, or inclusion bodies, have been found. Black granules, apparently produced by all myxobacteria, have been identified as polyphosphate (Voelz et al., 1966c). Electron-transparent granules are of at least two types: 1) granules that are pale gray, containing material that does not contrast well; these have been suggested to be polysaccharide (Reichenbach et al., 1969b), "slime vacuoles" (Schmidt-Lorenz and Kühlwein, 1969; Abadie, 1971b), or glycogen (Voelz et al., 1966c). This has been corroborated by light microscopy after performing the periodic-acid Schiff reaction (Schmidt-Lorenz and Kühlwein, 1969). 2) Completely empty, large, circular areas, which are sudanophilic under the light microscope, have been seen in the cells of *So. cellulosum* (Lampky, 1976) and may be lipid material. Also, poly-β-hydroxybutyric acid has been demonstrated chemically in *So. cellulosum* (H. Jansen, personal communication). In cells of *Mx. xanthus* grown under suboptimal conditions, large parts of the cytoplasm sometimes appear to have a crystalloid pattern (Voelz, 1966a; Voelz, 1967; Voelz, 1968).

In many myxobacteria, intracellular microtubules and fibrils may be seen (Schmidt-Lorenz and Kühlwein, 1968; Abadie, 1971b; MacRae and McCurdy, 1975; A. C. Burchard et al., 1977). The diameter of the microtubules is 10–16 nm (with *Cm. crocatus* 15–19 nm), and that of the fibrils is 4–5 nm (with *Cm. crocatus* 7.5–10 nm). Once it was thought that microtubules and fibrils were just two different aspects of the same structure depending on the plane of sectioning (Schmidt-Lorenz and Kühlwein, 1968), but this is not the

case. The fibrils are usually arranged in bundles that may be 40 nm wide and 3.5 μm long. The bundles usually lie close to the cytoplasmic membrane and often follow the long axis of the cell, sometimes to the cell poles, but they also may cross the cell from one side to the other. A herringbone-like periodicity with a 12-nm spacing has occasionally been seen (A. C. Burchard et al., 1977). Also, transverse bands with a very regular, crystal-like pattern may occur (A. C. Burchard et al., 1977). Originally, fibrils were found only in cells from swarming agar cultures (Schmidt-Lorenz and Kühlwein, 1968), but they were subsequently found in cells from liquid cultures as well (A. C. Burchard et al., 1977; H. Reichenbach, unpublished observations). These systems of fibrils and microtubules have been interpreted as contractile elements responsible for gliding motility by producing contraction waves (Schmidt-Lorenz and Kühlwein, 1968; A. C. Burchard et al., 1977), but there is no evidence that those structures can really contract, and they could have some other function or even be artifacts. In *Cm. crocatus*, those structures were assumed to be composed of ribosomes (paracrystalline system; Abadie, 1971b).

An extremely complex structure has been discovered in the surface layers of *Mx. fulvus*, just below the outer membrane (Lünsdorf and Reichenbach, 1989). One or several helical belts appear to be wrapped around the periphery of the cell, beginning close to the cell pole. From isolated material, it was deduced that the unit element consists of a long series of tiny rings at a regular, periodic distance, connected to each other by two longitudinal structures, perhaps two fibrils. Several of these unit elements, or strands, form a ribbon, and it could be shown that the rings in those ribbons can change their conformation, thereby producing a reduction of the transverse dimension of the ribbon by 40%. The belt is composed of about seven ribbons, and conceivably a coordination of the conformational changes occurring in the ribbons could produce a traveling wave in the belt and concomitantly in the cell surface, which would propel the cell. Nothing is known yet about the biochemistry of the system, and not even the structural details are fully understood. However, the components of the apparatus have been seen also in other myxobacteria, including *Mx. xanthus*.

Chemical Composition

While much has been learned about the chemical composition of the myxobacterial cell, the precise nature of the extracellular polysaccharides remains undetermined, as it is usually accompanied by a variety of other large and small excreted molecules (Sudo and Dworkin, 1972; Hanson and Dworkin, 1974). The excreted slime appears usually to be a heteropolysaccharide containing, among others, *N*-acetyl aminosugars (Sutherland and Thomson, 1975; Sutherland, 1979). Slime from fruiting bodies resembles in its overall composition that produced in vegetative cultures. A large extracellular protein-polysaccharide-lipid complex with proteolytic activity has been isolated from culture supernatants of *Mx. virescens* (Gnosspelius, 1978b). A glycopeptide with a heat-stable blood anticoagulant activity, named "myxaline," has been obtained from the culture broth of *Mx. xanthus* (El Akoum et al., 1987; Masson and Guespin-Michel, 1988). The glycan part contains *N*-acetyl aminosugars, and the peptide is rich in glutamic acid and serine. The excreted slime must be chemically different in the suborders of myxobacteria, since Congo red (0.01% [w/v] aqueous solution) stains the slime of the Cystobacterineae but not that of the Sorangineae/Nannocystineae (McCurdy, 1969b).

LIPOPOLYSACHARIDES AND CELL WALLS. There are several studies on the chemistry of the lipopolysaccharides (LPS) of various myxobacteria (Weckesser et al., 1971; Rosenfelder et al., 1974; Sutherland and Thomson, 1975; Sutherland, 1979). Contrary to the statement of Ruiz et al. (1987), myxobacterial LPS, including lipid A, can be readily and completely extracted from the cells by the usual phenol procedure (e.g., Sutherland and Smith, 1973; Rosenfelder et al., 1974; Sutherland and Thomson, 1975; Panasenko, 1985). Typical LPS yields are 0.5–1.0% (up to 1.9%) of the cell dry weight. Chemically, the LPS of the myxobacteria resembles that of the enterobacteria. The LPS of *Mx. fulvus* contains mannose, galactose, glucose, rhamnose, arabinose, glucosamine, and 3-*O*-methyl-D-xylose, a sugar only very rarely seen in bacterial LPS (Weckesser et al., 1971; Rosenfelder et al., 1974). The LPS of other myxobacteria may differ somewhat in sugar composition, but 2-keto-3-deoxyoctonic acid is always present, and heptose is never present. The 3-*O*-methyl-D-xylose may or may not be found, and in some cases, similar nonpolar but thus far unidentified sugars have been observed (Sutherland and Smith, 1973; Sutherland and Thomson, 1975; Sutherland, 1979). While the scant data do not really allow generalizations, a complete lack of rhamnose seems to be characteristic for *Sorangium* and may be another distinguishing characteristic of that suborder. The lipid A moiety contains glucosamine and the usual 3-hydroxy fatty acids, mostly *iso*-3-OH-C15 and *iso*-3-OH-C17 (Rosenfelder et al., 1974).

Man (1998) has carefully analyzed the LPS of *Mx. xanthus* and has found that the O-antigen portion consists of a small, repeating disaccha-

ride comprising → 4) -α -D-Gal*N*Ac*p* - (1 → 6)-α -D-Glcp - (1 →, where about 40% of the Gal*N*Ac*p* residues are methylated at their 6-positions. The "*p*" denotes pyranoside. The core LPS was found to consist of glucose, mannose, 2-acetamido-2-deoxy-galactose, and 2-acetamido-2-deoxy-6-*O*-methyl-galactose, in relative molar ratios of 1.00 : 0.41 : 0.40 : 0.32, respectively. Also present in the core LPS were small amounts of rhamnose, arabinose, xylose, galactose, and 2-acetamido-2-deoxyglucose.

Several mutations have been described that eliminate LPS O-antigen biosynthesis in *Mx. xanthus*. Fink et al. discovered that mutants resistant to phage Mx8 lack O-antigen and fail to react with monoclonal antibodies specific to O-antigen (Fink et al., 1989c). The genes containing these mutations have not been identified. The *wzm* and *wzt* gene products are predicted to encode an ATP-binding cassette (ABC) transporter involved in export of the O-antigen to the periplasm (Bowden and Kaplan, 1998). The *wbgA* and *wbgB* genes share similarity with bacterial glycosyltransferases and are suggested to be required for O-antigen synthesis (Bowden and Kaplan, 1998; Yang et al., 2000a). In all of these reports the authors noted deficiencies in motility, but there is some discrepancy as to whether the deficiencies are in A motility, S motility, or both. The consensus appears to be that both A and S motility are greatly reduced (Yang et al., 2000a). Man (1998) examined the LPS of two O-antigen mutants of *Mx. xanthus* (Fink and Zissler, 1989b) and found that both lacked the O-antigen repeating polysaccharide present in the wild-type parental strain. Man speculated that the corresponding reduction in cell surface hydrophilicity may be responsible for the motility defect.

During sporulation of *Mx. xanthus*, the LPS, or at least its polysaccharide part, is completely lost (Sutherland, 1976a), and a capsule composed of an α-1,3-glucan is produced (Sutherland and Mackenzie, 1977). That capsule is ruptured and discarded during germination rather than enzymatically solubilized (Voelz, 1966b). Monoclonal antibodies have also been obtained against O-antigens and against a core antigen of *Mx. xanthus* LPS (Gill and Dworkin, 1988; Fink and Zissler, 1989a). Several O-antigen mutants were defective in the A system but not in the S system of gliding. They were still able to form normal fruiting bodies (Fink and Zissler, 1989b). Endotoxin-like effects (anticomplement test and Shwartzman skin reaction) could be produced with whole cells and myxospores of *Mx. xanthus* (Ruiz et al., 1987).

Myxococcus xanthus, the only myxobacterium for which the organization of the cell wall has been investigated, has a thin peptidoglycan layer that seems to be organized in patches rather than in a homogeneous sacculus (White et al., 1968). The composition of the peptidoglycan resembles that of *E. coli*, with diaminopimelic acid at the cross-links. Apparently during sporulation, the degree of cross-linking increases. At the same time, galactosamine and glycine accumulate in the wall layer. The peptidoglycan content of vegetative cells and myxospores is, however, the same (0.6% of the dry weight).

LIPIDS. The fatty acid composition of the myxobacteria has not been thoroughly examined. However the data that are available suggest characteristic differences among the suborders (Fautz et al., 1981). While all myxobacteria contain substantial quantities of iso-branched fatty acids, mainly saturated C15:0 and C17:0, the fatty acid composition is dominated by them only in the suborder Cystobacterineae (usually between 50 and 70% total fatty acid; Schröder and Reichenbach, 1970; Ware and Dworkin, 1973; Fautz et al., 1979; Fautz et al., 1981; Yamanaka et al., 1988). *Nannocystis exedens* (suborder Nannocystineae) is the only myxobacterium so far in which larger amounts (about 30%) of *iso*-C15:1 and *iso*-C17:1 are found (Fautz et al., 1981). *Sorangium cellulosum*, suborder Sorangineae, has an intermediate phenotype with a more balanced representation of the saturated and unsaturated members of the iso odd family (Fautz et al., 1981).

The distribution of 2-hydroxy(OH) fatty acids also follows a trend, being high in the Cystobacterineae and virtually absent in the Nannocystineae and Sorangineae (Fautz et al., 1981). *Iso*-2-OH C17:0 was first seen as a contaminant from phospholipid in LPS preparations of *Mx. fulvus* and *Cb. ferrugineus* (Rosenfelder et al., 1974). Later, proof was presented for the chemical structure of the 2- and 3-OH fatty acids of *Mx. fulvus* and *C. ferrugineus*. In total cell hydrolysates, 2-OH fatty acids, mainly *iso*-2-OH C17:0, constitute 10–12% of the total fatty acids, while 3-OH fatty acids only play a minor role (2.5– 4.5%; Fautz et al., 1979). In members of the Cystobacterineae, two types of phosphatidylethanolamine have been demonstrated, one of which contains exclusively nonhydroxy fatty acids and the other 50–70% 2-OH fatty acids (Yamanaka et al., 1988).

Another major component of the fatty acid pattern of the Cystobacterineae is C16:1 (15–39%), which is greatly reduced in the Nannocystineae and Sorangineae. This fatty acid was later identified as 16:1 ω5c (which is extremely rare in nature; Kearns et al., 2001b). Anteiso fatty acids have also been reported in *Mx. xanthus* (Ruiz et al., 1985; Toal et al., 1995; Kearns et al., 2001b). In another study, a clear difference was seen between the fatty acid patterns of *Cor-*

allococcus and those of *Myxococcus* species (Monteoliva-Sanchez et al., 1987): branched and unbranched C17:0 fatty acids were present in large quantities in the latter (27–38%) but completely absent in the former. While such a clear difference would be useful for taxonomic reasons, it is not supported by other investigations (Yamanaka et al., 1988).

Myxococcus fulvus Mx f2 contains phosphatidylethanolamine as the main component (72%), with phosphatidylglycerol (9%), phosphatidylinositol (7%), and an unknown, ninhydrin-positive phospholipid (8%) as minor components (Kleinig, 1972). A small proportion of the phospholipids were alk-I-enyl-acyl (1–15%) and alkyl-acyl compounds; however, they comprised 22% of the phosphatidylinositol compounds. The phospholipid content of the isolated membranes was 25% (dry weight). The occurrence of two species of phosphatidylethanolamine in myxobacteria has already been mentioned (Yamanaka et al., 1988). The outer membrane of *Mx.* xanthus MD-2, an S motility mutant, showed an unusually low buoyant density (1.221 g/ml) which was probably due to an exceptionally high phospholipid content (Orndorff and Dworkin, 1980). The low buoyant density of the outer membrane was recently confirmed in the fully motile derivative DK1622 (Simunovic et al., 2003). The main phospholipid in both membranes was phosphatidylethanolamine (60–70% of total phospholipid). The other major phospholipids were phosphatidylglycerol and two unknown phospholipids. Phosphatidylserine and cardiolipin were found only in the cytoplasmic membrane. Substantial amounts of lysophospholipids were found in both membranes, although somewhat more in the outer membrane. They obviously indicate lipase activities, which seem not to be restricted to the outer membrane.

In *Sg. aurantiaca* the main phospholipid was phosphatidylethanolamine (50%), followed by phosphatidylinositol (20%) and phosphatidylglycerol (12%; Caillon et al., 1983). Very high amounts of lysophosphatidylethanolamine were also found. Each phospholipid had a different fatty acid composition. Alkyl ether linkages were common; phosphatidylinositol occurred only as the dialkyl compound. The structural details of an alk-I-enyl-acyl-phosphatidylethanolamine, i.e., a plasmalogen, from *Mx. stipitatus* have been elucidated (Stein and Budzikiewicz, 1987).

From the amount of phosphorus in the lipid fraction of *Sg. aurantiaca*, it has been estimated that only about 40% of the fatty acids are present in phospholipids (Schröder and Reichenbach, 1970). Ceramides and cerebrosides have been isolated from *Cystobacter fuscus* and chemically characterized (Eckau et al., 1984; Dill et al., 1985). Ceramides are long-chain, 1,3-dihydroxy-2-amino bases (sphinganine) with an amide-bound fatty acid. Cerebrosides are sphingolipids consisting of a ceramide with a C-1-sugar. The myxobacterial ceramides all contain 2-hydroxy fatty acids. A novel, long-chain base, 17-methylsphinga-4E,8E-dienine, was found in two of them. The cerebrosides are all galactosides. Cerebrosides are common in eukaryotes, where they play a role in cell-cell recognition and cell adhesion. They have also have been found to be fruiting-body inducers in basidiomycetes.

The capnoids, discovered as a characteristic constituent of the lipids of another group of gliding bacteria, the *Cytophaga*/*Flavobacterium* group, have not been found in myxobacteria (Godchaux and Leadbetter, 1983). The capnoids are C-1 sulfonic acids of sphinganine bases and their ceramides.

With one exception, *Methylococcus capsulatus* (Bird et al., 1971), myxobacteria are the only prokaryotes that are known to synthesize true steroids. *Nannocystis* strains produce cholestenols (Kohl et al., 1983; Zeggel, 1993); *Polyangium* (but not *Sorangium*) and *Cb. minus* produce lanosterol; and *Cb. violaceus*, *Sg. erecta* and *Sg. aurantiaca* contain cycloartenol (Bode et al., 2003). Squalene has been found in many myxobacteria. The cholestenol and squalene content of *Na. exedens* may be up to 0.4% of the dry weight. In contrast to most secondary metabolites, steroids are found in all strains of a species. Recently the key enzyme 2,3(S)-oxidosqualene oxidase has been demonstrated in *Sg. aurantiaca* (Bode et al., 2003).

PIGMENTS AND RESPIRATORY COMPOUNDS. Carotenoids are the main pigments of most myxobacteria. They have been extensively characterized by Kleinig et al. (1970) and especially in *Mx. fulvus* by Reichenbach and Kleinig (1971), who reported the presence of 50–60 different carotenoids and were able to identify 24 of these. Among the Cystobacterineae, the main compounds are always monocyclic carotenoid glycosides containing glucose with a fatty acid attached to the sugar via an ester bond (Kleinig et al., 1970; Kleinig et al., 1971; Reichenbach and Kleinig, 1971). While the Cystobacterineae have glycosides with keto but not with hydroxyl groups, members of the suborder Sorangineae produce glycosides with hydroxyl but not keto functions on the chromophore. *Sorangium* has also been shown to produce a nonesterified carotenoid rhamnoside (Kleinig et al., 1971). Four new carotenoids were recently identified in *Polyangium fumosum* (Jansen et al., 1995). *Nannocystis* contains mainly aromatic carotenoids and no glycosides. Thus apparently certain pigment types are characteristic for taxonomic subgroups (for a review, see Reichenbach and Kleinig, 1984b).

Carotenoid pigments forming esterified glycosides are rather unusual. The main pigment of many myxobacteria, including *Mx. xanthus*, is the deep-red myxobacton, which comprises 70% (by weight) of the total carotenoids of *Mx. fulvus*; it consists of 1′,2′-dihydro-1′-hydroxy-4-keto-torulene glucoside, esterified via glucose to a variety of unbranched, straight-chain fatty acids. This structure has been reinvestigated and corroborated (Eckau et al., 1984). An additional 10% of the total carotenoids consists of myxobacton esters, which contain 3,4-dehydro torulene rather than 4-keto torulene. In *Myxococcus*, complex mixtures of carotenoids are found, but only monocyclic and acyclic compounds occur. The carotenoid content of late log-phase cultures of *Mx. fulvus* Mx f2 has been determined. Acetone extracted carotenoids of such cells typically represent 0.003% of the dry weight of dark-grown cells and 0.03–0.06% of light-grown cells (Reichenbach and Kleinig, 1971). This difference between light- and dark-grown cells increases to 20-fold as the cells enter stationary phase. The carotenoids are exclusively located in the cytoplasmic membrane fraction, where they constitute up to 0.14% of the dry weight (Kleinig, 1972).

The effect of various inhibitors of the cyclization reaction of carotenoid synthesis has also been studied (Reichenbach and Kleinig, 1971; Kleinig, 1974a; Kleinig, 1975). In laboratory cultures, there is virtually no turnover or degradation of carotenoids. A system for the in vitro synthesis of C15 to C60 polyprenols from isopentenylpyrophosphate with a crude enzyme preparation from *Mx. fulvus* has been developed (Beyer and Kleinig, 1985).

The only demonstrated role of carotenoids in myxobacteria is to provide protection against photooxidation (Burchard and Dworkin, 1966b). Dark-grown cultures, which produce only small amounts of carotenoid, lyse quickly if illuminated upon entry to stationary phase of growth. If, however, the cells are exposed to light during exponential growth, carotenogenesis is photoinduced and the cells become resistant to photokilling. In *Mx. xanthus*, protoporphyrin IX was shown to be the photosensitizing pigment (Burchard and Dworkin, 1966b). Photokilling has also been demonstrated with carotenoid-free mutants of *Mx. fulvus* Mx f2. The killing effect is seen only if the mutants are illuminated with daylight intensities (about 40,000 lux; H. Reichenbach, unpublished observations). However, whether all myxobacteria accumulate protoporphyrin IX as a photosensitizer is unclear; protoporphyrin IX could not be detected, for example, in *Mx. fulvus* (H. Reichenbach, unpublished).

The carotenoid synthesis of myxobacteria is induced by light (Burchard and Dworkin, 1966b; Burchard and Hendricks, 1969; Reichenbach and Kleinig, 1971), and its control in *Mx. xanthus* involves a complex regulatory circuit (for reviews, see Hodgson and Murillo [1993] and Hodgson and Berry [1998]). Many carotenoid biosynthetic genes (*crt* genes) are clustered in the *crtEBDC* and *carB* operons, which are repressed in the dark by CarA. In outline, the reception of a blue light signal by CarF results eventually in the inactivation CarA. The light receptor, CarF, is remarkably similar to Kua, a family of proteins of unknown function that is widely distributed among eukaryotes (Fontes et al., 2003). But little is known about its interaction with protoporphyrin IX. Central to this response is the activation of the light-inducible promoter, *PcarQRS*, and the transcription of three downstream genes, *carQ*, *carR* and *carS* (Gorham, et al., 1996). CarQ is a member of the ECF (extracytoplasmic function) subfamily of RNA polymerase σ factors that is responsible for initiation at *PcarQRS*. CarR is an anti-σ factor that sequesters CarQ in a transcriptionally inactive complex. CarR was found to be unstable in illuminated stationary phase cells, providing a possible mechanism by which the CarR-CarQ complex is disrupted (Browning et al., 2003). In the light, CarS is synthesized and activates the *crtEBDC* operon by preventing CarA from binding to its operator (Whitworth and Hodgson, 2001). CarS is therefore an antirepressor. CarA is a novel protein consisting of a DNA-binding domain of the MerR family of transcriptional regulators, directly joined to a cobalamin-binding domain. The presence of vitamin B_{12} or some other cobalamin derivatives is absolutely required for activation of the *carB* promoter by light (Cervantes and Murillo, 2002).

Pigments other than carotenoids are also produced by myxobacteria. A pale-yellow pigment is found in *Mx. xanthus* (Gerth et al., 1983). On certain media, the colonies of *Cb. violaceus* (= *Ar. violaceum*) develop a deep purple-violet color, probably due to melanoid pigments (Kuhlwein and Gallwitz, 1958; Reichenbach, 1965c). A red pigment of *Cb. violaceus* is probably a dopachrome (Mayer, 1967). Melanoid pigments are also relatively often seen with individual strains of many other species, especially on peptone-containing media, e.g., with *Cystobacter*, *Archangium*, *Corallococcus* and *Stigmatella*. Liquid cultures of *Sg. aurantiaca* in peptone medium turn a deep black color within 1–2 h after reaching the stationary phase (Reichenbach and Dworkin, 1969a). The slimy swarms of some strains of *So. cellulosum* on CA2 agar become deep violet to black after 2–4 weeks. *Myxococcus virescens* and *Kofleria flava* are bright greenish-yellow, especially on protein-containing media. Several myxococci, especially

Mx. virescens, excrete a greenish-yellow diffusing pigment that appears to be myxochromid (Trowitzsch-Kienast et al., 1993). Plate cultures of *Mx. stipitatus* show under the UV lamp at 366 nm a bright yellow fluorescence (Lampky and Brockman, 1977) that is caused by phenylamides, or stipiamides (Kim et al., 1991; Trowitzsch-Kienast et al., 1992). Often the various shades observed with different strains and species are only due to differences in the quantitative proportions of the same pigments. Fruiting bodies, and especially the walls of the sporangioles, are often very intensely colored, but the chemical nature of these pigments is not known.

The respiratory quinones of the myxobacteria are exclusively menaquinones (Kleinig, 1972; Kleinig et al., 1974b). A survey of 11 genera and 20 species of both suborders showed that the menaquinone pattern of the myxobacteria is completely uniform and comprises virtually only MK-8 (Yamanaka et al., 1987; M.D. Collins, personal communication). Dworkin and Niederpruem (1964b) have characterized the terminal respiratory pigments of *Mx. xanthus* and have shown them to include cytochromes *a*, *b* and *c* in addition to presenting tentative evidence for the presence of flavin-linked enzymes.

MEMBRANES AND MEMBRANE PROTEINS. The membranes of *Mx. fulvus* consist of about 64% protein and 29% lipid, the latter comprising mainly (25%) phospholipid (Kleinig, 1972). The inner and outer membranes of *Mx. xanthus* strain DK1622 were fractionated (Simunovic et al., 2003). Membranes were enriched from spheroplasts of vegetative cells and then separated into three peaks on a three-step sucrose gradient. The high density fraction corresponded to inner membrane (IM), the medium density fraction corresponded to hybrid membrane (HM), and the low density fraction corresponded to outer membrane (OM). Each fraction was subjected to further separation on discontinuous sucrose gradients, resulting in the emergence of discrete protein peaks within each major fraction. The purity and origin of each peak were assessed using succinate dehydrogenase (SDH) activity as the IM marker and reactivity to lipopolysaccharide core and O-antigen monoclonal antibodies as the OM markers. As previously reported (Orndorff and Dworkin, 1980), the OM markers localized to the low density membrane fractions, while SDH localized to high density fractions (Simunovic et al., 2003). Immunoblotting was used to localize important motility and signaling proteins within the protein peaks. CsgA (the developmental C-signal producing protein) and FibA (a fibril-associated zinc metalloprotease) were localized in the IM. The motility lipoproteins Tgl and Cgl were localized in the OM. FrzCD (a methyl-accepting chemotaxis protein) was predominantly located in the IM, although lesser amounts were found in the OM. PilA (the major subunit of type IV pili) was distributed in all the fractions. Two-dimensional polyacrylamide gel electrophoresis illustrated the presence of proteins that were unique to the inner and outer membranes. Characterization of proteins within an unusually low density (ρ = 1.072–1.094 $g \cdot cm^{-3}$) membrane peak showed the presence of Ta-1 polyketide synthetase, which synthesizes the antibiotic myxovirescin (Simunovic et al., 2003).

Six of the membrane polypeptides changed substantially during aggregation (Orndorff and Dworkin, 1982): during the early stages of fruiting, protein T disappeared from the soluble fraction of *Mx. xanthus*, when proteins S and U first appeared, and then increased dramatically. Protein S (Inouye et al., 1979b) has been particularly well studied. In vegetative cells it amounts to less than 1% of the soluble protein, but during fruiting body formation, its rate of synthesis rises to 15% of total protein synthesis. In the presence of Ca^{+2}, protein S assembles on the surface of the maturing myxospores (Inouye et al., 1979a). Certain domains in the amino acid sequence of protein S show homologies to the Ca^{+2}-binding sites of calmodulin. The functional correspondence of those areas was demonstrated by site-specific mutations, which eliminated the Ca-binding property of protein S together with its ability to accumulate on the myxospores. Protein S also has some homology with the bovine-lens protein, α-crystallin (Inouye et al., 1983a; Teintze et al., 1988; Wistow, 1990).

Another development-specific protein is a myxobacterial hemagglutinin (MbhA), a lectin that is induced during the developmental aggregation of *Mx. xanthus* (Cumsky and Zusman, 1981). It appears to be localized in the periplasmic space or loosely bound to the cell surface and to recognize a galactose-containing, complex receptor. Its distribution over the cell surface is uneven, as it is found in patches mainly at the cell poles (Nelson et al., 1981). The 28-kDa protein contains four highly conserved domains of 67 amino acids each, which are reflected in four strong internal homologies in its gene (Romeo et al., 1986). MbhA is produced primarily by the peripheral rods, which never enter fruiting bodies or sporulate (O'Connor and Zusman, 1991a; O'Connor and Zusman, 1991b; O'Connor and Zusman, 1991c).

Using an iodination technique, substantial changes in the pattern of accessible surface proteins of *Mx. xanthus* were shown to take place during development (Maeba, 1983). A glycoprotein, probably a peripheral membrane protein that is present only in vegetative cells, was further characterized (Maeba, 1986). The 74-kDa

protein contained about 15% carbohydrate, mainly neutral sugars, but also some hexosamines and uronic acids. It made up 1% of the total cell protein and might play a role in cell-cell interactions.

A different approach to the study of the dynamics of the cell surface during development has been the use of monoclonal antibodies against cell-surface antigens (CSAs) of vegetative and developing cells (Gill et al., 1985). In this way, many different CSAs of *Mx. xanthus* have been identified (e.g., Gill and Dworkin, 1986; Gill and Dworkin, 1988). While most of the antibodies are directed against cell-surface proteins, some are directed against LPS or, in the case of CSA 1604, against a large, 150-kDa complex consisting of at least two protein subunits and LPS (Jarvis and Dworkin, 1989a; Jarvis and Dworkin, 1989b). Other monoclonal antibodies are directed against cell-surface proteins that appear uniquely during development (Gill and Dworkin, 1986).

Transfer and expression of myxobacterial genes in *E. coli* have identified some proteins. An 18.7-kDa basic protein coded by the *vegA* gene of *Mx. xanthus* turned out to have amino acid homologies with certain histones and may function as a DNA-binding protein. It is essential for vegetative growth (Komano et al., 1987). Also, the *Mx. xanthus* gene *fprA* was shown to code for a protein that binds flavin mononucleotide. Overexpression of the gene in *E. coli* leads to a spectacular increase in flavin biosynthesis, but the FprA protein seems to be an enzyme not in the flavin biosynthetic pathway (Shimkets, 1990b) but rather in vitamin B_6 biosynthesis (Lam and Winkler, 1992).

NUCLEIC ACIDS. The G+C content of myxobacterial DNA is between 67 and 72 mol% (as measured by buoyant density, thermal melting, and high pressure liquid chromatography [HPLC]; Mandel and Leadbetter, 1965; McCurdy and Wolf, 1967; Behrens et al., 1976; Mesbah et al., 1989).

As a group, the myxobacteria have among the largest bacterial genomes. The genome size of *Mx. xanthus* FB is 9450 kilobase pairs (kbp; Chen et al., 1990), double that of *E. coli* (4700 kbp). The genome size of *So. cellulosum* So ce56 is about 12.2 Mb (Pradella et al., 2002). Genome sequencing of these two species is in progress. The genome size of *Sg. aurantiaca* DW 4/3.1 is 9350 kbp (Neumann et al., 1992). Genome sizes of a variety of *Sg. aurantiaca* and *Sg. erecta* isolates were examined and ranged from 9.2 to 10.01 Mb. Thus the genome size of this group appears to be uniformly large. In the two species that have been examined, the genome is arranged in a single circular chromosome (Chen et al., 1990; Chen et al., 1991; Neumann et al., 1993).

The DNA of *Mx. xanthus* strain FB is methylated at cytosine sites (Yee and Inouye, 1982). In rapidly growing cells, the DNA is undermethylated, and the degree of methylation increases when growth slows. *Myxococcus xanthus* RNA polymerase shows the usual subunit composition, but two slightly different σ factors have been obtained from vegetative cells (Rudd and Zusman, 1982).

A most unusual kind of nucleic acid, msDNA, which was first discovered in *Mx. xanthus*, has raised many intriguing questions (Yee et al., 1984). Found in nearly all myxobacteria (Dhundale et al., 1985), msDNA is a single-stranded DNA and 500–700 copies of it are present per genome. The DNA is 162 bases long, and its 5′-end is linked via a 5′ to 2′-phosphodiester bond with a guanidine residue in an RNA (msdRNA), which is 77 bases long. The RNA has an extremely stable stem-and-loop structure. The coding regions for msDNA and msdRNA are found side by side at one specific site in the *Myxococcus* genome, but they are oriented in opposite directions and overlap with 8 bases at their 3′-ends (Dhundale et al., 1987). A very similar msDNA (81% sequence homology) with a corresponding genome structure has been found in *Sg. aurantiaca* (Furuichi et al., 1987a; Furuichi et al., 1987b). A second species of such DNA (mrDNA) was later discovered in *Mx. xanthus* (Dhundale et al., 1988b). However, mrDNA is much shorter, and it has a completely different DNA and RNA base sequence, but the RNA still has a similar secondary structure to that of msRNA. Also, there seem to be only 100 copies of mrDNA per genome.

The msDNA is synthesized by a reverse transcriptase with an associated ribonuclease H activity. The enzyme uses a folded RNA precursor as a primer and a template (Lampson et al., 1989). The gene of the reverse transcriptase has been found immediately downstream from the msdRNA region and codes for 485 amino acids, which show sequence homology with retroviral reverse transcriptases (Inouye et al., 1989). In fact, *Mx. xanthus* actually contains two reverse transcriptases (Inouye et al., 1990b). The wide distribution of msDNA in myxobacteria, as well as a comparison of codon usage in the reverse transcriptase gene and in other *Myxococcus* genes, suggests that the system is very old and was not acquired recently (in contrast to a similar system in certain *E. coli* strains). By deletion mutation, msDNA synthesis could be eliminated, but this had no significant effects on growth, motility and morphogenesis compared to the wild-type strain (Dhundale et al., 1988a).

No evidence has been found for the presence of a poly(dT-dG) · poly(dC-dA) (TG) element in *Mx. xanthus* (Morris et al., 1986).

While the half-life of mRNA is about 3.5 min in vegetative cells of *Mx. xanthus*, 5–10 species of mRNA with a much longer half-life (13–30 min) could be demonstrated during fruiting body formation (Smith and Dworkin, 1981; Nelson and Zusman, 1983). One of the long-lived mRNAs is for protein S. The stable mRNA can amount up to 30–40% of the total mRNA.

Although no clear evidence has been presented for free, self-replicating plasmids in myxobacteria, several early reports suggest that extrachromosomal DNA may be present. Thus, after chloramphenicol resistance was induced in *Mx. xanthus* RB, a burst of synthesis of circular, covalently linked, extrachromosomal DNA was observed (Brown and Parish, 1976a). The resistance was due to chloramphenicol acetylation (Burchard and Parish, 1975). Also, it was possible to transfer resistance to several different antibiotics from R factor-containing strains of *E. coli* to several *Myxococcus* species by conjugation (Parish, 1975). In some cases, extrachromosomal DNA could be demonstrated in the resistant *Myxococcus* strains, and the resistance could be transferred to other *Myxococcus* strains by conjugation.

PHAGES AND BACTERIOCINS. All myxophages that have been found thus far contain double-stranded DNA of around 50 kbp. The first myxophage to be isolated was *Mx. xanthus* phage MX-1. It came from a sample of cow dung and is a virulent phage resembling coli phage T2 with an icosahedral head and a contractile tail sheath. It has a latent period of 2–2.5 h (30°C), a 2-h rise, and a burst size of about 100 (Burchard and Dworkin, 1966a). MX-1 attacks *Mx. xanthus* as well as the related species *Mx. virescens* and *Mx. fulvus* (Tsopanakis and Parish, 1976). It has a genome size of 130–150 $\times$ 10^6 Da and a G+C content of 56 mol% (using buoyant density; a reinvestigation with a chemical method gave 50–52 mol%; Brown et al., 1976c).

MX-1 shows the classical pattern of infection and propagation known for *E. coli* phages (Voelz and Burchard, 1971). However, if cells are infected with MX-1 during glycerol induction of myxospores (see the section Functions of Myxospores and Fruiting Bodies in this Chapter), there is a brief period during which the phage may be taken up without interfering with myxospore induction. The phage is trapped in the myxospore and is released upon germination (Burchard and Dworkin, 1966a).

Defective phage particles, originally described as rhapidosomes (Reichenbach, 1965a), have been seen in several myxobacteria (Reichenbach, 1967; McCurdy and McRae, 1974; Brown et al., 1976b). When the ultracentrifugal pellets of culture supernatants of six strains of *Mx. xanthus*, *Mx. virescens* and *Mx. fulvus* were examined under the electron microscope, all six strains produced small quantities of phage particles (Brown et al., 1976b). An endogenous bacteriophage was detected in a derivative of *Mx. xanthus* FB through the use of transposon tagging (Starich et al., 1985). Transposon mutagenesis with Tn*5* revealed a class of bacterial mutants that transduced the transposon through the culture supernatant. Virus-like particles, 30–35 nm in diameter, co-purified with the transducing activity. A physical map of this region of the genome comprised multiple units of about 80 kbp each (Starich and Zissler, 1989). All but one of these has been deleted in *Mx. xanthus* DK1622, which was subjected to UV irradiation, resulting in a genome that is 220 kbp smaller than that of FB (Chen et al., 1990).

Restriction of phage MX-1 has been shown directly with a strain of *Mx. virescens* (Morris and Parish, 1976). The strain became fully sensitive after curing by UV irradiation. Also, two restriction endonucleases have been demonstrated in the restrictive strain. Such enzymes have been found in many different myxobacteria (Mayer and Reichenbach, 1978). Mx8 expresses a nonessential DNA methylase, Mox, which modifies adenine residues in *Xho*I and *Pst*I recognition sites, CTCGAG and CTGCAG, respectively, on both phage DNA and the host chromosome (Magrini et al., 1997). Mox may protect Mx8 phage DNA against restriction upon infection of some *Mx. xanthus* hosts.

A strategy was applied to isolate transducing phages from a collection of *Mx. xanthus* strains using certain sensitive strains as indicators, such as strain FB. Several *Mx. xanthus* phages have been isolated (Brown et al., 1976b; Campos et al., 1978; Geisselsoder et al., 1978; Martin et al., 1978), including morphological types either with very short tails, e.g., MX-4, or with long tails, e.g., MX-8 and MX-9. Also isolated have been λ-like myxophages (Rodriguez et al., 1980). MX-8, which has been particularly well characterized (e.g., Orndorff et al., 1983; Stellwag et al., 1985), is a generalized transducing phage with a linear, double-stranded DNA (56 kbp). The genome has a terminal redundancy of 8% and is circularly permuted over at least 40% of its length. The MX-8 prophage is integrated into the bacterial genome by a site-specific recombination between the *attB* and *attP* sites that has been extensively examined (Tojo et al., 1996; Salmi et al., 1998; Magrini et al., 1999a; Magrini et al., 1999b; Tojo and Komano, 2003). Another well-studied transducing phage is MX-4 (Campos et al., 1978; Geisselsoder et al., 1978).

Myxobacteria have been shown to produce bacteriocins, termed "myxocins." The first, xanthacin, was discovered in *Mx. xanthus* (McCurdy and McRae, 1974). This myxocin is produced

after mitomycin C induction, is active exclusively on a few myxobacteria, and consists of spherical bodies of somewhat variable size. It resembles membrane vesicles and may in fact have been a defective phage. Fulvocin C, one of three substances with bacteriocin activity from *Mx. fulvus* Mx f16 (Hirsch, 1977), turned out to be a protein consisting of 45 amino acid residues (molecular weight 4672 Da) with no lipid or carbohydrate attached. Disulfide bridges (probably four) make it a very compact molecule (Tsai and Hirsch, 1981). It was the first bacteriocin for which a complete structure could be presented. Fulvocin C, at a minimal inhibitory concentration of 0.1–0.25 μM, killed 16 out of 17 tested *Mx. fulvus* strains, the exception being the producer, and appeared to exert its effect on the cell membranes (Hirsch et al., 1978).

Gliding Motility

Gliding is the translocation of cells on surfaces without the use of flagella (McBride, 2001). The speed of gliding cells varies, depending on the substrate and the temperature; typically it is between 3 and 13 μm/min (*Mx. virescens*, 32°C, on peptone agar, measured in movie frames; Reichenbach, 1966). Gliding cells always deposit slime trails (Fig. 5). *Myxococcus xanthus* cells glide on solid surfaces in the direction of the long cell axis at rates of 1–2 μm/min (Kaiser and Crosby, 1983) and reverse their direction of movement every 6.8 min on average (Blackhart and Zusman, 1985).

Genetic analysis of *Mx. xanthus* motility has revealed the presence of two nearly separate motility systems enabling cell translocation over solid surfaces (Hodgkin and Kaiser [1979a] and Hodgkin and Kaiser [1979b]; for reviews, see Shimkets [1990b], Shi and Zusman [1993a], Hartzell and Youderian [1995], Youderian [1998], Ward and Zusman [1999], Kaiser [2000a], and Kearns and Shimkets [2001a]). The "A" system (for adventurous) enables cells to move as individuals without direct contact with other cells. In nature this system may allow cell dispersal for foraging. The A system also contributes to the movement of cells within groups. Thus far, 33 genes known to be essential for A motility have been identified and sequenced. From the frequency of mutagenesis and the target sizes of the genes it has been estimated that about half of the A motility genes have been discovered (Youderian et al., 2003). Six of these genes encode different homologues of the TolR, TolB and TolQ transport proteins, suggesting that A motility is dependent on biopolymer transport (Youderian et al., 2003).

The mechanism of A motility is unknown. One hypothesis is that A motility may involve extrusion of a propellant that swells as it hydrates, pushing the cell forward (Wolgemuth et al., 2002). Another hypothesis is suggested by the presence of a complex cell surface apparatus, revealed by electron microscopy in *Mx. xanthus*, *Mx. fulvus* and implied in *Sg. aurantiaca*. It has been suggested that the apparatus produces traveling waves along the surface of the cell which in turn may propel the cell (Freese et al., 1997; Lünsdorf and Reichenbach, 1989; Lünsdorf and Schairer, 2001). To this point, the A motility mutants have not been studied carefully enough to provide support for any particular model.

The "S" system (for social) enables cell movement only if cells are within one cell length of one another (Kaiser and Crosby, 1983). In nature this system plays an important role in multicellular behaviors like fruiting body formation. S motility is genetically and functionally similar to twitching motility of many Gram-negative bacteria including *Pseudomonas* and *Neisseria* (Kaiser, 2000a). Twitching and S motile cells

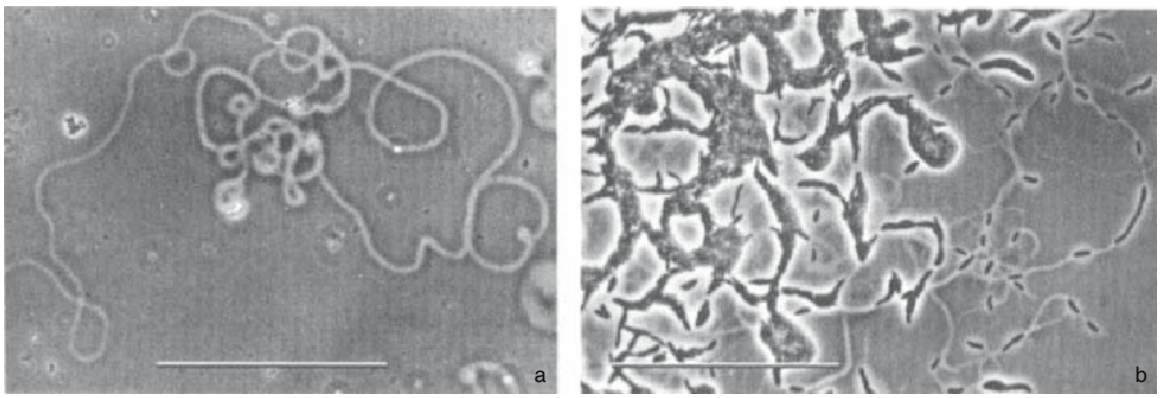


Fig. 5. Slime tracks as seen on thin agar layers in chamber cultures. a) *Nannocystis exedens*. b) *Myxococcus fulvus*; the spots on the left are cell clusters, not single cells. Bar = 100 μm. Phase contrast.

display tufts of type IV pili (also called "fimbriae") on the leading cell pole (Kaiser, 1979a; Henrichsen, 1983). Pili are 5–8 nm wide and may exceed the length of a cell (MacRae and McCurdy, 1975; MacRae and McCurdy, 1976; MacRae et al., 1977; Dobson and McCurdy, 1979; Arnold and Shimkets, 1988a). Fimbriae have been observed with many different species of both suborders; the only negative result was with *Nannocystis*, but this may have been due to technical problems. The fimbriae may be restricted to one of the two cell poles, and up to six have been seen per cell (Kaiser, 1979a). Tiny holes in the outer membrane, 6.5–8.5 nm wide and surrounded by a collar of 2.6 nm, which sometimes shows a ring of 12 spikes, have been proposed to be the insertion sites of the fimbriae (MacRae et al., 1977). Movement appears to result from pilus extension, pilus attachment, and pilus retraction to pull the cell forward like a grappling hook (Merz et al., 2000; Sun et al., 2000; Skerker and Berg, 2001). The pilus retraction motor, PilT, is the most powerful molecular motor yet examined. The force exerted by a single retracting *Neisseria* pilus was estimated to exceed 100 pN (pico Newtons; Maier et al., 2002). When a cell reverses direction, the pili on one cell pole are replaced by pili on the opposite cell pole (Sun et al., 2000). It has been suggested that the pili from one cell attach to cell surface appendages known as fibrils from another cell to mediate group movement (Li et al., 2003). There is an excellent correspondence between genetics and physiology with regard to S motility. The majority of the known S motility genes are involved in the production of type IV pili or the pilus receptor on the cell surface (Wu and Kaiser, 1995; Wu et al., 1997; Wu et al., 1998; Lancero et al., 2002; Li et al., 2003).

A mutant becomes nonmotile if it is A^-S^-, which normally requires two mutations. A single mutation in one gene, *mglA*, has the same effect, suggesting that it is shared between the two systems. The *mglA* gene encodes a 22-kDa GTPase that is thought to act as a switch between the two motility systems (Hartzell and Kaiser, 1991). MglA interacts with MasK, a protein of the STY kinase family (Thomasson et al., 2002). MasK, expressed in *E. coli*, was shown to phosphorylate tyrosine residue(s). The gain-of-function *masK815* mutant is restored in S motility and displayed increased production of cell surface fibrils. Attempts to eliminate the *masK* gene have been unsuccessful, suggesting that it is essential for growth. The A motility system also utilizes a tyrosine kinase, AgmO, although the role of this protein has not yet been determined (Youderian et al., 2003).

Myxococcus xanthus cells show directed movements towards glass and latex beads and clumps of prey bacteria (Dworkin, 1983a). The mechanism(s) of such long range directed movement remain a mystery. Elasticotaxis, a phenomenon first described by Roger Stanier, results from delicate structural disturbances in the substrate surface, such as are produced in agar plates by mechanical stress. Cells orient their movement perpendicular to stress forces within the agar (Stanier, 1942a). Elasticotaxis is mediated by the A motility system (Fontes and Kaiser, 1999). Cells also direct their movement to certain chemicals. Chemotaxis has been observed to complex chemical mixtures (Shi et al., 1993b) and to certain isomers of the major membrane lipid phosphatidylethanolamine (PE; Kearns and Shimkets, 1998).

Myxococcus xanthus exhibits the hallmarks of chemotactic behavior, excitation and adaptation in response to PE. Certain molecular PE species increase the reversal period as much as sixfold (excitation; Kearns and Shimkets, 1998). Cells return to the pre-stimulus reversal frequency in about an hour (adaptation; Kearns and Shimkets, 1998). Dilauroyl PE (PE-12:0/12:0), dioleoyl PE (PE-18:1ω9c/18:1ω9c), and PE with 16:1ω5c at both the *sn*-1 and *sn*-2 positions (PE-16:1ω5c/16:1ω5c) cause excitation, whereas many other species do not (Kearns and Shimkets, 1998). These results begin to bring into focus properties of the as yet unknown PE binding protein that serves as the receptor (hereafter called "PE receptor"). A major unresolved point is whether the PE receptor recognizes the *sn*-1 fatty acid, the *sn*-2 fatty acid, or both.

Chemosensory systems analogous to those found in the enteric bacteria appear to play roles in the response to PE. Excitation to PE appears to be mediated by the Dif signal transduction pathway. The *difA* gene encodes a methyl-accepting chemotaxis protein (MCP), *difC* encodes a CheW homolog, *difD* encodes a CheY homolog, and *difE* encodes a CheA homolog. Excitation to both PE-12:0/12:0 and PE-16:1ω5c/16:1ω5c requires DifA (Kearns et al., 2000). The DifA ligand is unknown, but binding of PE to DifA directly is unlikely. The periplasmic face of DifA is approximately 15 amino acids, much smaller than that of Tar (Kearns and Shimkets, 2001a). Thus understanding the nature of the stimulus for DifA is important.

Another set of chemosensory genes, the "*frizzy*" (*frz*) genes, also regulates the frequency with which the cells change the direction of their movement (Blackhart and Zusman, 1985) and the response to PE (Kearns and Shimkets, 1998). While wild type cells reverse their direction of movement every 6–7 min, some Frz mutants do so every 2 h or every 2 min. The genes and their gene products have been analyzed in considerable detail (for review, see Ward and Zusman,

1997). The FrzCD protein encodes the MCP (McBride et al., 1989b) and the FrzE protein is a fused CheA-CheY homolog (McCleary and Zusman, 1990a; McCleary and Zusman, 1990b). The *frzCD* and *frzE* mutants exhibit excitation to PE but had aberrant adaptation (Kearns and Shimkets, 1998). As in the enteric bacteria, the direction of *Mx. xanthus* movement is controlled by methylation and demethylation reactions in the Frz system (McCleary et al., 1990c).

Extracellular Matrix

An extracellular matrix composed of polysaccharide and protein connects bacteria living in biofilms. The extracellular matrix of *Mx. xanthus* appears to be extruded as long thin fibrils, about 30 nm in diameter, that emanate from many points on the cell surface. Fibrils have been visualized using a wide variety of fixation techniques and microscopy methods (Arnold and Shimkets, 1988b; Behmlander and Dworkin, 1991; Behmlander and Dworkin, 1994; Merroun et al., 2003), most notably transmission electron microscopy of freeze-substituted cell thin sections (Kim et al., 1999), which is the most advanced approach for examining surface layers (Beveridge, 1999). Fibril synthesis is induced by starvation (Arnold and Shimkets, 1988b), stimulated by cell contact (Behmlander and Dworkin, 1991) and Ca^{+2} (Kim et al., 1999), and repressed by the *stk* locus (Dana and Shimkets, 1993).

The extracellular matrix is composed of a polysaccharide core with noncovalently associated protein (Behmlander and Dworkin, 1994). Mutants lacking the ability to secrete the extracellular matrix polysaccharide fail to bind Congo red or calcofluor white (Arnold and Shimkets, 1988a; Arnold and Shimkets, 1988b; Dana and Shimkets, 1993; Ramaswamy et al., 1997; Kim et al., 1999). The most carefully studied of these mutants has been isolated twice; the first was based on loss of S motility, where it was called *dsp* (Shimkets, 1986), and the second based on loss of development, where it was called *dif* (Yang et al., 1998). Now clearly these two sets of mutants are defective in the same genes (Lancero et al., 2002). The phenotype of the *dsp*/*dif* mutants provides one level of insight into the many functions of the extracellular matrix. These mutants are defective in adhesion to surfaces, cohesion to other *Mx. xanthus* cells, S motility (also known as twitching in other Gram-negative bacteria), fruiting body morphogenesis, sporulation, developmental gene expression, and chemotaxis to phosphatidylethanolamine (PE). The *dsp*/*dif* mutants are blocked about 8 h into the developmental program and fail to express roughly one-third of the developmentally regulated genes (Li and Shimkets, 1993). The *dsp*/*dif* locus encodes components of a chemotaxis pathway with homology to the Che proteins of enteric bacteria (Yang et al., 1998).

Fibrils are extracted from cells with 0.5% sodium dodecylsulfate (SDS) and sedimented by low speed centrifugation (Behmlander and Dworkin, 1991). Fibrils can restore cohesion, chemotaxis, and fruiting body development to mutants that cannot produce fibrils by simply adding them back to cells (Chang and Dworkin, 1994; Yang et al., 2000b; Kearns et al., 2002). The functions of the many proteins in the matrix are as yet unknown. One fibril protein, a zinc metalloproteinase known as FibA, is essential for PE chemotaxis to dilauroyl PE and PE-16:1ω5c/16:1ω5c but not dioleoyl PE (Kearns et al., 2002).

The excreted polysaccharide appears to contain amino sugars, among others (Sutherland and Thomson, 1975; Sutherland, 1979). Slime from fruiting bodies resembles in its overall composition that produced in vegetative cultures. In *Mx. xanthus*, fibril polysaccharide is composed of galactose, glucosamine, glucose, rhamnose and xylose (Behmlander and Dworkin, 1994). A large extracellular protein-polysaccharide-lipid complex with proteolytic activity has been isolated from culture supernatants of *Mx. virescens* (Gnosspelius, 1978b). A glycopeptide with a heat-stable blood anticoagulant activity, named "myxaline," has been obtained from the culture broth of *Mx. xanthus* (El Akoum et al., 1987; Masson and Guespin-Michel, 1988). The glycan part contains *N*-acetyl amino sugars, and the peptide is rich in glutamic acid and serine. The excreted slime must be chemically different in the three suborders of myxobacteria, since Congo red (0.01% [w/v] aqueous solution) stains the slime of the Cystobacterineae but not that of the Sorangineae (McCurdy, 1969b) or the Nannocystineae.

Myxobacterial Colonies

On media with a relatively low nutrient content (like VY/2 and CY agar), colonies of myxobacteria spread over the agar surface. Such colonies are called "swarms." With a few exceptions the colony does not move as a whole but simply increases in diameter because of gliding motility and cell division.

The appearance of a swarm depends on the species and the medium. On rich media, such as those with a high peptone content (around 1%), myxobacterial colonies may remain small and more or less compact; sometimes they are even circular, convex, and with an entire edge like ordinary bacterial colonies. The typical myxobacterial colony is, however, a swarm sheet consisting of cells and excreted slime; much of the latter

is probably composed of interwoven slime trails. The swarms often contain conspicuous veins, rings, and ridges or even depressions in the agar surface. Such patterns can be characteristic for specific taxonomic groups (see below). The swarm sheet can sometimes be loosened from the agar surface with water (e.g., with *Cystobacter* and *Stigmatella*); sometimes, however, it sticks tenaciously to the agar surface, occasionally to such an extent that the agar is torn into pieces when the aging swarm sheet contracts (especially with *Sorangium*). In some cases, the swarm sheet becomes so tough that it is very difficult to cut with the inoculating loop (with *Archangium*, *Cystobacter* and *Melittangium*); in other cases, it is soft and slimy and can easily be scraped off the plate (with *Myxococcus*). Often the myxobacteria penetrate the agar (even 1.5% agar), and the swarm spreads below the agar surface, sometimes penetrating to the very bottom of the dish (often with *Polyangium*). In such cases, the swarm may completely disappear below the surface, and its former position can be recognized only by a shallow depression. All members of the Sorangineae and Nannocystineae tend to grow within the agar. However, some members of the Cystobacterineae also do so, although here it is often a consequence of swarm degeneration. Some myxobacteria corrode the agar surface to the extent that they produce long tunnels or large, cavern-like holes in the plate. This is typical of *Polyangium* and especially of *Nannocystis*.

The swarm edge usually shows characteristic flame-like protrusions, or flares. Sometimes wide, tongue-like depressions are produced (by *Chondromyces* and other members of the Sorangineae), or isolated narrow trenches radiate from the swarm center far into the surrounding agar surface (*Polyangium* and *Nannocystis*). Occasionally, massive ridges are also seen at the swarm edge (*Chondromyces*, *Polyangium* and *Nannocystis*). At the swarm edge, the swarm may consist of only one cell layer, but as a rule the swarm sheet is composed of many layers that quickly pile up behind the margin. When growing on very lean media, most of the swarm may be only one cell thick, although even then the bacteria tend to concentrate themselves in certain areas, such as along radial tracks. In large swarms, most of the cell population may be found in the outer regions, and if no fruiting bodies or myxospores have been produced, the interior may be completely deserted. The speed of the advancing edge is in the range of 1 mm in 9 h (*Cb. violaceus* at 22°C; Reichenbach, 1965b) to about 1 mm in 1.6 h (*Cystobacter* and *Stigmatella* strains at 30°C).

Various taxa may be recognized by their swarm morphology. When plate cultures are flooded with an aqueous 0.01% (w/v) Congo red solution for 5–10 min, the swarm sheets of the Cystobacterineae stain a deep violet-red, while those of the Sorangineae and Nannocystineae remain unstained (McCurdy, 1969b). The swarms of *Myxococcus* and *Corallococcus* typically develop meandering radial veins, which may be particularly conspicuous on CY agar; the swarm sheet usually remains soft and slimy. In contrast, long, more or less straight, branching veins are seen in the swarms of *Cystobacter*, *Archangium*, *Melittangium* and *Stigmatella*. On VY/2 agar, the swarms of most Sorangineae are sunk into the agar and appear as wide shallow depressions at the surface. *Chondromyces* and *Sorangium* often develop long, cable-like veins on the surface that sometimes curl into a ring at one end. Also, isolated rings are often seen in those swarms. With *Chondromyces* swarms, the swarm edge typically consists of a series of tongue- and shell-like shallow depressions. *Polyangium* swarms on VY/2 agar often penetrate the agar to the bottom and characteristically are surrounded by a more or less perpendicular, curtain-like zone composed of tiny, well-separated cell clumps. *Polyangium* and *Nannocystis* growing on streaks of autoclaved *E. coli* often produce long, radiating, narrow trenches in the agar surface, with small cell clusters (and, later on, fruiting bodies) at the end. The two can often be distinguished because the tracks and cell clumps of *Nannocystis* are more delicate. On mineral salts-glucose media, like CA2 and CK6 agar, *Sorangium* often produces swarms with a beautiful, dense network of branching cable-like veins.

Note that the swarm structures described above are usually only seen with strains that have recently been isolated. The swarms of strains that have been transferred many times, or that have been adapted to growth in liquid media, are often very different. Such swarms may be soft and slimy, lacking all morphological differentiation, and, in the case of the Cystobacterineae, sometimes no longer grow on the surface but are sunk into the agar.

A colony dimorphism has been described for *Cb. violaceus* and *Mx. xanthus* and is probably quite common among myxobacteria (Reichenbach, 1965c; Grimm and Kühlwein, 1973a; Grimm and Kühlwein, 1973b; Grimm and Kühlwein, 1973c; A. C. Burchard et al., 1977). This dimorphism may regulate cell motility and consequently the spreading behavior of the colonies. The dimorphism in *Cb. violaceus* affects the slime, the capacity to fruit, and the ability to grow as a homogeneous cell suspension. Also, pigmentation can be affected (deep and pale violet in *Cb. violaceus*, tan or yellow in *Mx. xanthus*). Usually one colony type is more stable, but segregation in both directions is possible, sug-

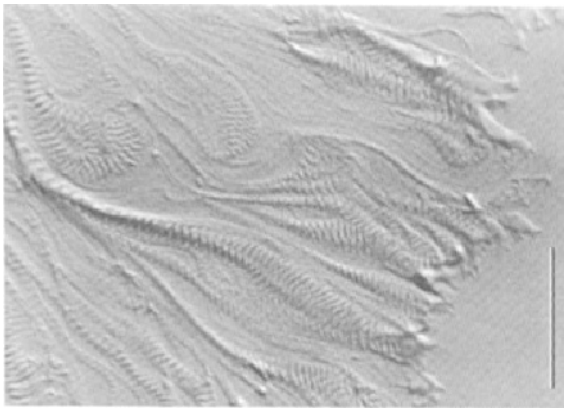


Fig. 6. Oscillating ridges or ripples. *Stigmatella erecta* on Casitone agar; dissecting microscope, oblique illumination. Bar = 200 μm.

gesting a phase variation. Both cell types may serve essential functions in nature. In *Mx. xanthus*, the tan cells preferentially make spores in spite of the fact that tan locked mutants are incapable of completing sporulation unless yellow cells are provided (Laue and Gill, 1995). The results suggest that the yellow cells provide a factor enabling spore maturation.

A striking feature of myxobacterial swarms is the ability of the cells to organize their movement in waves that have been referred to as "ripples," "oscillatory waves," or "traveling waves." Dense fields of regularly arranged ridges are found in long, parallel rays. They are positioned parallel to one another and perpendicular to the direction of the ray. The distance between the ridges is uniform and is approximately equal to the width of the ridges (Fig. 6). In *Mx. xanthus*, a distance of 45 μm from crest to crest has been measured (Shimkets and Kaiser, 1982). The phenomenon was discovered when movies of developing *Corallococcus* and *Myxococcus* swarms were first made. They showed that the ridges actually move, so that the whole field appears to oscillate; hence, the ridges were called "oscillating waves" (Reichenbach, 1965a; Reichenbach et al., 1965f; Reichenbach et al., 1965g). In fact, the movements of the ridges may even speed up and slow down, or pulsate, over a period of several hours. The movement of the ridges is fast enough to be recognized under the dissecting microscope; a speed of about 2 μm/min has been measured in *Mx. xanthus* (Shimkets and Kaiser, 1982). Traveling waves were later found in many other myxobacteria. They appear to occur with all Cystobacterineae and occasionally are also observed with members of the Sorangineae, e.g., *Chondromyces*. With *Sg. aurantiaca*, even waves travelling in opposite directions along the same path have been seen (Reichenbach et al., 1980).

The mechanism of cell movement in the traveling waves has been examined extensively in *Mx. xanthus* (Shimkets and Kaiser, 1982; Sager and Kaiser, 1994; Sager, 1996; Igoshin et al., 2001; Welch and Kaiser, 2001; Borner et al., 2002). Rippling can be induced by purified peptidoglycan and peptidoglycan components, such as *N*-acetyl-glucosamine, *N*-acetylmuramic acid, diaminopimelic acid, and D-alanine (Shimkets and Kaiser, 1982). The CsgA cell signaling protein is essential (Shimkets and Kaiser, 1982). Cell movement occurs along the same axis as the rippling movement and is periodic (Sager and Kaiser, 1994; Sager, 1996; Welch and Kaiser, 2001). The periodicity of individual cells matches the period of macroscopic rippling. When two cells moving in opposite directions meet end to end, they reverse their gliding direction. This periodicity is thought to be linked to the C signal, a cell contact-mediated signaling molecule. Mathematical models quantitatively reproduce the experimental observations and illustrate how intracellular dynamics, contact-mediated intercellular communication, and cell motility can coordinate to produce this behavior (Igoshin et al., 2001; Borner et al., 2002). These models of traveling waves illustrate how cell-contact interactions rather than reaction diffusion can regulate biological pattern formation.

Finally, the shape of myxobacterial swarms may become distorted by stress lines in the agar plate produced, for example, by mechanical pressure and stretching, a phenomenon known as "elasticotaxis" (Stanier, 1942). Elasticotaxis is due to A motility (Fontes and Kaiser, 1999).

Fruiting Bodies

The outstanding trait of the myxobacteria is their ability to form multicellular fruiting bodies (Figs. 7–10), which serve as the housing for the resistant resting cells, the myxospores. The processes that lead to their formation make the myxobacteria the object of considerable interest as model systems for the study of development. There are some excellent reviews focusing on different aspects of fruiting body and myxospore development: Kaiser (1989), Dworkin (1991), Dworkin (1996), White (1993), Shimkets and Dworkin (1997c), Shimkets (1999), and Ward and Zusman (2000).

DESCRIPTION OF FRUITING BODIES. The terminology used to describe the structural components of myxobacterial fruiting bodies is illustrated in Fig. 13. Although complete consistency with usage of terms in other biological systems is not possible, terms that have already acquired a well-defined meaning in other systems should be avoided if they are inappropriate. The term "fruiting body" has a long tradition of

use and may be acceptable in connection with myxobacteria, although in biology, fruiting usually implies sexual processes, and no such events seem to be involved in the case of the myxobacteria. The term "sporangium" has also been applied to these structures, but this term is even more suggestive of sexuality and is best avoided. Most types of fruiting bodies are composed of myxospores, and the walled portions containing these myxospores are in fact communal cysts; they have traditionally been referred to as "cysts." However, since the term "cyst" in microbiology is applied to quite a different structure (i.e., dormant, encapsulated single cells), we propose to use the term "sporangiole" instead and to accept its obvious shortcomings. The advantage of the term sporangiole over "sporangium" is that it is a relatively unusual term and has been used only in connection with a restricted group of fungi (Mucorales), where it is not even used with a consistent meaning. Its use here is thus less likely to cause confusion. Incidentally, the term sporangiole was applied to these myxobacterial structures by Link (1809) in the very first description of a myxobacterium, *Pl. vitellinum* (Fig. 14). The simplest myxobacterial fruiting bodies are mounds of either soft or hardened slime, in the latter case often having bizarre shapes (*Corallococcus* and *Archangium*) or in the former typically having a knob or head with a constriction or a stalk at the base (*Myxococcus*).

The size of myxobacterial fruiting bodies varies between 10 and 1000 µm or greater depending on the species. Many can be seen with the naked eye, especially as they are normally produced in large numbers and often are brightly colored in hues of yellow, orange, red, brown or black. The distribution of fruiting bodies over the swarm usually is random, but sometimes fruiting bodies arise in concentric rings or along radial veins or in various patterns controlled by elasticotaxis (Stanier, 1942a). Fruiting proceeds in the swarm from the center to the periphery. The shape, structure and color of fruiting bodies is species specific—in fact myxobacterial species are mainly defined by their fruiting bodies—but similar shapes were developed by completely unrelated species, e.g., *Stigmatella* and *Chondromyces*, or *Cystobacter*, *Polyangium*, *Byssophagia*, *Jahnia* and *Sorangium*. Also, the shape and color of fruiting bodies may vary substantially within the species.

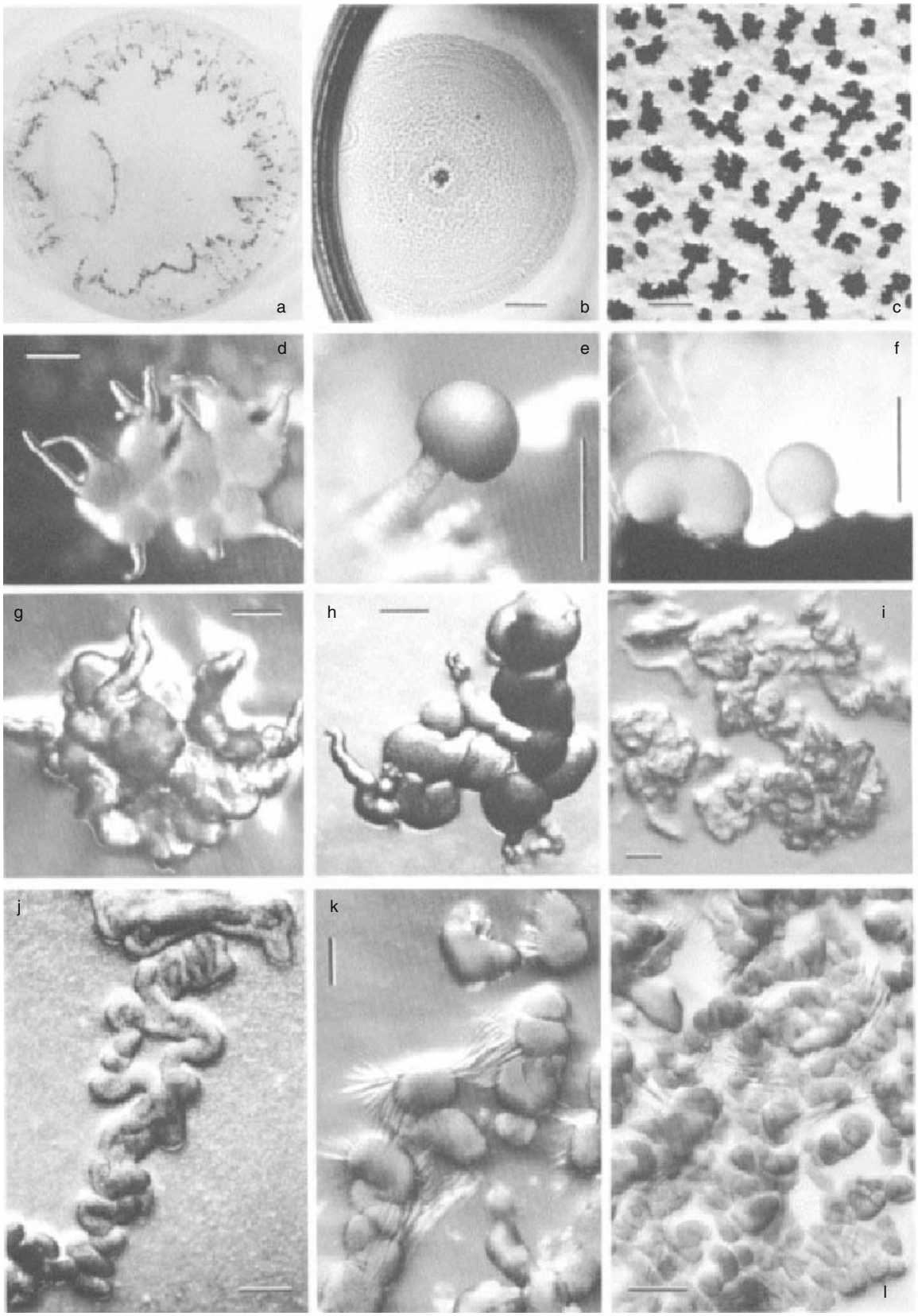
Most myxobacteria enclose their myxospores in sporangioles, i.e., containers with well-defined walls; the interior of the sporangioles is always hard and tough. The sporangioles may occur singly or in groups; they may rest either directly on or in the substratum or on simple or branched slime stalks.

The following key gives a survey of the different fruiting body types and their relation to the various myxobacterial taxa. Photographs of fruiting bodies of most species are provided for reference (Figs. 7–10). Keep in mind, however, that fruiting body structure and morphology often degenerate quickly after a few transfers upon isolation. Also, strains may be isolated that produce degenerate fruiting bodies from the very beginning. In such cases, only completely atypical knobs and ridges of slime and myxospores are formed. In addition, the fruiting bodies of several myxobacteria have also been studied with the scanning electron microscope, and some very impressive pictures have been obtained (e.g., Brockman and Todd, 1974; Stephens and White, 1980b; Galván et al., 1981; Stephens et al., 1982).

Key to the Fruiting Body Types of Myxobacteria

1. Naked masses of slime and myxospores 2
1′. Myxospores encased in sporangioles 3
2. Fruiting body soft-slimy . . . . . *Myxococcus* (*Kofleria*)
2′. Fruiting body hard, cartilaginous. . . . . *Corallococcus*, *Archangium*
3. Sporangioles single 4
3′. Sporangioles in groups 5
4. Single sporangiole located on or in the substrate . . . . . *Nannocystis*, *Haploangium*
4′. Single sporangiole on top of a stalk . . . . . *Melittangium*, *Stigmatella erecta*
5. Group of sporangioles on or in the substrate . . . . . *Cystobacter*, *Pyxicoccus*, *Polyangium*, *Sorangium*, *Nannocystis*, *Haliangium*, *Byssophaga*
5′. Group of small spherical sporangioles that are often empty and then look glassy transparent, arranged in extended, dense sheets, or often in chains. . . . . *Hyalangium*
5″. Coils of sporangioles sitting on a slime cushion or soft slime stalk . . . . . *Jahnia*
5‴. Cluster of sporangioles on top of an unbranched stalk . . . . . *Stigmatella aurantiaca*, most *Chondromyces* species
5⁗. Clusters of sporangioles on a branched stalk . . . . . *Chondromyces crocatus*

More or less the same morphological types of fruiting bodies are found in the suborders Cystobacterineae and Sorangineae (with the qualification that the Sorangineae produce only fruiting bodies with sporangioles). Still, the course of morphological differentiation may be different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with seemingly identical fruiting body types. Thus, *Chondromyces* and *Sg. aurantiaca* both form little treelets, but the stalks and sporangioles are produced in a different way in the two cases. While *Chondromyces* excretes a slime stalk that lifts the undifferentiated cell mass upward, and differentiation of the sporangioles occurs as the last step, *Sg. aurantiaca* piles up a mass of cells with approximately the shape and size of the final fruiting body, then the cells either withdraw from the stalk area or degenerate and lyse in that part, and the sporangioles mature (Fig. 11).

As has already been mentioned, stalks, sporangiole walls, and pigments are produced during fruiting but nothing is known about the chemical composition of these structures and substances. Under the electron microscope, the stalk of *Cm. crocatus* is seen to consist of numerous parallel tubes, which are approximately the diameter of a cell and run in the direction of the long axis of the stalk (McCurdy, 1969a; Abadie, 1971b). The stalk of *Sg. aurantiaca* was found to be composed of tubules wedged against each other and containing cell debris but only rarely a cell or myxospore; the stalk was surrounded by a thin, dense surface layer resembling the wall that surrounds the sporangioles (Voelz and Reichenbach, 1969). The stalk of *Sg. erecta* also was found to be cell free and to consist of slime, although in this case tubules could not be distinguished (Galván et al., 1987). In contrast, scanning electron micrographs suggested that the stalk of *Sg. aurantiaca* is cellular (Stephens and White, 1980b). The contradiction is not yet resolved; perhaps the fruiting bodies examined with the scanning electron microscope had not yet fully matured. In any case, under the light microscope the stalk of *Sg. aurantiaca* looks translucent, unpigmented, and white and thus completely different from the opaque, dark brown sporangioles.

The base of fruiting body stalks is often clearly disk-shaped. The disks apparently arise early during fruiting body development when the aggregated cells move on a circular path at the site of fruiting.

Little is known about the arrangement of the cells within the developing fruiting body, but it appears that this arrangement, and perhaps rearrangements by migrations of certain cell populations, may play a major role during fruiting body morphogenesis (O'Connor and Zusman, 1989; Sager and Kaiser, 1993). Many species surround the cell mass with a tough wall, often after a subdivision of the original mass into portions of approximately equal size, thus creating sporangioles, either single or in clusters. In *Me. boletus*, a very regular pattern of three or more layers of strictly parallel cells has been observed (Jahn, 1924). The cells are perpendicularly arranged on the outer surface of the fruiting body, and it has been suggested that they are responsible for the secretion of the sporangiole wall. In developing *Cb. fuscus* sporangioles, the cells in the outer layers lie parallel to the surface.

The wall of the sporangioles is very thin, about 30 nm in *Cm. crocatus* (Abadie, 1971b) and 200–300 nm in *Cystobacter* (Vahle, 1910; Jahn, 1924). It dissolves in potassium hypochlorite solution (Vahle, 1910) and is intensely colored by pigments that are not identical with those of the cells and cannot be extracted by any of the normal solvents (Vahle, 1910). The chemical composition and the origin of the wall are not known. It has been suggested that cells lying at the surface excrete the wall (Jahn, 1924). Or perhaps it is formed by fusion of the cells forming the top layer. In *Me. boletus*, the cells in the developing sporangiole are arranged in a regular pattern in rows of parallel rods standing perpendicular to the surface. Thus, in this case it seems that the wall is excreted from one cell pole only (Jahn, 1924). How the size of the sporangiole is determined within the undifferentiated cell mass is one of the many unsolved questions in myxobacterial morphogenesis. During germination the sporangiole wall is ruptured mechanically, and an empty husk remains after the cells leave.

Fruiting Body Morphogenesis

The fruiting bodies are produced by the coordinated movement of 10^5 to 10^7 swarm cells that

Fig. 7. Myxobacterial fruiting bodies. a) *Cystobacter ferrugineus* on VY/2-agar; culture is 4 weeks old; the organism has spread all over the plate and produced numerous dark brown fruiting bodies; the diameter of the plate is 9 cm. (b–d) *Corallococcus coralloides*: b) swarm on VY/2 agar, with numerous fruiting bodies; the delicate swarm edge and the clear lysis zone in the yeast agar are well recognizable; c) field of fruiting bodies at higher magnification; d) a single fruiting body, in situ on the agar surface. e) *Myxococcus stipitatus*, fruiting body at the edge of a piece of filter paper, in situ. f) *Myxococcus fulvus*, fruiting bodies on a soil crumb, in situ. (g–j) *Archangium*-like fruiting bodies: g) *Archangium gephyra*, large fruiting body in situ on agar surface; h) *Cystobacter ferrugineus*, fruiting body with only rudimentary differentiation into sporangioles, in situ on agar surface; i) *Cystobacter*, probably *Cb. ferrugineus*, with totally degenerated fruiting bodies, in situ on agar surface; j) *Cystobacter* strain with typical "*Archangium*" fruiting bodies, in situ on agar surface; k) *Cystobacter velatus*, fruiting bodies in situ on agar surface, covered with a delicately plicated slime sheet; and l) the same organism at lower magnification; one can see some of the enormous number of fruiting bodies that may be produced under suitable conditions. Bar = 5 mm in b), 500 µm in c), 200 µm in l), and 100 µm in the remaining micrographs.

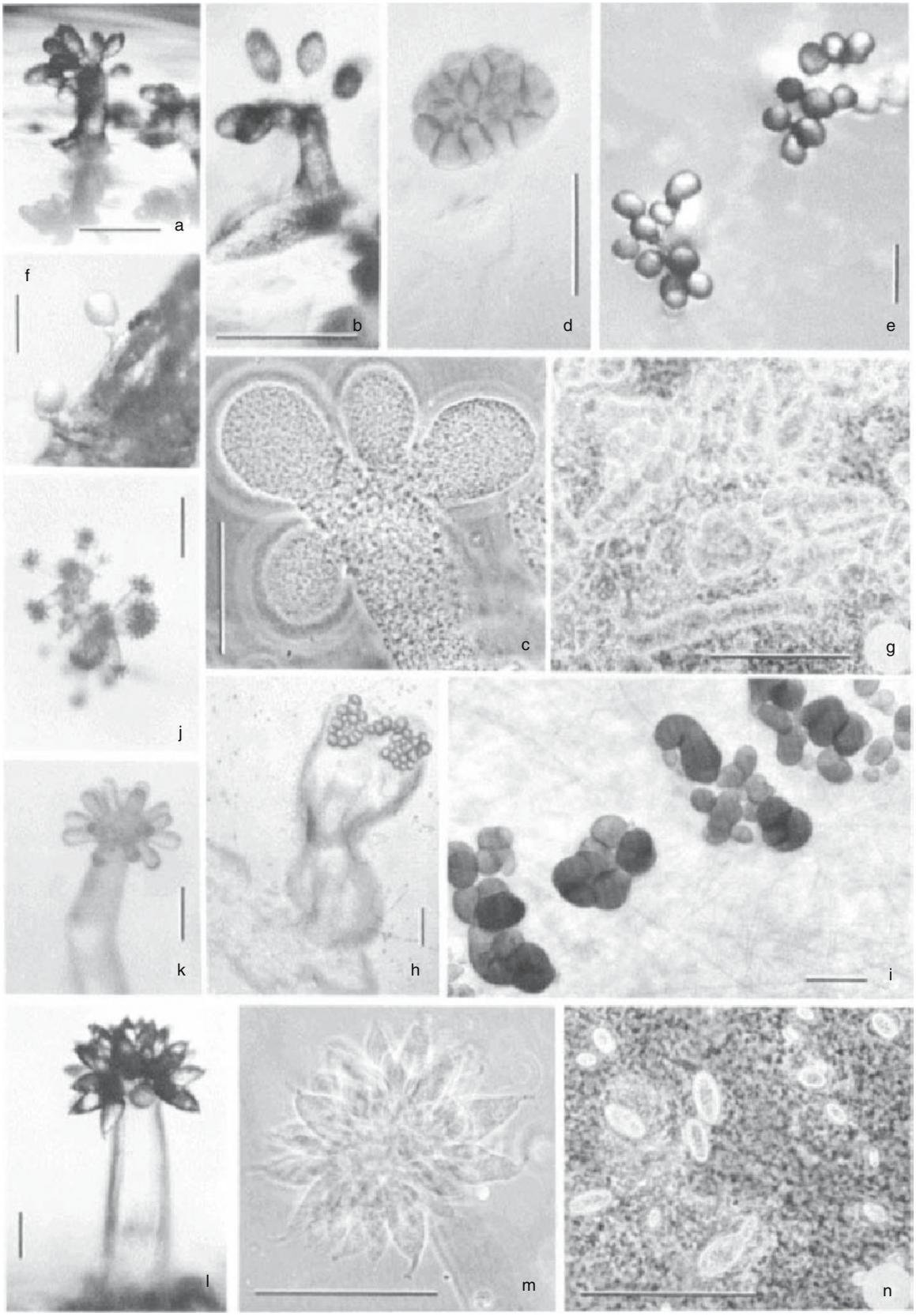
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Fig. 8. Myxobacterial fruiting bodies. (a–c) *Stigmatella aurantiaca*: a) fruiting body in situ on an agar surface; b) fruiting body with its sporangioles on exceptionally long peduncles, in situ; c) early developmental stage of a fruiting body, mounted in "Miracle Mount" (Cunningham, 1972) to make it transparent; the stalk still consists mainly of cells that later migrate into developing sporangioles or degenerate; the young sporangiole in the upper left corner shows the developing sporangiole wall that ends in a collar at the base of the sporangiole; phase contrast. d) Although this fruiting body strikingly resembles those of certain *Polyangium* (see h) or *Sorangium* species (see g), it still is that of a *Cystobacter*, *Cb. minus*, as is clearly shown by the shape of the vegetative cells; interference contrast. e) *Stigmatella erecta*, fruiting bodies in situ on agar surface. f) *Melittangium lichenicola*, fruiting bodies in situ on a piece of wood. g) *Sorangium cellulosum* on filter paper, cautiously squeezed slide mount; the shape of the fruiting bodies is often controlled by the orientation of the remnants of the wood fibers and tracheids, along and within which they develop; phase contrast. h) *Polyangium* sp., fruiting body in crude culture in situ on agar surface; note the deep path that has been etched into the agar surface by the migrating bacterial mass (compare with Fig. 2c) before it encased itself in sporangioles; the tiny dots all over the agar surface are encysted amoebae. i) *Jahnia thaxteri*, fruiting bodies on filter paper, in situ. (j–m) *Chondromyces apiculatus*: j) cluster of fruiting bodies on surface of filter paper, in situ; dissecting microscope; k) early (club) stage of sporangiole differentiation; dissecting microscope; l) mature fruiting body; m) small, mature, fruiting body in "Miracle Mount" (Cunningham, 1972) to make it transparent; the myxospores within the turnip-shaped sporangioles can just be recognized; the slime stalk, in contrast, is cell free; phase contrast. And n) *Nannocystis exedens*; the fruiting bodies are single sporangioles of very variable size and are embedded in the agar substrate; slide mount, phase contrast. Bar = 50 μm in (c), 200 μm in (i), 400 μm in (j), and 100 μm in the remaining pictures.

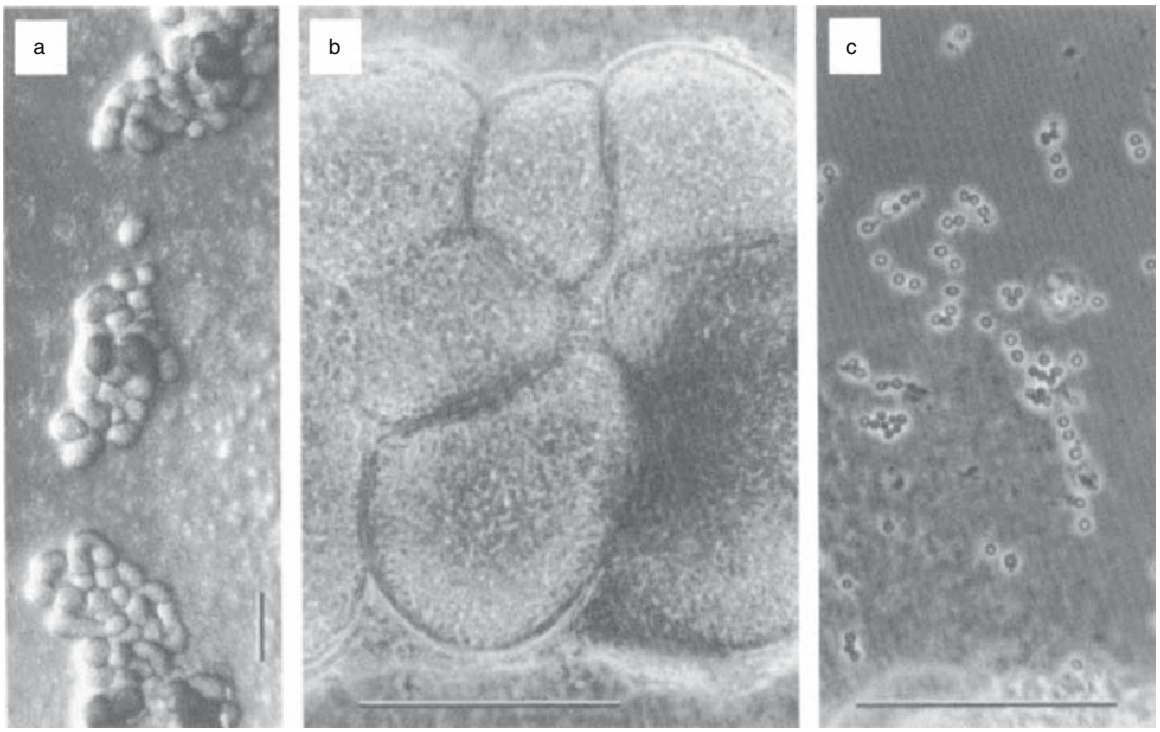

Fig. 9. Myxobacterial fruiting bodies: *Pyxicoccus fallax*. a) In crude cultures and on natural substrates the sporangioles tend to be disk-shaped and loosely stacked but, as shown here, in pure culture, they are more or less spherical and tightly attached to each other. b) At high magnification, the walls of the sporangioles, although only about 1 mm thick, become clearly recognizable. c) When the sporangioles are crushed, they release optically refractile, spherical myxospores, shown here at the same magnification as the sporangioles in (b), which look exactly like *Myxococcus* myxospores. (b) and (c) are in phase contrast. Bar = 100 μm in (a), 50 μm in (b) and (c).

retain their physical individuality throughout this process of cooperative morphogenesis (Fig. 12). Formation of fruiting bodies can schematically be divided into a number of developmental stages: 1) environmental signal(s) to direct the vegetatively growing cells to a developmental path (Kaiser, 2000b); 2) cell aggregation or accumulation; 3) emergence of molecules on the cell surface that mediate cell adherence; 4) rearrangement or clustering of the cells within the original undifferentiated mass; 5) creation of the specific shape of the fruiting body, perhaps the most fascinating event in the whole sequence; 6) production of special structural

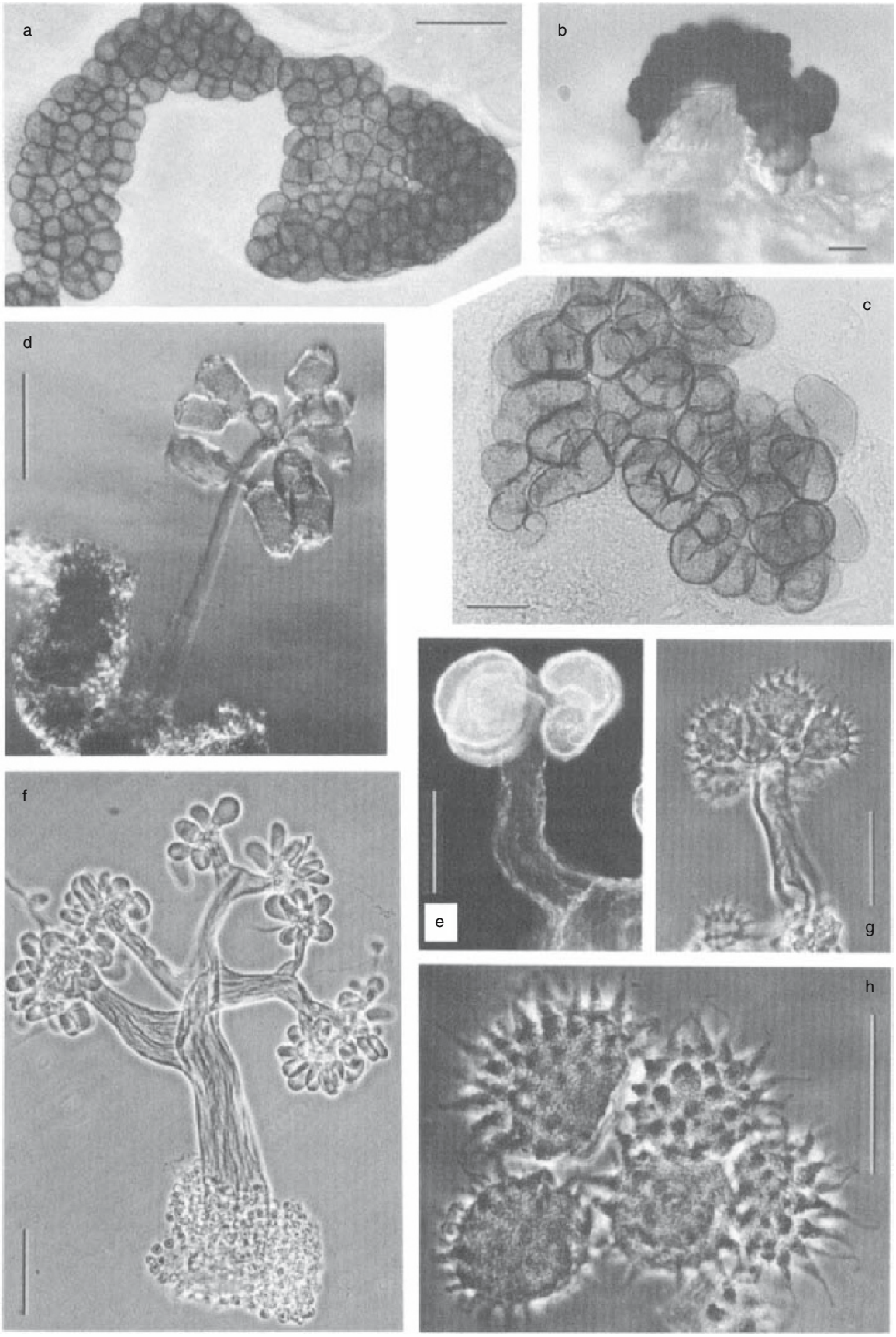
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Fig. 10. Myxobacterial fruiting bodies. a) *Sorangium cellulosum*, on VY/2 agar in situ (compare with Fig. 9). (b and c) *Jahnia thaxteri*; b) on agar surface in situ; a mass of tightly packed sporangioles is situated on a stalk-like cushion of slime: the whole fruiting body looks like a false morel; c) when mounted in "Miracle Mount" (Cunningham, 1972), the individual sporangioles can easily be distinguished. d) *Chondromyces pediculatus*, fruiting body on a soil crumb, embedded in Miracle Mount. (e and f) *Chondromyces crocatus*, both in Miracle Mount: e) a young, not yet fully differentiated stage; in dark field illumination, the translucent, cell-free stalk and the densely packed cell masses on its top are clearly distinguished; f) mature fruiting body; at the base of the stalk some encysted soil amoebae are seen. (g and h) *Chondromyces lanuginosus*, both in Miracle Mount: g) survey of the whole fruiting body; and h) view from above showing the end face of the sporangioles covered with little tails. (f to h) in phase contrast. All bars = 100 mm.

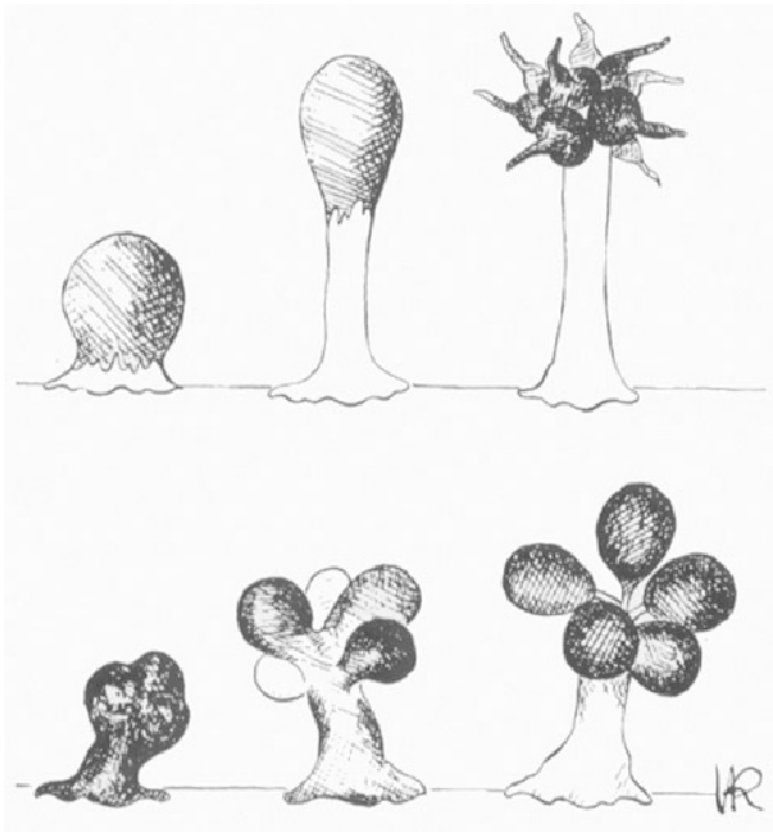


Fig. 11. Similar fruiting body shapes may be reached in different ways, as shown here for *Chondromyces apiculatus* (above) and *Stigmatella aurantiaca* (below), two organisms formerly thought to be closely related. The hatched areas indicate where the cells are located at the various stages.

elements (stalks and sporangiole walls; sporangial walls are produced only after the shape of the fruiting body has been obtained); and finally, 7) during the maturation phase, conversion of the vegetative cells into myxospores (White, 1993). In addition, cell-cell signals are exchanged throughout the entire process.

Under optimal conditions, the bacteria may go through the whole developmental cycle within 12–24 h. Fruiting body induction as well as the individual steps of differentiation are subject to control by environmental factors such as nutrients, pH, divalent cations, and temperature. In at least two cases (*Cm. apiculatus* and *Sg. aurantiaca*), normal development takes place only in illuminated cultures (Reichenbach, 1974a; Reichenbach, 1974b; Qualls et al., 1978; Stephens and White, 1980a). The course of fruiting body formation has been documented for different genera and species in several scientific films (Reichenbach et al., 1965d; Reichenbach et al., 1965e; Reichenbach et al., 1965f; Reichenbach et al., 1965g), which show the sequence of events during fruiting.

Fruiting body formation by *Mx. xanthus* has been the subject of the most intense scrutiny, and a great deal is known about the various signals that are exchanged during conversion of the vegetative cells to the simple *Myxococcus* fruiting body (Fig. 8). Five such signals, A through E, have been identified and are described in detail in Shimkets (1999). On the other hand, the signal(s) that directs movement of the vegetative cells into the aggregation center has remained mysterious. While it is intuitively plausible that the process is driven by chemotaxis, and indeed,

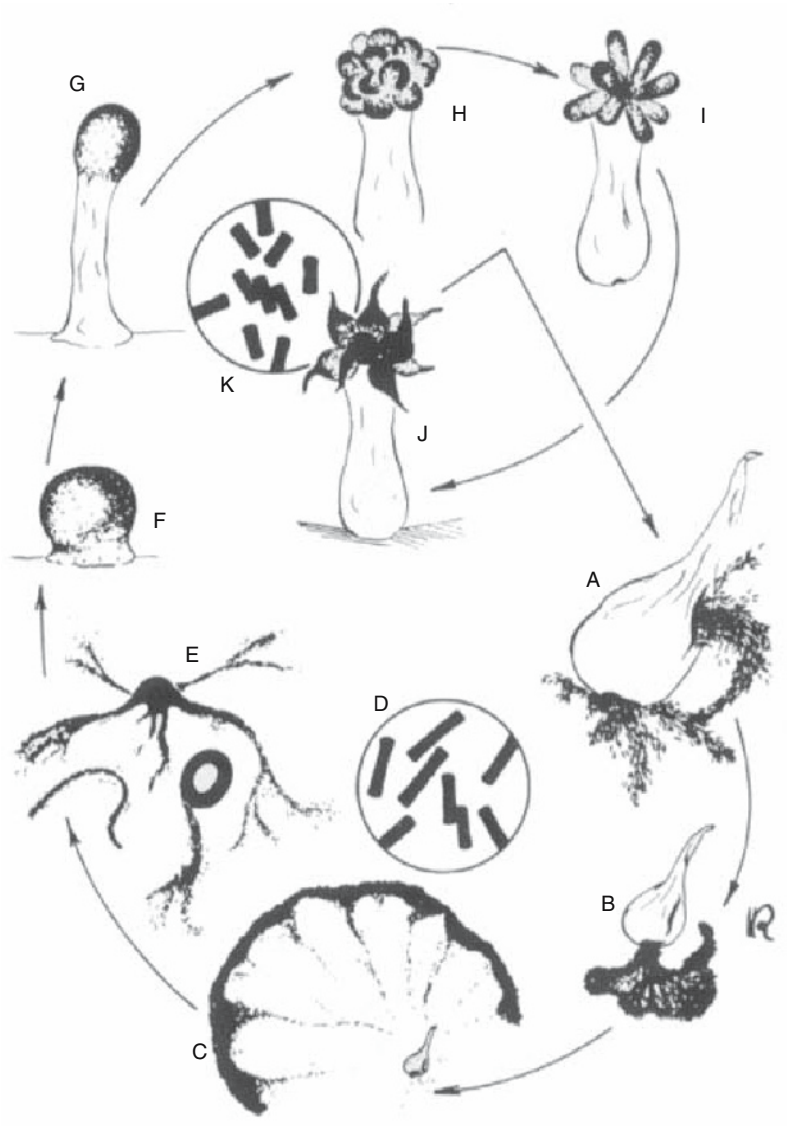


Fig. 12. Cooperative morphogenesis in myxobacteria. The organism shown in the diagram is *Chondromyces apiculatus*. Symbols: A, germinating sporangiole; B, C, development of a swarm colony; D, vegetative cells; E, aggregation of vegetative cells at various places within the swarm. F to J, fruiting body development; F, knob stage; G, excretion of the slime stalk, cells all concentrated in the terminal knob; H, the terminal mass begins to differentiate, forming wart-like protrusions which later elongate into (I) club-like structures and finally mature into (J) turnip-shaped sporangioles; K, myxospores.

chemotactic behavior has been demonstrated (see the section Gliding Motility in this Chapter), whether chemotaxis per se directs the cells into the centers has not been demonstrated.

Myxospores

Within the maturing fruiting body, a cellular morphogenesis takes place, i.e., the production of myxospores. In the suborder Cystobacterineae, this always means a rather dramatic shortening and fattening of the vegetative rods into spheres or at least into short, fat rods. The myxospores surround themselves with a capsule that may, however, be rather thin and recognizable only under the electron microscope. Also, they become optically refractile. In the suborders Sorangineae and Nannocystineae, the shape change is much less extensive. The vegetative cells may become somewhat shorter and slightly constricted in the middle. Often they also become optically refractile and may (e.g., in *Cm. crocatus*; Abadie, 1971b) or may not develop a thin capsule. The fine structure of myxospores, formerly also called "microcysts," has been studied with six organisms: *Mx. xanthus* (Voelz and Dworkin, 1962; Voelz, 1966a; Bacon and Eiserling, 1968; Kottel et al., 1975), *Sg. aurantiaca* (Reichenbach et al., 1969b; Voelz and Reichenbach, 1969), *Sg. erecta* (Galván et al., 1987), *Archangium gephyra* (Galván et al., 1992), *So. cellulosum* (Lampky, 1976), and *Cm. crocatus* (McCurdy, 1969a; Abadie, 1971b). *Myxococcus xanthus* has a very thick, layered capsule, which can be seen under the light microscope, especially after staining with India ink. The capsule appears to be reduced in thickness during germination and is left back by the emerging vegeta-

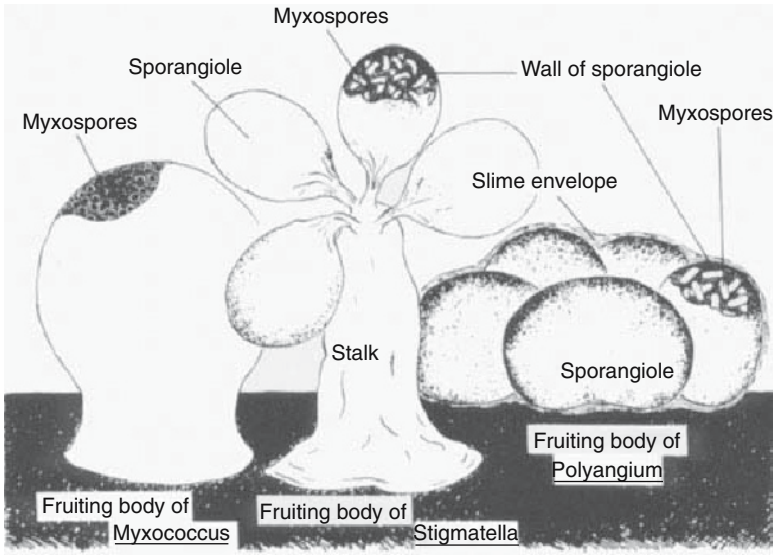


Fig. 13. The terminology for myxobacterial fruiting bodies and their structural components.

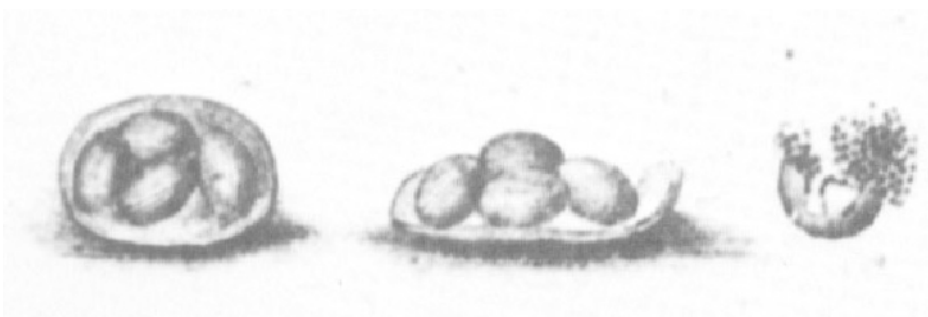


Fig. 14. *Polyangium vitellinum*, probably the first drawing of a myxobacterium to be published (Link, 1809). The original description of this still-valid genus and species reads as follows: "Among the Gasteromycetes in front of *Endogene* is to be inserted: *Polyangium*. Sporangium spherical or distortedly ovoid. Sporangioles very large in relation to the sporangium, ovoid, crammed full with a granular material. The sporangium consists of a thin, translucent, gelatinous, vesicular membrane enclosing four to six ovoid sporangioles which are filled with a colored granular mass. Sporidia could not be distinguished. *P. vitellinum*, with small yolk-colored spots, is to be counted among the smallest (species) of the order. It is found on old tree trunks, either singly or in groups of two or three, and can be recognized by its intense yolk color. Discovered by my friend Ditmar. Is to be inserted among the Gasteromycetes after *Tremella*." (Translated from the Latin.)

tive cell as an empty shell. The cell wall is retained intact during sporulation. In the cytoplasm, polyphosphate and polysaccharide granules can be seen. In glycerol-induced spores (see below), about 75% of the capsule is a polysaccharide consisting entirely of galactosamine and glucose (Kottel et al., 1975). Artificially induced myxospores fold their outer membrane into vesicular and tubular structures. An interesting open question is what happens with the gliding apparatus during shape change.

With S*g. aurantiaca* fruiting body myxospores, the outer membrane also becomes ruffled or folded; in artificially induced myxospores, these folds form densely packed membrane bodies between the cytoplasmic membrane and the capsule. Presumably, the outer membrane cannot be melted in during cell conversion and therefore is conserved in this way. As artificially induced cells change their shape much faster than cells in developing fruiting bodies, the phenomenon is more pronounced in the former. In both types of myxospores, polyphosphate and polysaccharide granules can be seen; the surface of the latter is densely covered by small, dark, ribosome-like particles. Essentially the same type of myxospores is found in fruiting bodies of *Sg. erecta* and *Ar. gephyra*; the latter may contain large, fusiform bodies with a crystalline internal structure, probably protein crystals. Fruiting body

myxospores of *So. cellulosum* seem to be devoid of a capsule. They are full of large, electron translucent granules, probably polysaccharide, which is also found near the poles of vegetative cells. In fact, phase (bright–pole) regions are observed under the phase contrast microscope in older vegetative cells. Mesosome-like membrane stacks are seen deep in the interior of the myxospores. Fruiting body myxospores of *Cm. crocatus* were found with and without a capsule. Also in this case mesosomes were present. Further, polysaccharide granules, slime vesicles, a ruffled outer membrane, and areas of vesicular or tubular outer membrane structures do occur. The arrangement of myxospores within the sporangioles is normally at random, but for *Me. boletus* an orientation strictly perpendicular to the outer wall has been described (Jahn, 1924). In *Cystobacter fuscus* sporangioles, a pattern of surface-parallel myxospores can be seen impressed on the inner face of the wall (Jahn, 1924). In cultures, myxospores are often produced free, outside of fruiting bodies.

With the discovery that the addition of glycerol to suspension cultures of *Mx. xanthus* induces the vegetative cells to convert synchronously within 90 min into myxospores (Dworkin and Gibson, 1964a), a well-defined experimental system for the study of morphogenesis in myxobacteria became available. This led to a host of studies on the events during induction and conversion. Nevertheless, as yet, there are no compelling insights into the mechanism of glycerol induction or sequence of the events leading to the formation of the myxospore. Details can be found in the reviews mentioned in the beginning of this section. Many other myxobacteria can also be induced to sporulate by adding chemicals to suspension cultures. *Stigmatella aurantiaca* can be induced by 40 different chemicals acting on at least three different receptors, as is suggested by reciprocal induction-resistant mutants (Gerth et al., 1993; Gerth and Reichenbach, 1994). Induction in *Sg. aurantiaca* is also possible by high temperatures just below the temperature maximum, and inductions by heat and by chemicals complement each other. This could mean that stress receptors and heat shock proteins play a role in myxospore induction, possibly within fruiting bodies, too (Heidelbach et al., 1993).

Myxospores are clearly designed for survival under unfavorable environmental conditions. Myxospores of *Mx. xanthus* are much more resistant to elevated temperatures (60°C), ultraviolet light, and sonic vibration than vegetative cells are (Sudo and Dworkin, 1969). In contrast to vegetative cells, myxospores are completely desiccation resistant and can be lyophilized. Dried myxospores may survive for at least 20 years at room temperature. In the dried state, temperature resistance of myxospores becomes especially impressive. Strains of *Myxococcus*, *Corallococcus*, *Archangium*, *Cystobacter*, *Polyangium* and *Sorangium* survived for 30 min at 140°C; *Corallococcus*, *Archangium*, *Polyangium* and *Sorangium* survived for 45 min; and *Archangium* and *Polyangium* even survived for 60 min. But 140°C for 240 min or 145°C for a short time killed all myxobacteria (H. Reichenbach, unpublished results).

Heat resistance of myxospores is often useful for the isolation of pure strains. To eliminate contaminants, fruiting body suspensions can be heated at 58°C for 15 or 40 min with good success. In *Mx. xanthus*, myxospore resistance to heat and desiccation appears to be accompanied by the accumulation of trehalose up to 1.1 μg/mg protein (McBride and Zusman, 1989a). To discuss here other details of the numerous studies on morphogenesis in myxobacteria is impractical; they will be found in several of the reviews mentioned at the beginning of this section (however, see also Sudo and Dworkin [1973], Shimkets [1987], Shimkets [2000], Ward and Zusman [2000], and White and Schairer [2000]).

Functions of Myxospores and Fruiting Bodies

The function of the myxospore is intuitively obvious. The metabolic quiescence of myxospores of *Mx. xanthus* (Dworkin and Niederpruem, 1964b) and their resistance to desiccation, temperature extremes, and UV light (Sudo and Dworkin, 1969) allow the organism to withstand environmental extremes and to persist for long periods during conditions that may not be optimal for growth. However, the function of the fruiting body is more subtle; "The most important question involving life history strategies is Why does one have fruiting bodies at all . . . ?" (Bonner, 1982).

While Bonner's question pertained to *Dictyostelium discoideum*, it is equally applicable to the myxobacteria. The function of the fruiting body, whether structurally simple as in *Myxococcus* or complex as in *Chondromyces*, relates ultimately to the "wolf pack" mode of feeding that is characteristic of all myxobacteria (Dworkin, 1973a). All the myxobacteria that have been so examined feed by means of excreting a battery of hydrolytic enzymes that enable them to degrade macromolecular debris or to lyse and feed on other cells (see the section Lysis of Other Bacteria in this Chapter). As a result, they are at the mercy of diffusion—of their enzymes away from the cell and of solublized nutrients toward the cell. Thus, feeding at a high cell density and thereby concentrating the hydrolytic enzymes and the resultant products is an advantage.

Rosenberg et al. (1977) has experimentally verified this hypothesis in *Mx. xanthus*. One may therefore view the fruiting body as a device for concentrating the cells, prior to their entrance into the resting state, so that upon germination they may immediately be present at a sufficiently high cell density to generate a swarm and thus to feed efficiently. The particular organization of the sporangioles that is characteristic for each species may simply reflect the optimal packaging of the myxospores, so that upon germination, multiple emerging swarms are poised to begin feeding at their optimal cell densities.

Lysis of Other Bacteria

The ability of the myxobacteria to lyse and feed on other cells and to hydrolyze a variety of insoluble macromolecules is well documented (Rosenberg and Varon, 1984), is characteristic of the myxobacteria, and accounts for the life style and multicellular proclivity of this group. This ability was observed early in the history of the myxobacteria, and the literature is full of such reports (e.g., Beebe, 1941; Singh, 1947; Nolte, 1957; Margalith, 1962; Bender, 1963; Raverdy, 1973). For this activity, a battery of cell-wall lytic enzymes, lipases, nucleases, polysaccharidases and nucleases as well as antibiotics and bacteriocins are produced (Rosenberg and Varon, 1984).

Studies on bacteriolytic activities led to the first studies on myxobacterial exoenzymes (e.g., Norén, 1960a; Bender, 1962; Hart and Zahler, 1966, Haskå, 1969; Hüttermann, 1969; Sudo and Dworkin, 1972). Some of the responsible enzymes have since been purified to varying degrees and have been biochemically characterized. While a complete bacteriolysis obviously requires many different enzymes, all of the studies on bacteriolytic enzymes of myxobacteria have concentrated on peptidoglycan-degrading enzymes. A glucosaminidase, an amidase, and an endopeptidase cleaving D-Ala/diaminopimelic acid and D-Ala/Lys bonds and proteases have been obtained from culture supernatants of *Mx. xanthus* (Sudo and Dworkin, 1972). Three bacteriolytic enzymes have been isolated and highly purified from *Mx. virescens* (Haskå, 1972a; Haskå, 1972b; Haskå, 1974); two of them proved to be endo-β-*N*-acetylglucosaminidases and the third an endopeptidase which could be purified by adsorption to montmorillonite followed by selective desorption (Haskå, 1974; Haskå, 1975). The fact that the bacteriolytic enzymes retain some of their activity when they are adsorbed to clay minerals may be of ecological relevance (Haskå, 1981). The addition of C11 through C15 fatty acids (about 2 mM) to suspensions of the target bacteria increased the efficiency of the bacteriolytic enzymes substantially (Haskå et al., 1972c). In contrast to the two myxococci, *Cc. coralloides* excreted an enzyme with a muramidase (i.e., lysozyme) activity (Harcke et al., 1972). (Additionally, the "*Sorangium*" isolate whose enzymes are often discussed in this connection was in reality a species of *Lysobacter*.)

Little is known about the enzymes with which microorganisms other than bacteria are attacked. Living cells of four yeast species could not be degraded by *Mx. xanthus* enzymes, but they were digested after a prior treatment with cysteine or organic solvents or after heating; in contrast, living cells of the yeast-like fungus *Pullularia* (*Dematium*) *pullulans* were partially decomposed (Bender, 1963). Isolated cell walls of *Saccharomyces cerevisiae* also could not be degraded by enzymes from *Mx. fulvus* Mx strain f80 without a brief heat treatment (Borchers, 1982). Apparently, the mannoprotein layer on the outside of the yeast cell wall protects the susceptible material. Two inducible enzymes have been demonstrated in the culture supernatant of *Mx. fulvus* Mx f80: 1) a laminarinase with a high specificity for β-1,3-glucosidic bonds cleaving laminaran endolytically with laminaritriose as the main product; and 2) a β-1,3-glucanase that can remove a high-molecular-weight polysaccharide other than β-1,3-glucan from the cell wall. The yeast cell wall is not completely broken down by *Mx. fulvus* Mx f80: The inner, fibrillar, glucan layer and the bud scars remain undigested, as can be seen under the electron microscope.

Several *Mx. xanthus* and *Mx. fulvus* strains were shown to control populations of the cyanobacterium, "*Phormidium luridum*," with predator-prey cycles of 9 days under experimental conditions. As few as 50 *Myxococcus* cells per 100 ml containing 10^7 cells of the prey were sufficient to start a lytic cycle (Burnham et al., 1984). The myxobacteria produced a lysozyme type enzyme (Daft et al., 1985). However, evidently because of insufficient supply of inorganic nutrients, myxobacteria cannot control natural blooms of cyanobacteria (Fraleigh and Burnham, 1988). In two cases myxobacteria have been observed to eat holes into the cell wall of the fungi *Cochleobolus* and *Rhizoctonia* (Homma, 1984) and of the green alga *Cladophora* (Geitler, 1924), respectively. The myxobacteria enter the cells and produce fruiting bodies inside.

Most myxobacteria excrete strong, diffusible proteases, as can be readily seen through the clearing action that occurs around colonies growing on skim milk agar. A few of these enzymes have been further characterized: From the culture supernatant of *Mx. virescens*, at least three proteases have been obtained (Gnosspelius,

1978b). One of them has been purified and was found to be an alkaline serine protease (26 kDa) that preferentially cleaved peptide bonds between hydrophobic amino acids. Three different proteases have also been demonstrated in the culture supernatant of *Mx. xanthus* FB (Coletta and Miller, 1986). They have tentatively been classified as alkaline serine proteases. There were qualitative differences among the proteases of different *Mx. xanthus* strains. A coagulase, myxocoagulase (57 kDa), has been isolated from the culture broth of *Mx. fulvus* strain NK35 (Bojary and Dhala, 1989). The enzyme was clearly different from *Staphylococcus* coagulase; it did not contain sugar and was active only on rabbit plasma.

While myxobacteria can hydrolyze many other macromolecules by means of diffusible exoenzymes, those enzymes have not been characterized so far. Thus, many myxobacteria decompose starch; quite a few are potent chitin degraders, e.g., all strains of *Cb. fuscus*, *Cb. ferrugineus*, *Sg. aurantiaca* and *Sg. erecta* and many strains of *Me. lichenicola* and *So. cellulosum*. Also, many myxobacteria appear to attack xylan. Two enzyme activities could be demonstrated in the culture supernatant of the cellulose degrader *So. compositum*, growing in a liquid medium with suspended cellulose powder. The enzymes produced only glucose and cellobiose from cellulose. One was a cellobiase and was destroyed by heating at 55°C for 10 min. After that, an enzyme releasing cellobiose from cellulose was still intact (Couke and Voets, 1968). These two enzymes probably did not constitute the entire cellulase complex of the organism; rather another endocellulolytic enzyme may exist that was not released from the cells. In fact, in plate cultures of *So. cellulosum* on filter paper or on cellulose powder agar, the lysis zone always exactly coincides with the size of the swarm colony. Thus, there seems to be no diffusible cellulase efficient enough to break down the crystalline cellulose completely.

During fruiting body formation of *Mx. xanthus* autolysis of a major part of the cell population takes place before sporulation begins. Approximately 85% of the vegetative cells in the population undergo lysis, the surviving cells completing myxospore formation and formation of the fruiting bodies (Wireman and Dworkin, 1977). Varon et al. (1986) have characterized the activities necessary for this developmental autolysis and have isolated five different autocides. Among these, AM I is a mixture of fatty acids and AM V is a phosphoethanolamine with unsaturated fatty acids (Gelvan et al., 1987). In the latter case, the cells release the fatty acids apparently through the action of a phospholipase; also in AM I, unsaturated fatty acids are the most efficient autocides. While AM I and AM V act specifically on *Myxococcus* species, the other autocides have wider specificity, acting on a variety of myxobacteria. Mutants that are resistant to autocides are no longer able to produce fruiting bodies.

Taxonomy and Identification

(Note: Much of the material in this section is taken verbatim from the recent revision of the taxonomy of the myxobacteria by Hans Reichenbach in the second edition of *Bergey's Manual of Systematic Bacteriology* [Reichenbach, 2005]).

All known myxobacteria are united in the single order Myxococcales. The name was changed from Myxobacterales (McCurdy, 1989) in accordance with Rule 9 of the International Code of Nomenclature of Bacteria (which requires that the name of an order be derived from the name of the type genus).

The phylogenetic position of the myxobacteria has been put on a solid base by 16S rRNA/DNA studies (Ludwig et al., 1983; Shimkets and Woese, 1992; Sproer et al., 1999). These studies have shown that the myxobacteria are a phylogenetically coherent group within the Deltaproteobacteria (Stackebrandt et al., 1988). Also apparent is that the order Myxococcales is divided into three suborders: a major division that separates the suborders Cystobacterineae and Sorangineae (Reichenbach, 1974b) and a second one separating the Nannocystineae (Reichenbach, 2005).

As discussed earlier in this chapter, the members of the suborders differ in cell shape, myxospore development, colony structure, fatty acid and carotenoid pattern, and the chemical composition of their slime (Congo red reaction). The suborders are divided into families based on the organization of the fruiting bodies and the shape of the myxospores. The definition of genera and species is based on the same characteristics and on a few complementary physiological facts. There are different possibilities for splitting and rearrangement, and in the absence of more compelling arguments, personal preference must still determine which route to follow. All in all, we can distinguish three suborders, five families, 17 genera and about 50 different species of myxobacteria, a number that while far from complete, is not likely to increase dramatically in the future. Note, however, that myxobacterial taxonomy cannot yet be regarded as settled and we are still confronted with major problems in distinguishing species, e.g., in the genera *Corallococcus*, *Archangium*, *Cystobacter*, *Nannocystis*, *Sorangium* and *Polyangium*.

Table 3. Taxonomic survey of the myxobacteria.[a]

Order: Myxococcales
Suborder: Cystobacterineae
Families and genera:
Myxococcaceae
Myxococcus
Corallococcus (formerly *Chondrococcus*)[2]
Pyxicoccus nov. gen.[b]
Cystobacteraceae
Archangium
Cystobacter
Melittangium
Stigmatella
Hyalangium nov. gen.[b]
Suborder: Sorangineae
Families and genera:
Polyangiaceae
Sorangium
Polyangium
Haploangium
Chondromyces
Byssophaga nov. gen.[b]
Jahnia nov. gen.[b]
Suborder: Nannocystineae
Families and genera:
Nannocystaceae
Nannocystis
Kofleriaceae
Kofleria nov. gen.[b]
Haliangium nov. gen.[b]

[a]Three recently described and unusual genera, *Anaeromyxobacter* (Sanford et al., 2002), *Enhygromyxa* (Iizuka et al., 2003b), and *Plestiocystis* (Iizuka et al., 2003a), are missing from Table 3 and the illustrated key (Fig. 15) because they have not yet been characterized thoroughly enough to accurately place them in this taxonomic scheme.

[b]These novel genera are validly described in Reichenbach (2004).

A survey of the taxonomy of myxobacteria derived from the taxonomy presented in *Bergey's Manual* (Reichenbach, 2005) is presented in Table 3. An annotated and illustrated key to the genera follows in Fig. 15. Note that three recently described genera, *Anaeromyxobacter* (Sanford et al., 2002), *Enhygromyxa* (Iizuka et al., 2003b) and *Plesiocystis* (Iizuka et al., 2003a), do not appear because they have not been characterized thoroughly enough for accurate placement.

Illustrated Key to the Genera of Myxobacteria

1. Vegetative cells are slender rods with more or less tapering ends, cigar- , boat- or needle-shaped, about 3.5–12 μm long and 0.6–0.8 μm wide. Swarms (e.g., on VY/2 agar) tend to remain thin, film-like, and often show a striking surface structure in form of radial veins and fields of tiny ridges or waves. Swarm edge usually has delicate fringes and flame-like protrusions. Myxospores arise through a striking cellular morphogenesis: the vegetative cells always shorten and fatten substantially. Myxospores always seem to have a capsule, which, however, is often seen only under the electron microscope. Slime and swarm sheets are stained with Congo red. The fatty acid pattern is dominated by branched-chain fatty acids; 2- and 3-hydroxy fatty acids are present in substantial amounts. All species are of the bacteriolytic type.

Suborder: Cystobacterineae 2

1′. Vegetative cells are stout, cylindrical rods, sometimes almost cube-shaped, with broadly rounded ends, about 2.5–8 μm long and 0.6–1.0 μm wide. Swarms (e.g., on VY/2 agar) tend to sink into and to penetrate the agar, sometimes to the bottom of the plate; they usually form shallow pits and bowl-like depressions; their surface structure is often less pronounced, although radial veins, ring-shaped ridges, and fanlike structures may be produced. The agar surface in the swarm area may be more or less corroded. Often, the cells concentrate at the swarm edge as a band or a massive ridge, or they form spherical or kidney-shaped clusters that migrate away from the center and leave deeply etched paths in the agar surface. Myxospores differ only slightly from vegetative cells in shape and seem to possess no capsule or, at the most, a very thin one; they are, however, optically refractile. Slime and colonies do not stain with Congo red. Branched-chain fatty acids are reduced in the fatty acid pattern, and hydroxy fatty acids are completely absent. Several species are cellulose decomposers.

Suborder: Sorangineae 5

1″. There are two types corresponding to two families. A. Swarm colonies without a slime sheet, on certain media tend to corrode the agar plate very deeply, transforming it into a spongy mass. Vegetative cells, short rods. Fruiting bodies tiny to moderately sized sporangioles, solitary or aggregated. Or, B. Vegetative cells are slender rods with rounded ends, like those of the Sorangineae but more delicate, 4–6 μm long and 0.6–0.7 μm wide. Swarm colonies with a well-developed slime sheet and massive, branching veins, resembling the colonies of the genus *Cystobacter*. Fruiting bodies not known.

Suborder Nannocystineae 10

2. Myxospores are regular spheres or ovoids with a smooth surface and a heavy capsule, diameter 1.2–2.5 μm. Vegetative cells are 3–6 μm long, boat- or cigar-shaped. Swarms often consist of a relatively soft slime sheet, although occasionally tough and tenacious sheets are also produced.

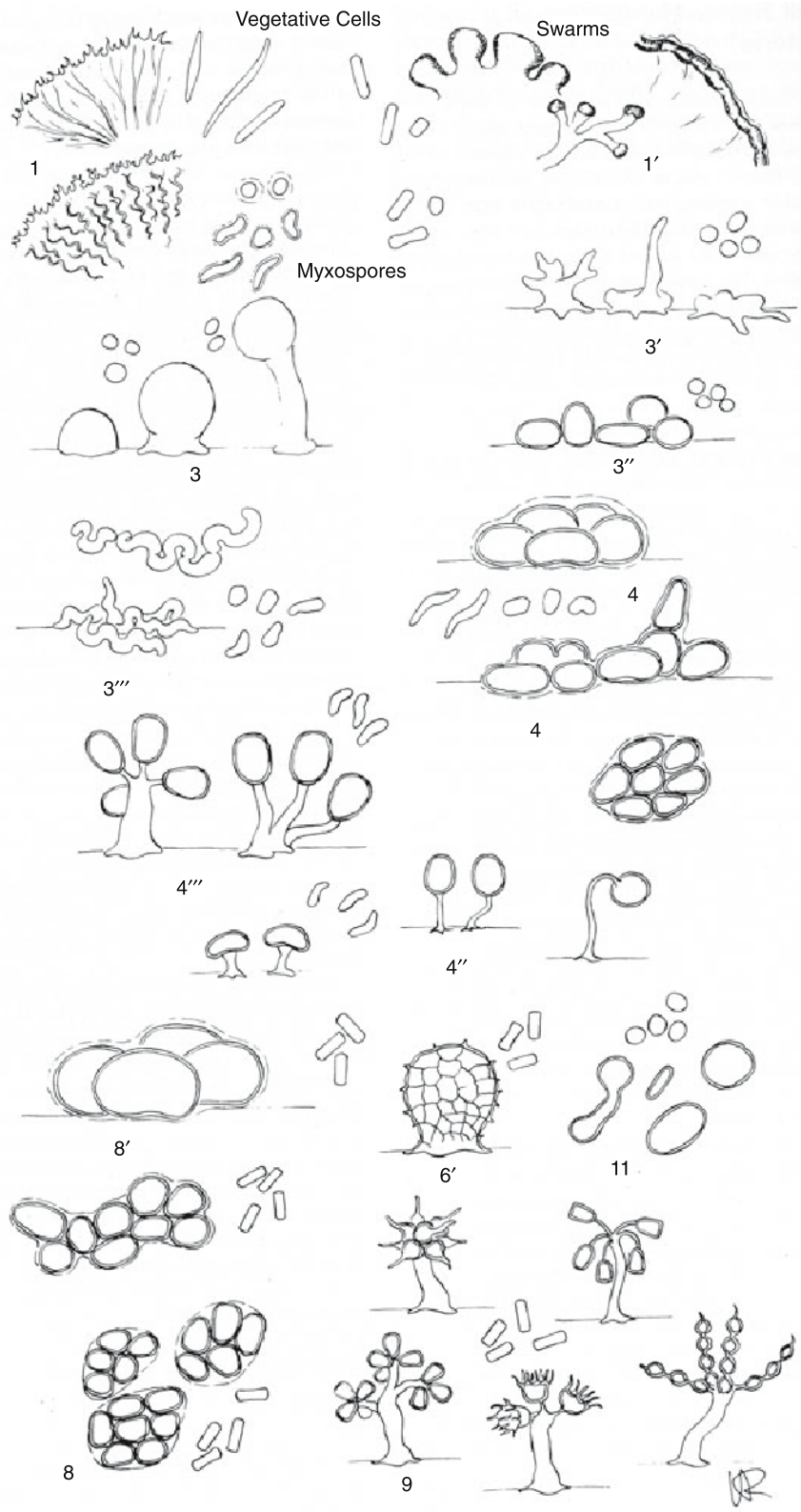


Fig. 15. A diagrammatic illustration of the key to the myxobacterial genera. The numbers in the figure refer to those in the key. The cells shown next to the fruiting bodies are myxospores.

The surface structure of the swarm is often rudimentary, but characteristic meandering radial veins may develop. Fruiting bodies are simple, spherical, soft-slimy mounds and heads or are cartilaginous columns and ridges that are sometimes branched, both without a distinct outer wall, or are sporangioles with a tough wall.

Family: Myxococcaceae 3

Comments: The differentiating characteristic of the family is the smooth, spherical myxospore. Myxospores that are nearly spherical are found with three other myxobacteria (*Archangium*, *Cystobacter* and *Nannocystis*), which can, however, easily be distinguished by the characters mentioned below.

3. Fruiting bodies are soft-slimy spherical heads or drops, often constricted at the base, in one species with a long slime stalk . . . . . *Myxococcus*

Comments: In agar cultures the shape of the fruiting body often deviates from the type, and only flat, cushion-like mounds or ridges are produced. There are four species: *Mx. fulvus* has white, pink, brick red, or even violet fruiting bodies with small myxospores (1.2–1.8 μm). *Mx. xanthus* and *Mx. virescens* produce orange and greenish yellow fruiting bodies, respectively, and have large myxospores (1.8–2.5 μm); they may be only one species, although, apparently, typical *Mx. virescens* strains produce very large myxospores and often gray, fruiting bodies on VY/2 agar. *Myxococcus stipitatus* has white-to-brownish fruiting bodies with a long slime stalk; these bodies are often found in groups on a common cushion-like base. Fruiting-body myxospores are small and they are often, but not always, clearly oval in shape. The swarms show a strong yellow fluorescence with UV light illumination at 366 nm; however, whether this quality is exclusive for the species is not yet established.

3′. Fruiting bodies are tough cartilaginous columns or ridges, sometimes staghorn- or coral-like, branched, very variable in shape, often more or less deeply rooted within the agar . . . . . *Corallococcus*

Comments: This genus has formerly been called *Chondrococcus*. The name is improper because it has been previously used for an alga. In *Bergey's Manual* (McCurdy, 1989), the genus is united with *Myxococcus*, but we prefer to retain it and suggest a new generic name. Because of its enormous variability, many species have been described in this genus, only two or three of which may be realistic. But a final decision will require more information. *Cc. coralloides* has small myxospores (1.2–1.8 μm) and produces relatively large pink or red fruiting bodies that are often branched or bear finger- or horn-like projections. Strains that also have small myxospores and produce large numbers of tiny, brownish fruiting bodies in dense fields have been described as *Cc. exiguus*. They represent either a species of their own or a variety of the former. *Chondrococcus macrosporus* has large myxospores (1.8–2.4 μm) and sturdy, yellow fruiting bodies. In contrast to the other two species, it is relatively rare.

3″. Fruiting bodies consist of sporangioles with a wall. Sporangioles are spherical, arranged in packages and sheets . . . . . *Pyxicoccus*

Comments: The third genus of the family, which is now called "*Pyxicoccus*," is somewhat confusing. The origin of the genus was a bacterium described by Thaxter (1904) as *Myxococcus disciformis*. As the spherical myxospores of the species are enclosed in sporangioles with a wall, Jahn (1924) created a new genus, *Angiococcus*, for it, apparently without ever having seen the organism. In spite of the fact that the same organism had been reported again from Poland (Krzemieniewska and Krzemieniewski, 1926), it was later concluded that *Angiococcus* does not exist (Peterson, 1966). But then the bacterium was rediscovered in the fossa of an alkaline bog (Hook et al., 1980). So the old species and genus names were reanimated, and a neotype strain of *Angiococcus disciformis* was deposited (ATCC 33172). The morphology of the organism suggests that it is really Thaxter's organism. But it does not belong to the Myxococcaceae. In the last edition of *Bergey's Manual* it was consequently classified as "*Cystobacter disciformis*." It has vegetative cells and a swarm morphology as is found in that genus. Its present classification is supported by 16S rRNA/DNA sequence data, which place the neotype strain close to *Cystobacter minus* (Sproer et al., 1999). There are, however, also organisms with spherical myxospores, a swarm morphology (as is characteristic for the Myxococcaceae), and a 16S rRNA/DNA sequence that position them close to *Myxococcus* (Sproer et al., 1999). For organisms of this type, exemplified by strain An d1, a new genus name, *Pyxicoccus*, will be used here. This avoids confusion, especially as it is not yet established whether the species *disciformis* really belongs to the genus *Cystobacter*. If not, the genus name *Angiococcus* may have to be used for it again. Note that *Pyxicoccus* fruiting bodies often degenerate into *Corallococcus*-like structures. The 16S rRNA/DNA study also proved that *Pyxicoccus* may easily be confused with other myxobacteria, as two of the three strains

tentatively identified as *Angiococcus disciformis* clustered in the Archangium group (Sproer et al., 1999).

3‴. Fruiting bodies consist of irregular masses of hardened slime (in which the myxospores are embedded) and lack an outer wall. They are either cushions with a bulging, brain-like surface or ridges that consist of meandering rolls often with rising, fingerlike projection . . . . . *Archangium*

Comments: The variability of *Archangium* fruiting bodies, and the degeneration of the fruiting bodies of other myxobacteria to *Archangium*-like structures, makes it impossible at the moment to distinguish different species. In fact, it is not even possible to recognize *Archangium* reliably, and it would be more honest to name such strains "*Archangium*-like myxobacteria." *Chondromyces serpens* is the first species for which *Archangium*-type fruiting bodies have been described, but because there are doubts about its identity, the later name, *Ar. gephyra* Jahn 1924, is used for relevant strains. This genus was formerly classified in a genus of its own, Archangiaeceae, but is now attached to the Cystobacteraceae. *Archangium violaceum* must be regarded as a degenerated *Cystobacter*, *Cb. violaceus*, under which name the organism was originally described.

2′. Fruiting bodies consist of sporangioles with a distinct outer wall, often with stalks. Myxospores are short, fat rods. Vegetative cells are slender rods with tapering ends, either boat- or needle-shaped.
Family: Cystobacteraceae 4

Comments: Aberrant fruiting bodies are common in cultures.

4. Fruiting bodies are clusters of sporangioles that sit directly on the substrate, often embedded in a clearly recognizable, common, transparent slime envelope or covered by a slime sheet . . . . . *Cystobacter*

Comments: There are myxobacteria with very similar fruiting bodies in the suborder Sorangineae, and some of these were originally united with the present *Cystobacter* species in a common genus, *Polyangium*, which, incidentally, is the oldest generic name in myxobacterial taxonomy (Link, 1809). The original family name was Polyangiaceae. Subsequently, it was realized that the genus *Polyangium* was heterogeneous. The genus was therefore split, and the Sorangineae-type species were united with species of the genus *Sorangium*. For reasons of priority, the name of the newly defined genus was *Polyangium*, and for the remaining species, the old generic name *Cystobacter* Schroeter 1886 was reactivated. The genus *Sorangium* disappeared completely, and as a consequence the family name Sorangiaceae was also eliminated and replaced by Polyangiaceae, which now, of course, had a completely different meaning than before. Here we redefine the genus *Sorangium* and replace it in the Polyangiaceae.

The species of the genus *Cystobacter* are not well studied, and it is not clear at the moment how many there are. The type species, *Cb. fuscus*, is readily recognizable from its large, glistening, chestnut brown sporangioles. *Cystobacter ferrugineus* has large, elongated, dull, dark brown sporangioles; they are often arranged in chains that tend to curve upwards in a fingerlike fashion. Both species have relatively long and slender, often slightly curved myxospores and are strongly chitinolytic. There are strains that produce light to reddish brown and often kidney-shaped sporangioles covered by a thin, translucent, and delicately plicated slime sheet; the myxospores are short, fat rods; these strains clearly represent a separate species, *Cb. velatus*. Other strains form somewhat smaller but still large, dull, dark brown, spherical sporangioles that contain myxospores in the shape of short, fat rods; they often produce a deep violet pigment and appear to constitute the species *Cb. violaceus*. Still other strains form tiny, light to golden brown, spherical sporangioles that are tightly packed together in small clusters and are often embedded in the agar substrate, resembling *Sorangium* or *Polyangium* fruiting bodies in all these respects; but the vegetative cells are long, slender needles, the myxospores are short, fat rods, and the swarms are of the *Cystobacter* type. These organisms belong to *Cb. minus*. The problem of *An. disciformis* has already been discussed.

4′. Vegetative cells are delicate, slender rods with tapering ends. Fruiting bodies consist of small spherical sporangioles that are often empty and then look glassy transparent, arranged in extended, dense sheets, or often, in chains. Myxospores are short rods to irregularly spherical, optically refractile. Swarm colonies have a thin but tough slime sheet with fine veins and adsorb Congo red, producing a purple red color. They are of the proteolytic–bacteriolytic nutritional type . . . . . *Hyalangium*

Comments: This organism differs substantially from the *Cystobacter* species discussed above and is therefore classified in a different, new genus. The distinguishing characteristics are as follows. The vegetative cells are shorter and more delicate than those of the *Cystobacter* spe-

cies. The sporangioles are small and often empty and glassy. They are arranged in monolayer sheets or in short chains and do not pile up or do so only slightly. The swarm colonies have a delicate slime sheet with very fine veins. The only species known so far resembles somewhat "*Cystobacter disciformis*," which, however, has much different fruiting bodies. Also, some traits are like those of *Polyangium minus* in the original description of Krzemieniewska and Krzemieniewski (1926). The identity with that organism cannot be ruled out with certainty, but even if the two organisms should be the same, a reclassification would be required.

4″. Fruiting body consists of a tiny sporangiole sitting on a delicate, white stalk . . . . . *Melittangium*

Comments: Most species were formerly attached to the genus *Podangium*. The type species of *Podangium*, *Pd. erectum*, has since been transferred to *Stigmatella*; the remaining species have been united in the genus *Melittangium*, so that the genus *Podangium* is now dissolved. There are at least three *Melittangium* species: *Me. boletus* has sporangioles that resemble mushroom caps and are located on very short stalks. *Mellitangium lichenicola* forms spherical, light brown sporangioles on short and relatively sturdy stalks. *Mellitangium alboraceum* is described as having a spherical sporangiole on a long and bent stalk. There are also strains that produce very tiny and delicate fruiting bodies; they may represent still another species, *Me. gracilipes*. The myxospores are always short, fat rods, and the vegetative cells are long, slender needles.

4‴. Fruiting bodies consist of dull, orange-brown to dark brown, ovoid sporangioles, which are arranged as either several on a common stalk or each on a stalk of its own . . . . . *Stigmatella*

Comments: *Stigmatella* is easily distinguished from *Melittangium*: its fruiting bodies are much coarser, and the vegetative cells are boat-shaped and of moderate length. The myxospores are short, fat rods and often S- or C-shaped. There are two species: *Sg. aurantiaca* with several sporangioles attached to a common stalk, often via tiny peduncles, and *Sg. erecta* with one sporangiole on each stalk. Clear as this may appear, the two species still are sometimes difficult to differentiate, because *Sg. erecta* often produces several fruiting bodies side by side on a common base and sometimes with partially fused stalks, so that the resulting structure may be confused with a *Sg. aurantiaca* fruiting body. These two species occupy different ecological niches: While *Sg. aurantiaca* almost always appears on rotting wood, *Sg. erecta* is found preferentially on dung and sometimes in soil. *Stigmatella erecta* was formerly classified as *Podangium erectum* (see "Comments" on *Melittangium*), *Sg. aurantiaca* as *Chondromyces aurantiacus*.

5. The suborder Sorangineae contains only one family. Vegetative cells are slender, cylindrical rods with blunt, rounded ends. Often containing optically bright granules of reserve material. The fruiting bodies always consist of sporangioles, that may be single or, more often, clustered in sedentary sori or borne on a slime stalk. In cultures, strains often refuse to produce fruiting bodies, yet degenerate fruiting bodies, as are so typical for the Cystobacteraceae, are only rarely formed. The myxospores in the fruiting bodies are morphologically not very different from vegetative cells, perhaps somewhat shorter and slightly constricted around the middle. But they are true, optically refractile resting cells, which are as desiccation and heat resistant as the myxospores of the two previous families. The swarm colonies tend to etch the agar, producing radial tracks, holes, and pits in the surface of the plate, and often penetrate the substrate deeply. In the genera *Polyangium* and *Byssophaga*, discontinuous swarm colonies with scattered cell clusters do also occur besides the more typical, coherent swarms with slime sheets and veins. Congo red is not adsorbed to the slime of the swarms. Most species are of the proteolytic–bacteriolytic nutritional type, but some are cellulose degraders and, in contrast to all other myxobacteria, may be cultivated on very simple media with an inorganic nitrogen source and a sugar as the only carbon source.

Family Polyangiaceae 6

6. Fruiting bodies consisting of several sporangioles clustered together 7

6′. Fruiting bodies consisting of large, solitary sporangioles, golden yellow, with a wrinkled or netlike surface structure when mature, found on bark and decaying wood . . . . . *Haploangium*

Comments: This genus is easily recognized by its peculiar fruiting bodies that so far have only been encountered on the bark of living or dead trees in North America and in Europe. It also has a special position in that these are the only myxobacteria that have resisted all efforts to cultivate them. (The "*Haploangium*" species, described and cultivated by Singh and Singh, 1971, were obviously misnamed because their vegetative cells were long, slender rods with tapering ends.) There may be two species that differ in size: *Ha. rugiseptum* with large sporangioles and *Ha. minus* with small ones.

7. Sporangioles located directly on or within the substrate 8

7′. Sporangioles borne on long, white stalks. 9

7″. Sporangioles orange to red brown, in convoluted, brain-like chains, often on a prominent slime cushion . . . . . *Jahnia*

8. Grows in the form of independently migrating cell companies, or pseudoplasmodia, that finally contract into intensely cinnabar- to carmine-red knob-like masses, resembling *Myxococcus* fruiting bodies. Fruiting bodies are clusters of large, red sporangioles, but are only exceptionally produced. Cellulose is decomposed . . . . . *Byssophaga*

8′. Fruiting bodies consist of tiny sporangioles that are tightly packed together and consequently are often polyhedral rather than spherical. A large number of the small parcels thus formed may lie together in more or less densely packed masses, but also the parcels themselves may become rather large. The fruiting bodies are often produced in enormous quantities, especially on digested filter paper, so that they determine the color of the swarm: bright yellow, orange, all shades of brown, and even pitch black. The vegetative swarm is usually more or less bright orange. Decomposes crystalline cellulose (filter paper) . . . . . *Sorangium*

Comments: We propose to restrict this genus to the cellulose decomposers. Cellulose degradation is such an unusual and stable character and in addition parallels a series of other morphological, physiological and biochemical characteristics in the respective strains that a separation of these cellulolytic species seems justified. The organisms have not been sufficiently studied to allow satisfactory classification of species. In addition, the original descriptions do not always indicate whether an organism was a cellulose decomposer, because not all strains have been cultivated. Also, the organisms are very variable. There appear to be at least three species: *Sorangium compositum* produces yellow-orange fruiting bodies, *So. cellulosum* brown or gray ones, and *So. nigrum* black ones, but this characteristic appears insufficient to differentiate the species reliably. There are many other morphological, chemosystematic, and physiological differences among strains, but the taxonomic relevance of these observations is presently still under study.

8″. Fruiting bodies consist of more or less spherical sporangioles that may be yellow, orange, brown or gray. Several of them may be embedded in a common, translucent slime envelope . . . . . *Polyangium*

Comments: For some time, this genus also contained the species of *Cystobacter* (see there for further comments). *Polyangium vitellinum* produces large, golden yellow sporangioles in groups of 1–20 in a common envelope. Apparently, in culture the color often changes to greenish yellow. *Polyangium luteum* has smaller, golden yellow sporangioles embedded in a yellow slime envelope. *Polyangium aureum* may be another species of this group of polyangia with orange sporangioles. There are further strains with small sporangioles that are densely clustered together in the same way as with certain *Cystobacter* and *Sorangium* strains. The fruiting bodies are light brown or gray in color and usually are embedded in the agar substrate. Probably, all strains of this type can be classified as *Pl. fumosum* and *Pl. spumosum. Polyangium parasiticum* seems to parasitize fresh-water algae; it produces red-brown sporangioles within the emptied alga cells. This species has not been reported since its first description in 1925, and its parasitic growth and even its appearance in freshwater may have been an exceptional situation. The polyangia are somewhat difficult to handle and are not well-studied organisms. There are some doubts whether the present genus is really homogeneous.

9. Fruiting bodies consist of a conspicuous, unbranched or branched white slime stalk, bearing a cluster of bright orange sporangioles. . . . . *Chondromyces*

Comments: The myxobacteria with the most beautiful and complex fruiting bodies are found in this genus. There are six species. One species, *Cm. aurantiacus*, had to be transferred to the genus *Stigmatella. Chondromyces crocatus* has a branched stalk with clusters of small, ovoid sporangioles at the ends of the branches. All other species have unbranched stalks. *Chondromyces apiculatus* produces conspicuous, turnip-shaped sporangioles, and *Cm. pediculatus* produces bell-shaped sporangioles, often drooping on long, delicate peduncles; both species are relatively common, the former especially on rotting wood, the latter often in soil and decaying plant material. *Chondromyces catenulatus* produces chains of sporangioles, and several chains originate from one stalk. *Chondromyces lanuginosus* (formerly *Synangium*) has a cluster of a few large sporangioles, each of which ends in a ring of long tips; the sporangiole thus resembles the young fruit of a hazelnut. *Chondromyces robustus* has large, fat, more or less spherical sporangioles with one to three tips.

10. Vegetative cells are short, stout, cylindrical to somewhat bulging rods with rounded to squarish ends. Fruiting bodies consist of spherical to ellipsoidal, sometimes irregular sporangioles. Most of the sporangioles are solitary, scattered on top and within the agar plate; many strains also produce dense clusters, sheets, and packets of sporangioles. The size of the sporangioles usually varies very widely. Myxospores are very short rods, ellipsoidal or spherical. Swarm colonies etch and corrode the agar more or less, depending on the medium, from shallow depressions and pits (on yeast agar) to deep holes and channels, often transforming the agar plate into a spongy mass down to the bottom of the dish.
Family Nannocystaceae 11

Comments: This family belongs to the third suborder of the Myxococcales, the Nannocystineae, together with the family Kofleriaceae. The suborder can presently be defined only by 16S rRNA/DNA sequence data, which clearly show a separate position of the organisms classified here (Sproer et al., 1999). The family, however, with only one genus, *Nannocystis*, is clearly defined by its phenotypic characteristics. Some of the more unusual myxobacteria appear to belong to this taxonomic group, in particular psychrophilic myxobacteria (Dawid et al., 1988).

10′. Vegetative cells are delicate, slender cylindrical rods with rounded ends. Swarm colonies consist of a slime sheet with more or less prominent radial veins, do not etch the agar, resemble the swarms of the Cystobacteraceae but do not adsorb Congo red. Produce usually many small to large globular knobs all over the swarm surface and dense, spherical cell aggregates within the agar resembling sporangioles, but without a wall, and sporangioles and myxospores have yet to be found. Besides terrestrial organisms also some marine, halophilic myxobacteria appear to belong to this group.
Family Kofleriaceae 12

11. Fruiting bodies are tiny, ovoid or spherical, solitary sporangioles that are produced in large numbers and are mostly embedded in the substrate. Myxospores are spherical or ovoid. Vegetative cells are often very short, stout, almost cube-shaped. Swarm is etched more or less deeply into the agar. The agar plate may become completely corroded . . . . . *Nannocystis*

Comments: Presently only two species, *Na. exedens* and *Na. pusilla*, are known, but probably other species do exist. *Nannocystis exedens* is easily recognized by its typical swarm pattern, which is especially conspicuous on water agar with streaks of living or autoclaved *E. coli*, and by its unique fruiting bodies. The latter may become considerably larger and very irregular in shape when they are produced on the agar surface. Some strains produce composite fruiting bodies that consist of clusters of small sporangioles that are more or less tightly squeezed together. Such strains may represent a different species ("*Na. aggregans*"). *Nannocystis* is perhaps the most common myxobacterium, although it is often overlooked; it is present in virtually every soil sample that contains any myxobacteria. While *Nannocystis* fits, in general, rather well with the other members of the order Myxococcales, it shows certain peculiarities that clearly set it apart, such as the nature of its carotenoid pigments and its synthesis of cholestenols.

12. Vegetative cells are long, slender, cylindrical rods with rounded ends. Swarm colonies have radial veins in a tough slime sheet, and there are numerous small to very large globular masses, or knobs, all over the swarm. Congo red is not adsorbed, and the agar is not etched. Mature fruiting bodies have not been observed but may consist of sporangioles. Cells within the knobs are long, cylindrical rods, optically refractile. They are of the proteolytic-bacteriolytic nutritional type, chitin degraders, and found in soil and similar substrates. Only one species, *Ko. flava*, is described. . . . . *Kofleria*

12′. Marine myxobacterium. Vegetative cells have rounded, blunt ends. Yellow colonies are spread and usually slightly sunk into the agar surface. Fruiting bodies are yellow to brown and consist of one or more sessile sporangioles in dense packs. They are moderately halophilic, requiring 1–3% NaCl for optimal growth (Fudou et al., 2002) . . . . . *Haliangium*

Applications

The most promising opportunity for biotechnological applications with myxobacteria is in the production of secondary metabolites that inhibit the growth of other organisms. The ability of the myxobacteria to produce antibiotics has been known for some time (Oxford, 1947; Norén, 1953; Kato, 1955; Norén and Raper, 1962). Apart from trivial fatty acids that inhibit the germination of fungal spores (Norén and Odham, 1973), until relatively recently, no chemical structure was proposed for any of these inhibitors. The first myxobacterial antibiotic for which the chemical structure has been elucidated was ambruticin, a potent antifungal compound from *So. cellulosum* (Ringel et al., 1977). In the past two decades, about 100 basic structures and 600 structural

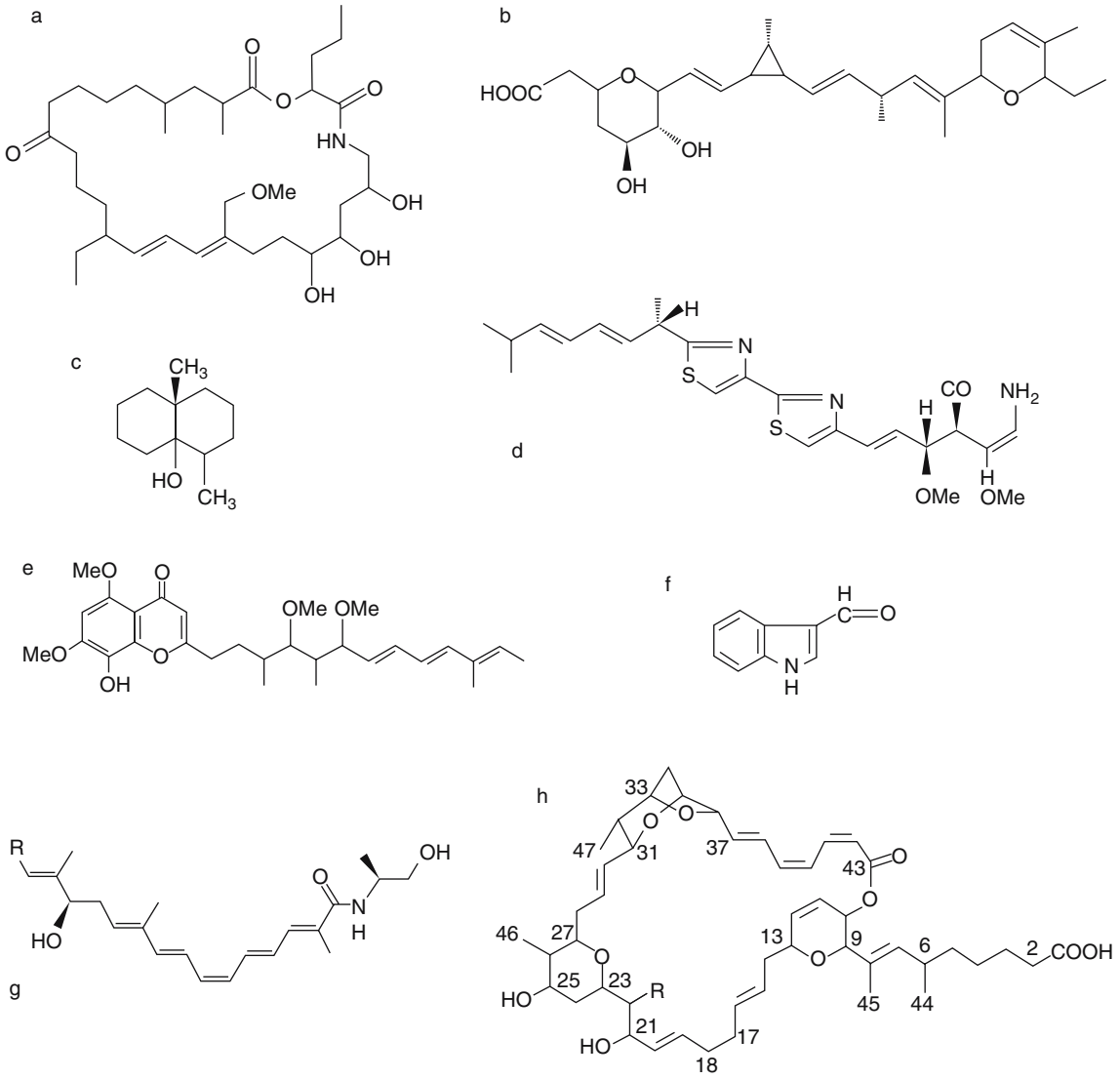


Fig. 16. A few examples of secondary metabolites produced by myxobacteria. a) Myxovirescin A; the compound, or variants of it, has been described as antibiotic TA and as megovalicins; b) ambruticin; c) geosmin; d) myxothiazol; e) stigmatellin; f) 3-formylindole; g) myxalamid; R is 2-butyl, 2-propyl, ethyl, and methyl for myxalamids A, B, C, and D, respectively; and h) sorangicin; R is OH and H for sorangicin A and B, respectively.

variants have been discovered in myxobacteria and have been fully characterized chemically (Reichenbach and Höfle, 1999; Reichenbach, 2001). The more significant insights can be summarized as follows: 1) A high proportion of myxobacterial strains (about 50% of the Cystobacterineae and almost 100% of the *Sorangium* strains) produce some kind of compound with biological activity. 2) The substances produced are chemically very different, including aromatic, heterocyclic, and polyenic compounds, alkaloids, macrocycles, polyethers, and peptides. A few examples are shown in Fig. 16. 3) Most of these compounds have not been isolated from other organisms and thus appear to be unique to the myxobacteria. The only exceptions are pyrrolnitrin and althiomycin; saframycin has been found with a new variant. 4) The ability to produce a specific compound is typical for particular but not all strains of that species. In fact, different strains of the same species may produce totally different antibiotics, but the same antibiotic may also be found in different species and even genera and families. Interestingly, the border between the suborders is also a division line for secondary metabolism, and so far only a few compounds have been found that are produced by representatives of the different suborders (e.g., pyrrolnitrin). 5) A strain may produce, at the same time, two or more compounds that are

totally different chemically. 6) Often a family of chemical variants of the same basic structure is synthesized by a strain; up to 40 different variants have been isolated from one single strain. 7) The initial yields are usually low (0.5–20 mg/liter), but with conventional methods, yield increases to more than 1 g/liter have already been achieved. 8) Interesting mechanisms of action have been discovered; compounds have been discovered that are specific inhibitors of eubacterial RNA and protein synthesis or are extremely efficient inhibitors of electron transport in respiration and photosynthesis. Two of the latter are used as specific biochemical probes and are commercially available (myxothiazol and stigmatellin). Among the compounds discovered also are iron transport metabolites of the myxobacteria (myxochelin and nannochelin). 9) An astonishing number of myxobacterial compounds interfere with the eukaryotic cytoskeleton, either actin (rhizopodin and chondramide) or tubulin (disorazol and epothilone).

At the moment, several of the myxobacterial inhibitors are being studied for potential application and large-scale production. The most promising of these is epothilone, which is currently being evaluated in phase three human clinical trials as an antitumor drug by three different pharmaceutical companies (Gerth et al., 1996). Epothilone isomers stabilize polymerized microtubules, leading to mitotic arrest at the G_2-M transition and cytotoxicity in proliferating cells (Bollag et al., 1995).

Antibiotic TA was discovered in *Mx. xanthus* strain TA (Rosenberg et al., 1973). Published chemical data, specifically NMR data (Rosenberg et al., 1982), suggest that the compound may be identical to one of the 20 variants of myxovirescin (Gerth et al., 1982; Trowitzsch et al., 1982; Trowitzsch-Kienast et al., 1989). This identification is further supported by the observation that myxovirescins are produced by many *Mx. xanthus* and *Mx. virescens* strains (H. Reichenbach, unpublished observations). In fact, the same antibiotic has been described a third time from another *Mx. xanthus* strain (Onishi et al., 1984). Furthermore, the megovalicins (Miyashiro et al., 1988; Takayama et al., 1988) are identical with previously published variants of myxovirescin; a new species, *Mx. flavescens*, has been created to describe the producing organism (Yamanaka et al., 1987), which cannot, however, be distinguished from existing species.

Another good opportunity for application may lie in the field of myxobacterial proteins and enzymes, a potential that has not yet been thoroughly investigated. Thus, restriction endonucleases (Morris and Parish, 1976; Mayer and Reichenbach, 1978) and special proteases could be of interest in the future. A lectin is produced by *Mx. xanthus* during development (Cumsky and Zusman, 1981; Nelson et al., 1981). *Myxococcus xanthus* also secretes a blood anticoagulant, myxaline, which is a heat-stable glycopeptide (El Akoum et al., 1987; Masson and Guespin-Michel, 1988). The same or a similar compound is also produced by other myxobacteria.

The efficient secretion of proteins observed with myxobacteria is unusual for Gram-negative bacteria and points to another conceivable application, as expression hosts for foreign proteins (Breton et al., 1984b; Breton et al., 1986; Nicaud et al., 1984). *Myxococcus xanthus* has been shown to secrete more than 50 different proteins into the medium, amounting to about 4% of the intracellular protein. This is impressive even if the actual number may be somewhat lower because some of the bands may have been due to proteolytic activity (Nicaud et al., 1984). Apparently, the outer membrane of the myxobacterial cell is less of a barrier than in most other cells, perhaps due to its more dynamic behavior. Two *E. coli* periplasmic enzymes, acid phosphatase and TEM2 β-lactamase, have been shown to leave the periplasm quickly when they are expressed in *Mx. xanthus* and appear in the extracellular medium (Breton and Guespin-Michel, 1987). The extracellular pectate lyases of *Erwinia* are also expressed in *Mx. xanthus* and secreted into the medium, although in this case the myxobacterial proteases inactivate the lyase (Breton et al., 1986).

There are no known myxobacterial pathogens for humans, animals or plants. The fish pathogen *Chondrococcus columnaris* is a *Cytophaga*-like bacterium and has been wrongly classified as a myxobacterium. Because of their many aggressive enzymes, myxobacteria may occasionally contribute to the deterioration of materials such as rawhide or cellulose fabrics (e.g., Heyn, 1957), but their importance in that respect is doubtful. However, although not yet demonstrated experimentally, myxobacteria reasonably play a substantial role in solubilizing large macromolecules, cell carcasses, and other biological detritus. As myxobacteria may reach rather high population densities in soil, it has been argued that they could become antagonistic to useful soil organisms like *Azotobacter* (Callao et al., 1966). However, although a suppression of *Azotobacter* by *Mx. xanthus* can indeed be demonstrated in the laboratory, whether this occurs in nature is an open question. Myxobacteria are obviously able to destroy cyanobacteria in mixed cultures in aqueous environments, and their possible use to control cyanobacterial water blooms has been suggested (Burnham et al., 1981; Burnham et al., 1984; Daft et al., 1985). But that potential has not yet been demonstrated under practical condi-

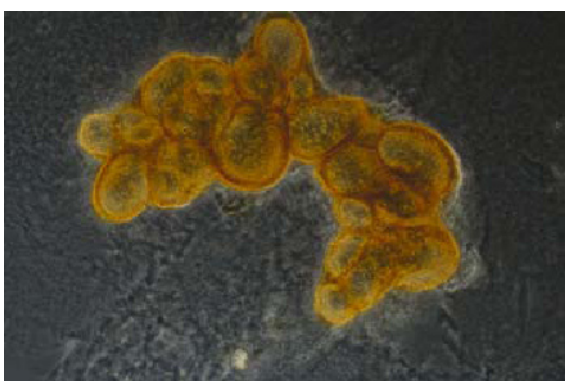
tions. Still another application of myxobacteria in environmental problems may be their use as pollution indicators (Gräf, 1975; Trzilová et al., 1980; Trzilová et al., 1981). This scheme is presently being perfected and may eventually become a useful addition to conventional biological water analysis.

Certain chemical compounds produced by myxobacteria may occasionally become troublesome. Many myxobacteria produce a strong earthy smell, which, in the case of *Na. exedens*, has been demonstrated to be due to geosmin (Trowitzsch et al., 1981). While that smell may be pleasant in connection with soil, it is disagreeable in drinking water, from which it has to be removed.

In conclusion, the economic potential of the myxobacteria is only beginning to be exploited and many potential products have yet to be adequately assessed.

Color Plates

1. *Pyxicoccus fallax* nov.gen., nov.spec., strain Ang D2, fruiting bodies on agar surface consisting of numerous small sporangioles.



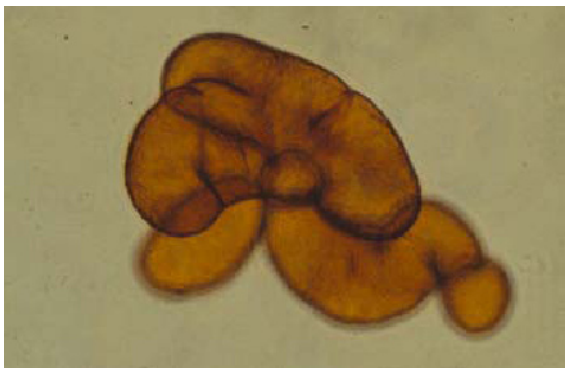
2. *Pyxicoccus fallax* nov.gen., nov.spec., fruiting body in slede mount at high magnification showing sporangioles with clear walls.

3. *Cystobacter fuscus*, mature fruiting body on agar surface, sporangioles with typically shining surface.

4. *Cystobacter fuscus*, developing fruiting bodies on agar surface.

5. *Cystobacter ferrugineus* strain Cb2056, fruiting body on agar surface with dull sporangioles.

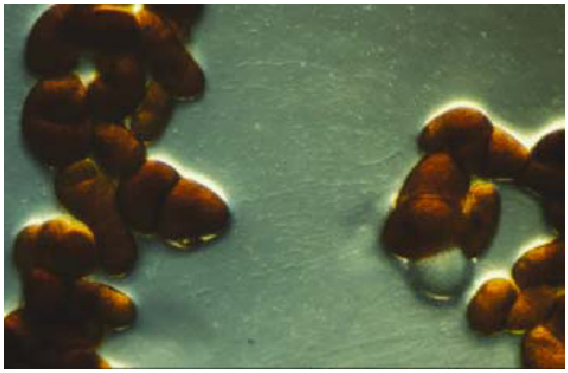
8. *Cystobacter velatus* strain Cb v37, fruiting bodies on agar surface with upward looping chains of sporangioles.



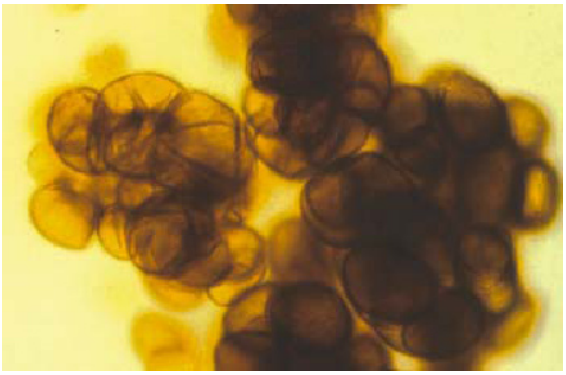
6. *Cystobacter ferrugineus* strain Cb2056, fruiting body in slide mount, several sporangioles show the elongated shape typical for that species.

7. *Cystobacter velatus*, fruiting bodies on agar surface, spoangioles covered with a delicate, plicated slime sheet.

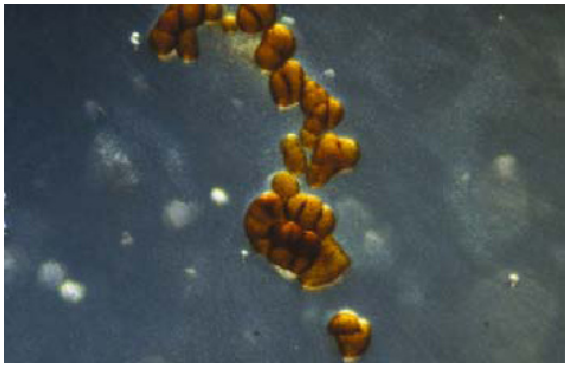
9. *Cystobacter violaceus*, original (now lost) type strain, violet swarm colonies on agar plate.



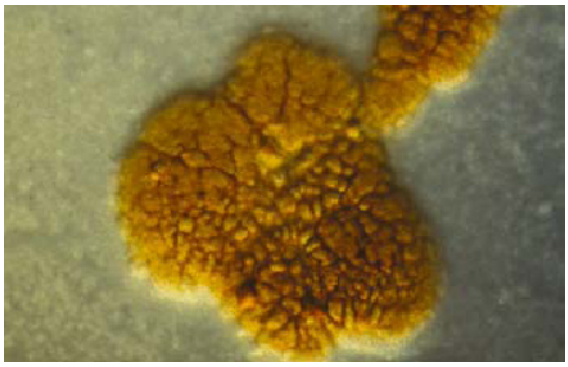
10. *Cystobacter violaceus* strain Cb vi67, fruiting body on agar surface.



13. *Cystobacter gracilis* nov.spec., strain Cb g53, fruiting body on agar surface under cover glass.

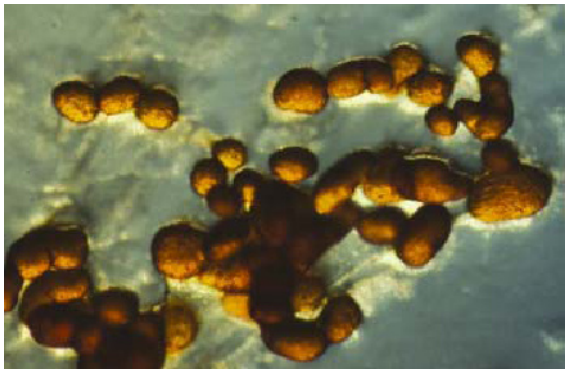


11. *Cystobacter* minor strain Cb m29, fruiting body on agar surface.



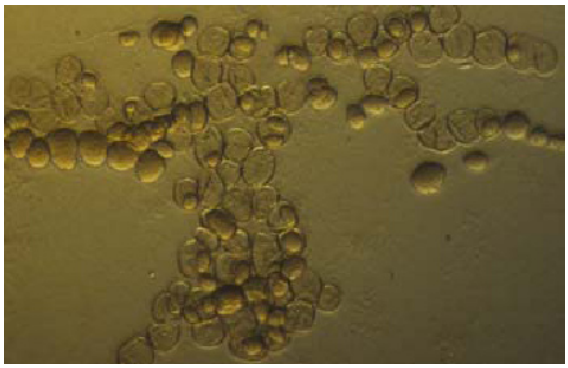
12. *Cystobacter* minor strain Cb m29, fruiting body on agar surface.

14. *Cystobacter miniatus* no.spec., strain Cb mi1, fruiting body consisting of densely packed sporangioles in a column-like, erect mass typical for that species.



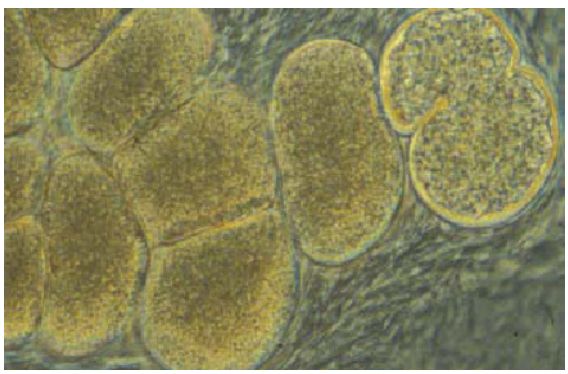
15. *Cystobacter badius* nov.spec., fruiting bodies on agar surface; besides chains of sporangioles, there also are found individual sporangioles, often on a short pedicel.

18. *Jahnia thaxteri* nov.gen., nov.spec., strain Pl t3, fruiting body on agar surface under cover glass; a wound chain of large sporangioles is sitting on a more or less obvious slime cushion.



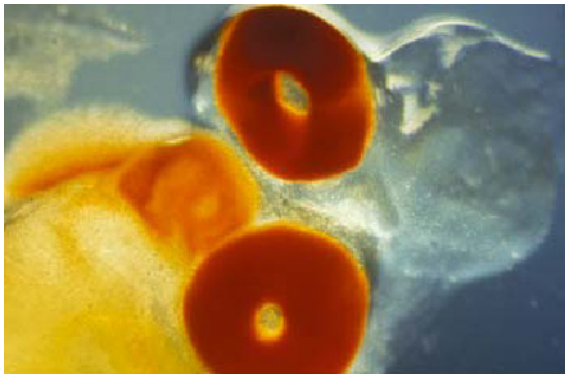
16. *Hyalangium minutum* nov.gen., nov.spec., strain Hy m3, fruiting bodies on agar surface under cover glass, with tiny sporangioles in a dense, mostly monolayer sheet; many sporangioles are empty looking, as if made of glass, as is typical for this organism.

19. *Byssophaga cruenta* nov.gen., nov.spec., strain By c1, swarm pseudoplasmodia on agar surface.

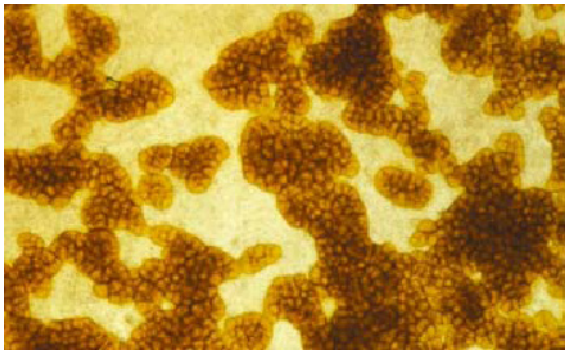


17. *Hyalangium minutum* nov.gen., nov.spec., strain NOCB-2, sporangioles in slide mount at high magnification, some of them showing invaginations of the sporangiole wall.

20. *Byssophaga cruenta* nov.gen., nov.spec., strain By c2, type strain), piled up cell mass on agar surface resembling a Myxococcus fruiting body.

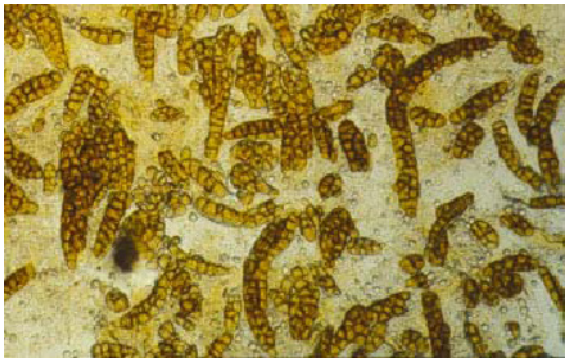


21. *Byssophaga cruenta* nov.gen., nov.spec., strain By c2 (type strain), cells assembled in massive rings on the agar surface.



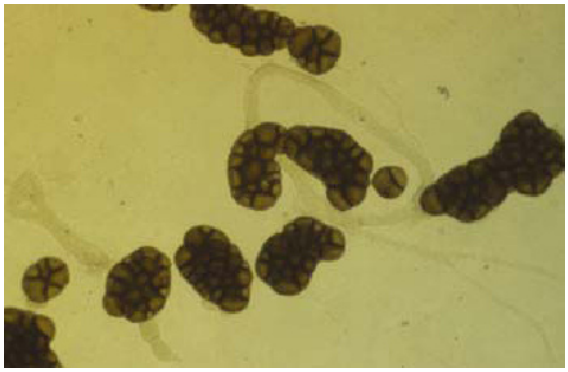
24. *Sorangium cellulosum* strain So ce1569, fruiting bodies on agar surface under cover glass.

22. *Byssophaga cruents* nov.gen., nov.spec., strain By c1, fruiting body on agar surface under cover glass consisting of large, densely packed sporangioles.

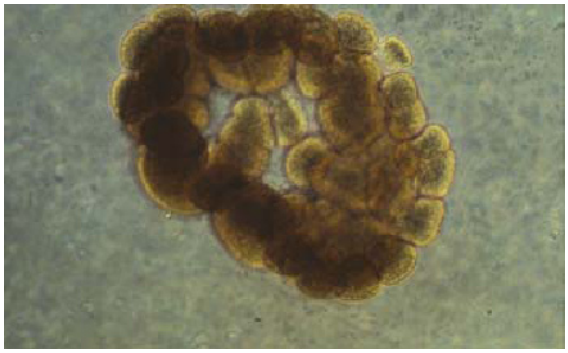


25. *Sorangium nigrum* nov.spec., strain So ce1600, fruiting bodies from macerated filter paper in slide mount.

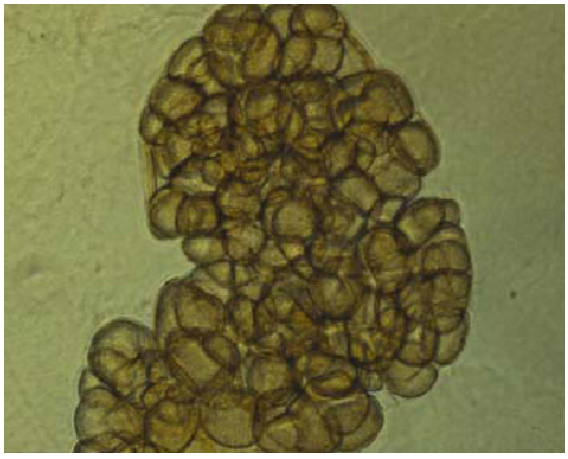
23. *Sorangium cellulosum* strain So ce1569, fruiting bodies from macerated filter paper in slide mount; the tiny sporangioles are often arranged in chains because they were produced along cellulose fibers.



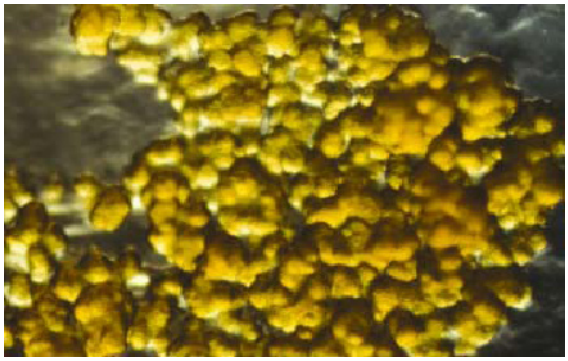
26. *Sorangium nigrum* nov.spec., strain So ce 1602, fruiting bodies on agar surface under cover glass.



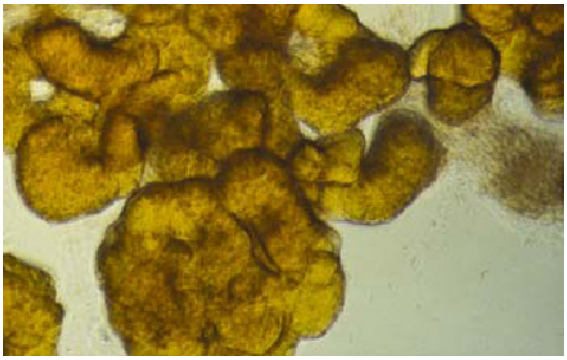
27. *Polyangium fumosum* strain Pl fu12, fruiting body on agar surface under cover glass.



28. *Polyangium spumosum* strain Pl sm4, fruiting body in slide mount.



29. *Kofleria flava* nov.gen., nov.spec., strain Pl vt10, growth on agar surface with numerous spherical knobs which are, however, not fruiting bodies.



30. *Kofleria flava* nov.gen., nov.spec., strain Pl vt10, nodules in slide mount.

Literature Cited

Abadie, M. 1967. Formation intracytoplasmiques du type "mesosome" chez Chondromyces crocatus Berkeley et Curtis. C. R. Acad. Sci. Paris, Sér. D 265:2132–2134.

Abadie, M. 1968. Mise en évidence des formations mésosomiques dans les cellules végétatives du Chondromyces apiculatus Thaxter. C. R. Acad. Sci. Paris, Sér. D 267:1538–1540.

Abadie, M. 1971a. Contribution à la connaissance des myxobactéries supérieures. I: Recherches culturales et microculturales sur le Chondromyces crocatus Berkeley et Curtis. Ann. Sci. Natur., Botanique (Paris), 12e Sér. 12:255–344.

Abadie, M. 1971b. Contribution à la connaissance des myxobactéries supérieures. II: Donnée ultrastructurales et morphogénétiques sur le Chondromyces crocatus Berkeley et Curtis. Ann. Sci. Natur., Botanique (Paris), 12e Sér. 12:345–428.

Agnihothrudu, V. G. C. S. Barua, and K. C. Barua. 1959. Occurrence of Chondromyces in the rhizosphere of plants. Indian Phytopathol. 12:158–160.

Arias, J. M., C. Rodriguez, and E. Montoya. 1979a. Biological activity of an antibiotic produced by Myxococcus coralloides. Microbios 24:123–131.

Arias, J. M., C. Rodriguez, and E. Montoya. 1979b. Purification and partial characterization of an antibiotic produced by Myxococcus coralloides. J. Antibiot. 32:205–211.

Arnold, J. W., and L. J. Shimkets. 1988a. Cell surface properties correlated with cohesion in Myxococcus xanthus. J. Bacteriol. 170:5771–5777.

Arnold, J. W., and L. J. Shimkets. 1988b. Inhibition of cell-cell interactions in Myxococcus xanthus by Congo red. J. Bacteriol. 170:5765–5770.

Aschner, M., and M. Chorin-Kirsch. 1970. Light-oriented locomotion in certain myxobacter species. Arch. Mikrobiol. 74:308–314.

Bacon, K., and F. A. Eiserling. 1968. A unique structure in microcysts of Myxococcus xanthus. J. Ultrastruct. Res. 21:378–382.

Balsalobre, J. M., R. Ruiz-Vázques, and F. J. Murillo. 1987. Light induction of gene expression in Myxococcus xanthus. Proc. Natl. Acad. Sci. USA 84:2359–2362.

Baur, E. 1905. Myxobakterien-Studien. Arch. Protistenk. 5:92–121.

Becker, K. 1990. Antibiotikca-Produktion mit trägerfixierten Myxobakterien [PhD thesis]. Technical University Braunschweig. Braunschweig, Germany.

Beebe, J. M. 1941. Studies on the myxobacteria. 2: The role of myxobacteria as bacterial parasites. Iowa State College J. Sci. 15:319–337.

Behmlander, R. M., and M. Dworkin. 1991. Extracellular fibrils and contact-mediated cell interactions in Myxococcus xanthus. J. Bacteriol. 173:7810–7821.

Behmlander, R., and M. Dworkin. 1994. Biochemical and structural analyses of the extracellular matrix fibrils of Myxococcus xanthus. J. Bacteriol. 176:6295–6303.

Behrens, J., J. Flossdorf, and H. Reichenbach. 1976. Base composition of deoxyribonucleic acid from Nannocystis exedens (Myxobacterales). Int. J. Syst. Bacteriol. 26:561–562.

Bender, H. 1962. Untersuchungen an Myxococcus xanthus. I: Bildungsbedingungen, Isolierung und Eigenschaften eines bakteriolytischen Enzymsystems. Arch. Mikrobiol. 43:262–279.

Bender, H. 1963. Untersuchungen an Myxococcus xanthus II. Partielle Lyse von Pullularia pullulans und einigen Hefen durch ein extrazelluläres Enzymsystem. Arch Mikrobiol. 45:407–422.

Berkeley, M. J. 1857. Introduction to Cryptogamic Botany [on Stigmatella and Chondromyces]. H. Bailliere. London, UK. 313–315.

Beveridge, T. J. 1999. Structures of Gram-negative cell walls and their derived membrane vesicles. J. Bacteriol. 181:4725–4733.

Beyer, P., and H. Kleinig. 1985. In vitro synthesis of C15-C60 polyprenols in a cell-free system of Myxococcus fulvus and determination of chain length by high-performance liquid chromatography. *In:* Methods in Enzymology. Academic Press. New York, NY. 110:299–303.

Bird. C. W., J. M. Lynch, F. J. Pirt, and W. W. Reid. 1971. Steroids and squalene in Methylococcus capsulatus grown on methane. Nature 230:473–474.

Blackhart, B. D., and D. Zusman. 1985. "Frizzy" genes of Myxococcus xanthus are involved in control of the frequency of reversal of gliding motility. Proc. Natl. Acad. Sci. USA 82:8767–8771.

Bode, H. B., B. Zeggel, B. Silakowski, S. C. Wenzel, H. Reichenbach, and R. Müller. 2003. Steroid biosynthesis in prokaryotes: identification of myxobacterial steroids and cloning of the first bacterial 2,3(S)-oxidosqualene cyclase from the myxobacterium Stigmatella aurantiaca. Molec. Microbiol. 47:471–481.

Bojary, M. R., and S. A. Dhala. 1989. Coagulase of Myxococcus fulvus NK35. 1: Purification and partial characterization. Zbl. Mikrobiol. 144:347–354.

Bollag, D. M., P. A. McQueney, J. Zhu, O. Hensens, L. Koupal, J. Liesch, M. Goetz, E. Lazarides, and C. M. Woods. 1995. Epothilones, a new class of microtubule-stabilizing agents with a taxol- like mechanism of action. Cancer Res. 55:2325–2333.

Bonner, J. T. 1982. Comparative Biology of Cellular Slime Molds. *In:* W. F. Loomis (Ed.). The Development of Dictyostelium discoideum. Academic Press. New York, NY. 1–33.

Bonner, P. J., and L. J. Shimkets. 2001. Piecing together a puzzling pathway: New insights into C-signaling. Trends Microbiol. 9:462–464.

Borchers, M. 1982. Isolierung und Charakterisierung hefelytischer Enzyme aus dem gleitenden Bakterium Myxococcus fulvus Mx f80 (Myxobacterales) [Ph.D thesis]. Technical University Braunschweig. Braunschweig, Germany.

Borner, U., A. Deutsch, H. Reichenbach, and M. Bar. 2002. Rippling patterns in aggregates of myxobacteria arise from cell-cell collisions. Phys. Rev. Lett. (078101-1-4) 89:1–4.

Bowden, M. G., and H. B. Kaplan. 1998. The Myxococcus xanthus lipopolysaccharide O-antigen is required for social motility and multicellular development. Molec. Microbiol. 30:275–284.

Brauss, R. W., I. Heyne-Katzenberger, H. Pech, and H. Barth. 1967. Beiträge zur Mikrobiologie von Binnengewässern (I). Arch. Hyg. Bakteriol. 150:716–724.

Brauss, F. W., I. Heyne-Katzenberger, and W. Heyne. 1968. Beiträge zur Mikrobiologie von Binnengewässern (II). Arch. Hyg. Bakteriol. 152:346–349.

Breton, A. M. 1984a. Transposon Tn5 confers streptomycin resistance to Myxococcus xanthus. FEMS Microbiol. Lett. 22:85–88.

Breton, A. M., J. M. Nicaud, G. Younes, and J. F. Guespin-Michel. 1984b. Myxococcus xanthus, a Gram-negative nonpathogenic bacterium, that secretes proteins into the extracellular medium growth, is a potential cloning host for excreted proteins production. *In:* 3rd European Congress Biotechnol. München. Verlag Chemie Weinheim. Weinheim, Germany. III:441–446.

Breton, A. M., S. Jaoua, and J. Guespin-Michel. 1985. Transfer of plasmid RP4 to Myxococcus xanthus and evidence for its integration into the chromosome. J. Bacteriol. 161:523–528.

Breton, A. M., G. Younes, F. van Gijsegem, and J. Guespin-Michel. 1986. Expression in Myxococcus xanthus of foreign genes coding for secreted pectate lyases of Erwinia chrysanthemi. J. Biotechnol. 4:303–311.

Breton, A. M., and J. F. Guespin-Michel. 1987. Escherichia coli pH 2.5 acid phosphatase and b-lactamase TEM2 are secreted into the medium by Myxococcus xanthus. FEMS Microbiol. Lett. 40:183–188.

Bretscher, A. P., and D. Kaiser. 1978. Nutrition of Myxococcus xanthus, a fruiting myxobacterium. J. Bacteriol. 133:763–768.

Brockman, E. R., and W. L. Boyd. 1963. Myxobacteria from soils of the Alaskan and Canadian arctic. J. Bacteriol. 86:605–606.

Brockman, E. R. 1967. Fruiting myxobacteria from the South Carolina coast. J. Bacteriol. 94:1253–1254.

Brockman, E. R., and R. L. Todd. 1974. Fruiting myxobacters as viewed with a scanning electron microscope. Int. J. Syst. Bacteriol. 24:118–124.

Brockman, E. R. 1976. Myxobacters from arid Mexican soil. Appl. Environ. Microbiol. 32:642–644.

Brown, N. L., and J. H. Parish. 1976a. Extrachromosomal DNA in chloramphenicol resistant Myxococcus strains. J. Gen. Microbiol. 93:63–68.

Brown, N. L., R. P. Burchard, D. W. Morris, J. H. Parish, N. D. Stow, and C. Tsopanakis. 1976b. Phage and defective phage of strains of Myxococcus. Arch. Microbiol. 108:271–279.

Brown, M. L., D. W. Morris, and J. H. Parish. 1976c. DNA of Myxococcus bacteriophage MX-1: macromolecular properties and restriction fragments. Arch. Microbiol. 108:221–226.

Browning, D. F., D. E. Whitworth, and D. A. Hodgson. 2003. Light-induced carotenogenesis in Myxococcus xanthus: functional characterization of the ECF sigma factor

CarQ and antisigma factor CarR. Molec. Microbiol. 48:237–251.

Brun, Y. V., and L. J. Shimkets (Eds.). 1999. Prokaryotic Development. American Society for Microbiology Press. Washington, DC.

Burchard, A. C., R. P. Burchard, and J. A. Kloetzel. 1977. Intracellular, periodic structures in the gliding bacterium Myxococcus xanthus. J. Bacteriol. 132:666–672.

Burchard, R. P., and M. Dworkin. 1966a. A bacteriophage for Myxococcus xanthus: Isolation, characterization and relation of infectivity to host morphogenesis. J. Bacteriol. 91:1305–1313.

Burchard, R. P., and M. Dworkin. 1966b. Light-induced lysis and carotenogenesis in Myxococcus xanthus. J. Bacteriol. 91:535–545.

Burchard, R. P., and S. B. Hendricks. 1969. Action spectrum for carotenogenesis in Myxococcus xanthus. J. Bacteriol. 97:1165–1168.

Burchard, R. P., and H. Voelz. 1972. Bacteriophage infection of Myxococcus xanthus during cellular differentiation and vegetative growth. Virology 48:555–556.

Burchard, R. P., and D. T. Brown. 1973. Surface structure of gliding bacteria after freeze-etching. J. Bacteriol. 114:1351–1355.

Burchard, R. P., and J. H. Parish. 1975. Mutants of Myxococcus xanthus insensitive to glycerol-induced myxospore formation. Arch. Microbiol. 104:289–292.

Burchard, R. P., and J. H. Parish. 1976. Chloramphenicol resistance in Myxococcus xanthus. Antimicrob. Agents Chemother. 7:233–238.

Burchard, R. P., A. C. Burchard, and J. H. Parish. 1977. Pigmentation phenotype instability in Myxococcus xanthus. Can. J. Microbiol. 23:1657–1662.

Burchard, R. P. 1980. Gliding motility of bacteria. Bio. Science 30:157–162.

Burchard, R. P. 1981. Gliding motility of prokaryotes: ultrastructure, physiology, and genetics. Ann. Rev. Microbiol. 35:497–529.

Burchard, R. P. 1984. Gliding motility and taxes. *In:* E. Rosenberg (Ed.). Myxobacteria: Development and Cell Interactions. Springer-Verlag. New York, NY. 139–161.

Burnham, J. C., S. A. Collart, and B. W. Highison. 1981. Entrapment and lysis of the cyanobacterium Phormidium luridum by aqueous colonies of Myxococcus xanthus PCO2. Arch. Microbiol. 129:285–294.

Burnham, J. C., S. A. Collart, and M. J. Daft. 1984. Myxococcal predation of the cyanobacterium Phormidium luridum in aqueous environments. Arch. Microbiol. 137:220–225.

Caillon, E., B. Lubochinsky, and D. Rigomier. 1983. Occurrence of dialkyl ether phospholipids in Stigmatella aurantiaca DW4. J. Bacteriol. 153:1348–1351.

Callao, V., R. Alvarado, A. Sedano, J. Olivares, and E. Montoya. 1966. Efecto antagonico del Myxococcus xanthus sobre los Azotobacter. Microbiol. Español 19:45–51.

Campos, J. M., J. Geisselsoder, and D. R. Zusman. 1978. Isolation of bacteriophage MX4, a generalized transducing phage for Myxococcus xanthus. J. Molec. Biol. 119:167–178.

Cervantes, M., and F. J. Murillo. 2002. Role for vitamin B(12) in light induction of gene expression in the bacterium Myxococcus xanthus. J. Bacteriol. 184:2215–2224.

Chang, B.-Y., and M. Dworkin. 1994. Isolated fibrils rescue cohesion and development in the dsp mutant of Myxococcus xanthus. J. Bacteriol. 176:7190–7196.

Chen, H., J. M. Keseler, and L. J. Shimkets. 1990. Genome size of Myxococcus xanthus determined by pulsed-field gel electrophoresis. J. Bacteriol. 172:4206–4213.

Chen, H.-W., A. Kuspa, I. Keseler, and L. J. Shimkets. 1991. Physical map of the M. xanthus chromosome. J. Bacteriol. 173:2109–2115.

Coletta, P. L., and P. G. G. Miller. 1986. The extracellular proteases of Myxococcus xanthus. FEMS Microbiol. Lett. 37:203–207.

Couke, P., and J. P. Voets. 1967. The mineral requirement of Polyangium cellulosum. Zeitschr. Allgem. Mikrobiol. 7:175–182.

Couke, P., and J. P. Voets. 1968. Etude de la cellulolyse enzymatique par Sorangium compositum. Ann. Inst. Pasteur. 115:549–560.

Couke, P. 1969. Morphology and morphogenesis of Sorangium compositum. J. Appl. Bacteriol. 32:24–29.

Crawford Jr., E. W., and L. J. Shimkets. 1999. Multicellular development in the myxobacteria Myxococcus xanthus and Stigmatella aurantiaca. *In:* V. E. A. Russo, D. J. Cove, L. G. Edgar, R. Jaenisch, and F. Salamini (Eds.). Development: Genetics, Epigenetics, and Environmental Regulation. Springer-Verlag. Heidelberg, Germany. 35–47.

Cumsky, M. G., and D. R. Zusman. 1981. Purification and characterization of myxobacterial hemagglutinin, a development-specific lectin of Myxococcus xanthus. J. Biol. Chem. 256:12581–12588.

Cunningham, J. L. 1972. A miracle mounting fluid for permanent whole-mounts of microfungi. Mycologia 64:906–911.

Daft, M. J., J. C. Burnham, and Y. Yamamoto. 1985. Lysis of Phormidium luridum by Myxococcus fulvus in continuous flow cultures. J. Appl. Bacteriol. 59:73–80.

Dana, J. R. and L. J. Shimkets. 1993. Regulation of cohesion-dependent cell interactions in Myxococcus xanthus. J. Bacteriol. 175:3636–3647.

Dawid, W. 1978. Fruchtkörper-bildende Myxobakterien in Böden Brasiliens. Zeitschr. Allgem. Mikrobiol. 18:83–93.

Dawid, W. 1979. Vorkommen und Verbreitung Fruchtkörperbildender Myxobakterien im Siebengebirge. Zeitschr. Allgem. Mikrobiol. 19:705–719.

Dawid, W. 1980. Fruchtkörperbildende Myxobakterien. VII: Die Chondromyces-Arten: Ch. apiculatus und Ch. lanuginosus. Entwicklungszyklus. Mikrokosmos 69:73–79.

Dawid, W. 1984. Myxobakterien in ungestörten Hochmooren des Hohen Venn (Hautes Fagnes, Belgien). Syst. Appl. Microbiol. 5:555–563.

Dawid, W., C. A. Gallikowski, and P. Hirsch. 1988. Psychrophilic myxobacteria from Antarctic soils. Polarforschung 58:271–278.

Dawid, W. 2000. Biology and global distribution of myxobacteria in soils. FEMS Microbiol. Rev. 24:403–427.

Dérijard, B., M. Ben A'ssa, B. Lubochinsky, and Y. Cenatiempo. 1989. Evidence for a membrane-associated GTP-binding protein in Stigmatella aurantiaca, a prokaryotic cell. Biochem. Biophys. Res. Commun. 158:562–568.

Devi, A. L., and H. D. McCurdy. 1984. Cyclic GMP and cyclic AMP binding proteins in Myxococcus xanthus. J. Gen. Microbiol. 130:1845–1849.

Dhundale, A. R., T. Furuichi, S. Inouye, and M. Inouye. 1985. Distribution of multicopy single-stranded DNA among myxobacteria and related species. J. Bacteriol. 164:914–917.

Dhundale, A., B. Lampson, T. Furuichi, M. Inouye, and S. Inouye. 1987. Structure of msDNA from Myxococcus xanthus: evidence for a long self-annealing RNA precursor for the covalently linked, branched RNA. Cell 51:1105–1112.

Dhundale, A., T. Furuichi, M. Inouye, and S. Inouye. 1988a. Mutations that affect production of branched RNA-linked msDNA in Myxococcus xanthus. J. Bacteriol. 170:5620–5624.

Dhundale, A., M. Inouye, and S. Inouye. 1988b. A new species of multicopy single-stranded DNA from Myxococcus xanthus with conserved structural features. J. Biol. Chem. 263:9055–9058.

Dill, D., H. Eckau, and H. Budzikiewicz. 1985. Neuartige Cerebroside aus Cystobacter fuscus (Myxobacterales). Zeitschr. Naturforsch. 40b:1738–1746.

Ditmar, L. P. F. 1814. Die Pilze Deutschlands. *In:* J. Sturm (Ed.). Deutschlands Flora, III. Abteilung, 1. Bändchen, Heft 2 [on Polyangium]. Nuremberg, Germany. 55–56 and plate 27.

Dobson, W. J., and H. D. McCurdy. 1979. The function of fimbriae in Myxococcus xanthus. I: Purification and properties of M. xanthus fimbriae. Can. J. Microbiol. 25:1152–1160.

Downard, J. S. 1988. Tn5-mediated transposition of plasmid DNA after transduction to Myxococcus xanthus. J. Bacteriol. 170:4939–4941.

Drews, G. 1974. Mikrobiologisches Praktikum, 2nd ed. Springer-Verlag. Berlin, Germany.

Dubos, R. 1928. The decomposition of cellulose by aerobic bacteria. J. Bacteriol. 15:223–234.

Dworkin, M. 1962. Nutritional requirements for vegetative growth of Myxococcus xanthus. J. Bacteriol. 84:250–257.

Dworkin, M., and S. M. Gibson. 1964a. A system for studying microbial morphogenesis: rapid formation of microcysts in Myxococcus xanthus. Science 146:243–244.

Dworkin, M., and D. J. Niederpruem. 1964b. Electron transport system in vegetative cells and microcysts of Myxococcus xanthus. J. Bacteriol. 87:316–322.

Dworkin, M., and W. Sadler. 1966. Induction of cellular morphogenesis in Myxococcus xanthus. I: General description. J. Bacteriol. 91:1516–1519.

Dworkin, M. 1969. Sensitivity of gliding bacteria to actinomycin D. J. Bacteriol. 98:851–852.

Dworkin, M. 1973a. Cell-cell interactions in the myxobacteria. Symp. Gen. Microbiol. 23:125–142.

Dworkin, M. 1973b. The Myxobacterales. *In:* A. I. Laskin and H. A. Lechevalier (Eds.). Handbook of Microbiology. CRC Press. Boca Raton, FL. I:191–202.

Dworkin, M. 1983a. Tactic behavior of Myxococcus xanthus. J. Bacteriol. 154:452–459.

Dworkin, M., and D. Eide. 1983b. Myxococcus xanthus does not respond chemotactically to moderate concentration gradients. J. Bacteriol. 154:437–442.

Dworkin, M., K. H. Keller, and D. Weisberg. 1983c. Experimental observations consistent with a surface tension model of gliding motility of Myxococcus xanthus. J. Bacteriol. 155:1367–1371.

Dworkin, M. 1984. Research on the myxobacteria: past, present, future. *In:* E. Rosenberg (Ed.). Myxobacteria. Development and Cell Interactions. Springer-Verlag. New York, NY. 222–245.

Dworkin, M. 1985a. Developmental Biology of the Bacteria. Benjamin/Cummings Publishing. Reading, MA.

Dworkin, M., and D. Kaiser. 1985b. Cell interactions in myxobacterial growth and development. Science 230:18–24.

Dworkin, M. 1991. Cell-cell interactions in Myxobacteria. *In:* M. Dworkin (Ed.). Microbial Cell-Cell Interactions. ASM Press. Washington, DC. 179–216.

Dworkin, M., and D. Kaiser (Eds.). 1993. Myxobacteria. American Society for Microbiology. Washington, DC.

Dworkin, M. 1996. Recent advances in the social and developmental biology of the Myxobacteria. Microbiol. Rev. 60:70–102.

Dworkin, M. 1999. Fibrils as extracellular appendages of bacteria: Their role in contact-mediated cell-cell interactions in Myxococcus xanthus. BioEssays 21:590–595.

Eckau, H., D. Dill, and H. Budzikiewicz. 1984. Neuartige Ceramide aus Cystobacter fuscus (Myxobacterales). Zeitschr. Naturforsch. 39c:1–9.

El Akoum, A., M. Vijayalakshmi, P. Cardon, B. Fournet, M. Sigot, and J. F. Guespin-Michel. 1987. Myxococcus xanthus produces an extracellular glycopeptide that displays blood anticoagulant properties. Enz. Microb. Technol. 9:426–429.

Fautz, E. , G. Rosenfelder, and L. Grotjahn. 1979. Isobranched 2- and 3-hydroxy fatty acids as characteristic lipid constituents of some gliding bacteria. J. Bacteriol. 140:852–858.

Fautz, E., L. Grotjahn, and H. Reichenbach. 1981. 2- and 3-hydroxy fatty acids as valuable chemosystematic markers in gliding bacteria. *In:* H. Reichenbach and O. B. Weeks (Eds.). The Flavobacterium-Cytophaga Group. Verlag Chemie Weinheim. Weinheim, Germany. 127–133.

Filer, D., E. Rosenberg, and S. H. Kindler. 1973. Aspartokinase of Myxococcus xanthus: "Feedback stimulation" by required amino acids. J. Bacteriol. 115:23–28.

Filer, D., S. H. Kindler, and E. Rosenberg. 1977. Myxospore coat synthesis in Myxococcus xanthus: Enzymes associated with uridine 5'-diphosphate-N-acetylgalactosamine formation during myxospore development. J. Bacteriol. 131:745–750.

Finck, G. 1950. Biologische und stoffwechselphysiologische Studien an Myxococcaceen. Arch. Mikrobiol. 15:358–388.

Fink, J. M., and J. F. Zissler. 1989a. Characterization of lipopolysaccharide from Myxococcus xanthus by use of monoclonal antibodies. J. Bacteriol. 171:2028–2032.

Fink, J. M., and J. F. Zissler. 1989b. Defects in motility and development of Myxococcus xanthus lipopolysaccharide mutants. J. Bacteriol. 171:2042–2048.

Fink, J. M., M. Kalos, and J. F. Zissler. 1989. Isolation of cell surface antigen mutants of Myxococcus xanthus by use of monoclonal antibodies. J. Bacteriol. 171:2033–2041.

Fluegel, W. 1963. Fruiting chemotaxis in Myxococcus fulvus (myxobacteria). Proc. Minnesota Acad. Sci. 30:120–123.

Fluegel, W. 1965. Fruiting body populations of Myxococcus fulvus (Myxobacterales). Growth 29:183–191.

Fontes, M., and D. Kaiser. 1999. Myxococcus cells respond to elastic forces in their substrate. Proc. Natl. Acad. Sci. USA 96:8052–8057.

Fontes, M., L. Galbis-Martinez, and F. J. Murillo. 2003. A novel regulatory gene for light-induced carotenoid synthesis in the bacterium Myxococcus xanthus. Molec. Microbiol. 47:561–571.

Fraleigh, P. C., and J. C. Burnham. 1988. Myxococcal predation on cyanobacterial populations—nutrient effects. Limnol. Oceanogr. 33:476–483.

Freese, A., H. Reichenbach, and H. Lunsdorf. 1997. Further characterization and in situ localization of chain-like aggregates of the gliding bacteria Myxococcus fulvus and Myxococcus xanthus. J. Bacteriol. 179:1246–1252.

Fudou, R., Y. Jojima, T. Iizuka, and S. Yamanaka. 2002. Haliangium ochraceum gen.nov., sp. nov. and Haliangium tepidum sp. nov.: Novel moderately halophilic myxobacteroa isolated from coastal saline environments. J. Gen. Appl. Microbiol. 48:109–115.

Furuichi, T., M. Inouye, and S. Inouye. 1985. Novel one-step cloning vector with a transposable element: Application to the Myxococcus xanthus genome. J. Bacteriol. 164:270–275.

Furuichi, T., A. Dhundale, M. Inouye, and S. Inouye. 1987a. Branched RNA covalently linked to the 5′ end of a single-stranded DNA in Stigmatella aurantiaca: structure of msDNA. Cell 48:47–53.

Furuichi, T., S. Inouye, and M. Inouye. 1987b. Biosynthesis and structure of stable branched RNA covalently linked to the 5' end of multicopy single-stranded DNA of Stigmatella aurantiaca. Cell 48:55–62.

Galván, A., R. Anadon, and F. de Castro. 1981. Observation de cuerpos fructiferos de Stigmatella erecta con el microscopio electronico de barrido. Bol. R. Espan. Hist. Nat. (Biol.) 79:259–264.

Galván, A., F. de Castro, and D. Fernández-Galiano. 1986. Ultrastructura de la célula vegetativa de Stigmatella erecta S12. Bol. R. Soc. Espan. Hist. Nat. (Biol.) 81:5–9.

Galván, A., F. de Castro, and D. Fernández-Galiano. 1987. Ultrastructure of the fruiting body of Stigmatella erecta (Myxobacterales). Trans. Am. Microscop. Soc. 106:89–93.

Galván, A., M.A. Marcotegui , and F. de Castro. 1992. Ultrastructure of natural and induced myxospores of Archangium gephyra. Can. J. Microbiol. 38:130–134.

Geisselsoder, J., J. Campos, and D. R. Zusman. 1978. Physical characterization of MX4, a generalized transducing phage for Myxococcus xanthus. J. Molec. Biol. 119:179–189.

Geitler, L. 1924. Über Polyangium parasiticum n. sp., eine submerse, parasitische Myxobacteriacee. Arch. Protistenk. 50:67–88.

Geitler, L. 1925. Über Polyangium parasiticum n. sp., eine submerse, parasitische Myxobacteriacee. Zeit. Botanik. 17:600–603.

Gelvan, I., M. Varon, and E. Rosenberg. 1987. Cell-density-dependent killing of Myxococcus xanthus by Autocide AmV. J. Bacteriol. 169:844–849.

Gerth, K. 1975. Untersuchungen über den molekularen Mechanismus der Induktion von Myxosporen bei Stigmatella aurantiaca Sg al (Myxobacterales) [PhD thesis]. University of Freiburg. Freiburg, Germany.

Gerth, K., and H. Reichenbach. 1978. Induction of myxospore formation in Stigmatella aurantiaca (Myxobacterales). I: General characterization of the system. Arch. Microbiol. 177:173–182.

Gerth, K., H. Irschik, H. Reichenbach, and W. Trowitzsch. 1980. Myxothiazol, an antibiotic from Myxococcus fulvus (Myxobacterales). I: Cultivation, isolation, physicochemical and biological properties. J. Antibiot. 33:1474–1479.

Gerth, K., H. Irschik, H. Reichenbach, and W. Trowitzsch. 1982. The myxovirescins, a family of antibiotics from Myxococcus virescens (Myxobacterales). J. Antibiot. 35:1454–1459.

Gerth, K., R. Jansen, G. Reifenstahl, G. Höfle, H. Irschik, B. Kunze, H. Reichenbach, and G. Thierbach. 1983. The myxalamids, new antibiotics from Myxococcus xanthus (Myxobacterales). I: Production, physico-chemical and biological properties and mechanism of action. J. Antibiot. 36:1150–1156.

Gerth, K., W. Trowitzsch, G. Piehl, R. Schultze, and J. Lehmann. 1984. Inexpensive media for mass cultivation of myxobacteria. Appl. Microbiol. Biotechnol. 19:23–28.

Gerth, K., and H. Reichenbach. 1986. Determination of bacterial ammonia pools using Myxococcus virescens as an example. Analyt. Biochem. 152:78–82.

Gerth, K., R. Metzger, and H. Reichenbach. 1993. Induction of myxospores in Stigmatella aurantiaca (Myxobacteria)—inducers and inhibitors of myxospore formation, and mutants with a changed sporulation behaviour. J. Gen. Microbiol. 139:865–871.

Gerth, K., and H. Reichenbach. 1994. Induction of myxospores in Stigmatella aurantiaca (Myxobacteria)—analysis of inducer–inducer and inducer-inhibitor interactions by dose-response curves. Microbiology 140:3241–3247.

Gerth, K., N. Bedorf, G. Höfle, H. Irschik, and H. Reichenbach. 1996. Epothilons A and B: antifungal and cytotoxic compounds from Sorangium cellulosum (Myxobacteria). Production, physico-chemical and biological properties. J. Antibiot. 49:560–563.

Gill, J., E. Stellwag, and M. Dworkin. 1985. Monoclonal antibodies against cell-surface antigens of developing cells of Myxococcus xanthus. Ann. Inst. Pasteur/Microbiol. 136A:11–18.

Gill, J. S., and M. Dworkin. 1986. Cell surface antigens during submerged development of Myxococcus xanthus examined with monoclonal antibodies. J. Bacteriol. 168:505–511.

Gill, J. S., and M. Dworkin. 1988. Isolation of additional monoclonal antibodies directed against cell surface antigens of Myxococcus xanthus cells undergoing submerged development. J. Bacteriol. 170:5953–5955.

Gnosspelius, G. 1978a. Myxobacterial slime and proteolytic activity. Arch. Microbiol. 116:51–59.

Gnosspelius, G. 1978b. Purification and properties of an extracellular protease from Myxococcus virescens. J. Bacteriol. 133:17–25.

Godchaux, W., and E. R. Leadbetter. 1983. Unusual sulfonolipids are characteristic of the Cytophaga-Flexibacter group. J. Bacteriol. 153:1238–1246.

Gorham, H. C., S. J. McGowan, P. R. Robson, and D. A. Hodgson. 1996. Light-induced carotenogenesis in Myxococcus xanthus: Light-dependent membrane sequestration of ECF sigma factor CarQ by anti-sigma factor CarR. Molec. Microbiol. 19:171–186.

Gräf, W. 1975. Myxobakterien der Gattung Myxococcus als indirekte Fäkalstoffindikatoren in Oberflächengewässern. Zbl. Bakteriol., 1. Abt. Orig., Reihe B 160:28–39.

Grilione, P. L. 1968. Serological reactions of some higher myxobacteria. J. Bacteriol. 95:1202–1204.

Grimm, K., H. K. Galle, and H. H. Heunert. 1971. Archangium violaceum (Myxobacteriales)—Bewegungsaktivität undo Kolonieform [film]. Institut für den

wissenschaftlichen Film. Göttingen Germany, Encyclopaedia Cinematographica E 1588.

Grimm, K., and H. Kühlwein. 1973a. Untersuchungen an spontanen Mutanten von Archangium violaceum (Myxobacterales). I: Bewegliche und unbewegliche Zellen von A. violaceum. Arch. Mikrobiol. 89:105–119.

Grimm, K., and H. Kühlwein. 1973b. Untersuchungen an spontanen Mutanten von Archangium violaceum (Myxobacterales). II: Über den Einfluß des Schleims auf die Bewegung der Zellen und die Entstehung stabiler Suspensionskulturen. Arch. Mikrobiol. 89:121–132.

Grimm, K., and H. Kühlwein. 1973c. Untersuchungen an spontanen Mutanten von Archangium violaceum (Myxobacterales). III: Über weitere Eigenschaften der K- und S-Zellen. Arch. Mikrobiol. 89:113–146.

Grimm, K. 1978. Comparison of spontaneous, UV-induced, and nitrosoguanidine-induced mutability to drug resistance in myxobacteria. J. Bacteriol. 135:748–753.

Hagen, D. C., A. P. Bretscher, and D. Kaiser. 1978. Synergism between morphogenetic mutants of Myxococcus xanthus. Devel. Biol. 64:284–296.

Hanson, C. W., and M. Dworkin. 1974. Intracellular and extracellular nucleotides and related compounds during the development of Myxococcus xanthus. J. Bacteriol. 118:486–496.

Harcke, E., A. Hüttermann, and H. Kühlwein. 1971. Studies on lytic activities of Chondrococcus coralloides (Myxobacterales). I: Purification and some properties of the bacteriolytic activity. Arch. Mikrobiol. 77:86–95.

Harcke, E., A. Hüttermann, and H. Kühlwein. 1972. Studies on lytic activities of Chondrococcus coralloides (Myxobacterales). II: Identification of the bacteriolytic enzyme as a muramidase. Arch. Mikrobiol. 85:6–12.

Hart, B. A., and S. A. Zahler. 1966. Lytic enzyme produced by Myxococcus xanthus. J. Bacteriol. 92:1632–1637.

Hartzell, P., and D. Kaiser. 1991. Function of MglA, a 22-kilodalton protein essential for gliding in Myxococcus xanthus. J. Bacteriol. 173:7615–7624.

Hartzell, P. L., and P. Youderian. 1995. Genetics of gliding motility and development in Myxococcus xanthus. Arch. Microbiol. 164:309–323.

Haskå, G. 1969. Production of lytic exoenzymes in casamino acids media by Myxococcus virescens. Physiol. Plant. 22:1074–1078.

Haskå, G., and S. Ståhl. 1971. Variants of Myxococcus virescens exhibiting dispersed growth. Growth and production of extracellular enzymes and slime. Physiol. Plant. 24:136–142.

Haskå, G. 1972a. Extracellular lytic enzymes of Myxococcus virescens. II: Purification of three bacteriolytic enzymes and determination of their molecular weights and isoelectric points. Physiol. Plant. 26:221–229.

Haskå, G. 1972b. Extracellular lytic enzymes of Myxococcus virescens. III: Characterization of two endo-β-N-acetylglucosaminidases. Physiol. Plant. 27:139–142.

Haskå, G., B. Norén, and G. Odham. 1972c. Effect of fatty acids on the activity of bacteriolytic enzymes. Physiol. Plant. 27:187–194.

Haskå, G. 1974. Extracellular lytic enzymes of Myxococcus virescens. IV: Purification and characterization of a D-alanyl-E-N-lysine endopeptidase. Physiol. Plant. 31:252–256.

Haskå, G. 1975. Influence of clay minerals on sorption of bacteriolytic enzymes. Microb. Ecol. 1:234–245.

Haskå, G. 1981. Activity of bacteriolytic enzymes adsorbed to clay. Microb. Ecol. 7:331–341.

Hecht, V., J. Vorlop, H. Kalbitz, K. Gerth, and J. Lehmann. 1987. Vortex chamber for in situ recovery of the antibiotic myxovirescin A in continuous cultivation. Biotechnol. Bioengin. 29:222–227.

Heidelbach, M., H. Skladny, and H. U. Schairer. 1993. Heat-shock and development induced synthesis of a low molecular weight stress-responsive protein in the myxobacterium Stigmatella aurantiaca. J. Bacteriol. 175:7479–7482.

Henrichsen, J. 1983. Twitching motility. Ann. Rev. Microbiol. 37:81–93.

Heunert, H. H. 1973. Präparationsmethoden für Vitalbeobachtungen an Mikroorganismen. Zeiss Informationen 20:40–49.

Heyn, A. N. J. 1957. Bacteriological studies on cotton. Textile Res. J. 27:591–603.

Hirsch, H. J. 1977. Bacteriocins from Myxococcus fulvus (Myxobacterales). Arch. Microbiol. 115:45–49.

Hirsch, H. J., H. Tsai, and I. Geffers. 1978. Purification and effects of fulvocin C, a bacteriocin from Myxococcus fulvus Mx f16. Arch. Microbiol. 119:279–286.

Hodgkin, J., and D. Kaiser. 1977. Cell-to-cell stimulation of movement in nonmotile mutants of Myxococcus. Proc. Natl. Acad. Sci. USA 74:2938–2942.

Hodgkin, J., and D. Kaiser. 1979a. Genetics of gliding motility in Myxococcus xanthus (Myxobacterales): Genes controlling movement of single cells. Molec. Gen. Genet. 171:167–176.

Hodgkin, J., and D. Kaiser. 1979b. Genetics of gliding motility in Myxococcus xanthus (Myxobacterales): Two gene systems control movement. Molec. Gen. Genet. 171:177–191.

Hodgson, D. A., and F. J. Murillo. 1993. Genetics of Regulation and Pathway of Synthesis of Carotenoids. *In:* M. Dworkin and D. Kaiser (Eds.). Myxobacteria. Springer-Verlag. New York, NY. II:157–181.

Hodgson, D. A., and A. E. Berry. 1998. Light regulation of carotenoid synthesis in Myxococcus xanthus. *In:* M. X. Caddick, S. Baumberg, D. A. Hodgson, and M. K. Phillips-Jones (Eds.). Microbial Responses to Light and Time. Cambridge University Press. Cambridge, UK. Society for General Microbiology Symposium Volume 56:185–211.

Homma, Y. 1984. Perforation and lysis of hyphae of Rhizoctonia solani and conidia of Cochliobolus miyabeanus by soil myxobacteria. Phytopathology 74:1234–1239.

Hook, L. A., J. M. Larkin, and E. R. Brockman. 1966. Isolation, characterization, and emendation of description of Angiococcus disciformis (Thaxter 1904) Jahn 1924 and proposal of a neotype strain. Internat. J. Syst. Bacteriol. 30:135–142.

Hook, L. A. 1977. Distribution of myxobacters in aquatic habitats in an alkaline bog. Appl. Environ. Microbiol. 34:333–335.

Hüttermann, A. 1969. Studies on bacteriolytic enzyme of Archangium violaceum (Myxobacterales). II: Partial purification and properties of the enzyme. Arch. Mikrobiol. 67:306–317.

Igoshin, O. A., A. Mogilner, R. D. Welch, D. Kaiser, and G. Oster. 2001. Pattern formation and traveling waves in myxobacteria: Theory and modeling. Proc. Nat. Acad. Sci. USA 98:14913–14918.

Iizuka, T., Y. Jojima, R. Fudou, and S. Yamanaka. 1998. Isolation of myxobacteria from the marine environment. FEMS Microbiol. Lett. 169:317–322.

Iizuka, T., Y. Jojima, R. Fudou, A. Hiraishi, J. W. Ahn, and S. Yamanaka. 2003a. Plesiocystis pacifica gen. nov., sp. nov., a marine myxobacterium that contains dihydrogenated menaquinone, isolated from the Pacific coasts of Japan. Int. J. Syst. Evol. Microbiol. 53:189–195.

Iizuka, T., Y. Jojima, R. Fudou, M. Tokura, A. Hiraishi, and S. Yamanaka. 2003b. Enhygromyxa salina gen. nov., sp. nov., a slightly halophilic myxobacterium isolated from the coastal areas of Japan. Syst. Appl. Microbiol. 26:189–196.

Imshenetski, A. A., L. Solntseva [name also transliterated as Imschenezki]. 1936. On aerobic cellulose-decomposing bacteria [in Russian, with English summary]. Izvestiia Akad. Nauk SSSR, Classe Sci. Math. Natl. Ser. Biol. 1936:1115–1172.

Imshenetski, A. A., and L. A. Kusjurina [name also transliterated as Imschenezki]. 1951. Bacteriotrophic microorganisms: The evolution of saprophytism and parasitism (in Russian). Mikrobiologiya 20:3–12.

Imschenetski, A. A. [name also transliterated as Imschenezki]. 1959. Mikrobiologie der Cellulose [translated from the Russian edition of 1953]. Akademie Verlag Berlin. Berlin, Germany.

Inouye, M., S. Inouye; D. R. Zusman. 1979a. Biosynthesis and self assembly of protein S, a development-specific protein of Myxococcus xanthus. Proc. Natl. Acad. Sci. USA 76:209–213.

Inouye, M., S. Inouye, and D. R. Zusman. 1979a. Gene expression during development of Myxococcus xanthus: Pattern of protein synthesis. Devel. Biol. 68:579–591.

Inouye, S., T. Franceschini, and M. Inouye. 1983a. Structural similarities between the development-specific protein S from a Gram-negative bacterium, Myxococcus xanthus, and calmodulin. Proc. Natl. Acad. Sci. USA 80:6829–6833.

Inouye, S., Y. Ike, and M. Inouye. 1983b. Tandem repeat of the genes for protein S, a development-specific protein of Myxococcus xanthus. J. Biol. Chem. 258:38–40.

Inouye, S. 1984. Identification of a development-specific promoter of Myxococcus xanthus. J. Molec. Biol. 174:113–120.

Inouye, S., T. Furuichi, A. Dhundale, and M. Inouye. 1987. Stable branched RNA covalently linked to the 5′ end of a single-stranded DNA of myxobacteria. *In:* M. Inouye and B. S. Dudock (Eds.). Molecular Biology of RNA: New Perspectives. Academic Press. San Diego, CA. 271–284.

Inouye, S., M. Y. Hsu, S. Eagle, and M. Inouye. 1989. Reverse transcriptase associated with the biosynthesis of the branched RNA-linked msDNA in Myxococcus xanthus. Cell 56:709–717.

Inouye, S. 1990a. Cloning and DNA sequence of the gene coding for the major sigma factor from Myxococcus xanthus. J. Bacteriol. 172:80–85.

Inouye, S., P. J. Herzer, and M. Inouye. 1990b. Two independent retrons with highly diverse transcriptases in Myxococcus xanthus. Proc. Natl. Acad. Sci. USA 87:942–945.

Irschik, H., K. Gerth, G. Höfle, W. Kohl, and H. Reichenbach. 1983a. The myxopyronins, new inhibitors of bacterial RNA synthesis from Myxococcus fulvus (Myxobacterales). J. Antibiot. 36:1651–1658.

Irschik, H., K. Gerth, T. Kemmer, H. Steinmetz, and H. Reichenbach. 1983b. The myxovalargins, new peptide antibiotics from Myxococcus fulvus (Myxobacterales). I: Cultivation, isolation and some chemical and biological properties. J. Antibiot. 36:6–12.

Irschik, H., and H. Reichenbach. 1985a. An unusual pattern of carbohydrate utilization in Corallococcus (Myxococcus) coralloides (Myxobacterales). Arch. Microbiol. 142:40–44.

Irschik, H., R. Jansen, G. Höfle, K. Gerth, and H. Reichenbach. 1985b. The corallopyronins, new inhibitors of bacterial RNA synthesis from myxobacteria. J. Antibiot. 38:145–152.

Irschik, H., R. Jansen, K. Gerth, G. Höfle, and H. Reichenbach. 1987. The sorangicins, novel and powerful inhibitors of eubacterial RNA polymerase isolated from myxobacteria. J. Antibiot. 40:7–13.

Jackson, D. J. 1959. The association of a slime bacterium with the inner envelope of the egg of Dytiscus marginalis (Coleoptera), and the less common occurrence of a similar bacterium on the egg of D. semisulcatus. Quart. J. Microscop. Sci. 100:433–443.

Jacobi, C. A., Assmus, B., Reichenbach, H., Stackebrandt, E. 1997. Molecular evidence for association between the sphingobacterium-like organism "Candidatus comitans" and the myxobacterium. Chondromyces crocatus 63:719–723.

Jahn, E. 1911. Myxobacteriales. Kryptogamenflora der Mark Brandenburg. Gebrüder Borntraeger Leipzig. Leipzig, Germany. 5:187–206.

Jahn, E. 1924. Beiträge zur botanischen Protistologie. I: Die Polyangiden. Gebrüder Borntraeger Leipzig. Leipzig, Germany.

Jansen, R., A. Nowak, B. Kunze, H. Reichenbach, and G. Höfle. 1995. Four new carotenoids from Polyangium fumosum (myxobacteria): 3,3,4,4'-tetradehydro-1,1′,2,2′-tetrahydro-1,1′dihydroxy-ψ,ψ-carotene (di-O-demethylspirilloxanthin), its β-glucoside and glucoside fatty acid. Liebigs Ann. 1995:873–876.

Janssen, G. R., J. W. Wireman, and M. Dworkin. 1977. Effect of temperature on the growth of Myxococcus xanthus. J. Bacteriol. 103:561–562.

Jaoua, S., J. F. Guespin-Michel, and A. M. Breton. 1987. Mode of insertion of the broad-host-range plasmid RP4 and its derivatives into the chromosome of Myxococcus xanthus. Plasmid 18:111–119.

Jaoua, S. , B. Letouvet-Pawlak, C. Monnier, and J. F. Guespin-Michel. 1990. Mechanism of integration of the broad-host-range plasmid RP4 into the chromosome of Myxococcus xanthus. Plasmid 23:183–193.

Jarvis, B. W., and M. Dworkin. 1989a. Purification and properties of Myxococcus xanthus cell surface antigen 1604. J. Bacteriol. 171:4655–4666.

Jarvis, B. W., and M. Dworkin. 1989b. Role of Myxococcus xanthus cell surface antigen 1604 in development. J. Bacteriol. 171:4667–4673.

Jeffers, E. E. 1964. Myxobacters of a freshwater lake and its environs. Int. Bull. Bact. Nomencl. Taxon. 14:115–136.

Johnson, J. L., and E. J. Ordal. 1969. Deoxyribonucleic acid homology among the fruiting myxobacteria. J. Bacteriol. 98:319–320.

Kaiser, D., and M. Dworkin. 1975. Gene transfer to a myxobacterium by Escherichia coli phage P1. Science 187:653–655.

Kaiser, D. 1979a. Social gliding is correlated with the presence of pili in Myxococcus xanthus. Proc. Natl. Acad. Sci. USA 76:5952–5956.

Kaiser, D., C. Manoil, and M. Dworkin. 1979b. Myxobacteria: Cell interactions, genetics, and development. Ann. Rev. Microbiol. 33:595–639.

Kaiser, D., and C. Crosby. 1983. Cell movement and its coordination in swarms of Myxococcus xanthus. Cell Motility 3:227–245.

Kaiser, D. 1984a. Genetics of myxobacteria. *In:* E. Rosenberg (Ed.). Myxobacteria: Development and Cell Interactions. Springer-Verlag. New York, NY. 163–184.

Kaiser, D. 1984b. Regulation of multicellular development in myxobacteria. *In:* R. Losick and L. Shapiro (Eds.). Microbial Development. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY. 197–218.

Kaiser, D., L. Kroos, and A. Kuspa. 1985. Cell interactions govern the temporal pattern of Myxococcus development. Cold Spring Harbor Symp. Quant. Biol. 50:823–830.

Kaiser, D. 1986. Control of multicellular development: Dictyostelium and Myxococcus. Ann. Rev. Genet. 20:539–566.

Kaiser, D. 1989. Multicellular development in myxobacteria. *In:* A. Hopwood and K. F. Chater (Eds.). Genetics of Bacterial Diversity. Academic Press. London, UK. 243–263.

Kaiser, D. 1993. Roland Thaxter's legacy and the origins of multicellular development. Genetics 135:249–254.

Kaiser, D. 1996. Bacteria also vote. Science 272:1598–1599.

Kaiser, D. 1998. How and why Myxobacteria talk to each other. Curr. Opin. Microbiol. 1:663–668.

Kaiser, D. 1999. Cell fate and organogenesis in bacteria. Trends Genet. 15:273–277.

Kaiser, D. 2000a. Bacterial motility: How do pili pull? Curr. Biol. 10:R777–R780.

Kaiser, D. 2000b. Cell-interactive sensing of the environment. *In:* Y. V. Brun and L. J. Shimkets (Eds.). Prokaryotic Development. ASM Press. Washington, DC. 263–275.

Kaiser, D. 2001. Building a multicellular organism. Ann. Rev. Genet. 35:103–123.

Karwowski, J. P., G. N. Sunga, S. Kadam, and J. B. McAlpine. 1996. A method for the selective isolation of Myxococcus directly from soil. J. Indust. Microbiol. 16:230–236.

Kashefi, K., and P. L. Hartzell. 1995. Genetic suppression and phenotypic masking of a Myxococcus xanthus frzF$^-$ defect. Molec. Microbiol. 15:483–494.

Kato, H. 1955. Notes on myxobacteria II. Antibacterial strains of Myxococcus fulvus. Ecol. Rev. 14:26–28.

Kearns, D. B., and L. J. Shimkets. 1998. Chemotaxis in a gliding bacterium. Proc. Natl. Acad. Sci. USA 95:11957–11962.

Kearns, D. B., B. D. Campbell, and L. J. Shimkets. 2000. Myxococcus xanthus fibril appendages are essential for excitation by a phospholipid attractant. Proc. Natl. Acad. Sci. USA 97:11505–11510.

Kearns, D. B., and L. J. Shimkets. 2001a. Lipid chemotaxis and signal transduction in Myxococcus xanthus. Trends Microbiol. 9:126–129.

Kearns, D. B., A. Venoit, P. J. Bonner, B. Stevens, G.-J. Boons, and L. J. Shimkets. 2001b. Identification of a developmental chemoattractant in Myxococcus xanthus through metabolic engineering. Proc. Natl. Acad. Sci. USA 98:13990–13994.

Kearns, D. B., P. J. Bonner, D. R. Smith, and L. J. Shimkets. 2002. An extracellular matrix-associated zinc metalloprotease is required for dilauroyl phosphatidylethanolamine chemotactic excitation in Myxococcus xanthus. J. Bacteriol. 184:1678–1684.

Keller, K. H., M. Grady, and M. Dworkin. 1983. Surface tension gradients: feasible model for gliding motility of Myxococcus xanthus. J. Bacteriol. 155:1358–1366.

Killeen, K. P., and D. R. Nelson. 1988. Acceleration of starvation- and glycerol-induced myxospore formation by prior heat shock in Myxococcus xanthus. J. Bacteriol. 170:5200–5207.

Kim, S. K., and D. Kaiser. 1990. C-factor: a cell-cell signaling protein required for fruiting body morphogenesis of M. xanthus. Cell 61:19–26.

Kim, S.-H., S. Ramaswamy, and J. Downard. 1999. Regulated exopolysaccharide production in Myxococcus xanthus. J. Bacteriol. 181:1496–1507.

Kim, Y. J., Furihata, K., Yamanaka, S., Fudo, R., and Seto, H. 1991. Isolation and structural elucidation of stipiamide, a new antibiotic effective to multidrug-resistant cancer cells. J. Antibiot. (Tokyo) 44:553–556.

Kimchi, A. E. Rosenberg. 1976. Linkages between deoxyribonucleic acid synthesis and cell division in Myxococcus xanthus. J. Bacteriol. 128:69–79.

Kleinig, H., H. Reichenbach, and H. Achenbach. 1970. Carotenoid pigments of Stigmatella aurantiaca (Myxobacterales). II: Acylated carotenoid glycosides. Arch. Mikrobiol. 74:223–234.

Kleinig, H., H. Reichenbach, H. Achenbach, and J. Stadler. 1971. Carotenoid pigments of Sorangium cellulosum (Myxobacterales) including two new carotenoid glycoside esters and two new carotenoid rhamnosides. Arch. Mikrobiol. 78:224–233.

Kleinig, H. 1972. Membranes from Myxococcus fulvus (Myxobacterales) containing carotenoid glucosides. I: Isolation and composition. Biochim. Biophys. Acta 274:489–498.

Kleinig, H., and H. Reichenbach. 1973. Biosynthesis of carotenoid glucoside esters in Myxococcus fulvus (Myxobacterales): Inhibition by nicotine and carotenoid turnover. Biochim. Biophys. Acta 306:249–256.

Kleinig, H. 1974a. Inhibition of carotenoid synthesis in Myxococcus fulvus (Myxobacterales). Arch. Microbiol. 97:217–226.

Kleinig, H., H. Reichenbach, N. Theobald, and H. Achenbach. 1974b. Flexibacter elegans and Myxococcus fulvus: Aerobic Gram-negative bacteria containing menaquinones as the only isoprenoid quinones. Arch. Microbiol. 101:91–93.

Kleinig, H. 1975. On the utilization in vivo of lycopene and phytoene as precursors for the formation of carotenoid glucoside ester and on the regulation of carotenoid biosynthesis in Myxococcus fulvus. Eur. J. Biochem. 57:301–308.

Kofler, L. 1913. Die Myxobakterien der Umgebung von Wien. Sitzber. Kais. Akad. Wiss. Wien, Math. Naturw. Klasse, Abt. 1 122:845–876.

Kohl, W., A. Gloe, and H. Reichenbach. 1983. Steroids from the myxobacterium Nannocystis exedens. J. Gen. Microbiol. 129:1629–1635.

Komano, T., T. Franceschini, and S. Inouye. 1987. Identification of a vegetative promoter in Myxococcus xanthus: A protein that has homology to histones. J. Molec. Biol. 196:517–524.

Kottel, R.H., K. Bacon, D. Clutter, and D. White. 1975. Coats from Myxococcus xanthus: Characterization and synthesis during myxospore differentiation. J. Bacteriol. 124:550–557.

Kroos, L., and D. Kaiser. 1984. Construction of Tn5 lac, a transposon that fuses lacZ expression to exogenous promoters, and its introduction into Myxococcus xanthus. Proc. Natl. Acad. Sci. USA 81:5816–5820.

Kroos, L., A. Kuspa, and D. Kaiser. 1986. A global analysis of developmentally regulated genes in Myxococcus xanthus. Devel. Biol. 17:252–266.

Kroos, L., A. Kuspa, and D. Kaiser. 1990. Defects in fruiting body development caused by Tn5 lac insertions in Myxococcus xanthus. J. Bacteriol. 172:484–487.

Krzemieniewska, H., and S. Krzemieniewski. 1926. Miksobakterje Polski [in Polish, with German summary]. Acta Soc. Botan. Polon. 4:1–54.

Krzemieniewska, H., and S. Krzemieniewski. 1927a. Miksobakterje Polski. Uzupelnienie [in Polish, with German summary]. Acta Soc. Botan. Polon. 5:79–98.

Krzemieniewska, H., and S. Krzemieniewski. 1927b. Rozsiedlenie miksobakteryj (Über die Verbreitung der Myxobakterien im Boden) [in Polish, with German summary]. Acta Soc. Botan. Polon. 5:102–139.

Krzemieniewska, H., and S. Krzemieniewski. 1928. Morphologja komorki miksobakteryj (Zur Morphologie der Myxobakterienzelle) [in Polish, with German summary]. Acta Soc. Botan. Polon. 5:46–90.

Krzemieniewska, H., and S. Krzemieniewski. 1930. Mikrobakterje Polski. Czesc trzecia (Die Myxobakterien von Polen. III. Teil) [in Polish, with German summary]. Acta Soc. Botan. Polon. 7:250–273.

Krzemieniewska, H., and S. Krzemieniewski. 1937a. Die zellulosezersetzenden Myxobakterien. Bull. Acad. Polon. Sci. Lettr. Classe Sci. Math. Nat. Sér. B (I):11–31.

Krzemieniewska, H., and S. Krzemieniewski. 1937b. Über die Zersetzung der Zellulose durch Myxobakterien. Bull. Acad. Polon. Sci. Lettr. Classe Sci. Math. Nat. Sér. B (I):33–59.

Krzemieniewska, H., and S. Krzemieniewski. 1946. Myxobacteria of the species Chondromyces Berkeley and Curtis. Bull. Acad. Polon. Sci. Lettr. Classe Sci. Math. Nat. Sér. B (I):31–48.

Kühlwein, H. 1950. Beiträge zur Biologie und Entwicklungsgeschichte der Myxobakterien. Arch. Mikrobiol. 14:678–704.

Kühlwein, H., and E. Gallwitz. 1958. Polyangium violaceum nov. spec.: Ein Beitrag zur Kenntnis der Myxobakterien. Arch. Mikrobiol. 31:139–145.

Kühlwein, H. 1960. Zur Systematik und Verbreitung der Myxobakterien. Zbl. Bakteriol., 2. Abt. 113:480–490.

Kühlwein, H., and H. Reichenbach. 1965. Anreicherung und Isolierung von Myxobakterien. Zbl. Bakteriol., 1. Abt. Orig. Suppl. 1:57–80.

Kühlwein, H. and H. Reichenbach. 1968a. Schwarmentwicklung und Morphogenese bei Myxobakterien Archangium-Myxococcus-Chondrococcus-Chondromyces. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segment #4:341–342 (Film C893; 1965).

Kühlwein, H. and H. Reichenbach. 1968b. Schwarmentwicklung und Morphogenese bei Myxobakterien Archangium-Myxococcus-Chondrococcus-Chondromyces. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segment #5:342.

Kühlwein, H. and H. Reichenbach. 1968c. Schwarmentwicklung und Morphogenese bei Myxobakterien Archangium-Myxococcus-Chondrococcus-Chondromyces. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segment #6:342.

Kühlwein, H. and H. Reichenbach. 1968d. Schwarmentwicklung und Morphogenese bei Myxobakterien Archangium-Myxococcus-Chondrococcus-Chondromyces. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segment #26:350.

Kühlwein, H. and H. Reichenbach. 1968e. Schwarmentwicklung und Morphogenese bei Myxobakterien Archangium-Myxococcus-Chondrococcus-Chondromyces. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segment #27:352.

Kühlwein, H. and H. Reichenbach. 1968f. Schwarmentwicklung und Morphogenese bei Myxobakterien Archangium-Myxococcus-Chondrococcus-Chondromyces. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segments #29–31:352–353.

Kühlwein, H. and H. Reichenbach. 1968g. Schwarmentwicklung und Morphogenese bei Myxobakterien Archangium-Myxococcus-Chondrococcus-Chondromyces. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segment #3:340–341.

Kühlwein, H. and H. Reichenbach. 1968h. Schwarmentwicklung und Morphogenese bei Myxobakterien Archangium-Myxococcus-Chondrococcus-Chondromyces. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segments #11–2:343–345.

Kühlwein, H. and H. Reichenbach. 1968i. Schwarmentwicklung und Morphogenese bei Myxobakterien Archangium-Myxococcus-Chondrococcus-Chondromyces. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segments #16–7:346–347.

Kühlwein, H. and H. Reichenbach. 1968j. Schwarmentwicklung und Morphogenese bei Myxobakterien Archangium-Myxococcus-Chondrococcus-Chondromyces. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segment #14:346.

Kühlwein, H., B. Schlicke, H. K. Galle, and H. H. Heunert. 1971a. Polyangium fuscum (Myxobacterales)—Cystenkeimung und Schwarmentwicklung [film]. Institut für den wissenschaftlichen Film. Göttingen, Germany. Encyclopaedia Cinematographica E 1582.

Kühlwein, H., B. Schlicke, H. K. Galle, and H. H. Heunert. 1971b. Polyangium fuscum (Myxobacterales)—Morphogenese. Institut für den wissenschaftlichen Film. Göttingen, Germany. Encyclopaedia Cinematographica E 1583.

Kuner, J. M., and D. Kaiser. 1981. Introduction of transposon Tn5 into Myxococcus for analysis of developmental and other nonselectable mutants. Proc. Natl. Acad. Sci. USA 78:425–429.

Kuner, J. M., and D. Kaiser. 1982. Fruiting body morphogenesis in submerged cultures of Myxococcus xanthus. J. Bacteriol. 151:458–461.

Kunze, B., T. Kemmer, G. Höfle, and H. Reichenbach. 1984. Stigmatellin, a new antibiotic from Stigmatella aurantiaca (Myxobacterales). I: Production, physico-chemical and biological properties. J. Antibiot. 37:454–461.

Kunze, B., W. Kohl, G. Höfle, and H. Reichenbach. 1985. Production, isolation, physicochemical and biological properties of angiolam A, a new antibiotic from Angio-

coccus disciformis (Myxobacterales). J. Antibiot. 38:1649–1654.

Kunze, B., G. Höfle, and H. Reichenbach. 1987. The aurachins, new quinoline antibiotics from myxobacteria: Production, physicochemical and biological properties. J. Antibiot. 40:258–265.

Kurtz, S., J. Rossi, L. Petko, and S. Lindquist. 1986. An ancient developmental induction: Heat-shock proteins induced in sporulation and oogenesis. Science 231:1154–1157.

Kuspa, A., D. Vollrath, Y. Cheng, and D. Kaiser. 1989. Physical mapping of the Myxococcus xanthus genome by random cloning in yeast artificial chromosomes. Proc. Natl. Acad. Sci. USA 86:8917–8921.

Lam, H.-M., and M. E. Winkler. 1992. Characterization of the complex pdxH-tyrS operon of Escherichia coli K12 and pleiotrophic phenotypes caused by pdxH insertion mutations. J. Bacteriol. 174:6033–6045.

Lampky, J. R. 1976. Ultrastructure of Polyangium cellulosum. J. Bacteriol. 126:1278–1284.

Lampky, J. R., and E. R. Brockman. 1977. Fluorescence of Myxococcus stipitatus. Int. J. Syst. Bacteriol. 27:161.

Lampson, B. C., M. Inouye, and S. Inouye. 1989. Reverse transcriptase with concomitant ribonuclease H. activity in the cell-free synthesis of branched RNA-linked msDNA of Myxococcus xanthus. Cell 56:701–707.

Lancero, H., J. E. Brofft, J. Downard, B. W. Birren, C. Nusbaum, J. Naylor, W. Shi, and L. J. Shimkets. 2002. Mapping of Myxococcus xanthus social motility dsp mutations to the dif genes. J. Bacteriol. 184:1462–1465.

Lau, J., S. Frykman, R. Regentin, S. Ou, H. Tsuruta, and P. Licari. 2002. Optimizing the heterologous production of epothilone D in Myxococcus xanthus. Biotechnol. Bioengin. 78:280–288.

Laue, B. E., and R. E. Gill. 1995. Using a phase-locked mutant of Myxococcus xanthus to study the role of phase variation in development. J. Bacteriol. 177:4089–4096.

Li, S.-F., and L. J. Shimkets. 1988. Site-specific integration and expression of a developmental promoter in Myxococcus xanthus. J. Bacteriol. 170:5552–5556.

Li, S.-F., and L. J. Shimkets. 1993. Effect of dsp mutations on the cell-to-cell transmission of CsgA in Myxococcus xanthus. J. Bacteriol. 175:3648–3652.

Li., Y.-Z., W. Hu, Y.-Q. Zhang, Z. Qiu, Y. Zhang, and B.-H. Wu. 2002. A simple method to isolate salt-tolerant myxobacteria from marine samples. J. Microbiol. Meth. 50:205–209.

Li, Y., H. Sun, X. Ma, A. Lu, R. Lux, D. Zusman, and W. Shi. 2003. Extracellular polysaccharides mediate pilus retraction during social motility of Myxococcus xanthus. Proc. Natl. Acad. Sci. USA 100:5443–5448.

Link, H. F. 1809. Observationes in Ordines plantarum naturales. Dissertatio Ima, complectens Anandrarum ordines Epiphytas, Mucedines Gastomycos et Fungos. Der Gesellschaft Naturforschender Freunde zu Berlin Magazin für die neuesten Entdeckungen in der gesamten Naturkunds 3:3–42 and Fig. 65.

Loebeck, M. E., and H. P. Klein. 1956. Substrates for Myxococcus virescens with special reference to eubacterial fractions. J. Gen. Microbiol. 14:281–289.

Lonergan, D. J., H. L. Jenter, J. D. Coates, E. J. Phillips, T. M. Schmidt, and D. R. Lovely. 1996. Phylogenetic analysis of dissimilatory Fe(III)-reducing bacteria. J. Bacteriol. 178:2402–2408.

Ludwig, W., H. H. Schleifer, H. Reichenbach, and E. Stackebrandt. 1983. A phylogenetic analysis of the myxobacteria Myxococcus fulvus, Stigmatella aurantiaca, Cystobacter fuscus, Sorangium cellulosum and Nannocystis exedens. Arch. Microbiol. 135:58–62.

Lünsdorf, H., and H. Reichenbach. 1989. Ultrastructural details of the apparatus of gliding motility of Myxococcus fulvus (Myxobacterales). J. Gen. Microbiol. 135:1633–1641.

Lünsdorf, H., and H. U. Schairer. 2001. Frozen motion of gliding bacteria outlines inherent features of the motility apparatus. Microbiology-UK 147:939–947.

MacRae, T. H., and H. D. McCurdy. 1975. Ultrastructural studies of Chondromyces crocatus vegetative cells. Can. J. Microbiol. 21:1815–1826.

MacRae, T. H., and H. D. McCurdy. 1976. Evidence for motility-related fimbriae in the gliding microorganism Myxococcus xanthus. Can. J. Microbiol. 22:1589–1593.

MacRae, T. H., W. J. Dobson, and H. D. McCurdy. 1977. Fimbriation in gliding bacteria. Can. J. Microbiol. 23:1096–1108.

Maeba, P. Y. 1983. Iodination of Myxococcus xanthus during development. J. Bacteriol. 155:1033–1041.

Maeba, P. Y. 1986. Isolation of a surface glycoprotein from Myxococcus xanthus. J. Bacteriol. 166:644–650.

Magrini, V., D. Salmi, D. Thomas, S. K. Herbert, P. L. Hartzell, and P. Youderian. 1997. Temperate Myxococcus xanthus phage Mx8 encodes a DNA adenine methylase, Mox. J. Bacteriol. 179:4254–4263.

Magrini, V., C. Creighton, and P. Youderian. 1999a. Site-specific recombination of temperate Myxococcus xanthus phage Mx8: Genetic elements required for integration. J. Bacteriol. 181:4050–4061.

Magrini, V., M. L. Storms, and P. Youderian. 1999b. Site-specific recombination of temperate Myxococcus xanthus phage Mx8: Regulation of integrase activity by reversible, covalent modification. J. Bacteriol. 181:4062–4070.

Maier, B., L. Potter, M. So, C. D. Long, H. S. Seifert, and M. P. Sheetz. 2002. Single pilus motor forces exceed 100 pN. Proc. Natl. Acad. Sci. USA 99:16012–16017.

Man, S. Z. 1998. Structural Characterization of the O-Antigen Polysaccharide from Wild-type Myxococcus xanthus Lipopolysaccharide and its Two Mutants by the Reductive Cleavage Method [PhD thesis]. University of Minnesota. Minneapolis, MN.

Mandel, M., and E. R. Leadbetter. 1965. Deoxyribonucleic acid base composition of myxobacteria. J. Bacteriol. 90:1795–1796.

Margalith, P. 1962. Bacteriolytic principles of Myxococcus fulvus. Nature 196:1335–1336.

Martin, S., E. Sodergren, T. Massuda, and D. Kaiser. 1978. Systematic isolation of transducing phages for Myxococcus xanthus. Virology 88:44–53.

Martinez-Laborda, A. , J. M. Balsalobre, M. Fontes, and F. J. Murillo. 1990. Accumulation of carotenoids in structural and regulatory mutants of the bacterium Myxococcus xanthus. Molec. Gen. Genet. 223:205–210.

Mason, J., and D. Powelson. 1958. Lysis of Myxococcus xanthus. J. Gen. Microbiol. 19:65–70.

Masson, P. J., and J. F. Guespin-Michel. 1988. An extracellular blood-anticoagulant glycopeptide produced exclusively during vegetative growth by Myxococcus xanthus and other myxobacteria is not co-regulated with other extracellular macromolecules. J. Gen. Microbiol. 134:801–806.

Mathew, S., and A. Dudani. 1955. Lysis of human pathogenic bacteria by myxobacteria. Nature 175:125.

Mayer, D. 1967. Ernährungsphysiologische Untersuchungen an Archangium violaceum. Arch. Mikrobiol. 58:186–200.

Mayer, H., and H. Reichenbach. 1978. Restriction endonucleases: General survey procedure and survey of gliding bacteria. J. Bacteriol. 136:708–713.

McBride, M. J., and D. R. Zusman. 1989a. Trehalose accumulation in vegetative cells and spores of Myxococcus xanthus. J. Bacteriol. 171:6383–6386.

McBride, M. J., R. A. Weinberg, and D. R. Zusman. 1989b. "Frizzy" aggregation genes of the gliding bacterium Myxococcus xanthus show sequence similarities to the chemotaxis genes of enteric bacteria. Proc. Natl. Acad. Sci. USA 86:424–428.

McBride, M. J. 2001. Bacterial gliding motility: Multiple mechanisms for cell movement over surfaces. Ann. Rev. Microbiol. 55:49–75.

McCleary, W., and D. Zusman. 1990a. FrzE of M. xanthus is homologous to both CheA and CheY of Salmonella typhimurium. Proc. Natl. Acad. Sci. USA 87:5898–5902.

McCleary, W. and D. Zusman. 1990b. Purification and characterization of the M. xanthus FrzE protein shows that it has autophosphorylation activity. J. Bacteriol. 172:6661–6668.

McCleary, W. R., M. J. McBride, and D. R. Zusman. 1990c. Developmental sensory transduction in Myxococcus xanthus involves methylation and demethylation of Frz CD. J. Bacteriol. 172:4877–4887.

McCurdy, H. D. 1963. A method for the isolation of myxobacteria in pure culture. Can. J. Microbiol. 9:282–285.

McCurdy, H. D., and S. Wolf. 1967. Deoxyribonucleic acid base compositions of fruiting Myxobacterales. Can. J. Microbiol. 13:1707–1708.

McCurdy, H. D. 1969a. Light and electron microscope studies on the fruiting bodies of Chondromyces crocatus. Arch. Mikrobiol. 65:380–390.

McCurdy, H. D. 1969b. Studies on the taxonomy of the Myxobacterales. I: Record of Canadian isolates and survey of methods. Can. J. Microbiol. 5:1453–1461.

McCurdy, H. D., and B. T. Khouw. 1969c. Studies on Stigmatella brunea. Can. J. Microbiol. 15:731–738.

McCurdy, H. D., and T. H. MacRae. 1973. Xanthacin. A bacteriocin of Myxococcus xanthus FB. Can. J. Microbiol. 20:131–135.

McCurdy, H. D. 1974. Myxobacterales. *In:* R. E. Buchanan and N. E. Gibbons (Eds.). Bergey's Manual of Determinative Bacteriology, 8th ed.. Williams and Wilkins. Baltimore, MD. 76–98.

McCurdy, H. D. 1989. Order Myxococcales Tchan, Pochon and Prévot 1948, 398 (with contributions of E. R. Brockman, H. Reichenbach, and D. White). *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (Eds.). Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 3:2139–2170.

McDonald, J. C., and J. E. Peterson. 1962. Liquid cultures of two members of the higher fruiting myxobacteria. Mycologia 54:368–373.

McDonald, J. C. 1967. Studies on the genus Archangium (Myxobacterales). II: The effect of temperature and carbohydrates on some physiological processes. Mycologia 59:1059–1068.

McVittie, A., and S. A. Zahler. 1962. Chemotaxis in Myxococcus. Nature 194:1299–1300.

Menne, B., and G. Rückert. 1988. Myxobakterien (Myxobacterales) in Höhlensedimenten des Hagengebirges (Nördliche Kalkalpen). Die Höhle 4:120–131.

Merroun, M. L., K. Ben Chekroun, M. T. Gonzalez-Munoz, and J. M. Arias. 2003. Lanthanum fixation by Myxococcus xanthus: cellular location and extracellular polysaccharide observation. Chemosphere 52:113–120.

Merz, A., M. So, and M. P. Sheetz. 2000. Pilus retraction powers bacterial twitching motility. Nature 407:98–101.

Mesbah, M., U. Premachandran, and W. B. Whitman. 1989. Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. Int. J. Syst. Bacteriol. 39:159–167.

Michoustine, E. [name also transliterated as Mishustin]. 1968. Microorganismes cellulolytiques des sols de l'U.S.S.R. Ann. Inst. Pasteur 115:596–603.

Miyashiro, S., S. Yamanaka, S. Takayama, and H. Shibai. 1988. Novel macrocyclic antibiotics: myxovalicins A, B, C, D, G and H. I: Screening of antibiotics-producing myxobacteria and production of megovalicins. J. Antibiot. 41:433–438.

Mizrahi, A., J. Arnan, G. Miller, Z. Liron, M. Manai, Y. Batus, and E. Rosenberg. 1977. Scaling-up process for production of antibiotic of Myxococcus xanthus TA. J. Appl. Chem. Biotechnol. 26:160–166.

Monteoliva-Sanchez, M., C. Ruiz, and A. Ramos-Cormenzana. 1987. Cellular fatty acid composition of Corallococcus coralloides. Curr. Microbiol. 15:269–271.

Morris, D. W., and J. H. Parish. 1976. Restriction in Myxococcus virescens. Arch. Microbiol. 108:227–230.

Morris, D. W., S. R. Ogden-Swift, V. Virrankowski-Castrodeza, K. Ainley, and J. H. Parish. 1978. Transduction of Myxococcus virescens by coliphage P1CM: Generation of plasmids containing both phage and Myxococcus genes. J. Gen. Microbiol. 107:73–83.

Morris, J., S. R. Kushner, and R. Ivarie. 1986. The simple repeat poly (dT-dG).poly (dC-dA) common to eukaryotes is absent from eubacteria and archaebacteria and rare in protozoans. Molec. Biol. Evol. 3:343–355.

Mullings, R., and J. H. Parish. 1984. Mesophilic aerobic Gram-negative cellulose degrading bacteria from aquatic habitats and soils. J. Appl. Bacteriol. 57:455–468.

Nellis, L. F., and H. R. Garner. 1964. Methods of isolation and purification of Chondromyces. J. Bacteriol. 87:230–231.

Nelson, D. R., M. G. Cumsky, and D. R. Zusman. 1981. Localization of myxobacterial hemagglutinin in the periplasmic space and on the cell surface of Myxococcus xanthus during developmental aggregation. J. Biol. Chem. 256:12589–12595.

Nelson, D. R., and D. R. Zusman. 1983. Evidence for a long-lived mRNA during fruiting body formation in Myxococcus xanthus. Proc. Natl. Acad. Sci. USA 80:1467–1471.

Nelson, D. R., and K. P. Killeen. 1986. Heat shock proteins of vegetative and fruiting Myxococcus xanthus. J. Bacteriol. 168:1100–1106.

Neumann, B., A. Pospiech, and H. U. Schairer. 1992. Size and stability of the genomes of the myxobacteria Stigmatella aurantiaca and Stigmatella erecta. J. Bacteriol. 174:6307–6310.

Neumann, B., A. Pospiech, and H. U. Schairer. 1993. A physical and genetic map of the Stigmatella aurantiaca DW4/3.1 chromosome. Molec. Microbiol. 10:1087–1099.

Nicaud, J. M., A. Breton, G. Younes, and J. Guespin-Michel. 1984. Mutants of Myxococcus xanthus impaired in pro-

tein secretion: An approach to study a secretory mechanism. Appl. Microbiol. Biotechnol. 20:344–350.

Nigam, J. N., J. Lehmann, K. Gerth, H. Piehl, R. Schultze, and W. Trowitzsch. 1984. Feeding strategy for the production of the new antibiotic myxovirescin A from Myxococcus virescens (Myxobacterales). Appl. Microbiol. Biotechnol. 19:157–160.

Nolte, E. M. 1957. Untersuchungen über Ernährung und Fruchtkörperbildung von Myxobakterien. Arch. Mikrobiol. 28:191–218.

Norén, B. 1950. Notes on myxobacteria in Sweden. Svensk Bot. Tidskr. 44:108–112.

Norén, B. 1952. Further notes on the distribution of myxobacteria in Swedish soils. Svensk Bot. Tidskr. 46:446–453.

Norén, B. 1953. On the production of antibiotics by myxobacteria. Svensk Bot. Tidskr. 47:402–410.

Norén, B. 1955. Studies on myxobacteria III. Organic factors in nutrition. Botaniska Notiser 108:81–134.

Norén, B. 1960a. Lytic activity on autoclaved and on intact eubacterial cells by a preparation UZD, obtained from a metabolic solution of Myxococcus virescens. Botaniska Notiser 113:320–336.

Norén, B. 1960b. Notes on the bacteriolytic activity of Myxococcus virescens. Svensk Bot. Tidskr. 54:550–560.

Norén, B., and K. B. Raper. 1962. Antibiotic activity of myxobacteria in relation to their bacteriolytic capacity. J. Bacteriol. 84:157–162.

Norén, B., and G. Odham. 1973. Antagonistic effects of Myxococcus xanthus on fungi. II: Isolation and characterization of inhibitory lipid factors. Lipids 8:573–583.

O'Connor, K. A., and D. R. Zusman. 1983. Coliphage P1-mediated transduction of cloned DNA from Escherichia coli to Myxococcus xanthus: Use for complementation and recombinational analyses. J. Bacteriol. 155:317–329.

O'Connor, K. A., and D. R. Zusman. 1989. Patterns of cellular interactions during fruiting body formation in Myxococcus xanthus. J. Bacteriol. 171:6013–6024.

O'Connor, K., and D. Zusman. 1991a. Analysis of Myxococcus xanthus cell types by two-dimensional polyacrylamide gel electrophoresis. J. Bacteriol. 173:3334–3341.

O'Connor, K., and D. Zusman. 1991b. Behavior of peripheral rods and their role in the life cycle of Myxococcus xanthus. J. Bacteriol. 173:3342–3355.

O'Connor, K., and D. Zusman. 1991c. Development in Myxococcus xanthus involves differentiation into two cell types, peripheral rods and spores. J. Bacteriol. 173:3318–3333.

Oetker, H. 1953. Untersuchungen über die Ernährung einiger Myxobakterien. Arch. Mikrobiol. 19:206–246.

Oka, M., Y. Nishiyama, S. Ohta, H. Kamei, M. Konishi, T. Miyaki, T. Oki, and H. Kawaguchi. 1988a. Glidobactins A, B and C, new antitumor antibiotics. I: Production, isolation, chemical properties and biological activity. J. Antibiot. 41:1331–1337.

Oka, M., H. Ohkuma, H. Kamei, M. Konishi, T. Oki, and H. Kawaguchi. 1988b. Glidobactins D, E, F, G and H; minor components of the antitumor antibiotic glidobactin. J. Antibiot. 41:1906–1909.

Oka, M., K. Yaginuma, K. Numata, M. Konishi, T. Oki, and H. Kawaguchi. 1988c. Glidobactins A, B and C, new antitumor antibiotics. II: Structure elucidation. J. Antibiot. 41:1338–1350.

Onishi, N., K. Izaki, and H. Takahashi. 1984. A macrocyclic antibiotic M-230B produced by Myxococcus xanthus: Isolation and characterization. J. Antibiot. 37:13–19.

Orlowski, M., and D. White. 1974. Intracellular proteolytic activity in developing myxospores of Myxococcus xanthus. Arch. Microbiol. 97:347–357.

Orlowski, M. 1980. Cyclic adenosine 3',5'-monophosphate binding protein in developing myxospores of Myxococcus xanthus. Can. J. Microbiol. 26:905–911.

Orndorff, P. E., and M. Dworkin. 1980. Separation and properties of the cytoplasmic and outer membranes of vegetative cells of Myxococcus xanthus. J. Bacteriol. 141:914–927.

Orndorff, P. E., and M. Dworkin. 1982. Synthesis of several membrane proteins during developmental aggregation in Myxococcus xanthus. J. Bacteriol. 149:29–39.

Orndorff, P. , E. Stellwag, T. Starich, M. Dworkin, and J. Zissler. 1983. Genetic and physical characterization of lysogeny by bacteriophage MX8 in Myxococcus xanthus. J. Bacteriol. 154:772–779.

Oxford, A. E., and B. N. Singh. 1946. Factors contributing to the bacteriolytic effect of species of myxococci upon viable eubacteria. Nature 1158:745.

Oxford, A. E. 1947. Observations concerning the growth and metabolic activities of myxococci in a simple protein-free liquid medium. J. Bacteriol. 53:129–138.

Oyaizu, H., and C. R. Woese. 1985. Phylogenetic relationships among the sulfate respiring bacteria, myxobacteria and purple bacteria. Syst. Appl. Microbiol. 6:257–263.

Panasenko, S. M. 1985. Methylation of macromolecules during development in Myxococcus xanthus. J. Bacteriol. 164:495–500.

Parish, J. H. 1975. Transfer of drug resistance to Myxococcus from bacteria carrying drug-resistance factors. J. Gen. Microbiol. 87:198–210.

Peterson, J. E. 1965. A group of strongly cellulolytic myxobacteria previously unreported in North American soils. Am. J. Bot. 52:636.

Peterson, J. E. 1966. The demise of the myxobacterial genus Angiococcus. Mycologia 58:962–965.

Peterson, J. E., and B. Norén. 1967. The occurrence of the cellulose-decomposing myxobacterium, Sorangium cellulosum, in Scandinavian soils. Am. J. Bot. 54:648.

Peterson, J. E. 1969. Isolation, cultivation and maintenance of the myxobacteria. *In:* J. R. Norris and D. W. Ribbons (Eds.). Methods in Microbiology. Academic Press. London, UK. 3B:185–210.

Pinoy, P.E. 1913. On the necessity of a bacterial association for the development of the myxobacterium, Chondromyces crocatus. Compt. Rend. Soc. Biol. 157:77–78.

Pradella, S., A. Hans, C. Sproer, H. Reichenbach, K. Gerth, and S. Beyer. 2002. Characterisation, genome size and genetic manipulation of the myxobacterium Sorangium cellulosum So ce56. Arch. Microbiol. 178:484–492.

Pronina, N. I,. 1962. A description of new species and varieties of cellulose decomposing myxobacteria. Microbiology 31:384–390.

Qualls, G. T., K. Stephens, and D. White. 1978. Light-stimulated morphogenesis in the fruiting myxobacterium Stigmatella aurantiaca. Science 201:444–445.

Quehl, A. 1906. Untersuchungen über die Myxobakterien. Zbl. Bakteriol., 2. Abt. 16:9–34.

Ramaswamy, S., M. Dworkin, and J. Downard. 1997. Identification and characterization of Myxococcus xanthus mutants deficient in calcofluor white binding. J. Bacteriol. 179:2863–2871.

Ramsey, W. S., and M. Dworkin. 1968. Microcyst germination in Myxococcus xanthus. J. Bacteriol. 95:2249–2257.

Raverdy, J. 1973. About the isolation and bacteriolytic activity of a few Myxobacteria isolated from water. Water Res. 7:687–693.

Reichenbach, H. 1965a. Rhapidosomen bei Myxobakterien. Arch. Mikrobiol. 50:246–255.

Reichenbach, H. 1965b. Rhythmische Vorgänge bei der Schwarmentfaltung von Myxobakterien. Ber. Deutsch. Bot. Ges. 78:102–105.

Reichenbach, H. 1965c. Untersuchungen an Archangium violaceum: Ein Beitrag zur Kenntnis der Myxobakterien. Arch. Mikrobiol. 52:376–403.

Reichenbach, H., H. H. Heunert, and H. Kuczka. 1965d. Archangium violaceum (Myxobacterales)—Schwarmentwicklung und Bildung von Protocysten [film]. Institut für den wissenschaftlichen Film. Göttingen, Germany. Encyclopaedia Cinematographica E 777.

Reichenbach, H., H. H. Heunert, and H. Kuczka. 1965e. Chondromyces apiculatus (Myxobacterales)—Schwarmentwicklung und Morphogenese [film]. Institut für den wissenschaftlichen Film. Göttingen, Germany. Encyclopaedia Cinematographica E 779.

Reichenbach, H., H. H. Heunert, and H. Kuczka. 1965f. Myxococcus spp. (Myxobacterales)—Schwarmentwicklung und Bildung von Protocysten [film]. Institut für den wissenschaftlichen Film. Göttingen, Germany. Encyclopaedia Cinematographica E 778.

Reichenbach, H., H. H. Heunert, and H. Kuczka. 1965g. Schwarmentwicklung und Morphogenese bei Myxobakterien—Archangium, Myxococcus, Chondrococcus, Chondromyces [film]. Institut für den wissenschaftlichen Film. Göttingen, Germany. Film C 893.

Reichenbach, H. 1966. Myxococcus spp. (Myxobacteriales): Schwarmentwicklung und Bildung von Protocysten [text with the film EC 778/1965]. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segment #1:570–571. (Film W778).

Reichenbach, H. 1967. Die wahre Natur der Myxobakterien-"Rhapidosomen." Arch. Mikrobiol. 56:371–383.

Reichenbach, H. 1968. Archangium violaceum (Myxobacteriales) (sic) Schwarmentwicklung und Bildung von Protocysten. *Ed.* G. Wolf. Göttingen. Encyclopaedia Cinematographica Institut für den wissenschaftlichen Film. Göttingen, Germany. Segment #24:330 (Film E777).

Reichenbach, H., and M. Dworkin. 1969a. Studies on Stigmatella aurantiaca (Myxobacterales). J. Gen. Microbiol. 58:3–14.

Reichenbach, H., H. Voelz, and M. Dworkin. 1969b. Structural changes in Stigmatella aurantiaca during myxospore induction. J. Bacteriol. 97:905–911.

Reichenbach, H., and M. Dworkin. 1970. Induction of myxospore formation in Stigmatella aurantiaca (Myxobacterales) by monovalent cations. J. Bacteriol. 101:325–326.

Reichenbach, H., and H. Kleinig. 1971. The carotenoids of Myxococcus fulvus (Myxobacterales). Arch. Mikrobiol. 76:364–380.

Reichenbach, H. 1974a. Chondromyces apiculatus (Myxobacterales)—Schwarmentwicklung und Morphogenese. Publiktionen zu wissenschaftlichen Filmen, Sektion Biologie 7. Institut für den wissenschaftlichen Film. Göttingen, Germany. 245–263.

Reichenbach, H. 1974b. Die Biologie der Myxobakterien. Biologie in unserer Zeit 4:33–45.

Reichenbach, H., H. K. Galle, and H. H. Heunert. 1980. Stigmatella aurantiaca (Myxobacterales): Schwarmentwicklung und Fruchtkörperbildung [film]. *In:* Encyclopaedia Cinematographica. Institut für den wissenschaftlichen Film. Göttingen, Germany. E2421.

Reichenbach, H. 1983. A simple method for the purification of myxobacteria. J. Microbiol. Meth. 1:77–79.

Reichenbach, H. 1984a. Myxobacteria: A most peculiar group of social prokaryotes. *In:* E. Rosenberg (Ed.). Myxobacteria. Development and Cell Interactions. Springer-Verlag. New York, NY. 1–50.

Reichenbach, H., and H. Kleinig. 1984b. Pigments of myxobacteria. *In:* E. Rosenberg (Ed.). Myxobacteria: Development and Cell Interactions. Springer-Verlag. New York, NY. 127–137.

Reichenbach, H. 1986. The myxobacteria: common organisms with uncommon behaviour. Microbiol. Sci. 3:268–274.

Reichenbach, H. 1988a. Gliding bacteria in biotechnology. *In:* H. J. Rehm and G. Reed (Eds.). Biotechnology. BCH Verlagsgesellschaft. Weinheim, Germany. 6b:673–696.

Reichenbach, H., K. Gerth, H. Irschik, B. Kunze, and G. Höfle. 1988b. Myxobacteria: A source of new antibiotics. Trends Biotechnol. 6:115–121.

Reichenbach, H., and G. Höfle. 1989. The gliding bacteria: a treasury of secondary metabolites. *In:* M. E. Bushell and U. Gräfe (Eds.). Bioactive Metabolites from Microorganisms. Elsevier Science Publishers. Amsterdam, The Netherlands. 79–100.

Reichenbach, H., and G. Höfle. 1993. Biologically active secondary metabolites from myxobacteria. Biotech. Adv. 11:219–277.

Reichenbach, H., and G. Höfle. 1998. Myxobacteria as producers of secondary metabolites. *In:* S. Grabley and R. Thiericke (Eds.). Drug Discovery from Nature. Springer-Verlag. Berlin, Germany. 149–179.

Reichenbach, H. 1999. The ecology of the myxobacteria. Environ. Microbiol. 1:15–21.

Reichenbach, H. 2001. Myxobacteria, producers of novel bioactive substances. J. Indust. Microbiol. Biotechnol. 27:149–156.

Reichenbach, H. and Dworkin, M. 2001. The myxobacteria. The Prokaryotes 4.

Reichenbach, H. 2005. The Myxococcales. *In:* G. M. Garrity (Ed.). Bergey's Manual of Systematic Bacteriology, 2nd edn, Part 3: The Beta-, Delta-, and Epsilonproteobacteria. Springer-Verlag. New York, NY.

Ringel, S. M., R. C. Greenough, S. Roemer, D. Connor, and M. von Strandtmann. 1977. Ambruticin (W 7783), a new antifungal antibiotic. J. Antibiot. 30:371–375.

Rodrigues, F. K., V. Virrankoski-Castrodeza, J. H. Parish, and K. Grimm. 1980. Isolation and characterization of new bacteriophages for Myxococcus xanthus. Arch. Microbiol. 126:175–180.

Romeo, J. M., B. Esmon, and D. R. Zusman. 1986. Nucleotide sequence of the myxobacterial hemagglutinin gene contains four homologous domains. Proc. Natl. Acad. Sci. USA 83:6332–6336.

Rosenberg, E., B. Vaks, and A. Zuckerberg. 1973. Bactericidal action of an antibiotic produced by Myxococcus xanthus. Antimicrob. Agents Chemother. 4:507–513.

Rosenberg, E., K. H. Keller, and M. Dworkin. 1977. Cell density-dependent growth of Myxococcus xanthus on casein. J. Bacteriol. 129:770–777.

Rosenberg, E., S. Fytlovitch, S. Carmeli, and Y. Kashman. 1982. Chemical properties of Myxococcus xanthus antibiotic TA. J. Antibiot. 35:788–793.

Rosenberg, E. (Ed.). 1984. Myxobacteria: Development and Cell Interactions. Springer-Verlag. New York, NY.

Rosenfelder, G., O. Lüderitz, and O. Westphal. 1974. Composition of lipopolysaccharides from Myxococcus fulvus and other fruiting and non-fruiting myxobacteria. Eur. J. Biochem. 44:411–420.

Rückert, G. 1975a. Koprochorie einiger Myxobakterien-Arten (Myxobacterales). Zeitschr. Allgem. Mikrobiol. 15:565–567.

Rückert, G. 1975b. Zur Verbreitung bakteriotropher Myxobakterien in Waldböden. Mitteilungen des Vereins für forstliche Standortskunde und Forstpflanzenzüchtung. 24:43–47.

Rückert, G. 1975c. Zur Verbreitung von Fruchtkörperbildenden Myxobakterien in europäischen Strand- und Dünenböden. Zbl. Bakteriol., 2. Abt. 130:343–347.

Rückert, G. 1976. Myxobakterien (Myxobacterales) in natürlichen und naturnahen Substraten aus der europäischen Subarktis. Acta Bot. Islandica 4:4–9.

Rückert, G., and G. Heym. 1977. Bakteriotrophe Myxobakterien (Myxobacterales) in ariden Substraten. Karlsruher Geographische Heft 8:101–111.

Rückert, G. 1978. Förderung der Fruchtkörper-Bildung von Myxococcus virescens Thaxter (Myxobacterales) in Rohkulturen durch Salzzusatz. Zeitschr. Allgem. Mikrobiol. 18:69–71.

Rückert, G. 1979. Myxobakterien-Artenspektren von Böden in Abhängigkeit von bodenbildenden Faktoren unter besonderer Berücksichtigung der Bodenreaktion. Zeitschr. Pflanzenernähr. Bodenk. 42:330–343.

Rückert, G. 1981. Myxobakterien (Myxobacterales) auf Blattoberflächen. Zeitschr. Allgem. Mikrobiol. 21:761–763.

Rückert, G. 1983. Myxobakterien in Böden extremer Biotope. Mitt. Deutsch. Bodenkundl. Gesellsch. 38:355–360.

Rückert, G. 1984. Untersuchungen zum Vorkommen von Myxobakterien in von Meerwasser beeinflussten Substraten unter besonderer Berücksichtigung der Insel Helgoland. Helgoländer Meeresunters. 38:179–184.

Rückert, G. 1985. Myxobacteria from Antarctic soils. Biol. Fert. Soils 1:215–216.

Rudd, K. E., and D. R. Zusman. 1982. RNA polymerase of Myxococcus xanthus: Purification and selective transcription in vitro with bacteriophage templates. J. Bacteriol. 151:89–105.

Ruiz, C., M. Monteoliva-Sanchez, and A. Ramos-Cormenzana. 1985. Effect of culture age on the cellular fatty acid composition of two strains of Myxococcus xanthus. Microbios Lett. 30:95–99.

Ruiz, C., A. Ruiz-Bravo, A. Ramos-Cormenzana. 1987. Endotoxin-like activities in Myxococcus xanthus. Curr. Microbiol. 15:343–345.

Ryding, N. J., and L. J. Shimkets. 2000. Myxococcus, genetics. Encyclopedia of Microbiology. Academic Press. New York, NY. 3:363–372.

Sabados-Saric, A. 1957. Istrazivanja o miksobacterijama u thina Jugoslavije [Myxobacteria in different types of soils in Yugoslavia: in Serbo-Croatian, with English summary]. Yugoslavenska Akademija Znanosti i Umjetnosti, Zagreb 312:5–29.

Sadler, W., and M. Dworkin. 1966. Induction of cellular morphogenesis in Myxococcus xanthus. II: Macromolecular synthesis and mechanism of inducer action. J. Bacteriol. 91:1520–1525.

Sager, B., and D. Kaiser. 1993. Two cell-density domains within the Myxococcus xanthus fruiting body. Proc. Natl. Acad. Sci. USA. 90:3690–3694.

Sager, B., and D. Kaiser. 1994. Intercellular C-signaling and the traveling waves of Myxococcus. Genes Dev. 8:2793–2804.

Sager, B. M. 1996. Propagation of traveling waves in excitable media. Genes Dev. 10:2237–2250.

Salmi, D., V. Magrini, P. L. Hartzell, and P. Youderian. 1998. Genetic determinants of immunity and integration of temperate Myxococcus xanthus phage Mx8. J. Bacteriol. 180:614–621.

Sanford, R. A., J. R. Cole, and J. M. Tiedje. 2002. Characterization and description of Anaeromyxobacter dehalogenans gen. nov., sp. nov., an aryl-halorespiring facultative anaerobic myxobacterium. Appl. Environ. Microbiol. 68:893–890.

Sarao, R., H. D. McCurdy, and L. Passador. 1985. Enzymes of the intermediary carbohydrate metabolism of Polyangium cellulosum. Can. J. Microbiol. 31:1142–1146.

Schmidt-Lorenz, W., and H. Kühlwein. 1968. Intracelluläre Bewegungsorganellen bei Myxobakterien. Arch. Mikrobiol. 60:95–98.

Schmidt-Lorenz, W. , and H. Kühlwein. 1969. Beiträge zur Kenntnis der Myxobakterienzelle. 2: Mitteilung. Oberflächenstrukturen der Schwarmzellen. Arch. Mikrobiol. 68:405–426.

Schröder, J., and H. Reichenbach. 1970. The fatty acid composition of vegetative cells and myxospores of Stigmatella aurantiaca (Myxobacterales). Arch. Mikrobiol. 71:384–390.

Schürmann, C. 1967. Growth of myxococci in suspension in liquid media. Appl. Microbiol. 15:971–974.

Sharpe, A. N., and D. C. Kilsby. 1971. A rapid, inexpensive bacterial count technique using agar droplets. J. Appl. Bacteriol. 34:435–440.

Shi, W., and D. R. Zusman. 1993a. Fatal attraction. Nature 366:414–415.

Shi, W., T. Kohler, and D. R. Zusman. 1993b. Chemotaxis plays a role in the social behavior of Myxococcus xanthus. Molec. Microbiol. 9:601–611.

Shimkets, L. J., and D. Kaiser. 1982. Induction of coordinated movement of Myxococcus xanthus cells. J. Bacteriol. 152:451–461.

Shimkets, L. J., R. E. Gill, and D. Kaiser. 1983. Developmental cell interactions in Myxococcus xanthus and the spoC locus. Proc. Natl. Acad. Sci. USA 80:1406–1410.

Shimkets, L. J. 1984. Nutrition, metabolism, and the initiation of development. *In:* E. Rosenberg (Ed.). Myxobacteria: Development and Cell Interactions. Springer-Verlag. New York, NY. 91–107.

Shimkets, L. J. 1986. Correlation of energy-dependent cell cohesion with social motility in Myxococcus xanthus. J. Bacteriol. 166:837–841.

Shimkets, L. J. 1987. Control of morphogenesis in myxobacteria. Crit. Rev. Microbiol. 14:195–227.

Shimkets, L. J., and S. J. Asher. 1988. Use of recombination techniques to examine the structure of the csg locus of Myxococcus xanthus. Molec. Gen. Genet. 211:63–71.

Shimkets, L. J. 1990a. Social and developmental biology of the myxobacteria. Microbiol. Rev. 54:473–501.

Shimkets, L. J. 1990b. The Myxococcus xanthus FprA protein causes increased flavin biosynthesis in Escherichia coli. J. Bacteriol. 172:24–30.

Shimkets, L., and C. R. Woese. 1992. A phylogenetic analysis of the myxobacteria: Basis for their classification. Proc. Natl. Acad. Sci. USA 89:9459–9463.

Shimkets, L. J. 1997a. Myxococcus xanthus DK1622. *In:* F. J. de Bruijn, J. R. Lupski, and G. M. Weinstock (Eds.). Bacterial Genomes: Physical Structure and Analysis. Chapman & Hall. New York, NY. 695–701.

Shimkets, L. J. 1997b. Structure and sizes of genomes of the Archaea and Bacteria. *In:* F. J. de Bruijn, J. R. Lupski, and G. M. Weinstock (Eds.). Bacterial Genomes: Physical Structure and Analysis. Chapman & Hall. New York, NY. 5–11.

Shimkets, L. J., and M. Dworkin. 1997c. Myxobacterial Multicellularity. *In:* J. A. Shapiro and M. Dworkin (Eds.). Bacteria as Multicellar Organisms. Oxford University Press. Oxford, UK. 220–244.

Shimkets, L. J. 1999. Intercellular signaling during fruiting body development of Myxococcus xanthus. Ann. Rev. Microbiol. 53:525–549.

Shimkets, L. J. 2000. Growth, sporulation, and other tough decisions. *In:* Y. Brun and L. J. Shimkets (Eds.). Prokaryotic Development. American Society for Microbiology. Washington, DC. 277–284.

Simunovic, V., F. C. Gherardini, and L. J. Shimkets. 2003. Membrane localization of motility, signaling and polyketide synthetase proteins in Myxococcus xanthus. J. Bacteriol. 185:5066–5075.

Singh, B. N. 1947. Myxobacteria in soils and composts: Their distribution, number and lytic action on bacteria. J. Gen. Microbiol. 1:1–10.

Singh, B. N., and N. B. Singh. 1971. Distribution of fruiting myxobacteria in Indian soils, bark of trees and dung of herbivorous animals. Indian J. Microbiol. 11:47–92.

Skerker, J. M., and H. C. Berg. 2001. Direct observation of extension and retraction of type IV pili. Proc. Natl. Acad. Sci. USA 98:6901–6904.

Smith, B.A., and M. Dworkin. 1981. Myxococcus xanthus synthesizes a stabilized messenger RNA during fruiting body formation. Curr. Microbiol. 6:95–100.

Smith, D. R., and M. Dworkin. 1994. Territorial interactions between two Myxococcus species. J. Bacteriol. 176:1201–1205.

Spröer, C., H. Reichenbach, and E. Stackebrandt. 1999. The correlation between morphological and phylogenetic classification of myxobacteria. Int. J. Syst. Bacteriol. 49:1255–1262.

Stackebrandt, E., R. G. E. Murray, and H. G. Trüper. 1988. Proteobacteria classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives." Int. J. Syst. Bacteriol. 38:321–325.

Stanier, R. Y. 1942a. A note on elasticotaxis in myxobacteria. J. Bacteriol. 44:405–412.

Stanier, R. Y. 1942b. The Cytophaga group: a contribution to the biology of myxobacteria. Bacteriol. Rev. 6:143–196.

Starich, T., P. Cordes, and J. Zissler. 1985. Transposon tagging to detect a latent virus in Myxococcus xanthus. Science 230:541–543.

Starich, T., and J. Zissler. 1989. Movement of multiple DNA units between Myxococcus xanthus cells. J. Bacteriol. 171:2323–2336.

Stein, J., and H. Budzikiewicz. 1987. 1-O-(13-Methyl-1-Z-tetradecenyl)2-O-(13-methyltetradecanoyl)-glycero-3-phospho-ethanolamine, ein Plasmalogen aus Myxococcus stipitatus. Zeitschr. Naturforsch. 42b:1017–1020.

Stellwag, E., J. M. Fink, and J. Zissler. 1985. Physical characterization of the genome of the Myxococcus xanthus bacteriophage MX-8. Molec. Gen. Genet. 199:123–132.

Stephens, K., and D. White. 1980a. Morphogenetic effects of light and guanine derivatives on the fruiting myxobacterium Stigmatella aurantiaca. J. Bacteriol. 144:322–326.

Stephens, K., and D. White. 1980b. Scanning electron micrographs of fruiting bodies of the myxobacterium Stigmatella aurantiaca lacking a coat and revealing a cellular stalk. FEMS Microbiol. Lett. 9:189–192.

Stephens, K., G. D. Hegeman, and D. White. 1982. Pheromone produced by the myxobacterium Stigmatella aurantiaca. J. Bacteriol. 149:739–747.

Sudo, S. Z., and M. Dworkin. 1969. Resistance of vegetative cells and microcysts of Myxococcus xanthus. J. Bacteriol. 98:883–887.

Sudo, S., and M. Dworkin. 1972. Bacteriolytic enzymes produced by Myxococcus xanthus. J. Bacteriol. 110:236–245.

Sudo, S. Z., and M. Dworkin. 1973. Comparative biology of prokaryotic resting cells. Adv. Microbial Physiol. 9:153–223.

Sun, H., D. R. Zusman, and W. Shi. 2000. Type IV pilus of Myxococcus xanthus is a motility apparatus controlled by the frz chemosensory system. Curr. Biol. 10:1143–1146.

Sutherland, I. W., and M. L. Smith. 1973. The lipopolysaccharides of fruiting and non-fruiting myxobacteria. J. Gen. Microbiol. 74:259–266.

Sutherland, I. W., and S. Thomson. 1975. Comparison of polysaccharides produced by Myxococcus strains. J. Gen. Microbiol. 89:124–132.

Sutherland, I. W. 1976a. Novel surface polymer changes in development of Myxococcus spp. Nature 259:46–47.

Sutherland, I. W. 1976b. Ultrasonication—an enrichment technique for microcyst-forming bacteria. J. Appl. Bacteriol. 41:185–188.

Sutherland, I. W., and C. L. Mackenzie. 1977. Glucan common to the microcyst walls of cyst-forming bacteria. J. Bacteriol. 129:599–605.

Sutherland, I. W. 1979. Polysaccharides produced by Cystobacter, Archangium, Sorangium, and Stigmatella species. J. Gen. Microbiol. 111:211–216.

Takayama, S., S. Yamanaka, S. Miyashiro, Y. Yokokawa, and H. Shibai. 1988. Novel macrocyclic antibiotics: megovalicins A, B, C, D, G and H. II. Isolation and chemical structures of megovalicins. J. Antibiot. 41:439–445.

Teintze, M., M. Inouye, and S. Inouye. 1988. Characterization of calcium-binding sites in development-specific protein S of Myxococcus xanthus using site-specific mutagenesis. J. Biol. Chem. 263:1199–1203.

Thaxter, R. 1892. On the Myxobacteriaceae, a new order of Schizomycetes. Bot. Gaz. 17:389–406.

Thaxter, R. 1893. A new order of Schizomycetes. Bot. Gaz. 18:29–30.

Thaxter, R. 1904. Notes on the Myxobacteriaceae. Bot. Gaz. 37:405–416 (including two plates).

Thaxter, T. 1897. Further observations on the Myxobacteriaceae. Bot. Gaz. 23:395–411.

Thomasson, B., J. Link, A. G. Stassinopoulos, N. Burke, L. Plamann, and P. L. Hartzell. 2002. MglA, a small GTPase, interacts with a tyrosine kinase to control type IV pili-mediated motility and development of Myxococcus xanthus. Molec. Microbiol. 46:1399–1413.

Toal, D. R., S. W. Clifton, B. R. Roe, and J. Downard. 1995. The esg locus of Myxococcus xanthus encodes the E1α

and E1β subunits of a branched-chain keto acid dehydrogenase. Molec. Microbiol. 16:177–189.

Tojo, N., K. Sanmiya, H. Sugawara, S. Inouye, and T. Komano. 1996. Integration of bacteriophage Mx8 into the Myxococcus xanthus chromosome causes a structural alteration at the C-terminal region of the IntP protein. J. Bacteriol. 178:4004–4011.

Tojo, N., and T. Komano. 2003. The IntP C-terminal segment is not required for excision of bacteriophage Mx8 from the Myxococcus xanthus chromosome. J. Bacteriol. 185:2187–2193.

Trowitzsch, W., L. Witte, and H. Reichenbach. 1981. Geosmin from earthy smelling cultures of Nannocystis exedens (Myxobacterales). FEMS Microbiol. Lett. 12:257–260.

Trowitzsch, W., V. Wray, K. Gerth, and G. Höfle. 1982. Structure of myxovirescin A, a new macrocyclic antibiotic from gliding bacteria. J. Chem. Soc. Chem. Commun. 1982:1340–1342.

Trowitzsch-Kienast, W., K. Schober, V. Wray, K. Gerth, H. Reichenbach, and G. Höfle. 1989. Zur Konstitution der Myxovirescine B-T und Biogenese des Myxovirescins A. Liebigs Ann. Chem. 1989:345–355.

Trowitzsch-Kienast, W., Forche, E., Wray, V., Jurkiewicz, E., Hunsmann, G., Reichenbach, H. and G. Höfle. 1992. HIV-1-inhibitoren aus Myxococcus stipitatus Mx s40. Liebigs Ann. Chem. 1992:659–664.

Trowitzsch-Kienast, W., V. Wray, W., K. Gerth, H. Reichenbach, and G. Höfle. 1993. Myxochromid A: ein hochengesattigtes lipopeptidlacton aus Myxococcus virescens. Liebigs Ann. Chem. 1993:1233–1237.

Trzilová, B., L. Miklosovicová, E. Golaisová, and M. Bobková. 1980. Indikatoren der Verunreinigung des Donauwassers im tschechoslowakischem Abschnitte. Acta Ecologica (Bratislava) 8:91–102.

Trzilová, B., L. Mikloöovicová, G. Morhácová, and E. Golaisová. 1981. Die Wasserqualitat der Zuflüsse des tschechoslowakischen Donauabschnittes von limnologischer und hygienischer Sicht. Biolgia (Bratislava) 36:765–774.

Tsai, H., and H. J. Hirsch. 1981. The primary structure of fulvocin C from Myxococcus fulvus. Biochim. Biophys. Acta 667:213–217.

Tsopanakis, C., and J. H. Parish. 1976. Bacteriophage MX-1: Properties of the phage and its structural proteins. J. Gen. Virol. 30:99–112.

Vacheron, M. J., N. Arpin, and G. Michel. 1970. Isolement d'esters de phleixanthophylle de Nocardia kirovani. C. R. Acad. Sci., Sér. C 271:881–884.

Vahle, C. 1910. Vergleichende Untersuchungen über die Myxobakteriazeen und Bakteriazeen, sowie die Rhodobakteriazeen und Spirillazeen. Zbl. Bakteriol., 2. Abt. 25:178–260.

Varon, M., A. Tietz, and E. Rosenberg. 1986. Myxococcus xanthus autocide AMI. J. Bacteriol. 167:356–361.

Voelz, H., and M. Dworkin. 1962. Fine structure of Myxococcus xanthus during morphogenesis. J. Bacteriol. 84:943–952.

Voelz, H. 1964. Sites of adenosine triphosphatase: Activity in bacteria. J. Bacteriol. 88:1196–1198.

Voelz, H. 1965. Formation and structure of mesosomes in Myxococcus xanthus. Arch. Mikrobiol. 51:60–70.

Voelz, H. 1966a. In vivo induction of "polysomes" by limiting phosphate and the structural consequences in Myxococcus xanthus. *In:* 6th International Congress Electron Microscopy. Tokyo: Maruzen. Kyoto, Japan. 255–256.

Voelz, H. 1966b. The fate of the cell envelopes of Myxococcus xanthus during microcyst germination. Arch. Mikrobiol. 55:110–115.

Voelz, H., U. Voelz, and R. O. Ortigoza. 1966c. The "polyphosphate overplus" phenomenon in Myxococcus xanthus and its influence on the architecture of the cell. Arch. Mikrobiol. 53:371–388.

Voelz, H. 1967. The physical organization of the cytoplasm in Myxococcus xanthus and the fine structure of its components. Arch. Mikrobiol. 57:181–195.

Voelz, H. 1968. Structural comparison between intramitochondrial and bacterial crystalloids. J. Ultrastruct. Res. 25:29–36.

Voelz, H., and H. Reichenbach. 1969. Fine structure of fruiting bodies of Stigmatella aurantiaca (Myxobacterales). J. Bacteriol. 99:856–866.

Voelz, H., and R. P. Burchard. 1971. Fine structure of bacteriophage-infected Myxococcus xanthus. I: The lytic cycle in vegetative cells. Virology 43:243–250.

Vuillemard, J. C., S. Terré, S. Benoit, and J. Amiot. 1988. Protease production by immobilized growing cells of Serratia marcescens and Myxococcus xanthus in calcium alginate gel beads. Appl. Microbiol. Biotechnol. 27:423–431.

Ward, M. J., and D. R. Zusman. 1997. Regulation of directed motility in Myxococcus xanthus. Molec. Microbiol. 24:885–893.

Ward, M. J., and D. R. Zusman. 1999. Motility in Myxococcus xanthus and its role in developmental aggregation. Curr. Opin. Microbiol. 2:624–629.

Ward, M. J., and D. R. Zusman. 2000. Developmental aggregation and fruiting body formation in the gliding bacterium Myxococcus xanthus. *In:* Y. V. Brun and L. J. Shimkets (Eds.). Prokaryotic Development. American Society for Microbiology Press. Washington, DC. 243–262.

Ware J. C., and M. Dworkin. 1973. Fatty acids of Myxococcus xanthus. J. Bacteriol. 115:253–261.

Watson, B. F., and M. Dworkin. 1968. Comparative intermediary metabolism of vegetative cells and microcysts of Myxococcus xanthus. J. Bacteriol. 96:1465–1473.

Weckesser, J., G. Rosenfelder, H. Mayer, and O. Lüderitz. 1971. The identification of 3-O-methyl-D-xylose and 3-O-methyl-L-xylose as constituents of the lipopolysaccharides of Myxococcus fulvus and Rhodopseudomonas viridis, respectively. Eur. J. Biochem. 24:112–115.

Weinberg, R. A., and D. R. Zusman. 1989. Evidence that the Myxococcus xanthus frz genes are developmentally regulated. J. Bacteriol. 171:6174–6186.

Welch, R., and D. Kaiser. 2001. Cell behavior in traveling wave patterns of myxobacteria. Proc. Natl. Acad. Sci. USA 98:14907–14912.

White, D., M. Dworkin, and D. J. Tipper. 1968. Peptidoglycan of Myxococcus xanthus: Structure and relation to morphogenesis. J. Bacteriol. 95:2186–2197.

White, D. 1993. Myxospore and fruiting body morphogenesis. *In:* M. Dworkin and D. Kaiser (Eds.). Myxobacteria. American Society for Microbiology. Washington, DC. II:307–332.

White, D., and H. U. Schairer. 2000. Development of Stigmatella. *In:* Y. Brun and L. J. Shimkets (Eds.). Prokaryotic Development. Washington, DC. 285–294.

Whitworth, D. E., and D. A. Hodgson. 2001. Light-induced carotenogenesis in Myxococcus xanthus: evidence that CarS acts as an anti-repressor of CarA. Molec. Microbiol. 42:809–819.

Wireman, J. W., and M. Dworkin. 1975. Morphogenesis and developmental interactions in myxobacteria. Science 189:516–523.

Wireman, J. W., and M. Dworkin. 1977. Developmentally induced autolysis during fruiting body formation by Myxococcus xanthus. J. Bacteriol. 129:796–802.

Wistow, G. 1990. Evolution of a protein superfamily: Relationships between vertebrate lens crystallins and microorganism dormancy proteins. J. Molec. Evol. 30:140–145.

Witkin, S., and E. Rosenberg. 1970. Induction of morphogenesis by methionine starvation in Myxococcus xanthus. Polyamine control. J. Bacteriol. 103:641–649.

Wolgemuth, C., E. Hoiczyk, D. Kaiser, and G. Oster. 2002. How myxobacteria glide. Curr. Biol. 12:369–377.

Woods, N. A. 1948. Studies on the Myxobacteria [MS thesis]. University of Washington. Seattle, WA.

Wu, S. S., and D. Kaiser. 1995. Genetic and functional evidence that Type IV pili are required for social gliding motility in Myxococcus xanthus. Molec. Microbiol. 18:547–558.

Wu, S. S., J. Wu, and D. Kaiser. 1997. The Myxococcus xanthus pilT locus is required for social gliding motility although pili are still produced. Molec. Microbiol. 23:109–121.

Wu, S. S., J. Wu, Y. Cheng, and D. Kaiser. 1998. The pilH gene encodes an ABC transporter homologue required for type IV pilus biogenesis and social gliding motility in Myxococcus xanthus. Molec. Microbiol. 29:1249–1261.

Yamanaka, S., A. Kawaguchi, and K. Komagata. 1987. Isolation and identification of myxobacteria from soils and plant materials, with special reference to DNA base composition, quinone system, and cellular fatty acid composition, and with a description of a new species, Myxococcus flavescens. J. Gen. Appl. Microbiol. 33:247–265.

Yamanaka, S. , R. Fudo, A. Kawaguchi, and K. Komagata. 1988. Taxonomic significance of hydroxy fatty acids in myxobacteria with special reference to 2-hydroxy fatty acids in phospholipids. J. Gen. Appl. Microbiol. 34:57–66.

Yang, Z., Y. Geng, D. Zu, H. B. Kaplan, and W. Shi. 1998. A new set of chemotaxis homologues is essential for Myxococcus xanthus social motility. Molec. Microbiol. 30:1123–1130.

Yang, Z. M., D. C. Guo, M. G. Bowden, H. Sun, L. M. Tong, Z. Li, A. E. Brown, H. B. Kaplan, and W. Shi. 2000a. The Myxococcus xanthus wbgB gene encodes a glycosyltransferase homologue required for lipopolysaccharide O-antigen biosynthesis. Archiv.Microbiol. 174:399–405.

Yang, Z., X. Ma, T. Leming H. B. Kaplan, and W. Shi. 2000b. Myxococcus xanthus dif genes are required for biogenesis of cell surface fibrils essential for social gliding motility. J. Bacteriol. 182:5793–5798.

Yee, T., and M. Inouye. 1981. Reexamination of the genome size of myxobacteria, including the use of a new method for genome size analysis. J. Bacteriol. 145:1257–1265.

Yee, T., and M. Inouye. 1982. Two-dimensional DNA electrophoresis applied to the study of DNA methylation and the analysis of genome size in Myxococcus xanthus. J. Molec. Biol. 154:181–196.

Yee, T., T. Furuichi, S. Inouye, and M. Inouye. 1984. Multicopy single-stranded DNA isolated from a Gram-negative bacterium, Myxococcus xanthus. Cell 38:203–209.

Youderian, P. 1998. Bacterial motility: Secretory secrets of gliding bacteria. Curr. Biol. 8:R408–R411.

Youderian, P., N. Burke, D. J. White, and P. L. Hartzell. 2003. Identification of genes required for adventurous gliding motility in Myxococcus xanthus with the transposable element mariner. Molec. Microbiol. 49:555–570.

Younes, G., J. M. Nicaud, and J. Guespin-Michel. 1984. Enhancement of extracellular enzymatic activities produced by immobilized growing cells of Myxococcus xanthus. Appl. Microbiol. Biotechnol. 19:67–69.

Younes, G., A. M. Breton, and J. Guespin-Michel. 1987. Production of extracellular native and foreign proteins by immobilized growing cells of Myxococcus xanthus. Appl. Microbiol. Biotechnol. 25:507–512.

Zeggel, B. 1993. Steroids bei Myxobakteria [doctoral thesis]. Technical University Braunschweig. Braunschweig, Germany. 134.

Zukal, H. 1896. Myxobotrys variabilis Zuk., als Repräsentant einer neuen Myxomyceten-Ordnung. Ber. Deutsch. Bot. Gesellsch. 14:340–347.

Zukal, H. 1897. Über die Myxobacterien. Ber. Deutsch. Bot. Gesellsch. 15:542–552.

Zusman, D., and E. Rosenberg. 1968. Deoxyribonucleic acid synthesis during microcyst germination in Myxococcus xanthus. J. Bacteriol. 96:981–986.

Zusman, D. R., D. M. Krotoski, and M. Cumsky. 1978. Chromosome replication in Myxococcus xanthus. J. Bacteriol. 133:122–129.

Zusman, D. R. 1980. Genetic approaches to the study of development in the myxobacteria. *In:* T. Leighton and W. F. Loomis (Eds.). The Molecular Genetics of Development: An Introduction to Recent Research in Experimental Systems. Academic Press. New York, NY. 41–78.

Zusman, D. R. 1984. Cell-cell interactions and development in Myxococcus xanthus. Quart. Rev. Microbiol. 59:119–138.

Zusman, D. R. 1991. Cellular aggregation during fruiting body formation in Myxococcus xanthus. *In:* M. Inouye, J. Campisi, D. Cunningham, and M. Riley (Eds.). Gene Expression and Regulation of Cell Growth and Development. John Wiley & Sons. New York, NY. 65–72.

SECTION 3.5

Epsilon Subclass

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CHAPTER 3.5.1

The Genus *Campylobacter*

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Introduction

The genus *Campylobacter* comprises a diverse group of Gram-negative bacteria, which colonize the mucosal surfaces of the intestinal tracts, oral cavities, or urogenital tracts of a wide range of bird and animal hosts. Both commensals and pathogens are represented within this group of organisms.

Phylogeny

The genus *Campylobacter* is placed in the Epsilonproteobacteria (Trust et al., 1994) and now classified in the bacterial family Campylobacteraceae (Vandamme and De Ley, 1991a), which includes the genera *Campylobacter*, *Arcobacter* and *Sulfurospirillum* (formally known as free-living campylobacters) and certain misclassified *Bacteroides* species. The closest genetically related genera are *Helicobacter* and *Wolinella*, which together form the family, Helicobacteraceae (Vandamme, 2000).

Currently, the genus *Campylobacter* comprises 15 species (one of which is still disputed) and 6 subspecies: *Campylobacter coli*, *C. concisus*, *C. curvus*, *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, *C. gracilis*, *C. helveticus*, *C. hyoilei* (controversial), *C. hyointestinalis* subsp. *hyointestinalis*, *C. hyointestinalis* subsp. *lawsonii*, *C. jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, *C. lari*, *C. mucosalis*, *C. rectus*, *C. showae*, *C. sputorum* and *C. upsaliensis* (Fig. 1). Recently a new species was described as *Campylobacter lanienae* sp. nov. The type strain for the genus is *Campylobacter fetus* subsp. *fetus*. The most commonly isolated pathogenic species are (in order of frequency) *C. jejuni*, *C. coli* and *C. fetus*.

Taxonomy

Historical Overview

Originally, campylobacters were described as members of the genus *Vibrio*. The first citation in the literature (McFadyean and Stockman, 1913) described the isolation of "*Vibrio fetus*" from an aborted lamb. This organism became *C. fetus* subsp. *fetus*, which is the type species for the genus. Later other veterinary pathogens were isolated. The first campylobacter, then called *Vibrio jejuni*, was isolated by Jones and Little (1931) from the jejunum of calves with enteritis, followed by *V. coli* isolated from pigs by Doyle (1944). The first report of an isolate from human gastrointestinal disease was by Levy (1946). However, one can argue that Escherich, in 1889, was the first to describe small vibrios in the large intestinal mucus of infants who had died of "cholera infantum," presumably caused by *C. jejuni* or *C. coli*. Important bacteriological progress was made by King (1957), who cultured microaerophilic vibrios at 42°C. She correctly hypothesized that the organism was involved in diarrheal illness (King, 1962). The genus *Campylobacter* was introduced in 1963 (Sebald and Veron, 1963) to differentiate, from the traditional members of the genus *Vibrio*, those organisms with a low G+C DNA content, a strict microaerophilic nature, and a nonsaccharolytic biochemistry. In 1973, Veron and Chatelain (1973) published the first accepted taxonomy of the genus *Campylobacter*.

The human gastric pathogen *Campylobacter pyloridis* (later corrected to *C. pylori*) was added to the genus (Marshall et al., 1984) in 1984. Five years later, this organism and related organisms were relocated into the new genus *Helicobacter* on the basis of 5S and 16S RNA sequence data and DNA-DNA hybridization experiments (Goodwin et al., 1989).

Campylobacter hyointestinalis was added to the genus in 1983 (Gebhart et al., 1983). Subsequently this species was divided into *C. hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonii* (On et al., 1995). *Campylobacter upsaliensis* was included in the genus in 1991 (Vandamme et al., 1991b).

Ribosomal sequence data resulted in a recent reclassification of certain *Wolinella* and *Bacteroides* species as part of the genus *Campylobacter*. *Wolinella curva* was described in 1984 and added

to the genus *Campylobacter* as *C. curvus* (also referred to as *C. curva*) and *Wollinella rectus* was added to the genus as *C. rectus* (also *C. recta*) in 1991 (Vandamme et al., 1991b). *Bacterioides gracilis* was included in the genus as *C. gracilis* in 1995 (Vandamme et al., 1995).

In 1985, *C. sputorum* subsp. *mucosalis* was renamed *C. mucosalis* (Roop et al., 1985a). The other subspecies of *C. sputorum* (*C. sputorum* subsp. *sputorum* and *C. sputorum* subsp. *bubulus*) and "*C. fecalis*" are classified as biovars of *C. sputorum* (Roop et al., 1985b).

Campylobacter helveticus sp. nov. was described in 1992 (Stanley et al., 1992). *Campylobacter showae* was described in 1993 (Etoh et al., 1993). In 2000, a new species was isolated from the feces of healthy abattoir workers. The proposed name for this species is "*Campylobacter lanienae* sp. nov." (Logan et al., 2000).

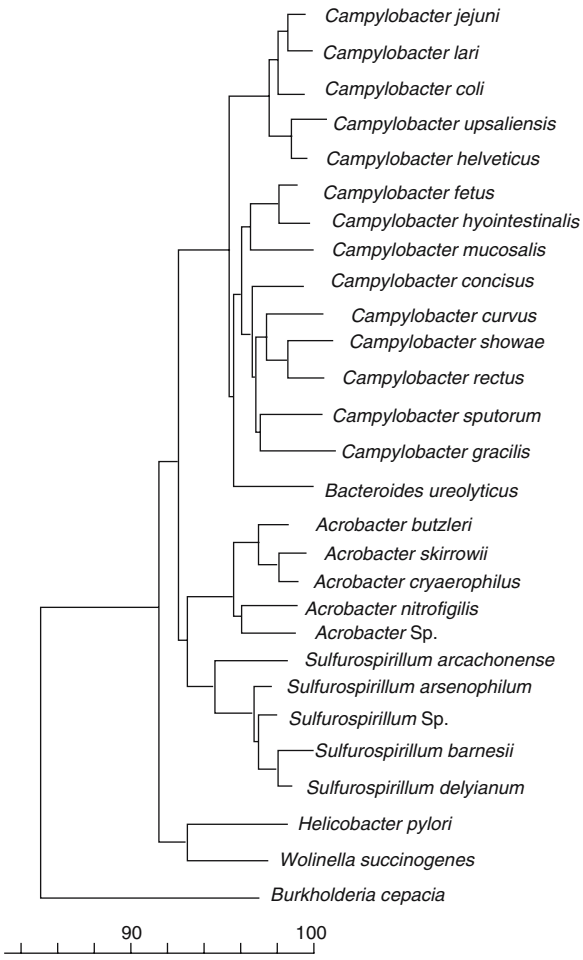

Fig. 1. Phylogenetic tree of the family Campylobacteraceae and its closest neighbors, based on the percent 16S rRNA gene sequence similarity. *Burkholderia cepacia* was used as an outgroup organism. From Vandamme (2000), with permission.

In 1991, a number of putative campylobacters were moved to the new genus *Arcobacter* or *Helicobacter* on the basis of ribosomal sequence analysis (Vandamme et al., 1991a; Vandamme and DeLey, 1991b). *Campylobacter cryaerophila* was added to the genus *Campylobacter* in 1985 and subsequently became *Arcobacter cryaerophilus*. *Campylobacter nitrofigilis* was added in 1983 and became *Arcobacter nitrofigilis*. *Campylobacter fennelliae* was added in 1988 and became *Helicobacter fennelliae*. In addition, *Campylobacter cinaedi* was reassigned as *Helicobacter cinaedi*.

The relatedness of the 15 species and 6 subspecies within the genus *Campylobacter* is shown (Fig. 1). In 1994, minimal standards were proposed for describing new species of the family Campylobacteraceae (Ursing et al., 1994). Despite these recommendations, some debate remains over the designation of *C. hyoilei* (Alderton et al., 1995), the characteristics of which may be identical to those of *C. coli* (Vandamme et al., 1997). The position of *Bacteroides ureolyticus*, formerly known as "*C. ureolyticus*" (or urease-positive thermophilic campylobacter), is also debatable (Vandamme et al., 1995).

Physiology

Table 1 summarizes the biochemical characteristics of the various Campylobacter species. All members of the genus have a low G+C content (29–47 mol%) and an optimal growth temperature (30–42°C). All the species are nonspore-forming, are sensitive to 3% or more NaCl, reduce fumarate to succinate, are oxidase-positive (except for *C. gracilis*), are indole-negative and reduce nitrate (except *C. jejuni* subsp. *doylei*).

All campylobacters are microaerophiles (optimal concentrations of O_2 [5–10%] and CO_2 [3–5%]). Some species (*C. gracilis*, *C. hyointestinalis*, *C. showae* and *C. sputorum* bv. *fecalis*) can grow under anaerobic conditions. *Campylobacter concisus*, *C. mucosalis*, *C. rectus*, *C. curvus*, *C. showae* and *C. gracilis* also require H_2 and/or formate or fumarate for growth. Energy metabolism occurs by respiration, not by fermentation or oxidation of carbohydrates.

The bacterial cells are slender, helical or curved rods (0.2–0.8 μm × 0.5–5.0 μm; Fig. 2). Certain species have cells that are straighter rods (i.e., *C. showae*), and in *C. jejuni* strains, cells are occasionally straight rods. Helical or curved rods can be observed in short or longer chains that can be V-, S-, or comma-shaped. In old cultures or cultures exposed to environmental stresses such as atmospheric oxygen, the cells can change to spherical or coccal forms.

Table 1. Biochemical differentiation of species within the Campylobacteraceae.

Characteristics[a]	*C. jejuni jejuni*	*C. jejuni doylei*	*C. coli*	*C. lari*	*C. upsaliensis*	*C. concisis*	*C. fetus*[b]	*C. hyointestinalis*[c]	*C. rectus*	*C. curvus*	*C. mucosalis*	*C. gracilis*	*C. helveticus*	*C. sputorum*[d]	*C. showas*
Growth 25°/42°	–/+	–/– (–/w)	–/+	–/+	–/v (–/+)	–/+ (–/v)	+/v (+/–)[b]	v/+ (w/+ or +/+)	–/w (–/v)	–/v (–/+)	–/+ (+/+)	–/v (–/–)	–/+	–/+ (–/v)	–/+ (–/v)
H_2 or fumarate/formate required for growth	–	–	–	–	–	+	–	v	+	+	+	NR	–	–	+
H_2S on TSI[f]	–	–	v (–)	– (+)	–	+ (– at 3 days)	–	+	+ (–)	+ (v)	+	–	–	+	– (v)
Catalase	+	v	+	+	w/–	–	+	+	– (v)	–	–	v	–	–[d]	+
Urease	–	–	–	v	–	–	–	–	–	–	–	–	–	–	–
Hippurate hydrolysis	+[g]	+ (v)	–	–	–	–	–	–	–	– (v)[h]	–	–	–	–	–
Nitrate reduction	+	–	+	+	+	+ (v)	+	+	+	+	+ (–)	– (v)	+	+	+
Nitrite reduction	–	–	– (v)[i]	–	–	+	–	–	+	+	+ (v)	+	–	+[d]	+
Indoxyl acetate hydrolysis	+	+ (–)[h]	+	–	+ (–)[h]	–	–	–	+	+ (v)	–	v (–)	+	–	+ (v)
Growth in 1% glycine	+	+ (v)	+	+	v (+)	+ (v or –)[h]	+[j] –[b,k]	+[c]	+	+	+ (v)	+ (–)	v (–)	+	v
Growth in bile esculin	NR	NR	NR	NR	NR	NR	–	NR	NR	NR	NR	NR	NR	+	NR
Growth in 3.5% NaCl	–	–	–	–	–	–	–	–	–	–	–	–	– (v)[h]	+[d]	–

Symbols: +, positive reaction; –, negative reaction; v, variation in reaction depending on strain; w, weak reaction; NR, not reported.

Abbreviations: H_2S on TSI, hydrogen sulfide production on triple sugar iron medium.

[a]The data represent a consensus from various tables published in the last 10 years, with significant divergence from the consensus (more than once reported) indicated in parentheses. (*Bergey's Manual*, 1994; Cowan and Steel, 1993; On, 1996; Tenover and Fenell, 1992; Skirrow, 1990; Topley, 2000; Nachamkin, 1995; Vandamme, 2000).

[b]*Campylobacter fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* are identical in most tests but for growth on glycine, which is negative for *C. venerealis*. Although this test is regarded as discriminative, the outcome can be unreliable since glycine-tolerant *C. venerealis* does occur. Growth at 42°C is negative for *C. venerealis*.

[c]*Campylobacter hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonii* are identical in most tests but for growth in 1% glycine, which is variable for *C. lawsonii*.

[d]All three biovars of *C. sputorum* are identical in most tests except catalase, which is positive for subsp. *fecalis*; nitrite reduction, which is sometimes reported variable for subsp. *sputorum* and *bubulus*; and growth in 3.5% NaCl, which is negative for subsp. *sputorum* and sometimes reported as negative for subsp. *fecalis*.

[f]In some publications this test is performed according to Roop et al., 1984, and in those cases, *C. jejuni* subsp. *jejuni*, *coli*, and *lari* are reported positive.

[g]Hippurate-negative *C. jejuni* subsp. *jujuni* has been reported (Totten et al., 1987).

[h]Only reported once, so that this entry may be erroneous.

[i]*Campylobacter hyoilei* is reported nitrite-reduction positive but is regarded as a *C. coli* strain on the basis of overall characteristics.

[j]*Campylobacter fetus* subsp. *fetus*.

[k]*Campylobacter fetus* subsp. *venerealis*.

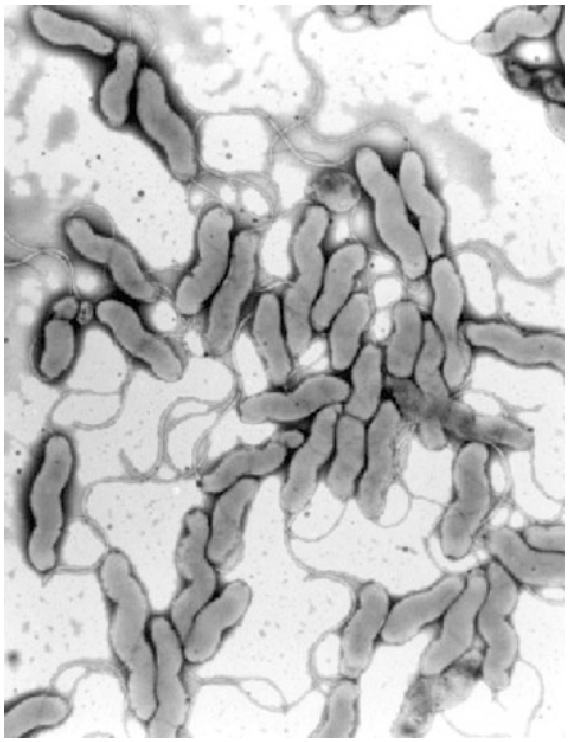

Fig. 2. Electronmicrograph of *Campylobacter jejuni*.

In general, campylobacters have a characteristic darting, corkscrew-like motility conferred by a single, polar non-sheathed flagellum. However, *C. showae* has a unipolar bundle of flagella, and *C. gracilis* is nonmotile and lacks flagella (Vandamme and De Ley, 1991a; Vandamme, 2000). Both *C. jejuni* and *C. coli* can produce nonmotile variants, especially on frequent subculture.

Habitat

Campylobacter species colonize the mucous overlying the mucosal surfaces of mammals and birds. Their darting motility enables them to maintain station in the mucous flow.

The thermophilic organism *C. jejuni* subsp. *jejuni* appears to have evolved to optimally colonize the intestinal mucosa, especially the cecum, of birds (body temperature 42°C). In this habitat, the organism appears to act as a commensal. It is most frequently isolated from broiler chickens. However, *C. jejuni* also colonizes the intestinal mucosa of mammals with lower body temperatures, where it acts as a pathogen. In some humans, particularly those who are immunocompromised, survival and growth at extraintestinal sites can also occur. The microaerophilic nature and temperature dependence of these organisms preclude growth outside their intestinal niches. Nevertheless, these species can be isolated from fecally contaminated environmental sources, such as surface water and animal products, including meat and milk.

The intestinal mucosa is also the usual habitat of most other *Campylobacter* species. Some species are frequently, but not exclusively, host-associated. *Campylobacter coli*, *C. mucosalis* and *C. hyointestinalis* are most frequently isolated from the intestinal contents of pigs. *Campylobacter lari* is usually isolated from wild birds, although it has also been isolated from shellfish, presumably from the filtration of water fecally contaminated by sea gulls. *Campylobacter upsaliensis* and *C. helveticus* are found in the intestines of cats and dogs.

Campylobacter fetus subsp. *fetus* also colonizes the intestinal tract, usually of cattle and sheep and sometimes of humans, but can translocate from the gut to cause systemic infection. In particular this organism appears to have a tropism for the urogenital tract, where it can colonize the uterine mucosa and fetal tissues. In contrast, *C. fetus* subsp. *venerealis* has bovine host specificity and has only rarely been recovered from the intestine. This organism specifically colonizes the prepuce of bulls and the vagina and fetal tissues of venereally infected cows. *Campylobacter sputorum* bv. *bubulus* is also found primarily in these bovine urogenital sites, whereas *C. sputorum* bv. *fecalis* colonizes bovine genitalia and the intestines of various host species.

Several *Campylobacter* species specifically colonize the oral cavity. *Campylobacter sputorum* subsp. *sputorum*, *C. concisus*, *C. rectus*, *C. curvus* and *C. showae* are recoverable from human oral flora. *Campylobacter gracilis* has been isolated from gingival crevices. Some of these bacteria are also recoverable from feces, though whether this reflects intestinal tract colonization or transient passage is debatable.

Isolation

Isolation methods for *Campylobacter* spp. have improved considerably over the last few decades. Isolation of clinically relevant thermophilic campylobacters from stool samples is generally based on selective antibiotics and growth at 42°C to inhibit contaminating flora. The isolation of bacteria from food and environmental samples, or any sample with low numbers of stressed bacteria potentially present, usually involves pre-enrichment procedures followed by culture on selective media. One drawback of this is the possible overgrowth and inhibition by competing bacteria during the pre-enrichment period. A further problem is that different culture tech-

niques recover different species and strains. Available isolation procedures and media have recently been reviewed (Corry et al., 1995). The most recent strategies for isolation from food and water are reviewed elsewhere (Hunt and Abeyta, 1995; International Organization for Standardization, 1995; Jacobs-Reitsma, 2000).

Most enrichment media for *Campylobacter* are based on selective antimicrobials (especially cefoperazone, cycloheximide, trimethoprim and vancomycin), which are sometimes added with delay to allow resuscitation of damaged organisms. Campylobacters, especially stressed organisms, are very sensitive to peroxides despite their possession of superoxide dismutase and catalase. Such organisms benefit from media with radical scavengers (e.g. charcoal or sheep or horse blood) and from improved atmospheric conditions (H_2 in combination with formate). The use of selective antibiotics and a high incubation temperature may prevent isolation of certain sensitive, less-common *Campylobacter* species, e.g., *C. hyointestinalis*, *C. upsaliensis*, *C. mucosalis* or *C. jejuni* subsp. *doylei*. Many of these species are slower growing and incubation periods should be extended for their isolation.

A nonselective protocol for the isolation and identification of campylobacters and related organisms from stools and blood, primarily of human origin, is described below (see the section Identification in Stools and Blood Samples: The Cape Town Protocol in this Chapter).

Genetic Detection

The polymerase chain reaction (PCR) has recently been applied to the detection of campylobacters. Such genetic detection techniques can be very sensitive; however, depending on the type of sample, they generate a number of problems. In samples with low bacterial numbers (i.e., foods), pre-enrichment is required. This greatly reduces the benefit of PCR as a fast one-step method. The presence of potential PCR inhibitors ensures the need for robust PCR protocols (Thunberg et al., 2000). Purification procedures to remove PCR inhibitors are effective but add extra work (Wang et al., 1999). In feces, numbers of campylobacters may vary and the presence of significant numbers of other microorganisms demands a high specificity. The desirability of detecting viable but not culturable forms in the environment is questionable. At the moment, the tendency is to ignore such damaged organisms because they are insignificant in terms of infectivity (Fearnley et al., 1996). However, the debate is not yet closed (Cappelier et al., 1999). Some PCR detection methods developed for *Campylobacter* spp. detect directly at the species level; others do not differentiate a number of species. (See the section Genetic Identification: Methods for Speciation in this Chapter.)

Identification

Biochemical Identification

A comparison of the recent literature regarding the systematic bacteriology of campylobacters (Skirrow, 1990; Tenover and Fenell, 1992; Cowan and Steel, 1993; *Bergey's Manual*, 1994; Nachamkin, 1995; On, 1996; Topley, 2000; Vandamme, 2000) reveals many inconsistencies in their biochemical properties. This variation, largely a consequence of experimental variation and the subjective nature of such tests, highlights the need for standardization. A consensus of biochemical and growth properties of all *Campylobacter* species is given in Table 1. Such tables should be treated circumspectly as rigid adherence to these properties may result in mistyping. Some strains have anomalous properties, e.g., hippurate-negative *C. jejuni* has been reported (Totten et al., 1987). Some tests give inconsistent results, i.e., H_2S production on triple sugar iron (TSI) medium is extremely inconsistent for *C. jejuni*, *C. coli*, *C. lari*, *C. concisus*, *C. hyointestinalis* and *C. sputorum*. Some properties are dependent on the age of the culture and the test conditions applied.

For the speciation of clinically relevant isolates, distinguishing campylobacters from other organisms with similar morphology and ecology (especially certain helicobacters and arcobacters) is important. Because *C. jejuni/coli* and *C. fetus* are thought to be the most relevant pathogens, identification of campylobacters, other than these three, is not always carried out. Indeed in most studies *C. jejuni* and *C. coli* are by far the most commonly isolated pathogens. However this may, in part, reflect the use of selective antibiotics, which does not allow growth of most other campylobacters. When a filter technique is used, without antimicrobial selection, significantly higher numbers of other campylobacters are detected.

Identification in Stools and Blood Samples: The Cape Town Protocol

One of the best protocols to isolate every possible member of the genus is the Cape Town protocol (developed at the Red Cross Children's Hospital in Cape Town, South Africa; Le Roux and Lastovica, 1998). In this laboratory, most *Campylobacter* species (with the notable exception of *C. sputorum* subsp. *sputorum*) have been isolated from blood of patients with bacteremia. One therefore may speculate that, under certain

conditions, every *Campylobacter* species may be pathogenic. However, these findings may be a consequence of the socio-economic, hygienic and nutritional status of the patients. A brief version of the protocol is included here with the permission of Dr. Albert Lastovica.

For stool samples, a watery emulsion of fecal material in saline should be applied, without pressure, to 0.6-µm pore filters placed directly on agar plates containing 10% horse blood. The filter is flooded three times with emulsified specimen. Blood samples are applied directly to the plate without filtration. Primary culture plates should be incubated in an H_2-containing atmosphere for 6 days, checking for growth every 2 days. Plates should not be checked within the first 24 h to prevent lethal damage to fastidious organisms. An H_2-containing atmosphere can be generated by anaerobic Gas Pack without using a catalyst, alternatively by the application of a gas mixture of 15% CO_2 and 85% H_2. *Campylobacter* colonies are mostly buff colored or dirty yellow. *Campylobacter helveticus*, *Helicobacter fennelliae*, *H. cinaedi* and *H. rappini* spread, forming thin, flat films, which often become visible after 6 days. *Arcobacter* colonies are whiter. A Gram stain should confirm the cell morphology. The cells of *H. fennelliae* and *H. cinaedi* are thin, long spirals; those of *C. mucosalis* are short and stubby. *Campylobacter hyointestinalis* and *Arcobacter* are big and slightly curved bacteria, whereas *C. concisus*, *C. rectus*, *C. curvus* and *C. ureolyticus* are straight or hardly curved.

Putative campylobacters are subcultured on two tryptose blood agar plates (TBA containing 10% horse blood). One plate is incubated in the H_2-containing atmosphere, the other in 13% CO_2 in air. All subsequent tests are performed using a 48-h old culture from the culture grown in the H_2-containing atmosphere, with the exceptions of the lead acetate (PbAc) and TSI tests, which could give false-positive results under such culture conditions. The CO_2 culture is used for these tests. Growth in the plate incubated in a CO_2-containing-atmosphere is checked for up to 4 days. Table 2 lists the organisms expected to grow in the CO_2-containing atmosphere.

The tests performed for species identification are listed in Table 2. Inconsistencies appear between Tables 1 and 2; the former is a compilation of available determination tables from the literature; however, the test results of Table 2 were obtained from one laboratory.

Identification in Veterinary Venereal Specimens

Campylobacter fetus infection in bovines is a challenging diagnostic problem. *Campylobacter fetus* subsp. *venerealis* can be venereally transmitted from bulls to cause infertility and abortion in susceptible cows. This infection is of statutory significance for import-export regulations in many countries. This is of major importance in the international trade of bovine semen and embryos. The major diagnostic problem, however, occurs because other *Campylobacter* species and *C. fetus* subspecies may also be present in bovine genital tract and fetal samples. One simple method of differentiation of *C. fetus* from the commensal, *C. sputorum*, is the rapid H_2S test and growth in bile esculin (Table 1). Some regulations call for freedom from all *C. fetus* and others for freedom from *C. fetus* subsp. *venerealis* only. Thus, differentiation between subspecies may be crucial. Currently subspeciation is based on growth tolerance to 1% glycine (Table 1). However, tolerant variants of *C. fetus* subsp. *venerealis* occur. There is little serotypic diversity within the species: *C. fetus* subsp. *fetus* comprises two serotypes (A and B), whereas *C. fetus* subsp. *venerealis* is always of serotype A (Marsh and Firehammer, 1953). Complementary genotypic methods have now been developed for the subspeciation of *C. fetus*.

Genetic Identification: Methods for Speciation

Tests have now been described to identify *Campylobacter* species by means of genetic methods. Most of these tests are based on PCR and some commercial PCR tests are now available. Potentially PCR should be easier to perform and more reliable than biochemical tests, but PCR identification for campylobacters has not been accepted for routine use. Available genetic identification methods have been reviewed recently (On, 1996).

Some PCR detection methods developed for *Campylobacter* spp. detect the thermophilic campylobacters directly at the species level with variable levels of discrimination (Table 3). Most of these methods are based on amplification of (fragments of) flagellin genes or ribosomal genes. In some of the methods, the target gene for species-specific amplification has been selected by hybridization experiments and has not been further characterized (Korolik et al., 1995). Species-specific hybridization of PCR products obtained with degenerate primers is also used (Al Rashid et al., 2000). The methods described vary in complexity from a single PCR directly from the sample to pre-enrichment and/or filtering, amplification, and gel electrophoresis followed by Southern blotting (or spot blots) and hybridization. The relative efficacy of these methods has yet to be determined. In one comparative study, speciation by PCR was at least as

Table 2. The Cape Town protocol for the differentiation of clinically relevant campylobacters and related organisms.

Table 2A. Grow on CO_2

Growth on CO_2	+									
	C. jejuni jejuni (biotypes 1 and 2), *C. jejuni doylei*,[a] *C. coli*, *C. lari*, *C. fetus*,[a,b] *C. upsaliensis*,[a] *C. hyoilei*, *Arcobacter butzleri*, *A. skirrowi*, *A. cryaerophila*									
Indoxyl acetate	+									–
	C. jejuni jejuni, *C. jejuni doylei*, *C. coli*, *C. hyoilei*, *C. upsaliensis*, *A.r butzleri*, *A. skirrowi*, *A. cryaerophila*									*C. lari*, *C. fetus*
Nitrate reductase	+							–		+
	C. jejuni jejuni, *C. coli*, *C. hyoilei*, *C. upsaliensis*, *A. butzleri*, *A. skirrowi*, *Acryaerophila*							*C. jejuni doylei*		*C. lari*, *C. fetus*
Catalase	+					Var *C. upsaliensis*, *A. butzleri*		Var		+
	C. jejuni jejuni, *C. coli*, *C. hyoilei*, *A. cryaerophila*, *A. skirrowi*									*C. lari*, *C. fetus*
Pyrazin amidase	+		–		+	–	+	+	–	
	C. jejuni jejuni (biotypes 1 and 2), *C. coli*, *C. hyoilei*		*A. cryaerophila*, *A. skirrowi*		*C. upsaliensis*	*A. butzleri*		*C. lari*	*C. fetus*	
Arylsulfatase	+	–	–		–	–	–	–	–	
	C. jejuni jejuni biotype 2	*C. jejuni jejuni* biotype 1, *C. coli*, *C. hyoilei*	*A. cryaerophila*, *A. skirrowi*							
Hippurate	+	+	–		–	–	+	–	–	
		C. jejuni jejuni biotype 1	*C. coli*, *C. hyoilei*	*A. cryaerophila*, *A. skirrowi*						
MacConkey	+	+	+	+ *A. cryaerophila*	– *A. skirrowi*	–	+	–	–	+
1.5% NaCl	–	–	–	+	+	–	+	–	+	–
Aerobic at RT	–	–	–	+	+	–	+	–	–	–
Rapid H_2S	+	–	–	–	–	–	–	–	(+)	–
PbAc H_2S	–	++	+++	–	–	Var	–	–	++	Var
TSI H_2S	–	–	+ –	–	–	–	–	–	–	–
Result	*C. jejuni jejuni* biotype 2	*C. jejuni jejuni* biotype 1	*C. hyoilei*	*C. coli*	*A. cryaerophila*	*A. skirrowi*	*C. upsaliensis*	*A. butzleri*	*C. jejuni doylei*	*C. lari*	*C. fetus*[b]

Symbols: +, ++ and +++, positive, very positive and extremely positive; –, negative; and var, variable.

Abbreviations: RT, room temperature; PbAc H_2S, hydrogen sulfide production in lead acetate medium; and TSI H_2S, hydrogen sulfide production in triple sugar iron medium.

[a]Veterinary isolates often grow weak or not at all on CO_2.

[b]To differentiate *C. fetus* from *C. venerealis*, see section above.

Table 2B. No growth on CO_2

No growth on CO_2	–												
	C. helvetica, *C. hyointestinalis*, *C. mucosalis*, *C. concisus*, *C. sputorum* (bv *fecalis*, *sputorum*, *bubulus*), *C. ureolyticus*, *C. gracilis*, *C. showae*, *C. curvus*, *C. rectus*, *Helicobacter cinaedi*, *H. fennelliae*, *H. rappini*											
Indoxyl Acetate	+				–								
	C. helvetica, *C. curvus*, *C. rectus*, *H. fennelliae*				*C. hyointestinalis*, *C. concisus*, *C. ureolyticus*, *C. mucosalis*, *C. sputorum*, *H. cinaedi* (variable), *H. rappini*								
Nitrate reductase	+		–	–	+								
	C. helveticus, *C. curvus*, *C. rectus*		*H. fennelliae*	*H. rappini*	*C. hyointestinalis*, *C. concisus*, *C. ureolyticus*, *C. mucosalis*, *C. sputorum*, *H. cinaedi*								
Catalase	+		–	+	–	+			–				
	C. curvus, *C. rectus*		*C. helveticus*		*C. hyointestinalis*, *C. sputorum fecalis*, *H. cinaedi*				*C. concisus*, *C. ureolyticus*, *C. mucosalis*, *C. sputorum bubulus*, *C. sputorum sputorum*				
Pyrazin amidase	+		+	–	–	+	–		+			–	
	C. curvus, *C. rectus*					*C. sputorum fecalis*	*C. hyointestinalis*, *H. cinaedi*		*C. sputorum sputorum*, *C. concisis*, *C. ureolyticus*			*C. sputorum bubulus*, *C. mucosalis*	
Arylsulfatase	+		–	+	–	+	–		+			+	–
	C. curvus, *C. rectus*						*C. hyointestinalis*, *H. cinaedi*		*C. sputorum sputorum*, *C. concisis*, *C. ureolyticus*			*C. sputurum bubulus*	*C. mucosalis*
Hippurate	–		–	–	–	–	–		–			–	–
	C. curvus, *C. rectus*						*C. hyointestinalis*, *H. cinaedi*		*C. sputorum sputorum*, *C. concisis*, *C. ureolyticus*				
MacConkey	+	–	–	–	–	+	+	–	(+)	*C. concisis*, *C. ureolyticus*		–	(+)
	C. curvus	*C. rectus*					*C. hyointestinalis*	*H. cinaedi*	*C. sputorum sputorum*				
1.5% NaCl	–	+	–	–	–	+	–	–	+	+	+	+	+
Urease	–	–	–	–	+	–	–	–	–	+	–	–	–
										C. ureolyticus	*C. concisis*		
Rapid H_2S	–	–	–	–	–	+	–	–	+	–	–	+	–
PbAc H_2S	+++	+++	–	+	–	+++	+++	–	+++	++	+++	+++	+++
TSI H_2S	+	+	–	–	–	+	+	–	+	+	+	+	+
Result	*C. curvus*	*C. rectus*	*C. helveticus*	*H. fennelliae*	*H. rappini*	*C. sputorum fecalis*	*C. hyointestinalis*	*H. cinaedi*	*C. sputorum sputorum*	*C. ureolyticus*	*C. concisis*	*C. sputurum bubulus*	*C. mucosalis*

Symbols: see footnote in Table 2A.
Abbreviations: see footnote in Table 2A.

Table 3. A comparison of molecular methods described for detection and speciation of thermophilic *Campylobacter* spp.

Sample material	Species[a]	Target gene	Detection limit	Remarks	Reference
PCR-dependent methods:					
Dairy products	*C. jejuni* + *coli*	*flaA* + *B*	With raw milk, more sensitive than culturing	Food samples were treated to liberate bacteria but not enriched	Allmann, 1995
Stool, water	*C. jejuni* + *coli*	*flaA*	30–60 CFU/assay in stool, 10–100 CFU/100 ml water	For water filtration is required	Oyofa, 1992
Chicken litter	*C. jejuni*	*flaA*	With dried litter, more sensitive than culturing	Enrichment required	Itoh, 1995
Water	*C. jejuni* + *coli*	*flaA* + *B*	10–20 CFU/ml	Filtration required	Kirk, 1994
Water	*C. jejuni*	*flaA*	30 CFU/100 ml	Enrichment required	Hernandez, 1995
Chicken meat washes	*C. jejuni* + *coli* + *lari*	16S rRNA	25 CFU/g meat	Enrichment and hybridization required	Giesendorf, 1992
DNA from pure cultures	*C. jejuni* + *coli* + *lari* + *upsaliensis* or *C. jejuni*, *coli*, *lari*, *upsaliensis*	23S rRNA	12 CFU/assay	Speciation is dependent on choice of primers	Eyers, 1993 Fermer, 1999
Chicken meat	*C. jejuni, coli*	rRNA intergenic spacer		Hybridization required for speciation	O'Sullivan, 2000
Cell lysates from pure cultures	*C. jejuni*	Membrane protein gene *mapA*	24 CFU/assay	The protein encoded by this gene is also immunogenic	Stucki, 1995
DNA from pure cultures	*C. jejuni*, *coli*	Hippuricase, aspartokinase		A three-step PCR for detection and speciation	Linton, 1997
DNA from pure cultures	*C. jejuni*, *coli*, *lari*, *upsaliensis*, *Arcobacter*	*glyA*	200 CFU/assay	Degenerate primers are used for detection, and hybridization for speciation	A Rashid et al., 2000
Lysed cells from enriched carcass washes	*C. jejuni*	U	NA	Filtration, culturing required	Winters, 1995
DNA from pure cultures	*C. jejuni*	U	1 CFU	Hybridization required	Stonnet, 1993
DNA from pure cultures	*C. jejuni*, *coli*, *lari*	U	NA	RAPD PCR followed by hybridization	Giesendorf, 1993
PCR independent methods:					
Pure cultures	*C. jejuni* + *coli* + *lari* or *jejuni*, *coli*, *lari*	16S rRNA	6 CFU in presence of 4×10^6 CFU Gram-negative bacteria	NASBA followed by hybridization, speciation is dependent on probe	Uyttendaele et al., 1994
Pure cultures	*C. jejuni* + *coli* + *lari*	NS	NAp	One-step DNA hybridization	Tenover et al., 1990
DNA from pure cultures	*C. jejuni*, *coli*	NAp	NAp	Slot blot hybridization to speciate atypical Campylobacters	Ng et al., 1987
DNA from pure cultures	*C. jejuni*, *coli*	U	NAp	Southern blot hybridization	Korolik et al., 1995

Abbreviations: PCR, polymerase chain reaction; CFU, colony-forming units; NA, not available; RAPD PCR, randomly amplified polymorphic DNA-PCR; NASBA, nucleic acid sequence-based amplification; NS, not specified; NAp, not applicable.

[a]When multiple species are detected that cannot be differentiated this is indicated by "+." When a single species is given the method is specific for that species. When the method allows differentiation between species, these are separated by ","

sensitive as classical biochemical techniques (Steinbrückner et al., 1999).

Genetic identification methods (independent of PCR) have also been described, for instance, hybridization of Southern blots with species-specific probes derived from the rRNA genes (Tenover et al., 1990). The technique known as "NASBA" ("nucleic acid sequence-based amplification") is based on Taq-independent, room temperature amplification, and this can be used for speciation when it is followed by hybridization (Uyttendaele et al., 1994). Atypical thermophilic campylobacters can be identified as *C. jejuni* or *C. coli* (or neither of these) by slot-blot hybridization (Ng et al., 1987). Positive identification of *Campylobacter* species should be confirmed, ideally, by DNA-DNA hybridization with the type-strain. DNA hybridization has revealed the existence of hippurate-negative *C. jejuni* isolates (Totten et al., 1987). Such techniques are generally considered too complex to be applied on a routine basis.

Isolates specified by classical biochemical identification are sometimes found more likely to be members of different species based on their genotype (Duim et al., 2001; Waino et al., 2003). Such observations indicate that biochemical identification is often unreliable when not backed up with DNA-DNA hybridization or other genetic methods. On the other hand, some genetic tests do not have a sufficient level of discrimination to be used for species identification. An example of the latter is the flagellin gene restriction fragment length polymorphism (RFLP), in which *C. coli* cannot be distinguished from *C. jejuni* because both species can share identical flagellin genotypes.

The subspeciation of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* has been addressed by genotyping techniques. Pulsed field gel electrophoresis (PFGE) of chromosomal DNA enables differentiation of the two subspecies, both on the basis of restriction enzyme banding patterns (Fujita et al., 1995a) and on their difference in genome size (*C. fetus* subsp. fetus has 1.1 Mb and *C. fetus* subsp. *venerealis* 1.3 Mb; Salama et al., 1992). In addition, a PCR test has been described that apparently can discriminate between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* (Hum et al., 1997). Finally, amplified fragment length polymorphism (AFLP) is also proving an extremely useful discriminatory method for these subspecies (Wagenaar et al., 2000).

Phenotypic Methods for Subtyping

Subtyping is a routine tool for epidemiological investigation, particularly tracing food-borne bacteria, such as *C. jejuni*/*coli*, through the food chain. Serotyping has been the most commonly used method of phenotyping. Early studies demonstrated considerable serologic variation in both the heat-stable (Penner and Hennessy, 1980) and heat-labile antigens (Lior et al., 1982) of *C. jejuni*/*coli* strains. The lipopolysaccharide (LPS) is considered the heat-stable antigenic component (Moran and Penner, 1999), but recent studies suggest that a surface polysaccharide may also be involved (Karlyshev et al., 2000). Similarly the heat-labile antigen may be a mixture of flagellin polypeptides modified by strain-specific posttranslational glycosylation (Doig et al., 1996a). Recently a further serotyping scheme has been developed for routine use in the United Kingdom (Frost et al., 1998). Although serotyping has been a useful tool, the poor availability and expense of production and quality control of antisera have generally precluded the extensive use of this method. In addition, a considerable proportion (up to 40%) of isolates are not typeable by this method.

Phage typing has also been developed (Grajewski et al., 1985; Salama et al., 1990; Khakhria and Lior, 1992). Recently a layered strategy combining serotyping and phage typing (Frost et al., 1999) has been applied to the typing of human campylobacter strains in the United Kingdom (Newell et al., 2000).

Other phenotyping techniques applied to campylobacters have included fatty acid profiling and sodium dodecyl sulfate polyacrylamide gel electrophoresis protein profiling, but such techniques rarely have the discriminatory power required for epidemiological purposes.

Genetic Methods for Subtyping

Many genetic methods have been described for the subtyping of Campylobacter strains, and these were recently reviewed (Wassenaar and Newell, 2000). Genetic subtyping studies have revealed an enormous genetic diversity within the species *C. jejuni* and *C. coli* and to a lesser extent within *C. lari*, *C. consisus* and *C. upsaliensis*. Genetic typing methods (such as PFGE [of digested chromosomal DNA], ribotyping, and AFLP) have been found satisfactory for epidemiological investigations and have given insight into the population structure of *C. jejuni* and *C. coli*. The findings suggest a complex, weakly clonal population which has been confirmed by multilocus enzyme electrophoresis studies (Meinersmann et al., 2002) and multilocus sequence typing (MLST; Dingle et al., 2002; Manning et al., 2003). Genetic characterization of isolates is potentially hampered by frequent exchange of DNA between strains (as suggested by MLST), possibly by the property of natural competence to take up and incorporate chromosomal DNA (Wang and Taylor, 1990). The fre-

quent exchange of (parts of) genes or loci may preclude recognition of particular pathotypes (Meinersmann et al., 2002). In addition to exchange of alleles, *Campylobacter* genomes may undergo inversions and translocations, rendering their genotypes unstable. This has been observed by changes in PFGE genotypes and seems to be strain-dependent rather than a general characteristic (Wassenaar et al., 1998). Finally, experimental mixed infections in animals have resulted in the selection of novel genotypes (Boer et al., 2002). These observations suggest that genotypic identification of isolates requires careful interpretation.

Preservation

Campylobacter cultures age quickly and rapidly, become coccoidal in form, and lose viability. Short-term storage (up to 7 days) of plate cultures is possible at 4°C under microaerobic conditions. Long-term storage should be of actively growing cultures. Campylobacters can be freeze-dried but specialist skills and equipment are required, and recovery is sometimes poor. In most routine laboratories, campylobacters are frozen in media containing a cryopreservative, such as 10% glycerol, and stored in –70° to –80°C freezers or in liquid nitrogen. Strains stored in this way have remained viable for over 20 years. Recovery from frozen stocks may require pre-enrichment for recovery of damaged cells.

Epidemiology

Epidemiology of *C. jejuni* and *C. coli*

Campylobacter jejuni and the closely related organism *C. coli* are the most common cause (worldwide) of acute bacterial enteritis (Tauxe, 1992). The clinical outcome of infection varies throughout the world. In industrialized countries, such as the United States and United Kingdom, infection results in acute watery or bloody diarrhea. The required infectious dose for disease, as determined by volunteer studies, was large (10^4 colony forming units; Black et al., 1992) but asymptomatic infection occurs at lower doses, and the outcome of disease is dependent on the immune status of the host. In nonindustrialized countries, diarrhea associated with infection is usually apparent only in children under the age of 2 years. Thereafter, infection appears to be asymptomatic. The reason for this difference is not clear but may reflect differences in the immune status of individuals in the non-industrialized world.

Surveillance of infection has been in place in England and Wales since the early 1980s. The data demonstrate a clearly increasing trend in reported cases. Initially this trend may have reflected improved diagnosis. The cause of the more recent increases is, as yet, unknown. In 1998, 58,000 cases were reported. Community-based studies (Tompkins et al., 1999) suggest that over 8 times as many cases are unreported. This indicates that in this geographical region there are half a million cases per annum. The World Health Organization (WHO) estimates approximately 1% of the population is infected each year. The social and economic cost of this disease is considerable.

The sources and routes of human infection are still debatable. Human-to-human spread rarely occurs. The organisms are ubiquitous in the environment and can be recovered from most food-producing and domestic animals and birds, as well as from surface waters, soil, etc. The vast majority of infections are sporadic. Outbreaks are occasionally detected. In large outbreaks, contaminated water and milk appear to be major vehicles. For sporadic cases, the vehicles are very difficult to detect. However, some case control studies indicate that the consumption or handling of raw or undercooked poultry meat is a major risk factor. Other recognized risk factors include young domestic pets (kittens or puppies with diarrhea), unpasteurized milk, swimming in surface waters, and barbecues.

The epidemiology of these organisms in mammalian species other than humans is unknown. Most young mammals become colonized early, presumably with organisms transmitted by occasional excretion from dams. These infections are rarely symptomatic.

Many poultry flocks are colonized asymptomatically with *C. jejuni* or *C. coli*. This infection is usually only detectable when the birds are 2–3 weeks of age. Once detected, 100% of chickens become infected within 72 h. The sources of infection for these birds are unknown, and although organisms can be recovered from the oviducts, vertical transmission is not considered a major source. Most likely birds are infected from organisms around the external environment of the poultry house.

Epidemiology of *C. fetus*

Campylobacter fetus subsp. *venerealis* is venereally transmitted and is a frequent infectious cause of bovine infertility in those countries where natural mating, rather than artificial insemination (AI), is standard agricultural practice (Garcia et al., 1983). For the purposes of AI, bulls standing at AI centers are screened for infection and semen is antibiotic treated. This has

significantly reduced the risk of transmission of bovine *C. fetus* subsp. *venerealis* infection. The statutory testing of bulls, semen and embryos for import and export is in place in most countries to protect cattle herds.

Campylobacters, mostly *C. fetus*, can cause fetopathology, leading to fetal death, in both cattle and sheep. In the United Kingdom Campylobacter infections in cattle represent a significant proportion (7%) of diagnosed infectious causes of bovine fetopathy and infertility (VIDA, 1996) and constitute the third most common cause of all ovine abortions (10%) diagnosed at veterinary investigation centers (VIDA, 1996). Serious economic loss results from abortion, which can affect up to 70% of a flock. Ovine *C. fetus* abortion is reported throughout the world and is of particular importance in Middle Eastern countries such as Jordan (Aldomy, 1992). In New Zealand, where it is the most common cause of ovine abortion, *C. fetus* subsp. *fetus* is responsible for 60–70% of cases (Skirrow, 1994). *Campylobacter fetus* abortion may be underdiagnosed in the United States (Hansen et al., 1990). This organism is not venereally transmitted but readily colonizes the intestinal tract. Infection appears to be transmitted via contact with active fecal shedders. Non-ovine reservoirs of infection may exist in some wildlife, including birds.

Especially in immunocompromised individuals, *C. fetus* subsp. *fetus* can cause severe human infection, which is thought to occur by the ingestion of fecally contaminated food, for example beef or lamb. The incidence of human disease is unknown.

Pathogenicity

Disease Caused by *C. jejuni* and *C. coli*

Human infection with *C. jejuni* or *C. coli* usually presents with fever followed by diarrhea. Intestinal illness may be accompanied by bacteremia and systemic infection, especially in persons at the extremes of age or who are immunocompromised. However, in most cases, infection is confined to the intestine. Colonic biopsies demonstrate an acute inflammatory response with infiltration of the epithelium and lamina propria by neutrophils and mononuclear cells. The diarrheic stools of affected individuals from industrialized countries usually contain leukocytes and erythrocytes, even when the stools are watery and not grossly bloody. Thus, *Campylobacter* colitis and enteritis should be considered an inflammatory illness. The disease is generally self-limiting and antibiotic treatment is not normally required. The duration of diarrhea is usually less than one week; however, excretion of the organism may continue for several weeks. In up to 20% of cases, a relapse, usually mild, may occur within a few days of spontaneous remission. Such cases may indicate an incomplete immune response. Immunocompromised hosts, including patients infected with human immunodeficiency virus, often have severe, extraintestinal, prolonged and relapsing illnesses, sometimes with a permanent inability to clear the organism (Melamed et al., 1983; Perlman et al., 1988).

An important sequelum of *C. jejuni* infections is the development of the Guillain-Barré Syndrome (GBS), an acute neurological disease marked by ascending paralysis resulting from demyelination of peripheral nerves (for a recent review, see Nachamkin et al. [1998]). In the United States, an estimated 0.05% of *C. jejuni* infections induce GBS (Nachamkin et al., 1998). The symptoms are evident about 7–21 days following onset of infection. This timing suggests that the host immune response to C. jejuni, rather than the acute toxicity of the infection, is responsible for the disease. Certain heat-stable serotypes, especially O:19 (in the United States and in Japan; Kuroki et al., 1993) and O:41 (in South Africa; Lastovica et al., 1997) are predominantly linked to the onset of GBS. However, in European countries the correlation with certain serotypes is less clear (Endtz et al., 2000). Autoimmune responses, as a consequence of mimicry of host neurological tissue antigens by bacterial antigenic components (especially LPS) of these serotypes, are considered to be the trigger for GBS.

Pathogenicity of *C. jejuni/coli*

The pathogenic mechanism(s) by which *C. jejuni* and *C. coli* cause diarrheal disease remains surprisingly obscure. Although a number of virulence factors have been implicated, a satisfying explanation for this most common outcome of infection cannot be given. The main reason for this is the absence of suitable laboratory animal models of disease (Newell, 2001).

The complete genome sequence of strain *C. jejuni* 11168 confirmed the absence of an identifiable virulence-associated pathogenicity island; however, this strain may not be representative of the species. Furthermore, virulence factors (enterotoxin, shiga-toxin, or hemolysin genes, and invasion loci) recognized as key in other enteropathogens were all absent (Parkhill et al., 2000). As recognized by genotypic analysis, *C. jejuni/coli* are diverse species (see the section Genetic Methods for Subtyping in this Chapter). This diversity is apparently reflected in pathogenic phenotype: many different toxins have been described (for a review, see Wassenaar

[1998]); fimbriae and other adhesion structures can be present under specific growth conditions (Doig et al., 1996b), and invasion properties and mechanisms vary or may be absent (Lindblom et al., 1990; Wassenaar and Blaser, 1999). This resembles the situation with *Escherichia coli* and *Salmonella enterica*, where many different pathotypes exist. However, in *C. jejuni/coli*, different pathotypes have not yet been sufficiently characterized; tissue tropism or host-specificity of strains within these bacterial species seems to be absent, and the genetic basis for the observed phenotypic variation is largely unknown.

All strains of *C. jejuni* and *C. coli* can produce flagella, and motility has long been recognized as a virulence property. Inactivation of the structural genes for flagellin results in loss or attenuation of colonization potential (Nachamkin et al., 1993; Wassenaar et al., 1993). Phase variation results in colonies of nonmotile cells and is regulated at the level of transcription of flagellin (Nuijten et al., 1989). The two tandem flagellin genes, present in a single locus, have different roles, with the first gene being predominantly expressed (Wassenaar et al., 1994). Flagellin protein is immunodominant during infection and can elicit a protective immune response for homologous but not heterologous strains (Pavlovskis et al., 1991).

The role of adhesive structures, like fimbriae, is unclear. Fimbrial-like protrusions (Doig et al., 1996a) are now considered an artifact of exposure to bile salts (Gaynor et al., 2001). No structural genes for fimbrial proteins have been identified. Both LPS and flagella have been shown to have adhesive properties. Proteinaceous adhesins have also been identified (reviewed in Konkel et al. [2000]). Adhesion may not be required for colonization because motility enables the bacteria to overcome peristaltic flow. However, invasion depends on prior adhesion to the mucosal cells. Different invasion mechanisms have been described that may depend on actin polymerization (reviewed in Konkel et al. [2000]). Either microfilament-dependent pseudopods are formed, or the microfilaments do not reorganize; instead, microtubules are involved in the uptake mechanism (Oelschlaeger et al., 1993). Both clathrin-coated pits (Oelschlaeger et al., 1993) and clathrin-independent caveolae (Wooldridge et al., 1996) have been implicated in endocytosis. These different findings may reflect strain and pathotype differences.

Cell damage, as a result of bacterial invasion, is probably one of the major factors resulting in diarrhea. This damage, combined with invasion into deeper tissue, would account for the inflammatory response. The bacteria may enter the bloodstream where they are quickly cleared by complement activation. Some *C. jejuni* strains that are highly resistant to serum have been detected, although the mechanisms for this resistance have not been clarified (Blaser et al., 1986). Most circulating bacteria eventually will be phagocytosed by leukocytes or macrophages of the reticuloendothelial system. Recent studies on the behavior of *C. jejuni* after phagocytosis in vitro using monocytes stimulated with cytokines showed that macrophages are able to kill all tested strains with high efficiency (Wassenaar et al., 1997). These findings are in agreement with the self-limiting nature of most *C. jejuni* infections.

One of the mechanisms for virulence proposed in the early days of *Campylobacter* research was the production of toxins. Research concentrated on exotoxins (as opposed to the toxic effects of LPS), which were called either "enterotoxins" or "cytotoxins." Cytotoxic activity with similarity (both in mode of action and immunological crossreaction) to that observed with the enterotoxins of *Vibrio cholera* and *E. coli* was detected. Nevertheless, enterotoxin genes are absent in *C. jejuni* (Perez-Perez et al., 1992). The cytotoxic activity is most likely the result of one or several cytotoxins. In a recent review (Wassenaar, 1997), at least six different toxins, or classes of toxins, were recognized. Of these, the cytolethal distending toxin (Cdt) is the first toxin for which the genetic locus has been cloned and its sequence analyzed (Pickett et al., 1996). This toxin blocks HeLa (a line derived from human adenocarcinoma) cell division in the G_2/M phase, ultimately leading to cell death (Whitehouse et al., 1998). The direct role of Cdt in disease remains to be demonstrated. All *C. jejuni* isolates studied to date possess the *cdt* gene; however, differences in expression of Cdt have been demonstrated (Johnson and Lior, 1988; Pickett et al., 1996). The genetic basis for this variation in levels of expression is not yet known; however, Cdt negative strains naturally occur as a result of deletions or point mutations (AbuOun et al., 2004). The strong conservation of the *cdt* locus in Cdt-negative strains suggests that toxicity is not the sole function of this locus.

As with other Gram-negative bacteria, the lipid A component of *C. jejuni* lipopolysaccharide (LPS) has endotoxic activity (Moran, 1996). Systemic infection can lead to sepsis and shock, presumably as a result of LPS release. The role of LPS in the development of GBS is discussed in "Disease Caused by *C. jejuni* and *C. coli*."

Since *C. jejuni* and *C. coli* are frequently associated with inflammatory diarrhea, a role for the immune system of the host can not be excluded. A number of cytokines are activated in host cells in the presence of campylobacter antigens

(Mellitis et al., 2002; Jones et al., 2003). That asymptomatic colonization of chickens, which occurs together with induced apoptosis of lymphocytes, further suggests a role of active lymphocytes in symptomatic human colonization (Zhu et al., 1999).

Human Disease Caused by *C. fetus*

Campylobacter fetus subsp. *fetus* infections in man have been well documented (Blaser, 1998). Infection is usually associated with systemic disease and is often reported in immunocompromised individuals. Clinical presentations include diarrhea, bacteremia, endocarditis, meningitis, arthritis, peritonitis, cellulitis, prosthetic infections, and even abortion (Blaser, 1998). The pathology of abortion resembles that previously observed in sheep (Simor et al., 1986). The recent development of a model of ovine abortion has enabled some understanding of the pathogenic mechanisms involved in systemic infections (Grogono-Thomas et al., 2000). Oral ingestion probably results in colonization of the intestinal tract, and if the organisms invade, portal bacteremia may result. In normal hosts, phagocytosis of the bacteria by the reticuloendothelial system in the liver may result in transient bacteremia. Immunocompromised individuals may be unable to prevent systemic bacteremia, which may lead to sepsis or the seeding of distant foci to produce at a secondary site disease that is often associated with a high mortality rate (Skirrow, 1990). Some have speculated that an ageing population combined with a global Acquired Immune Deficiency Syndrome epidemic could precipitate an increased incidence of *C. fetus* subsp. *fetus* infections (Blaser, 1998).

Bovine Infertility and Abortion

Campylobacter fetus subsp. *venerealis* is the causal agent of bovine venereal campylobacteriosis. The organism asymptomatically colonizes the epithelial crypts of the penis and prepuce of bulls. Infection is transferred to cows at coitus, where the primary sites of infection are the uterine glands. Mild endometritis results in infertility. Abortion can occur and usually happens in the fifth or sixth month of gestation. Infection is usually cleared from the uterine tubes and uterus presumably by the host immune response, but the organism can persist in the vagina for several years, during which time a new bull could be infected (Osburn and Hoskins, 1970; Corbeil et al., 1981). Unlike *C. fetus* subsp. *venerealis*, *C. fetus* subsp. *fetus* is commonly isolated from the bovine gut (Atabay and Corry, 1998). If bacteremia follows ingestion of *C. fetus* subsp *fetus* during the last three months of pregnancy, then abortion may follow; however, the organism is not venereally transmitted.

Ovine Abortion

Ovine abortion is a consequence of bacteremia following ingestion of material contaminated with *C. fetus* subsp. *fetus* during the last three months of pregnancy. Pathologic changes are limited to the uterus and its contents. The placenta is not retained, which is in contrast to cattle. Many of the pathological changes associated with *C. fetus* infection are not distinctive. However, the presence of pale necrotic foci in the enlarged and hemorrhagic livers of aborted fetuses is indicative (Gilmour, 1991; Grogono-Thomas et al., 2000). Extensive edema is often seen in the uterus, placentomes and chorion.

Pathogenicity of *C. fetus*

Myers et al. (1970) described the only well-defined virulence factor of *C. fetus* as a "rather loosely attached capsular envelope protein." This material has been characterized as a microcapsular protein existing in a complex with LPS (Pei et al., 1988). Recently this microcapsule has been shown to be a paracrystalline S-layer. The S-layer proteins confer serum-resistance by inhibiting binding of complement (C3b) to the cell surface, preventing the formation of the terminal C5–C9 membrane attack complex, and opsonization via the C3b cellular receptors (Blaser et al., 1988). Moreover, the S-layer covers the underlying immunogenic LPS layer (Fogg et al., 1990), rendering it inaccessible or undetectable by the host. These proteins are essential for the induction of ovine abortion (Grogono-Thomas et al., 2000).

The S-layer proteins are products of surface array protein (*sap*) genes. The *sap* locus apparently contains eight homologues (*sapA*, *A1–A7*) in type A strains and a parallel family in type B strains (Tummuru and Blaser, 1992; Fujita et al., 1995b). A single promotor that is invertible between flanking *sap* genes regulates *sap* gene expression (Tummuru and Blaser, 1992). The *sap* gene cassettes rearrange around this promotor by RecA-dependent reciprocal recombination, resulting in alternate expression (Dworkin and Blaser, 1996; Dworkin and Blaser, 1997a, 1997b). However, RecA-independent recombination can also occur (Ray et al., 2000).

The eight homologues of the *sap* gene appear to encode proteins of three different apparent molecular masses (97, 127 and 149 kDa) but with different antigenicities (Dubreuil et al., 1990; Wang et al., 1993). Experimental infections in sheep indicate that this variation occurs in vivo with time (Grogono-Thomas et al., 2000) and delays the onset of host immune response. Such

antigenic variation may provide a bacterial mechanism for survival in an immunologically hostile host environment and enable chronic infection.

Genetics

The complete genome of strain *C. jejuni* subsp. *jejuni* 11168 has been sequenced (Parkhill et al., 2000). Within the genome 1654 open reading frames (ORFs), 3 rRNA loci and 54 tRNA's were identified. Two regions with relatively low G+C content encoded genes for lipo-oligosaccharide (LOS) and extracellular polysaccharide (EP) biosynthesis clusters, respectively. A surprising finding was the presence of 23 polymorphic homonucleotide repeats (mainly G or C tracts) that varied in length within a locus. These interrupted ORFs in 14 cases and fused (or separated) 2 ORFs in 6 cases. Many of these hypervariable sites cluster in regions containing genes responsible for LOS biosynthesis, EP biosynthesis, and flagellar modification (Parkhill et al., 2000). Such hypervariable regions have since been detected experimentally in other strains of *C. jejuni* (Wassenaar et al., 2002).

The elucidation of the genome sequence has allowed the construction of *C. jejuni*-specific microarrays, printed either with complete ORFs (produced by gene-specific PCR; Pearson et al., 2003; Stinzi, 2003) or with the shot-gun clones used for the sequence analysis (Dorrell et al., 2001).

Unfortunately, the strain (NCTC11168) used for the genome sequence has an unusual phenotype in that it is nonmotile, has a straight and not a curved bacterial morphology, is poorly invasive, and has a low colonization capacity in chickens. Meanwhile, variants of this strain have been characterized that have a more typical phenotype (Carrillo et al., 2004; Gaynor et al., 2004). Transcriptome comparison of the original isolate and the genome sequence variant (Gaynor et al., 2004) has shown that metabolic adaptation to increased oxygen tension contributes to the changed phenotype, including differences in colonization potential, possibly in combination with mutations in sigma factors. Similar studies using two other variants indicate that the flagellar regulatory system of the sequenced variant of 11168 may be impaired (Carrillo et al., 2004).

The genome sequence indicated a relatively low number of operons in which flanking genes are involved in similar processes. Compared to other microbial genomes, that of *C. jejuni* seems more scrambled, although operons are not completely absent. The presence of only three sigma factors and few regulatory proteins makes coordinate gene expression a subject of interest. Gene expression during the transition of growth from 37°C to 42°C was investigated by microarray analysis (Stinzi, 2003). A transient global change in expression was detected for 20% of the genes. Although either temperature is permissive, a temporary down regulation of ribosomal genes, and an upregulation of chaperonins, suggests a temporary halt in growth. Interestingly, expression of genes involved in membrane structure displayed temperature-specific activity (Stinzi, 2003).

Previous laboratory observations that few *Campylobacter* genes are expressed well in *E. coli* and vice versa can be explained by the finding of an atypical -35 promoter region in front of *C. jejuni* genes (Peterson et al., 2003). The absence of this *C. jejuni*-specific promoter sequence in front of many genes with an intergenic upstream region <100 bp suggests that operon transcription may not be uncommon. However, such co-expressed genes may not have putative roles in common biochemical pathways as in *E. coli*.

With the first genome sequence available, the search has begun to identify the gene set characteristic of all isolates within the species *C. jejuni* (core gene complement) rather than genes that are more or less strain-specific. DNA micro arrays are now being extensively used to compare the genetic content of many strains (Dorrell et al., 2001; Pearson et al., 2003). These investigations suggest that at least 15% of the gene content can vary between strains, notably genes encoding surface structures. However, genes not present on the arrays, which still largely comprise the gene complement of strain 11168, cannot be detected. In contrast, the technique of subtractive hybridization can be used for strain comparison (Ahmed et al., 2002) and has been successfully used to identify novel genes in *C. jejuni*. The genome of *C. jejuni* RM1221, a strain with a more typical phenotype, is currently being annotated and will become available in future.

Literature Cited

AbuOun, M., G. Manning, S. A. Cawthraw, A. Ridley, I. Ahmed, T. M. Wassenaar and D. G. Newell. 2004. Cytolethal distending toxin (CDT) negative Campylobacter jejuni strains and anti-CDT antibodies induced during human infection. (in press).

Ahmed, I. H., G. Manning, T. M. Wassenaar, S. Cawthraw, and D. G. Newell. 2002. Identification of genetic differences between two Campylobacter jejuni strains with different colonization potentials. Microbiology 148: 1203–1212.

Alderton, M. R., V. Korolik, P. J. Coloe, F. J. Dewhirst, and B. J. Paster. 1995. Campylobacter hyoilei sp. nov., asso-

ciated with porcine proliferative enteritis. Int. J. Syst. Bacteriol. 4:61–66.

Aldomy, F. M. M. A. 1992. A Study of Perinatal Mortality in Small Ruminants in Jordan (PhD Thesis). University of London. London, UK.

Al Rashid, S., I. Dakuna, H. Louie, D. Ng, P. Vandamme, W. Johnson, and V. L. Chan. 2000. Identification of Campylobacter jejuni, C. coli, C. lari, C. upsaliensis, Arcobacter butzleri, and A. butzleri-like species based on the glyA gene. J. Clin. Microbiol. 38:1488–1494.

Anon. 1996. Veterinary Investigation Data Analysis. Veterinary Laboratories Agency. Weybridge, UK.

Atabay, H. I., and J. E. L. Corry. 1998. The isolation and prevalence of campylobacters from dairy cattle using a variety of methods. J. Appl. Microbiol. 84:733–740.

1994. Group 2: Aerobic/microaerophilic, motile, helical/vibroid Gram-negative bacteria. *In:* J. G. Holt, N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams (Eds.) Bergey's Manual of Determinative Bacteriology, 9th ed. Williams & Wilkins. Baltimore, MD. 39–63.

Black, R. E., M. M. Levine, M. L. Clements, M. M. Levine, and M. J. Blaser. 1992. Human volunteer studies with Campylobacter jejuni. *In:* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (Eds.)Campylobacter Jejuni: Current Status and Future Trends. American Society for Microbiology. Washington, DC. 207–215.

Blaser, M. J., G. Perez-Perez, P. F. Smith, C. Patton, F. C. Tenover, A. J. Lastovica, and W. L. Wang. 1986. Extraintestinal Campylobacter jejuni and Campylobacter coli infections: Host factors and strain characteristics. J. Infect. Dis. 153:552–559.

Blaser, M. J., P. F. Smith, J. E. Repine, and K. A. Joiner. 1988. Pathogenesis of Campylobacter fetus infections: Failure of encapsulated Campylobacter fetus to bind C3b explains serum and phagocytosis resistance. J. Clin. Invest. 81:1434–1444.

Blaser, M. J. 1998. Campylobacter fetus—emerging infection and model system for bacterial pathogenesis at mucosal surfaces. Clin. Infect. Dis. 27:256–258.

Boer, P., J. A. Wagenaar, R. P. Achterberg, J. P. van Putten, L. M. Schouls, and B. Duim. 2002. Generation of Campylobacter jejuni genetic diversity in vivo. Molec. Microbiol. 44:351–359.

Cappelier, J. M., J. Minet, C. Magras, R. R. Colwell, and M. Federighi. 1999. Recovery in embryonated eggs of viable but nonculturable Campylobacter jejuni cells and maintenance of ability to adhere to HeLa cells after resuscitation. Appl. Environ. Microbiol. 65:5154–5157.

Carrillo, C. D., E. Taboada, J. H. Nash, P. Lanthier, J. Kelly, P. Lau, R. Verhulp, O. Mykytczuk, J. Sy, W. Findlay, K. Amoako, S. Gomis, P. Willson, J. W. Austin, A. Potter, L. Babiuk, B. Allan, and C. M. Szymanski. 2004. Genome-wide expression analyses of campylobacter jejuni NCTC11168 reveals coordinate regulation of motility and virulence by flhA. J. Biol. Chem. 279:20327–20338.

Corbeil, L. B., G. G. Schurig, J. R. Duncan, B. N. Wilkie, andA. J. Winter. 1981. Immunity in the female bovine reproductive tract based on the response to "Campylobacter fetus." Adv. Exp. Med. Biol. 137:729–743.

Corry, J. E. L., D. E. Post, P. Colin, and M. J. Laisney. 1995. Culture media for the isolation of campylobacters. Int. J. Food Microbiol. 26:43–76.

Cowan and Steel. 1993. Characters of Gram-negative bacteria: Section 7.10. Cowan and Steel's Manual for the Identification of Medical Bacteria, 3rd ed. Cambridge University Press. Cambridge, UK. 158–160.

Dingle, K. E., F. M. Colles, R. Ure, J. A. Wagenaar, B. Duim, F. J. Bolton, A. J. Fox, D. R. Wareing, and M. C. Maiden. 2002. Molecular characterization of Campylobacter jejuni clones: A basis for epidemiologic investigation. Emerg. Infect. Dis. 8:949–955.

Doig, P., N. Kinsella, P. Guerry, and T. J. Trust. 1996a. Characterisation of a post-translational modification of Campylobacter flagellin: Identification of a sero-specific glycosyl moiety. Molec. Microbiol. 19:379–387.

Doig, P., R. Yao, D. H. Burr, P. Guerry, and T. J. Trust. 1996b. An environmentally regulated pilus-like appendage involved in Campylobacter pathogenesis. Molec. Microbiol. 20:885–894.

Dorrell, N., J. A. Mangan, K. G. Laing, J. Hinds, D. Linton, H. Al-Ghusein, B. G. Barrell, J. Parkhill, N. G. Stoker, A. V. Karlyshev, P. D. Butcher, and B. W. Wren. 2001. Whole genome comparison of Campylobacter jejuni human isolates using a low-cost microarray reveals extensive genetic diversity. Genome Res. 11:1706–1715.

Doyle, L. P. 1944. A vibrio associated with swine dysentery. Am. J. Vet. Res. 5:3–5.

Dubreuil, J. D., M. Kostrzynska, J. W. Austin, and T. J. Trust. 1990. Antigenic differences among Campylobacter fetus S-layer proteins. J. Bacteriol. 172:5035–5043.

Duim, B., P. A. Vandamme, A. Rigter, S. Laevens, J. R. Dijkstra, and J. A. Wagenaar. 2001. Differentiation of Campylobacter species by AFLP fingerprinting. Microbiology 147:2729–2737.

Dworkin, J., and M. J. Blaser. 1996. Generation of Campylobacter fetus S-layer protein diversity utilizes a single promoter on an invertible DNA segment. Molec. Microbiol. 19:1241–1253.

Dworkin, J., and M. J. Blaser. 1997a. Molecular mechanisms of Campylobacter fetus surface layer protein expression. Molec. Microbiol. 26:433–440.

Dworkin, J., O. L. Shedd, and M. J. Blaser. 1997b. Nested DNA inversion of Campylobacter fetus S-layer genes is recA dependent. J. Bacteriol. 179:7523–7529.

Endtz, H. P., C. W. Ang, N. van Den Braak, B. Duim, A. Rigter, L. J. Price, D. L. Woodward, F. G. Rodgers, W. M. Johnson, J. A. Wagenaar, B. C. Jacobs, H. A. Verbrugh, and A. van Belkum. 2000. Molecular characterization of Campylobacter jejuni from patients with Guillain-Barré and Miller Fisher syndromes. Clin. Microbiol. 38:2297–2301.

Etoh, Y., F. E. Dewhirst, B. J. Paster, A. Yamamoto, and N. Goto. 1993. Campylobacter showae sp. nov., isolated from the human oral cavity. Int. J. Syst. Bacteriol. 43:631–639.

Fearnley, C., R. Ayling, S. Cawthraw, and D. G. Newell. 1996. The formation of viable but nonculturable Campylobacter jejuni and their failure to colonise one-day-old chicks. *In:* D. G. Newell, J. M. Ketley, and R. A. Feldman (Eds.) Campylobacters, Helicobacters and Related Organisms. Plenum Press. London, UK. 101–104.

Fogg, G. C., L. Y. Yang, E. Wang, and M. J. Blaser. 1990. Surface array proteins of Campylobacter fetus block lectin-mediated binding to type A lipopolysaccharide. Infect. Immunol. 58:2738–2744.

Fouts, D. E., E. F. Mongodin, R. E. Mandrell, W. G. Miller, D. A. Rasko, J. Ravel, L. M. Brinkac, R. T. Deboy, C. T. Parker, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, S. A. Sullivan, J. U. Shetty, M. A. Ayodeji, A. Shvartsbeyn, M. C. Schatz, J. H. Badger, C. M. Fraser, and K. E. Nelson. 2005. Major structural differences and novel potential virulence mechanisms from the genomes

of multiple campylobacter species. PLoS Biol. 3:e15. Epub 2005 Jan 04.

Frost, J. A., A. N. Oza, R. T. Thwaites, and B. Rowe. 1998. Serotyping scheme for Campylobacter jejuni and Campylobacter coli based on direct agglutination of heat-stable antigens. J. Clin. Microbiol. 36:335–339.

Frost, J. A., J. M. Kramer, and S. A. Gillanders. 1999. Phage typing of Campylobacter jejuni and Campylobacter coli and its use as an adjunct to serotyping. Epidemiol. Infect. 123:47–55.

Fujita, M., S. Fujimoto, T. Morooka, and K. Amako. 1995a. Analysis of strains of Campylobacter fetus by pulsed-field gel electrophoresis. J. Clin. Microbiol. 33:1676–1678.

Fujita, M., T. Morooka, S. Fuijmoto, T. Moriya, and K. Amako. 1995b. Southern blotting analyses of strains of Campylobacter fetus using the conserved region of sapA. Arch. Microbiol. 164:444–447.

Garcia, M. M., M. D. Eaglesome, and C. Rigby. 1983. Campylobacters important in veterinary medicine. Vet. Bull. 53:793–818.

Gaynor, E. C., N. Ghori, and S. Falkow. 2001. Bile-induced "pili" in Campylobacter jejuni are bacteria-independent artifacts of the culture medium. Molec. Microbiol. 39:1546–1549.

Gaynor, E. C., S. Cawthraw, G. Manning, J. K. MacKichan, S. Falkow, and D. G. Newell. 2004. The genome-sequenced variant of Campylobacter jejuni NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. J. Bacteriol. 186:503–517.

Gebhart, C. J., G. E. Ward, K. Chang, and H. J. Kurtz. 1983. Campylobacter hyointestinalis (new species) isolated from swine with lesions of proliferative enteritis. Am. J. Vet. Res. 44:361–367.

Gilmour, N. J. L. 1991. Campylobacteriosis. *In:* W. B. Martin and I. D. Aitken (Eds.) Diseases of Sheep. Blackwell Scientific Publications. Oxford, UK. 80–82.

Goodwin, C. S., J. A. Armstrong, T. Chilvers, M. Peters, M. D. Collins, L. Sly, M. McConnell, and W. E. S. Harper. 1989. Transfer of Campylobacter pylori and Campylobacter mustelae to Helicobacter gen. nov. as Helicobacter pylori comb. nov, and Helicobacter mustelae comb. nov., respectively. Int. J. Syst. Bacteriol. 39:397–405.

Grajewski, B. A., J. W. Kusek, and H. M. Gelfand. 1985. Development of a bacteriophage typing system for Campylobacter jejuni and Campylobacter coli. J. Clin. Microbiol. 22:13–18.

Grogono-Thomas, R., J. Dworkin, M. J. Blaser, and D. G. Newell. 2000. Roles of the surface layer proteins of Campylobacter fetus subsp. fetus in ovine abortion. Infect. Immunol. 68:1687–1169.

Grogono-Thomas, R., M. J. Blaser, M. Ahmadi, and D. G. Newell. 2003. The role of S-layer protein antigenic diversity in the immune responses of sheep experimentally challenged with Campylobacter fetus subsp. fetus. Infect. Immun. 71:147–154.

Hansen, D. E., O. R. Hedstrom, R. J. Sonn, and S. P. Snyder. 1990. Efficacy of a vaccine to prevent Chlamydia- or Campylobacter-induced abortions in ewes. J. Am. Vet. Med. Assoc. 196:731–734.

Hum, S., K. Quinn, J. Brunner, and S. L. On. 1997. Evaluation of a PCR assay for identification and differentiation of Campylobacter fetus subspecies. Aust. Vet. J. 75:827–831.

Hunt, J. M., and C. Abeyta. 1995. Campylobacter. *In:* Food and Drug Administration (Eds.)Bacteriological Analytical Manual, 8th ed.. AOAC International. Gaithersburg, MD. 7.01–7.21.

International Organization for Standardization. 1995. Microbiology of Food and Animal Feeding Stuffs: Horizontal Method for Detection of Thermotolerant Campylobacter. International Organization for Standardization. Geneva, Switzerland. ISO 10727.

Jacobs-Reitsma, W.. 2000. Campylobacter in the food supply. *In:* I. Nachamkin and M. J. Blaser (Eds.)Campylobacter, 2nd ed. ASM Press. Washington, DC. 467–481.

Johnson, W. M., and H. Lior. 1988. A new heat-labile cytolethal distending toxin (CLDT) produced by Campylobacter spp. Microb. Pathog. 4:115–126.

Jones, F. S., and R. B. Little. 1931. The etiology of infectious diarrhea (winter scours) in cattle. J. Exp. Med. 53:835–851.

Jones, M. A., S. Totemeyer, D. J. Maskell, C. E. Bryant, and P. A. Barrow. 2003. Induction of proinflammatory responses in the human monocytic cell THP-1 by Campylobacter jejuni. Infect. Immun. 71:2626–2633.

Karlyshev, A. V., D. Linton, N. A. Gregson, A. Lastovica, and B. W. Wren. 2000. Genetic and biochemical evidence of a Campylobacter jejuni capsular polysaccharide that accounts for Penner serotype specificity. Molec. Microbiol. 35:529–541.

Khakhria, R., and H. Lior. 1992. Extended phage-typing scheme for Campylobacter jejuni and Campylobacter coli. Epidemiol. Infect. 108:403–414.

King, E. O. 1957. Human infections with Vibrio fetus and a closely related Vibrio. J. Infect. Dis. 101:119–128.

King, E. O. 1962. The laboratory recognition of Vibrio fetus and a closely related Vibrio isolated from cases of human vibriosis. Ann. NY Acad. Sci. 98:700–711.

Konkel, M. E., L. A. Joens, and P. F. Mixter. 2000. Molecular characterization of Campylobacter jejuni virulence determinants. *In:* I. Nachamkin and M. J. Blaser (Eds.) Campylobacter, 2nd ed. ASM Press. Washington, DC. 217–240.

Korolik V., L. Moorthy, and P. J. Coloe. 1995. Differentiation of Campylobacter jejuni and Campylobacter coli strains by using restriction endonuclease DNA profiles and DNA fragment polymorphisms. J. Clin. Microbiol. 33:1136–1140.

Kuroki, S., T. Saida, M. Nukina, et al. 1993. Campylobacter jejuni strains from patients with Guillain-Barré syndrome belong mostly to Penner serogroup 19 and contain N-acetylglucosamine residues. Ann. Neurol. 33:243–247.

Lastovica, A. J., E. A. Goddard, and A. C. Argent. 1997. Guillain-Barré syndrome in South Africa associated with Campylobacter jejuni O:41 strains. J. Infect. Dis. 176 (Suppl. 2):S139–S143.

Le Roux, E., and A. J. Lastovica. 1998. The Cape Town Protocol: How to isolate the most Calmpylobacters for your dollar, pound, franc, yen, etc. *In:* A. J. Lastovica, D. G. Newell, and E. E. Lastovica (Eds.) Campylobacter, Helicobacter and Related Organisms. Institute of Child Heath, University of Cape Town. Cape Town, South Africa. 30–33.

Levy, A. J. 1946. A gastroenteritis outbreak probably due to a bovine strain of Vibrio. Yale J. Biol. Med. 18:243–247.

Lindblom, G.-B., L.-E. Cervantes, E. Sjögren, B. Kaijser, and G. M. Ruiz-Palacios. 1990. Adherence, enterotoxigenicity, invasiveness and serogroups in Campylobacter jejuni

and Campylobacter coli strains from adult humans with acute enterocolitis. APMIS 98:179–184.

Lior, H., D. L. Woodward, J. A. Edgar, J. Laroche, and P. Gill. 1982. Serotyping of Calmpylobacter jejuni by slide agglutiantion based on heat-labile antigenic factors. J. Clin. Microbiol. 15:761–768.

Manning, G., C. G. Dowson, M. C. Bagnall, I. H. Ahmed, M. West, and D. G. Newell. 2003. Multilocus sequence typing for comparison of veterinary and human isolates of Campylobacter jejuni. Appl. Environ. Microbiol. 69:6370–6379.

Marsh, H., and B. D. Firehammer. 1953. Serological relationships of twenty-three ovine and three bovine strians of Vibrio fetus. Am. J. Vet. Res. 14:396–398.

Marshall, B. J., H. Royce, D. I. Annear, C. S. Goodwin, J. W. Pearman, J. R. Warren, and A. Armstrong. 1984. Original isolation of Campylobacter pyloridis from human gastric mucosa. Microbiol. Lett. 25:83–88.

McFadyean, J., and S. Stockman. 1913. Report of the Departmental Committee Appointed by the Board of Agriculture and Fisheries to Enquire into Epizootic Abortion. Part III: Abortion in Sheep. HMSO. London, UK. 1–64.

Meinersmann, R. J., C. M. Patton, G. M. Evins, I. K. Wachsmuth, and P. I. Fields. 2002. Genetic diversity and relationships of Campylobacter species and subspecies. Int. J. Syst. Evol. Microbiol. 52:1789–1797.

Melamed, I., Y. Bujanover, Y. S. Igra, D. Schwartz, V. Zakuth, and Z. Spirer. 1983. Campylobacter enteritis in normal and immunodeficient children. Am. J. Dis. Child. 137: 752–753.

Mellitis, K. H., J. Mullen, M. Wand, G. Armbruster, A. Patel, P. L. Connerton, M. Skelly, and I. F. Connerton. 2002. Activation of the transcription factor NF-kappaB by Campylobacter. Microbiology 148:2753–2763.

Moran, A. P. 1996. Biological and serological characterization of Campylobacter jejuni lipopolisaccharides with deviating core and lipid A structures. FEMS Immunol. Med. Microbiol. 11:121–130.

Moran, A. P., and J. L. Penner. 1999. Serotyping of Campylobacter jejuni based on heat-stable antigens: Relevance, molecular basis and implications in pathogenesis. J. Appl. Microbiol. 86:361–377.

Myers, L. L., R. I. Berg, and B. D. Firehammer. 1970. Immunoserologic properties of antigens from Vibrio fetus of ovine origin. Am. J. Vet. Res. 31:1773–1777.

Nachamkin, I., X. H. Yang, and N. J. Stern. 1993. Role of Campylobacter jejuni flagella as colonization factors for three-day-old chicks: Analysis with flagella mutants. Appl. Environ. Microbol. 59:1269–1273.

Nachamkin, I. 1995. Campylobacter and Arcobacter. *In:* P. R. Murray (Ed.) Manual of Clinical Microbiology, 6th ed. American Society for Microbiology. Washington, DC. 483–498.

Nachamkin, I., B. Mishu Allos, and T. Ho. 1998. Campylobacter species and Guillain-Barré syndrome. Clin. Microbiol. Rev. 11:555–567.

Newell, D. G., J. A. Frost, B. Duim, J. A. Wagenaar, R. H. Madden, J. van der Plas, and S. L. W. On. 2000. New developments in the subtyping of Campylobacter species. *In:* I. Nachamkin and M. J. Blaser (Eds.) Campylobacter, 2nd ed.. ASM Press. Washington DC, 27–44.

Newell, D. G. 2001. Animal models of Campylobacter jejuni colonisation and disease and the lessons to be learned from similar Helicobacter pylori models. J. Appl. Microbiol. 90:57S–67S.

Ng, L.-K., M. E. Stiles, and D. I. Taylor. 1987. Classification of Campylobacter strains using DNA probes. Molec. Cell Probes 1:233–243.

Nuijten, P. J. M., N. M. C. Bleumink-Pluym, W. Gaastra, and B. A. M. Van der Zeijst. 1989. Flagellin expression in Campylobacter jejuni is regulated at the transcriptional level. Infect. Immunol. 57:1084–1088.

Oelschlaeger, T. A., P. Guerry, and D. J. Kopecko. 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by Campylobacter jejuni and Citrobacter freundii. Proc. Natl. Acad. Sci. USA 90:6884–6888.

On, S. L. W., B. Bloch, B. Holmes, B. Hoste, and P. Vandamme. 1995. Campylobacter hyointestinalis subsp. lawsonii subsp. nov., isolated from the porcine stomach, and an emended description of Campylobacter hyointestinalis. Int. J. Syst. Bacteriol. 45:767–774.

On, S. L. W.. 1996. Identification methods for Campylobacters, Helicobacters, and related organisms. Clin. Microbiol. Rev. 9:405–422.

Osburn, B. I., and R. K. Hoskins. 1970. Experimentally induced Vibrio fetus var. intestinalis infection in pregnant cows. Am. J. Vet. Res. 1733–1741.

Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell. 2000. The genome sequence of the foodborne pathogen Campylobacter jejuni reveals hypervariable sequence. Nature 403:665–668.

Pavlovskis, O. R., D. M. Rollins, R. L. Haberberger, A. E. Green, L. Habash, S. Strocko, and R. I. Walker. 1991. Significance of flagella in colonization resistance of rabbits immunized with Campylobacter spp. Infect. Immunol. 59:2259–2264.

Pearson, B. M., C. Pin, J. Wright, K. I'Anson, T. Humphrey, and J. M. Wells. 2003. Comparative genome analysis of Campylobacter jejuni using whole genome DNA microarrays. FEBS Lett. 554:224–230.

Pei, Z., R. T. Ellison 3rd, R. V. Lewis, and M. J. Blaser. 1988. Purification and characterization of a family of high molecular weight surface-array proteins from Campylobacter fetus. J. Biol. Chem. 263:6416–6420.

Perez-Perez, G. I., D. N. Taylor, P. D. Echeverria, and M. J. Blaser. 1992. Lack of evidence of enterotoxin involvement in pathogenesis of Camulobacter diarrhea. *In:* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (Eds.) Campylobacter jejuni: Current Status and Future Trends. American Society for Microbiology. Washington, DC. 184–192.

Perlman, D. M., N. M. Ampel, R. B. Schifman, D. L. Cohn, C. M. Patton, M. L. Aguirre, W. L. Wang, and M. J. Blaser. 1988. Persistent Campylobacter jejuni infections in patients infected with the human immunodeficiency virus: Association with abnormal serological response to C. jejuni and emergence of erythromycin resistance during therapy. Ann. Int. Med. 108:540–546.

Petersen, L., T. S. Larsen, D. W. Ussery, S. L. On, and A. Krogh. 2003. RpoD promoters in Campylobacter jejuni exhibit a strong periodic signal instead of a -35 box. J. Molec. Biol. 326:1361–1372.

Pickett, C. L., E. C. Pesci, D. L. Cottle, G. Russell, A. N. Erdem, and H. Zeytin. 1996. Prevalence of cytolethal distending toxin production in Campylobacter jejuni and relatedness of Campylobacter sp. cdtB genes. Infect. Immunol. 64:2070–2078.

Ray, K., T. Zheng-Chao, R. Grogono-Thomas, D. G. Newell, S. A. Thompson, and M. J. Blaser. 2000. Campylobacter fetus inversion occurs in the absence of Rec A function. Infect. Immun. 68:5663–5667.

Roop, R. M., R. M. Smibert, J. L. Johnson, and N. R. Krieg. 1985a. Campylobacter mucosalis (Lawson, Leaver, Pettigrew, and Rowland 1981) comb. nov.: Emended description. Int. J. Syst. Bacteriol. 35:189–192.

Roop, R. M., R. M. Smibert, J. L. Johnson, and N. R. Krieg. 1985b. DNA homology studies of the catalase-negative campylobacters and "Campylobacter fecalis" an emended description of Campylobacter sputorum, and proposal of the neotype strain of Campylobacter sputorum. Can. J. Microbiol. 31:823–831.

Salama, S. M., F. J. Bolton, and D. N. Hutchinson. 1990. Application of a new phagetyping scheme to campylobacters isolated during outbreaks. Epidemiol. Infect. 104:405–411.

Salama, S. M., M. M. Garcia, and D. E. Taylor. 1992. Differentiation of the subspecies of Campylobacter fetus by genomic sizing. Int. J. Syst. Bacteriol. 42:446–450.

Sebald, M., and M. Veron. 1963. Teneur en bases de l'ADN et classification des vibrions. Ann. Inst. Pasteur 105:897–910.

Simor, A. E., M. A. Karmali, T. Jadavji, and M. Roscoe. 1986. Abortion and perinatal sepsis associated with Campylobacter infection. Rev. Infect. Dis. 8:397–402.

Skirrow, M. B. 1990. Campylobacter, Helicobacter and other motile cured Gram-negative rods. *In:* Topley and Wilson's Principles of Bacteriology, Virology and Immunity: Systematic Bacteriology, 8th Ed.. Hodder & Stoughton. London, UK. 531–550.

Skirrow, M. B. 1994. Diseases due to Campylobacter, Helicobacter and related bacteria. J. Comp. Pathol. 111:113–149.

Stanley, J., A. P. Burnens, D. Linton, S. L. W. On, M. Costas, and R. J. Owen. 1992. Campylobacter helveticus sp. nov., a new thermophilic species from domestic animals: Characterization and cloning of a species-specific DNA probe. J. Gen. Microbiol. 138:2293–2303.

Steinbrückner, B., G. Härter, K. Pelz, and M. Kist. 1999. Routine identification of Campylobacter jejuni and Campylobacter coli from human stool samples. FEMS Microbiol. Lett. 179:227–232.

Stintzi, A. 2003. Gene expression profile of Campylobacter jejuni in response to growth temperature variation. J. Bacteriol. 185:2009–2016.

Tauxe, R. V. 1992. Epidemiology of Campylobacter jejuni infections in the United States and other industrialized nations. *In:* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (Eds.) Campylobacter jejuni: Current Status and Future Trends. American Society of Microbiology. Washington DC, 9–10.

Tenover, F. C., L. Carlson, S. Barbagallo, and I. Nachamkin. 1990. DNA probe culture confirmation assay for identification of thermophilic Campylobacter species. J. Clin. Microbiol. 28:1284–1287.

Tenover, F. C., and C. L. Fennell. 1992. The genera Campylobacter and Helicobacter. In: A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 3488–3511.

Thunberg, R. L., T. T. Tran, and M. O. Walderhaug. 2000. Detection of thermophilic Campylobacter spp. in blood-free enriched samples of inoculated foods by the polymerase chain reaction. J. Food Prot. 63:299–303.

Tompkins, D. S., M. J. Hudson, H. R. Smith, R. P. Eglin, J. G. Wheeler, M. M. Brett, R. J. Owen, J. S. Brazier, P. Cumberland, V. King, and P. E. Cook. 1999. A study of infectious intestinal disease in England: Microbiological findings in cases and controls. Commun. Dis. Public Health 2:108–113.

Topley online. 2000. (http://www.topleyonline.com).

Totten, P. A., C. M. Patton, F. C. Tenover, T. J. Barett, W. E. Stamm, A. G. Steigerwalt, J. Y. Lin, K. K. Holmes, and D. J. Brenner. 1987. Prevalence and characterisation of hippurate-negative Campylobacter jejuni in King County, Washington. J. Clin. Microbiol. 25:1747–1752.

Trust, T. J., S. M. Logan, C. E. Gustafson, P. J. Romaniuk, N. W. Kim, V. L. Chan, M. A. Ragan, P. Guerry, and R. R. Gutell. 1994. Phylogenetic and molecular characterization of a 23S rRNA gene positions the genus Campylobacter in the epsilon subdivision of the Proteobacteria and shows that the presence of transcribed spacers is common in Campylobacter spp. J. Bacteriol. 176:4597–4609.

Tummuru, M. K., and M. J. Blaser. 1992. Characterization of the Campylobacter fetus sapA promoter: Evidence that the sapA promoter is deleted in spontaneous mutant strains. J. Bacteriol. 174:5916–5922.

Ursing, J. B., H. Lior, and R. J. Owen. 1994. Proposal of minimal standards for describing new species of the family Campylobacteraceae. Int. J. Syst. Bacteriol. 44:842–845.

Uyttendaele, M., R. Schukkink, B. van Gemen, and J. Debevere. 1994. Identification of Campylobacter jejuni, Campylobacter coli and Campylobacter lari by the nucleic acid amplification system NASBA. J. Appl. Bacteriol. 77:694–701.

Vandamme, P., and J. De Ley. 1991a. Proposal for a new family, Campylobacteraceae. Int. J. Syst. Bacteriol. 41:451–455.

Vandamme, P., E. Falsen, R. Rossau, B. Hoste, P. Segers, R. Tytgat, and J. De Ley. 1991b. Revision of Campylobacter, Helicobacter, and Wolinella taxonomy: Emendation of generic descriptions and proposal of Arcobacter gen. nov. Int. J. Syst. Bacteriol. 41:88–103.

Vandamme, P., M. I. Daneshvar, F. E. Dewhirst, B. J. Paster, K. Kersters, H. Goossens, and C. W. Moss. 1995. Chemotaxonomic analyses of Bacteroides gracilis and Bacteroides ureolyticus and reclassification of B. gracilis as Campylobacter gracilis comb. nov. Int. J. Syst. Bacteriol. 45:145–152.

Vandamme, P., L.-J. van Doorn, S. T. Al Rashid, W. G. V. Quint, J., J. van der Plas, V. L. Chan, and S. L. W. On. 1997. Camplylobacter hyoilei Alderton et al. 1995 and Campylobacter coli Veron and Chatelain 1973 are subjective synonyms. Int. J. Syst. Bacteriol. 47:1055–1060.

Vandamme, P. 2000. Taxonomy of the family Campylobacteraceae. *In:* I. Nachamkin and M. J. Blaser (Eds.) Campylobacter, 2nd ed. ASM Press. Washington, DC. 3–26.

Veron, M., and R. Chatelain. 1973. Taxonomic study of the genus Campylobacter Sebald and Veron and designation of the neotype strain for the type species Campylobacter fetus (Smith and Taylor) Sebald and Veron. Int. J. Syst. Bacteriol. 23:122–134.

Wagenaar, J. A., M. A. P. van Bergen, D. G. Newell, R. Grogono-Thomas, and B. Duim. 2000. Comparative study using AFLP-genotyping and phenotyping for dif-

ferentiation of Campylobacter fetus strains. Appl. Environ. Microbiol.

Waino, M., D. D. Bang, M. Lund, S. Nordentoft, J. S. Andersen, K. Pedersen, and M. Madsen. 2003. Identification of campylobacteria isolated from Danish broilers by phenotypic tests and species-specific PCR assays. J. Appl. Microbiol. 95:649–655.

Wang, Y., and D. E. Taylor. 1990. Natural transformation in Campylobacter species. J. Bacteriol. 172:949–955.

Wang, E., M. M. Garcia, M. S. Blake, Z. Pei, and M. J. Blaser. 1993. Shift in S-layer protein expression responsible for antigenic variation in Campylobacter fetus. J. Bacteriol. 175:4979–4984.

Wang, H., J. M. Farber, N. Malik, and G. Sanders. 1999. Improved PCR detection of Campylobacter jejuni from chicken rinses by a simple sample preparation procedure. Int. J. Food Microbiol. 52:39–45.

Wassenaar, T. M., B. A. van der Zeijst, R. Ayling, and D. G. Newell. 1993. Colonization of chicks by motility mutants of Campylobacter jejuni deomisntrates the importance of flagellin A expression. J. Gen. Microbiol. 139:1171–1175.

Wassenaar, T. M., N. M. C. Bleumink-Pluijm, D. G. Newell, P. J. M. Nuijten, and B. A. M. van der Zeijst. 1994. Differential flagellin expression in a flaAflaB+ mutant of Campylobacter jejuni. Infect. Immunol. 62:3901–3906.

Wassenaar, T. M., M. Engelskirchen, S. Park, and A. Lastovica. 1997. Uptake and killing of Campylobacter jejuni by human peripheral monocytes/macrophages. Med. Microbiol. Immunol. 186:139–144.

Wassenaar, T. M. 1998. Toxin production by Campylobacter spp. Rev. Clin. Microbiol. 10:466–476.

Wassenaar, T. M., B. Geilhausen, and D. G. Newell. 1998. Evidence of genomic instability in Campylobacter jejuni isolated from poultry. Appl. Environ. Microbiol. 64: 1816–1821.

Wassenaar, T. M., and M. J. Blaser. 1999. Pathophysiology of Campylobacter infections of humans. Microb. Infect. 1:1023–1033.

Wassenaar, T. M., and D. G. Newell. 2000. Genotyping of Campylobacter spp. Appl. Environ. Microbiol. 66:1–9.

Wassenaar, T. M., J. A. Wagenaar, A. Rigter, C. Fearnley, D. G. Newell, and B. Duim. 2002. Homonucleotide stretches in chromosomal DNA of Campylobacter jejuni display high frequency polymorphism as detected by direct PCR analysis. FEMS Microbiol. Lett. 212:77–85.

Whitehouse, C. A., P. B. Balbo, E. C. Pesci, D. L. Cottle, P. M. Mirabito, and C. L. Pickett. 1998. Campylobacter jejuni cytolethal distending toxin causes a G2-phase cell cycle block. Infect. Immunol. 66:1934–1940.

Wooldridge, K. G., P. H. Williams, and J. M. Ketley. 1996. Host signal transduction and endocytosis of Campylobacter jejuni. Microb. Pathogen. 21:299–305.

Zhu, J., R. J. Meinersmann, K. L. Hiett, and D. L. Evans. 1999. Apoptotic effect of outer-membrane proteins from Campylobacter jejuni on chicken lymphocytes. Curr. Microbiol. 38:244–249.

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CHAPTER 3.5.2

The Genus *Helicobacter*

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Historical Background

Spiral-shaped bacteria were first seen in human gastric mucosa early in the twentieth century (Krienitz, 1906) and were subsequently described by several investigators (reviewed in Marshall, 1989). Similar organisms were also seen in the gastric mucosa of dogs, cats and a variety of other animals (Rappin, 1881; Bizzozero, 1892; Salomon, 1896; Lim, 1920; Doenges, 1939). The significance of these organisms was alternately debated and forgotten until Marshall and Warren isolated a Gram-negative, microaerophilic bacterium from human gastric biopsies and proposed that the organism may be the cause of chronic gastritis and peptic ulcer disease (Marshall and Warren, 1984a). The bacterium was thought originally to be a member of the genus *Campylobacter* and named "*Campylobacter pyloridis*," later corrected to "*Campylobacter pylori*." Subsequent 16S rRNA sequence analysis showed that the distance between the true campylobacters and *C. pylori* was sufficient to exclude it from the *Campylobacter* genus (Romaniuk et al., 1987), and it was renamed "*Helicobacter pylori*" (Goodwin et al., 1989a), the first member of the new genus *Helicobacter*.

Description of the *Helicobacter* Genus

The *Helicobacter* genus consists of a group of organisms that colonize the mucus layer covering the epithelial surface of the gastrointestinal tract of humans and a variety of animal species. There are currently 6 validated *Helicobacter* species isolated from gastric tissue (Tables 1 and 3) and 16 validated enterohepatic species (Tables 2 and 3). Some *Helicobacter* species may be commonly (*Helicobacter aurati*) or occasionally (*Helicobacter bilis* and *Helicobacter muridarum*) isolated from both gastric and enterohepatic sites. There is, in addition, a growing list of candidate and unvalidated species (Tables 4 and 5).

Cellular Morphology and Ultrastructure

In general, *Helicobacter* spp. are spiral/helical-shaped, although variant forms include short or tapered rods, and they are motile by means of flagella (Fig. 1). These characteristics are thought to be advantageous for survival in their niche of gastrointestinal mucus. Ultrastructural studies reveal bacteria with a typical Gram-negative cell wall structure, i.e., outer and inner membranes separated by a 30-nm periplasmic space and a dense cytoplasm containing nucleoid material and ribosomes (Fig. 2). Electron-lucent areas and a "polar membrane," an additional electron-dense band 6–8 nm thick located 20 nm below the plasma membrane, are often seen at the terminal region of organisms (Lee and O'Rourke, 1993a; Figs. 2A, C–E). A similar membrane has been seen in other spiral-shaped bacteria, where it has been found to be an assembly of ATPase molecules and, as such, has been implicated in energy production for motility or cell wall synthesis (Murray and Birch-Andersen, 1963; Ferris et al., 1984; Brock and Murray, 1988). Such a membrane was not seen in *H. mustela*; instead, O'Rourke et al. identified a large electron dense area, 80 × 100 nm, adjacent to or below the flagella insertion point, and proposed this may perform a similar function to the polar membrane seen in the other helicobacters (O'Rourke et al., 1992; Fig. 2B). Another feature associated with energy supply for these organisms is intracellular granules, which in several instances have been identified as polyphosphate (Bode et al., 1993; Fig. 2F).

A number of cell surface structures have been identified on *H. pylori*, including urease (Bode et al., 1989; Hawtin et al., 1990), a GroEL homolog (Hp60K; Austin et al., 1992), and outer membrane proteins, including lipopolysaccharide, porins and proteins of unknown function (Doig and Trust, 1994). Electron microscopic studies have also revealed that the structure of the vacuolating cytotoxin (VacA) of *H. pylori* resembles that of the A–B toxins seen in a number of other bacteria (Lupetti et al., 1996; Cover et al., 1997).

Table 1. Gastric *Helicobacter* taxa.

Taxon	Natural host	Strain/clone	GenBank 16S rRNA accession no.	References
H. acinonychis	Cheetah	ATCC 51101^T	M88148	Eaton et al., 1993
H. bizzozeronii	Dog	CCUG 35545^T	Y09404	Hanninen et al., 1996
H. felis	Cat, dog	ATCC 49179^T	M37642	Paster et al., 1991
H. mustelae	Ferret	ATCC 43772^T	M35048	Fox et al., 1988 Goodwin et al., 1989
H. nemestrinae[a]	Pigtailed macaque	ATCC 49396^T	X67854	Bronsdon et al., 1991
H. pylori	Human, rhesus macaque	ATCC 43505^T	M88157	Goodwin et al., 1989
H. salomonis	Dog	CCUG 37845^T	U89351	Jalava et al., 1997

Abbreviations: ATCC, American Type Culture Collection; ^{T}, type strain; and CCUG, Culture Collection, University of Göteborg, Dept. of Clinical Bacteriology, Göteborg, Sweden.
[a]*H. nemestrinae* has now been classified as a junior heterotypic synonym of *H. pylori* (see text; Suerbaum et al., 2002).

Table 2. Enterohepatic *Helicobacter* taxa.

Taxon	Natural host	Strain	GenBank 16S rRNA accession no.	References
H. aurati	Hamster	ATCC BAA-1^T	AF297868	Patterson et al., 2000
H. bilis	Mice, dog, and human	ATCC 51630^T	U18766	Fox et al., 1995
H. canadensis	Human	ATCC 700968^T	AF262037	Fox et al., 2000
H. canis	Dog, human	ATCC 51401^T	L13464	Stanley et al., 1993
H. cholecystus	Hamster	ATCC 700242^T	U46129	Franklin et al., 1996
H. cinaedi	Human, hamster, and rhesus monkey	CCUG 18818^T	M88150	Totten et al., 1985 Vandamme et al., 1991
H. fennelliae	Human	ATCC 35684^T	M88154	Totten et al., 1985 Vandamme et al., 1991
H. ganmani	Mice	CCUG 43526^T	AF000221	Robertson et al., 2001
H. hepaticus	Mice	ATCC 51448^T	U07574	Fox et al., 1994
H. mesocricetorum	Hamster	ATCC 700932^T	AF072471	Simmons et al., 2000
H. muridarum	Mice, rat	ATCC 49282^T	M80205	Lee et al., 1992
H. pametensis	Bird, swine	ATCC 51478^T	M88147	Dewhirst et al., 1994
H. pullorum	Chicken, human	ATCC 51801^T	L36141	Stanley et al., 1994
H. rodentium	Mice	ATCC 700285^T	U96296	Shen et al., 1997
H. trogontum	Rat	ATCC 700114^T	U65103	Mendes et al., 1996
H. typhlonius	Mice	MU96-1^T	AF061104	Fox et al., 1999 Franklin et al., 1999

Abbreviations: Please refer to footnote in Table 1.

All helicobacters possess flagella, which in most cases are sheathed, with configurations ranging from single polar flagella to bipolar tufts of up to 20 flagella. The flagella of *H. pylori* are 30 nm in diameter with an internal filament (~12 nm in diameter) surrounded by a sheath whose outer membrane is continuous with the outer membrane of the cell (Goodwin et al., 1985; Jones et al., 1985). Periplasmic fibers, located just below the outer membrane in the periplasmic space, have been reported in a number of *Helicobacter* species (On et al., 2002). They are distinct from the flagella and have a striated appearance due to electron dense banding. It has been suggested they are contractile proteins involved in motility (Lee and O'Rourke, 1993a).

Methods for Distinguishing *Helicobacter* Species

Classical Phenotypic Characteristics

General biochemical reactivity, morphology and growth characteristics are listed in Tables 3 and 5. The basic biochemical tests used for the identification and differentiation of all *Campylobacter*-like organisms are generally used to identify *Helicobacter* strains. The lack of application of highly standardized procedures and the well-known biochemical inertness of *Campylobacter*-like organisms render biochemical identification of all of these bacteria very difficult. There are no biochemical characteristics to separate members of the genus *Helicobacter*

Table 3. Characteristics of cultivated and validated *Helicobacter* species.

Taxon	Catalase production	Nitrate reduction	Alkaline phosphatase hydrolysis	Urease	Indoxyl acetate hydrolysis	γ-Glutamyl transferase	Growth at 42°C	Growth with 1% glycine	Susceptibility to: Nalidixic acid (30-µg disc)	Susceptibility to: Cephalothin (30-µg disc)	Periplasmic fibers	No. of flagella	Distribution of flagella	G+C content (mol%)
Gastric:														
H. acinonychis	+	–	+	+	–	+	–	–	R	S	–	2–5	Bipolar	30
H. bizzozeronii	+	+	+	+	+	+	+	–	R	S	–	10–20	Bipolar	ND
H. felis	+	+	+	+	–	+	+	–	R	S	+	14–20	Bipolar	42
H. mustelae	+	+	+	+	+	+	+	–	S	R	–	4–8	Peritrichous	36–41
H. nemestrinae[a]	+	–	+	+	–	ND	+	–	R	S	–	4–8	Bipolar	24
H. pylori	+	–	+	+	–	+	–	–	R	S	–	4–8	Bipolar	37–39
H. salomonis	+	+	+	+	+	+	–	ND	R	S	–	10–23	Bipolar	ND
Enterohepatic:														
H. aurati	+	–	–	+	+	+	+	–	S	R	+	7–10	Bipolar	ND
H. bilis	+	+	ND	+	–	ND	+	V	R	R	+	3–14	Bipolar	ND
H. canadensis	+	V	–	–	+	–	+	+	R	R	–	1–2	Bipolar	ND
H. canis	–	–	+	–	+	ND	+	–	S	I	–	2	Bipolar	48
H. cholecystus	+	+	+	–	–	–	+	+	I	R	–	1	Monopolar	ND
H. cinaedi	+	+	v	–	–	–	V	+	V	V	–	1–2	Bipolar	37–38
H. fennelliae	+	–	v	–	+	–	V	+	S	S	–	2	Bipolar	37–38
H. ganmani	V	+	–	–	–	ND	–	–	S	R	–	2	Bipolar	ND
H. hepaticus	+	+	ND	+	+	ND	–	+	R	R	–	2	Bipolar	ND
H. mesocricetorum	+	+	+	–	ND	–	+	–	S	R	–	2	Bipolar	ND
H. muridarum	+	–	+	+	+	+	–	–	R	R	+	10–14	Bipolar	34
H. pametensis	+	+	+	–	–	–	+	+	S	S	–	2	Bipolar	38
H. pullorum	+	+	–	–	–	ND	+	–	S	R	–	1	Monopolar	34–35
H. rodentium	+	+	–	–	–	–	+	+	R	R	–	2	Bipolar	ND
H. trogontum	+	+	–	+	ND	+	+	–	R	R	+	5–7	Bipolar	ND
H. typhlonius	+	+	–	–	–	–	–	+	ND	ND	–	2	Bipolar	ND

Symbols and abbreviations: +, positive reaction; –, negative reaction; S, susceptible; R, resistant; I, intermediate; ND, not determined; and V, variable.
[a]Unpublished data suggest that *H. nemestrinae* may be identical to *H. pylori* (see text).

Table 4. Candidate and unvalidated taxa.

Taxon	Natural host	Strain/clone	GenBank 16S rRNA accession no.	References
"*Candidatus* Helicobacter bovis"	Cattle	Clone R2XA	AF127027	DeGroote et al., 1999b
"*Helicobacter* sp."				
Bird-B	Terns	CCUG 29256	M88139	Seymour et al., 1994
Bird-C	House sparrow	CCUG 29261	M88144	
"*Helicobacter* sp. CLO-3"	Human	CCUG 14564	M88151	Fennell et al., 1984
"*H. colifelis*"	Cat	NA	AF142062	Foley et al., 1998
"*Helicobacter* sp. Cotton-top"	Cotton-top tamarin	MIT 97-6194-5	AF107494	Saunders et al., 1999
"*Helicobacter cetorum*"	Dolphin	MIT 99-5657	AF292377	Harper et al., 2000
"*Helicobacter* sp. flexispira" (taxon 5)	Sheep, dog, human, and mouse	ATCC 43966	M88137	Dewhirst et al., 2000 Hanninen et al., 2001 Kirkbride et al., 1985
"*H. heilmannii*"	Human			
type 1		Clone G1A1	L10079	Solnick et al., 1993
type 2		Clone G2A9	L10080	
"*Helicobacter* sp. rhesus"	Rhesus macaque			
type 1		MIT 99-5501	AF333338	Fox et al., 2001
type 2		MIT 99-5507	AF333340	
"*Candidatus* Helicobacter suis"	Swine	Clone V2BXA	AF127028	De Groote et al., 1999a
"*H. suncus*"	House musk shrew	Kaz-1	AB006147	Goto et al., 1998
"*H. winghamensis*"	Human	NLEP 97-1090	AF246984	Melito et al., 2001
"*H. muricola*"	Wild mouse	w-06^T	AF264783	
"*H. marmotae*"	Woodchuck	MIT 98-6070^T	AF333341	

Abbreviations: MIT, Massachusetts Institue of Technology; NA, not available; NLEP, National Laboratory for Enteric Pathogens; and for all other abbreviations, see footnote in Table 1.

[a]"*H. heilmannii*" 1 and *Candidatus* H. suis are probably identical. Bacteria with the 16S rRNA sequence present in "*H. heilmannii*" 1 have to date been identified in humans, pigs, and nonhuman primates (O'Rourke et al., 2001a).

from the genus *Campylobacter*. (*Arcobacter* strains can be differentiated from campylobacters and helicobacters by their ability to grow in air and at low temperatures.) Therefore, genus level identification is only achieved through the identification of strains at the species level. As a consequence, one has to differentiate over 35 validly named species and subspecies and various unnamed taxa. It should be mentioned that minimal standards for the description of new *Helicobacter* species have been described (Dewhirst et al., 2000b). These minimal standards outline tests (biochemical tests and others) and test procedures that are recommended for the description and hence differentiation of helicobacters.

One of the remarkable features of helicobacters is the extensive variability in cellular morphology (see above). It should be emphasized that although morphology may be helpful to guide the identification process, many species are morphologically indistinguishable. In this context it is worth mentioning two morphologically defined *Helicobacter* taxa: "*Flexispira rappini*" and "*Gastrospirillum*." The former is a provisional name given to Gram-negative, microaerobic, motile, spindle-shaped bacteria with spiral periplasmic fibers and bipolar tufts of sheathed flagella (see below). Strains with this distinctive morphology have been isolated from various sources and belong to the genus *Helicobacter* by phylogenetic analysis. They share their unusual morphological features with several named *Helicobacter* species, including *H. bilis*, *H. trogontum* and *H. aurati*. Extensive polymorphism in 16S rRNA gene sequences of "*Flexispira rappini*" strains and substantial taxonomic diversity among strains with this distinctive morphotype have been described (Dewhirst et al., 2000a). A recent analysis has shown that "*F. rappini*" phylotypes 1, 4 and 5 represent the same species, for which the name "*Helicobacter rappini*" has been proposed (Hänninen et al., 2001).

Similar nomenclatural and taxonomic problems exist for the group of gastric bacteria known as "gastrospirilla" or "*H. heilmannii*." These organisms too are primarily characterized by their cell morphology (large, tightly coiled rods without periplasmic fibers) and such cells have been observed in gastric biopsies of many hosts including humans, cats, dogs, pigs, monkeys, rats, and various captive exotic carnivores. Gastrospirilla are also characterized by their extremely fastidious growth requirements. The uncertainty concerning their taxonomic status is a result of the inability of investigators to culture the strains in vitro. The taxonomy of these gastrospirilla is discussed in detail below.

Table 5. Characteristics of cultivated but unvalidated *Helicobacter* taxa.

Taxon	Catalase production	Nitrate reduction	Alkaline phosphatase hydrolysis	Urease	Indoxyl acetate hydrolysis	γ-Glutamyl transferase	Growth at 42°C	Growth with 1% glycine	Susceptibility to: Nalidixic acid (30-μg disc)	Susceptibility to: Cephalothin (30-μg disc)	Periplasmic fibers	No. of flagella	Distribution of flagella	G+C content (mol%)
Gastric:														
"*H. suncus*"	+	+	+	+	–	–	ND	ND	R	R	–	2	Bipolar	ND
Enterohepatic:														
"*Helicobacter* sp. Bird-B"	+	+	+	+	+	–	+	+	S	R	–	2	Bipolar	31
"*Helicobacter* sp. Bird-C"	+	+	+	+	+	–	+	+	MS	R	–	2	Bipolar	30
"*Helicobacter* sp. CLO-3"	+	–	+	–	+	–	+	+	S	R	–	1	Polar	45
"*Helicobacter* sp. Cotton top"	+	–	–	–	–	–	+	+	R	R	+	6–12	Bipolar	ND
"*Helicobacter cetorum*"	+	–	–	+	–	+	+	+	S	S	–	>2	Bipolar	ND
"*Helicobacter* sp. flexispira" 5	+	–	–	+	–	+	+	–	R	R	+	10–20	Bipolar	ND
"*Helicobacter* sp. rhesus" type 1	+	–	–	–	–	–	+	ND	R	R	–	2	Bipolar	ND
"*Helicobacter* sp. rhesus" type 2	+	+	–	–	–	–	+	ND	R	S	–	2	Bipolar	ND
"*Helicobacter winghamenis*"	–	–	–	–	+	ND	–	ND	V	V	–	1–2	Bipolar	ND
"*Helicobacter muricola*"	+	+	–	+	–	–	–	–	S	R	–	2	Bipolar	ND
"*Helicobacter marmotae*"	+	–	+	+	–	–	–	+	R	R	–	2	Bipolar	ND

Symbols and abbreviations: +, positive reaction; –, negative reaction; S, susceptible; MS, marked sensitivity; R, resistant; I, intermediate; ND, not determined; and V, variable.

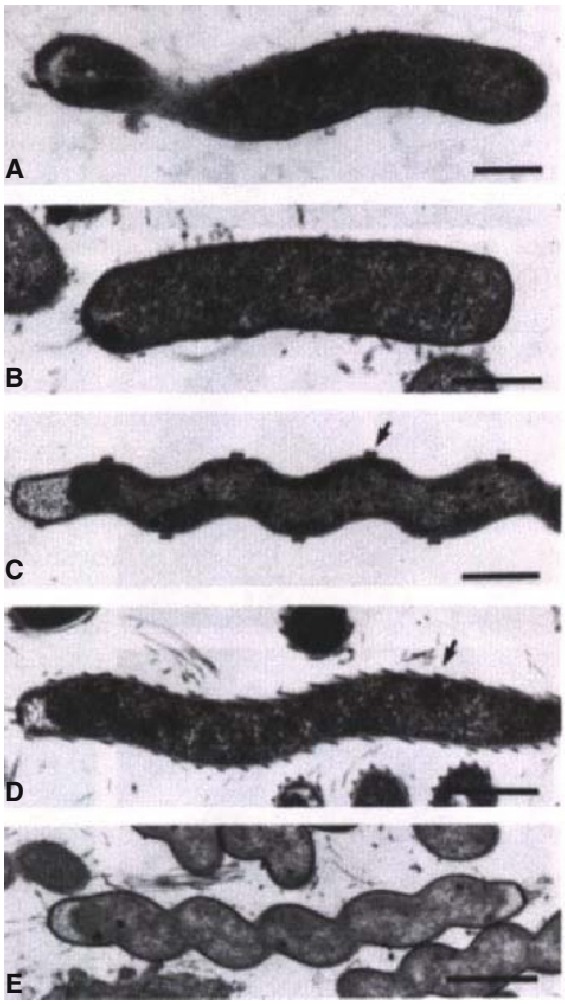

Fig. 1. Transmission electron micrographs of thin sections of *Helicobacter* species showing detail of their morphology. A) *H. pylori*: S-shaped with 2–3 turns, B) *H. mustelae*: straight or curved rods with lateral and polar flagella, C) *H. felis*: tight helical shape with 5–7 turns and periplasmic fibers entwining the cell singularly or in pairs (arrow), D) *H. muridarum*: S-shaped with 2–3 turns and periplasmic fibers entwining the whole cell (arrow), and E) "*H. heilmannii*": tight helical shape with 5–7 turns. Bars = 0.5 μm. (Reproduced with permission from Lee and O'Rourke, 1993a.)

DNA-DNA Hybridization

Whole-genomic DNA-DNA hybridization is the major cornerstone of bacterial species determination in current taxonomic practice (Wayne et al., 1987). However, the method is not widely used because it is not easily implemented. The fastidious growth characteristics of many *Helicobacter* species are a serious hindrance for the isolation of sufficient quantities of highly purified high-molecular-weight DNA required for these hybridization experiments. Therefore, relatively few DNA-DNA hybridization studies have been performed among *Helicobacter* species. Significant levels of DNA-DNA hybridization have been reported between 1) *H. pylori* and *H. mustelae* (Fox et al., 1989); 2) *H. cinaedi*, *H. fennelliae* and *H. canis* (Stanley et al., 1993); and 3) *H. felis*, *H. bizzozeronii* and *H. salomonis* (Jalava et al., 1997). Most other species have not been included in quantitative DNA-DNA hybridization studies.

Chemotaxonomic Methods

It is obviously not practical to implement DNA-DNA hybridization in a routine laboratory or to use it for routine identification in a reference laboratory. The need for an alternative but equivalent method for species delineation prompted researchers in the 1980s and 1990s to evaluate the discriminatory power of chemotaxonomic methods, including whole-cell protein and fatty acid analyses.

The comparison of whole-cell protein profiles obtained by highly standardized sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) has proven to be extremely reliable to screen and identify large numbers of strains. Numerous studies revealed a correlation between high similarity in whole-cell protein content and level of DNA-DNA hybridization (Vandamme et al., 1996). PAGE of whole-cell proteins has been a most successful method for the differentiation of helicobacters (Vandamme et al., 1990; Vandamme et al., 1991b; Vandamme et al., 2000; Costas et al., 1993; van der Ven et al., 1996; Jalava et al., 1998; Sorlin et al., 1999), and strains of all *Helicobacter* species are readily distinguished by means of simple numerical analysis. However, this method is not appropriate for routine identification studies because it is laborious, time-consuming and technically demanding to run the patterns in a sufficiently standardized way.

The total cellular fatty acid methyl ester (FAME) composition is a stable parameter, provided cells are grown in highly standardized conditions. The methylated fatty acids are typically separated by gas-liquid chromatography, and both the occurrence and the relative amounts characterize the fatty acid profiles. The procedure is cheap, rapid, and has reached a high degree of automatization as developed by Microbial Identification Systems (MIS, Newark, Delaware, United States). Isolates can be identified by using libraries supplied by the manufacturer. Only a few of the presently known *Helicobacter* species have been included in published FAME studies, but the results are very promising (Lambert et al., 1987; Goodwin et al., 1989b). *Helicobacter cinaedi*, *H. fennelliae*, *H. pylori*, *H. mustelae* and *H. nemestrinae* were readily distinguished (Lambert et al., 1987; Goodwin et al., 1989b).

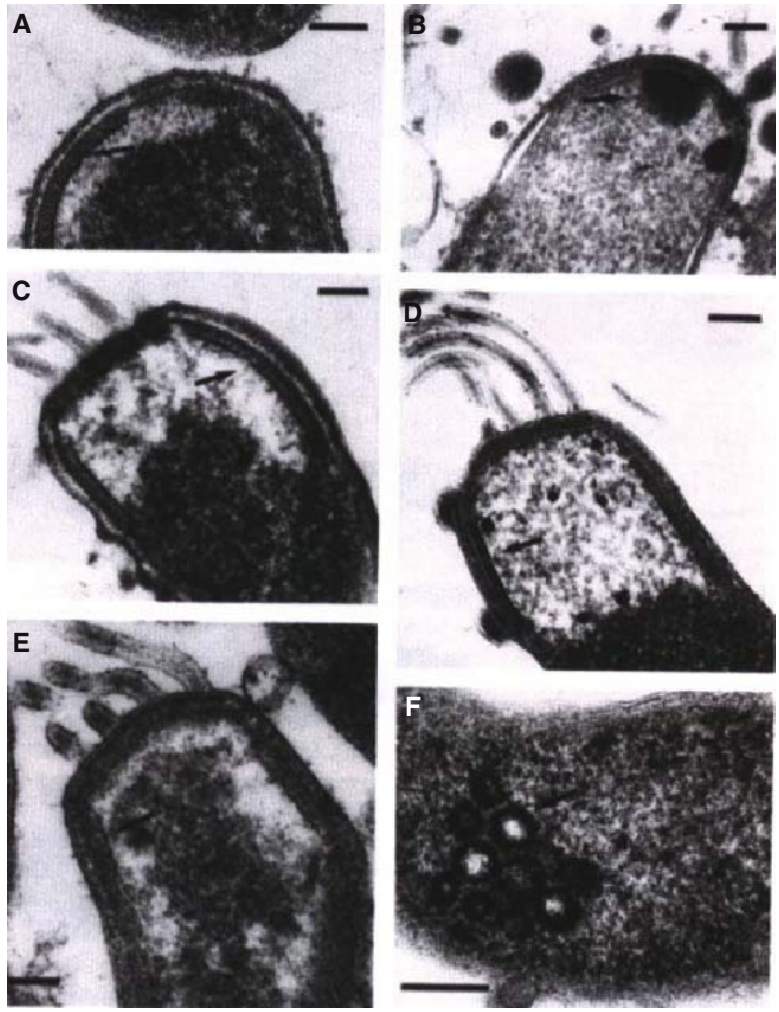

Fig. 2. Transmission electron micrographs of thin sections of *Helicobacter* species showing their cell wall and internal appearance. A) *H. pylori*, B) *H. mustelae*, C) *H. muridarum*, D) and F) *H. felis* and E) "*H. heilmanni*." All organisms exhibit the typical cell wall structure of Gram-negative bacteria with a "polar membrane" visible (arrow) on those organisms with polar flagella only (A, C, D and E). Large electron-dense bodies are noted beneath the periplasmic membrane, near flagella insertion points in *H. mustelae* (B, arrow). An example of intracellular granules occasionally noted in some preparations is shown in F. Bars = 0.1 µm. (Reproduced with permission from Lee and O'Rourke [1993a] and Fox et al. [1986].)

Sequence Analysis of the Small Subunit rDNA

Comparison of (near) entire 16S rDNA sequences is one of the most powerful procedures to establish the phylogenetic neighborhood of an unknown organism. Commercial identification services based on sequence analysis of ribosomal RNA genes have become readily available. With organisms that are otherwise difficult to identify, such as helicobacters, it has become common practice to determine the 16S rDNA sequence (or a fragment of it) of isolates that are of special interest and to compare this sequence with sequences available in public databases. Many taxonomic studies have revealed that this approach is often not sensitive enough to identify strains to the species level. The reported intraspecies diversity in 16S rDNA sequences of *Campylobacter hyointestinalis* and *H. cinaedi* was up to 4.5% of the total 16S rDNA sequence, which is exceptionally high (Harrington and On, 1999; Vandamme et al., 2000). Similar diversity was seen among phylotypes 1, 4 and 5 strains of "*F. rappini*," which were shown to represent a single species (Hänninen et al., 2001). In phylogenetic trees, strains of such species may cluster far apart and erroneously suggest that they represent different species.

On the other hand, 16S rDNA sequences may be too conserved to reveal diversity among species. This has been documented in many groups of bacteria. In *Helicobacter*, this is illustrated by comparison of 16S rDNA gene sequences of multiple *H. felis*, *H. bizzozeronii* and *H. salomonis* strains (Jalava et al., 1997). Although DNA-DNA hybridization studies demonstrated unambiguously that these three taxa represent three distinct species, 16S rDNA sequence analysis showed that all three species have virtually identical 16S rDNA genes. An example of a phylogenetic tree derived from the 16S rDNA sequences of a number of the *Helicobacter* species is shown in Fig. 3.

It should be clear that identification strategies by means of whole-cell protein or fatty acid analysis, extended biochemical testing, or restriction

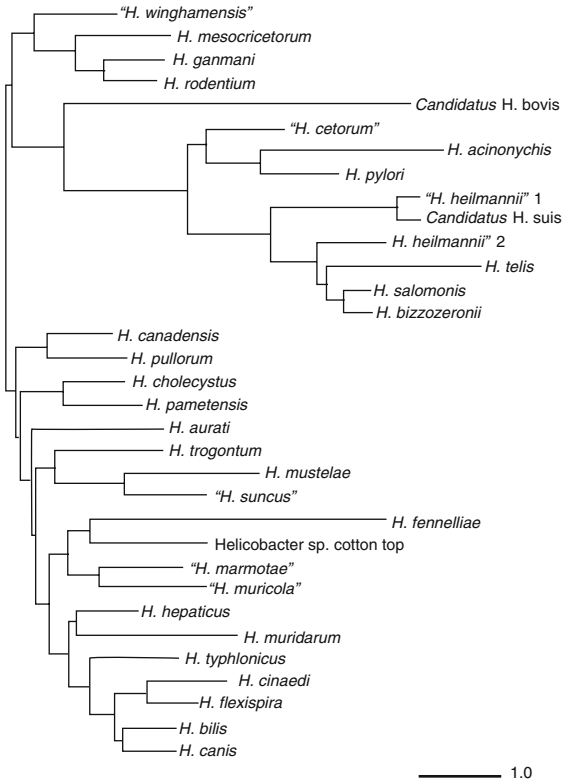

Fig. 3. Phylogenetic tree for 22 validated *Helicobacter* species and for provisional species selected on the basis of 16S rRNA sequences. Sequences from strains shown in Tables 1 and 2, and selected sequences from Table 4, were aligned with PILEUP and compared with DISTANCES (Wisconsin Sequence Analysis Package; Genetics Computer Group). Only sequence positions for which data were available for at least 90% of strains were included in the analysis (*E. coli* positions 28 to 1473). Intervening sequences were removed from *H. bilis*, *H. typhlonicus*, and *Helicobacter* sp. Cotton top. Percentages of difference were corrected for multiple base changes by the method of Jukes and Cantor. The tree was constructed from the distances matrix using TREEVIEW (Page, 1996). The scale bar represents a 1% difference in nucleotide sequence as determined by measuring the lengths of horizontal lines connecting any two species.

profile analysis of polymerase chain reaction (PCR) amplicons derived from conserved genes (see below) should be considered to endorse tentative identification results obtained by comparison of complete 16S rDNA genes. As useful as the 16S rDNA sequence analysis method is, present data clearly indicate that it cannot be regarded as the "gold standard" for species-level identification of helicobacters and other epsilon Proteobacteria.

Molecular Diagnostics

The information content of the rRNA cistrons and other genomic information has been used in several alternative ways for the identification of bacteria by the development of a range of DNA or RNA probes and amplification assays. Although the overall rDNA sequence similarity may be very high, the presence of variable regions in 16S or 23S RNA genes can provide the basis for specific and sensitive targets for identification procedures.

A range of specific oligonucleotide probes and PCR assays have been described for *H. pylori* (On, 1996) and several other *Helicobacter* species including *H. pametensis*, the *Helicobacter* Bird-B and Bird-C groups, *H. felis*, *H. hepaticus*, *H. pullorum*, *H. bilis*, *H. trogontum*, *H. canis*, "*Candidatus H. suis*" and "*Candidatus* H. bovis" (Stanley et al., 1993; Stanley et al., 1994; Dewhirst et al., 1994; Battles et al., 1995; Fox et al., 1995; Fox et al., 1998b; Mendes et al., 1996a; Germani et al., 1997; De Groote et al., 1999a; De Groote et al., 2000; Roosendaal et al., 2000; Shen et al., 2000; Choi et al., 2001; Ge et al., 2001). It should be stressed that because of the constant developments in the taxonomy of *Helicobacter*, the specificity and sensitivity of these probes and PCR assays have not been fully evaluated against all species described to date. Alternatively, polymorphisms can also be revealed by restriction profile analysis of PCR amplicons derived from conserved genes such as 16S and 23S rRNA genes. Restriction fragment length polymorphism (RFLP) analysis of 23S rRNA genes (Hurtado and Owen, 1997) was shown to differentiate 13 *Helicobacter* species (including "*F. rappini*") but not *H. felis*, *H. bizzozeronii* and *H. salomonis* (Jalava et al., 1999b). RFLP analysis of 16S rRNA genes was successfully used by Shen et al. (Shen et al., 2000; Shen et al., 2001) to differentiate strains from 10 enterohepatic species. ("*Flexispira rappini*" strains could not be differentiated from *H. bilis* strains.) The same workers reported on preliminary results of gastric species, indicating that the same approach was useful for the discrimination of some gastric species as well. Riley et al. (1996) used a similar approach, but amplified a rather small (374 bp) portion of the 16S rDNA to examine rodent isolates of *H. hepaticus*, *H. bilis* and *H. muridarum.* Several other studies have focused on rDNA-RFLP analysis of clinical isolates, but did not include balanced sets of reference strains of established *Helicobacter* species. Recently, O'Rourke et al. (2001a) have highlighted the possibility of differentiating between closely related species on the basis of analysis of a protein-encoding gene. Sequence analysis of a section of the urease gene complex enabled these workers to differentiate between the closely related gastric species *H. felis*, *H. bizzozeronii*, *H. salomonis* and "*H. heilmannii*."

Isolation, Culture and Preservation

Specimens

Gastric *Helicobacter* species are most commonly isolated by culture of endoscopic biopsies from the antral portion of gastric mucosa. For human patients, recent administration of antibiotics, including bismuth compounds, will decrease the yield of culture. Under conditions of acid suppression, such as in persons treated with proton pump inhibitors or H2 antagonists, *H. pylori* culture may also be less sensitive, and the bacteria may be found in larger numbers in the gastric corpus. Infection is usually spotty, so several biopsies must be obtained for optimal results. Cultivation of gastric juice by aspiration or through use of the string test (Torres et al., 2001) may yield positive results, but usually with lower sensitivity. *Helicobacter pylori* may also be cultivated from other sites within the gastrointestinal tract, such as areas of gastric metaplasia in the duodenum and occasionally from other sites of ectopic gastric mucosa. Recent evidence suggests that *H. pylori* may be found in large numbers in vomitus from infected persons, which may serve as a route for transmission (Parsonnet et al., 1999). Although gastric *Helicobacter* species have typically not been cultivated from feces, relative achlorhydria (Fox et al., 1992) and rapid transit times may favor positive fecal cultures (Parsonnet et al., 1999). *Helicobacter pylori* has occasionally been identified from human liver tissue by PCR, with one recent report of a positive culture (Nilsson et al., 2000; Queiroz and Santos, 2001).

The lower bowel is the natural reservoir of enterohepatic *Helicobacter* species, which are most commonly cultivated from feces or mucosal biopsies from the colon or cecum; however, isolation from liver and gallbladder is also common. Occasionally enterohepatic *Helicobacter* species may migrate to the stomach (Lee et al., 1993b) or cause bacteremia, particularly in persons infected with the human immunodeficiency virus or other immunocompromised hosts (Mammen et al., 1995; Weir et al., 1999; Cuccherini et al., 2000).

Transport and Sample Preparation

Gastric biopsies may be transported immediately to the laboratory with or without media such as Stuarts transport medium or brucella broth. Phosphate buffered saline is less desirable as a transport medium, especially if a delay from biopsy to cultivation is expected. Excellent recovery of *H. pylori* can also be obtained from gastric biopsies stored without transport medium for days to weeks at –20°C or preferably –80°C. Biopsies may be plated by direct inoculation onto agar plates followed by spreading in the conventional manner or by first grinding the tissue and then plating. Quantitative culture results (colony forming units [CFU]/g tissue) can be obtained by placing biopsies in preweighed vials that contain media, homogenizing the tissue, and plating serial dilutions. This method is effective for some species such as *H. pylori*, but not for others such as *H. felis* and many enterohepatic species that typically do not form colonies. Optimum cultivation of the other gastric helicobacters is achieved by placing fresh gastric specimens onto moist agar plates as soon as possible. Enterohepatic helicobacters are typically isolated directly from intestinal mucus or homogenates, fresh fecal slurries, or freshly homogenized liver tissue.

Selective and Nonselective Media

Primary isolation of gastric *Helicobacter* species is generally performed with solid media supplemented with 5–10% horse or sheep blood or with bovine calf serum. Brucella or Columbia agar is commonly used as the agar base, although brain heart infusion (BHI), trypticase soy agar and others are also adequate. If blood-free culture media are required, agar may be supplemented with cyclodextran (Olivieri et al., 1993) or egg yolk emulsion (Westblom et al., 1991). Defined media have also been developed for cultivation of *H. pylori* (Reynolds and Penn, 1994; Albertson et al., 1998), including Hams F-12 nutrient mixture supplemented with cholesterol or cyclodextran (Testerman et al., 2001). Most *Helicobacter* species grow poorly or not at all in liquid media, although *H. pylori* and some others can often be adapted to grow in liquid culture.

The yield from culture of gastric tissue may be improved if both selective and nonselective agar are used. Commonly used antibiotic supplements are those of Skirrow (1977) and Dent (Dent and McNulty, 1988):

Skirrows Supplement

Vancomycin	10 mg/liter
Trimethoprim	5 mg/liter
Polymyxin B	2,500 IU/liter

Dents Supplement

Vancomycin	10 mg/liter
Trimethoprim	5 mg/liter
Cefsulodin	5 mg/liter
Amphotericin B	5 mg/liter

Commercially prepared agar plates are also available (BBL, Cockeysville, MD) that contain 10% defibrinated whole sheep blood with an antibiotic supplement described by Blaser (Blaser et al., 1979) for isolation of *Campylobacter jejuni*.

Blasers Supplement

Cephalothin	15 mg/liter
Polymyxin B	2,500 IU/liter
Trimethoprim	5 mg/liter
Vancomycin	10 mg/liter
Amphotericin B	2 mg/liter

Enterohepatic *Helicobacter* species are typically isolated using media and antibiotic supplements similar to those used for isolation of gastric helicobacters. When enterohepatic helicobacters are isolated from feces or cecal contents, a slurry is usually inoculated onto a 0.45- or 0.65-μm filter that is placed directly onto either selective or nonselective agar. The plates are incubated for approximately two hours in a CO_2 incubator, if possible. The filter is then removed and the plates are incubated under the appropriate conditions. This same technique is also useful for the cultivation of possible novel species obtained from either gastric or intestinal mucus samples. Preliminary studies suggest that the combination of the filtration method with nonselective media and high hydrogen concentration may improve the recovery of fastidious helicobacters from stool (Lastovica and le Roux, 2000a; Lastovica and Skirrow, 2000b).

Growth Conditions

Nearly all *Helicobacter* species are microaerobic, exhibiting growth in 3–7% O_2. No growth is observed under aerobic conditions, although occasional species will grow in anaerobic as well as microaerobic conditions (Fox et al., 1994; Shen et al., 1997; Robertson et al., 2001). Hydrogen is required for some enterohepatic species and may promote growth of others, sometimes in a strain-dependent fashion. For example, isolates originally described as "*Helicobacter westmeadii*" were subsequently identified as hydrogen-requiring *Helicobacter cinaedi* (Vandamme et al., 2000). Many laboratories routinely use anaerobic jars with commercial gas generating envelopes (CampyPak Plus, Becton Dickinson, Cockeysville, MD; Campylobacter Gas Generating Kits, Oxoid, Basingstoke, United Kingdom) for culture of helicobacters. After primary isolation, some helicobacters can be adapted to grow in 5–10% CO_2, although this is not usually reliable for primary culture and is not effective for all species.

The majority of the *Helicobacter* species are fastidious with respect to isolation and cultivation and require freshly poured plates incubated with lids uppermost to maintain a moist environment. *Helicobacter pylori* is less demanding in this regard than many of the other species. A high degree of humidity during incubation, obtained by placing wet paper towel in anaerobe jars or by setting CO_2 incubators to run at 95% humidity, is often essential for culture of many different species. Culture is routinely performed at 37°C.

Identification

Helicobacter species grow as small (0.5–2 mm) translucent colonies or as a thin watery film, which typically appear after 3–7 or occasionally up to 10 days of incubation. Organisms are Gram-negative and often pleomorphic. After prolonged incubation, most helicobacters assume a coccoid morphology, the significance of which remains controversial (Cellini et al., 1994; Eaton et al., 1995; Kusters et al., 1997; Nilsson et al., 2002). Routine identification of many *Helicobacter* species relies on their positive tests for urease, catalase, and oxidase, although many enterohepatic species are urease negative. As discussed above, definitive identification requires a polyphasic approach to taxonomy, but there is an obvious role for molecular diagnostic tests provided their specificity and sensitivity have been evaluated using balanced and well-chosen sets of reference strains. 16S rRNA sequence analysis may be insufficiently sensitive to distinguish different species, while in other cases it may exaggerate differences that are not confirmed by other methods.

Preservation

Helicobacter species can be preserved in brucella broth with 20% (vol/vol) glycerol at –80°C.

Gastric *Helicobacter* Species

Validated gastric *Helicobacter* taxa are listed in Table 2, along with the type strain and GenBank 16S rRNA accession number. Characteristics of cultivated and validated gastric *Helicobacter* species are shown in Table 3.

Helicobacter pylori

Helicobacter pylori was initially isolated from the gastric mucosa of humans presenting with dyspepsia (Marshall et al., 1984b; Goodwin et al., 1989a). *Helicbacter pylori* has also been detected in certain primate species, particularly rhesus monkeys (Dubois et al., 1994; Solnick et al., 1996), in sheep (Dore et al., 2001) and in a closed colony of barrier-maintained cats (Handt et al., 1994). Morphologically the bacterial cells are curved to S-shaped rods, 0.5 μm × 2.5–5 μm (Fig. 4). They are motile by means of 4–8 unipolar or bipolar, sheathed flagella. The bacteria grow as small, translucent colonies (1–2 mm) or a spreading film with increased periods of incubation.

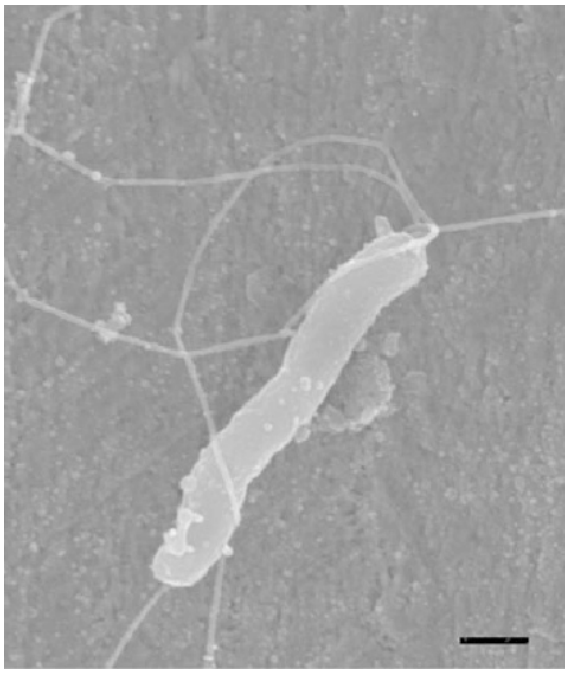

Fig. 4. Field emission scanning electron micrograph of a critical point dried preparation of *Helicobacter pylori* showing its S-shaped morphology and bipolar, sheathed flagella. Bar = 0.5 μm. (Micrograph courtesy of L.Thompson, University of New South Wales, Sydney, Australia).

Optimum growth is obtained under microaerobic conditions at 37°C with no growth obtained at 42°C or under anaerobic conditions. For primary isolation, 4–5 days of incubation are required, which can be reduced to 2 days for subsequent subculture.

All strains have been reported to be urease, catalase and oxidase positive. They do not reduce nitrate and do not hydrolyze indoxyl acetate or hippurate. Activities for γ-glutamyl transpeptidase, leucine arylamidase, and alkaline phosphatase are present. *Helicobacter pylori* is sensitive to 1% glycine, 1.5% NaCl, and cephalothin. It is resistant to nalidixic acid, with variable resistance to metronidazole. The G+C content is 39 mol%. The major fatty acid of *H. pylori* is $C_{14:0}$ with smaller amounts of $C_{18:0}$, $C_{18:1}$ and $C_{19:0}$ cyclic, and the major isoprenoid is MK-6 (Goodwin et al., 1989b; Moss et al., 1990). *Helicobacter pylori* was originally thought to be asaccharolytic, on the basis of data obtained using standard methods to detect carbohydrate metabolism; however, recently it has been shown to utilize glucose via the pentose phosphate and Entner-Doudoroff pathways. Glycolysis is also utilized with fermentation of glucose, possibly leading to the production of mixed acid products or the provision of metabolites for the Krebs cycle (Mendz et al., 1993; Mendz et al., 1994; Chalk et al., 1994). *Helicobacter pylori* also has a requirement for amino acids whose deamination could lead to the production of nitrogen, carbon and energy for the cell (Nedenskov, 1994; Reynolds and Penn, 1994). Features of both aerobic and anaerobic respiration have been reported (Hazell and Mendz, 1997).

The complete DNA sequences of two *H. pylori* strains have been published (Tomb et al., 1997; Alm et al., 1999). *Helicobacter pylori* 26695 has a single, circular chromosome of 1,667,867 bp with 1,590 predicted coding regions, of which 1,091 match known database entries (Tomb et al., 1997). Comparison of the two sequenced genomes revealed that the overall genomic organization and predicted proteins were quite similar, although a group of genes (about 7%) in one hypervariable region was unique to each strain (Alm et al., 1999). This relative similarity between these two sequenced strains was in contrast to previous studies employing pulse field gel electrophoresis (PFEG; Taylor et al., 1992), random amplified polymorphic DNA (RAPD) PCR (Akopyanz et al., 1992a), restriction fragment length polymorphism (RFLP; Akopyanz et al., 1992b), and multilocus electrophoresis (Go et al., 1996), all of which showed a high degree of genomic variability among strains. Recently, a whole-genome microarray analysis showed that 22% of the genes present in *H. pylori* 26695 and J99 were absent in one or more of 15 clinical isolates (Salama et al., 2000). This is consistent with marked genetic heterogeneity among *H. pylori* isolates and suggests a panmictic population structure (Suerbaum et al., 1998). Approximately 50% of *H. pylori* strains harbor cryptic plasmids that range in size from 3.5–>148 kb (Tjia et al., 1987; Graham et al., 1988; Penfold et al., 1988).

Helicobacter mustelae

Helicobacter mustelae was isolated from the gastric mucosa of ferrets (Fox et al., 1986; Goodwin et al., 1989a). Morphologically the bacterial cells are small rods, 0.5–1 μm × 2–5 μm (Fig. 5). They are motile by means of bipolar and lateral sheathed flagella. The bacteria grow as small (1–2 mm), translucent colonies or as a spreading film under microaerobic conditions at 37°C. Growth also occurs at 42°C and under anaerobic conditions. For primary isolation, 3–5 days of incubation are required, which can be reduced to 2 days for subsequent subculture.

All strains have been reported to be urease, catalase and oxidase positive. They are able to reduce nitrate, hydrolyze indoxyl acetate, but not hippurate, and have no activity for leucine arylamidase. *Helicobacter mustelae* is sensitive to 1.5% NaCl, metronidazole, and nalidixic acid

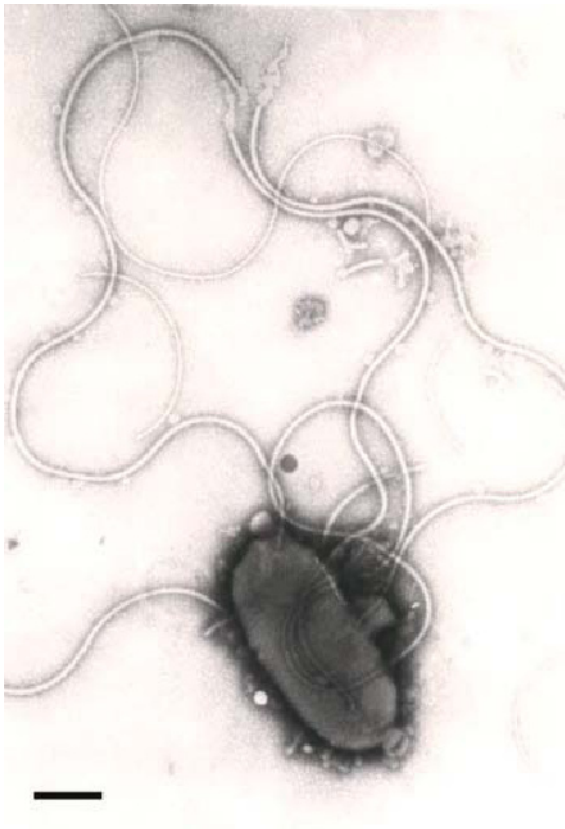

Fig. 5. Transmission electron micrograph of a negatively stained preparation of *Helicobacter mustelae* showing its short rod shape with lateral and polar flagella. Bar = 0.4 μm.

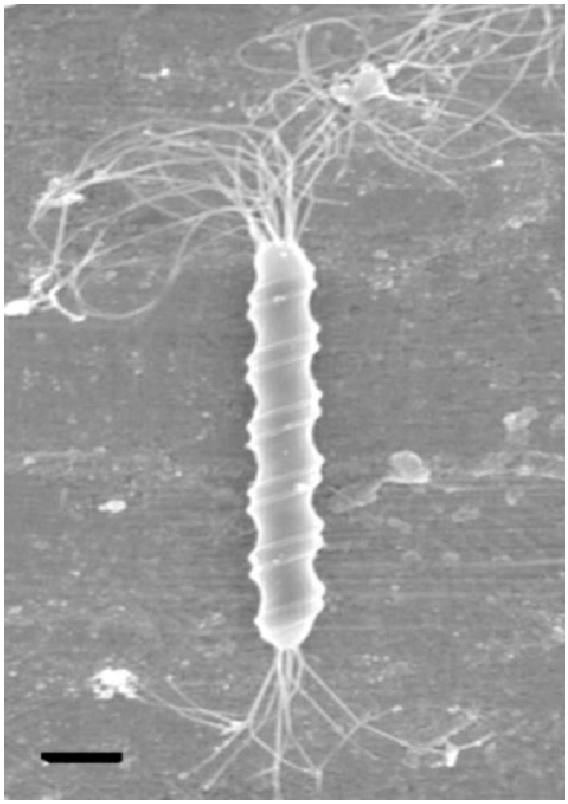

Fig. 6. Field emission scanning electron micrograph of a critical point dried preparation of *Helicobacter felis* showing its tight helical morphology with bipolar tufts of flagella and periplasmic fibers entwining the cell in pairs. Bar = 0.7 μm. (Micrograph courtesy of L. Thompson, University of New South Wales, Sydney, Australia).

and resistant to cephalothin and 5-fluorouracil. There is variable growth on media containing 1% glycine. *Helicobacter mustelae* has a G+C content of 36 mol%. The major fatty acid of *H. mustelae* is $C_{16:0}$ with small amounts of $C_{14:0}$, $C_{18:1}$ and $C_{19:0}$ cyclic.

The genome size of *H. mustelae* is approximately 1.7 Mb (Taylor et al., 1994), which is very similar to that of *H. pylori*. In contrast to the marked heterogeneity among *H. pylori* strains, there is significant genomic conservation among isolates of *H. mustelae* (Morgan and Owen, 1990; Taylor et al., 1994).

Helicobacter felis

Helicobacter felis was isolated from the gastric mucosa of cats and dogs (Lee et al., 1988; Paster et al., 1991). Morphologically the bacterial cells are tightly coiled helical rods (0.4 μm × 5–7.5 μm; Fig. 6). Most strains are surrounded by periplasmic fibers, in pairs, triplets or singly. They have a rapid corkscrew-like motility by means of bipolar tuffs of sheathed flagella. The bacteria grow as a thin, spreading nonhemolytic film, occasionally with pinpoint colonies. Optimum growth is obtained under microaerobic conditions at 37°C, with growth also possible at 42°C and under anaerobic conditions. For primary isolation, 3–5 days of incubation are required, which can be reduced to 2 days for subsequent subculture.

All strains have been reported to be urease, catalase and oxidase positive. They are able to reduce nitrate but not TTC (2,3,5-triphenyltetrazolium chloride), do not hydrolyze indoxyl acetate or hippurate, and exhibit activities for γ-glutamyl transpeptidase, alkaline phosphatase, arginine aminopeptidase, leucine aminopeptidase and histidine aminopeptidase. *Helicobacter felis* is sensitive to 1% bile, 1% glycine, 1.5% NaCl, metronidazole, cephalothin, erythromycin and ampicillin and is resistant to nalidixic acid and 5-fluorouracil.

Genetically, *H. felis* forms a very tight cluster with *H. bizzozeronii*, *H. salomonis*, "*Candidatus* Helicobacter suis" and "*H. heilmannii*." These organisms show greater than 97% sequence similarity in their 16S rRNA genes, although further discrimination is hampered by the inability to culture "*H. heilmannii*" and "*Candidatus* Helico-

bacter suis" in vitro. *Helicobacter felis*, *H. bizzozeronii* and *H. salomonis* can be differentiated by morphology, protein profiling, and DNA-DNA hybridization assay (Jalava et al., 1998) but not by biochemical testing or 23S rRNA RFLP analysis (Jalava et al., 1999b). Strains of *H. felis* have been shown by various techniques (pulsed field gel electrophoresis [PFGE], plasmid profiling and ribotyping) to be heterogeneous with no association to host species or country of origin (Jalava et al., 1999a). *Helicobacter felis* has a genome size of 1.6 Mb and G+C content of 42.5 mol%. Most strains have multiple plasmids, ranging from 2 kb to more than 16 kb.

Helicobacter acinonychis

Helicobacter acinonychis (formerly *H. acinonyx*) was isolated from the gastric mucosa of cheetahs (*Acinonyx jubilatus*) and tigers (Eaton et al., 1993a; Schroder et al., 1998). Morphologically it is a small spiral rod (0.3 μm × 1.5–2 μm; Fig. 7). The bacteria are motile and possess 2–5 unipolar, sheathed flagella. Primary culture was performed by blind subculture for 3 days after an initial 4–5 days of incubation (Eaton et al., 1993a). *Helicobacter acinonychis* will not grow under anaerobic conditions or at 25 or 42°C.

All strains have been reported to be urease, catalase and oxidase positive. They exhibit activities for γ-glutamyl transpeptidase and alkaline phosphatase but do not reduce nitrate or hydrolyze indoxyl acetate or hippurate. *Helicobacter acinonychis* is sensitive to 1% glycine, 1.5% NaCl, metronidazole and cephalothin but is resistant to nalidixic acid. *Helicobacter acinonychis* appears to be most closely related to *H. pylori* by analysis of protein profiles and sequencing of 16S rRNA genes; however, *H. acinonychis* has a lower G+C content (30 mol%) than that of *H. pylori* (39 mol%).

Helicobacter bizzozeronii

Helicobacter bizzozeronii was isolated from the gastric mucosa of dogs (Hänninen et al., 1996) and named in honor of Guilio Bizzozero, who provided one of the first descriptions of spiral bacteria in the gastric environment (Bizzozero, 1892). Recently a similar bacterium cultivated from a human patient has been identified as *H. bizzozeronii* (Jalava et al., 2001). Morphologically the bacterial cells are tight helical rods (0.3 μm × 5–10 μm), and they exhibit a rapid corkscrew-like motility by means of bipolar tuffs of sheathed flagella (Fig. 8). The bacteria grow as a thin, spreading nonhemolytic film and do not readily form discrete colonies. Optimum growth is obtained on fresh, moist brain heart infusion (BHI) blood agar under microaerobic conditions

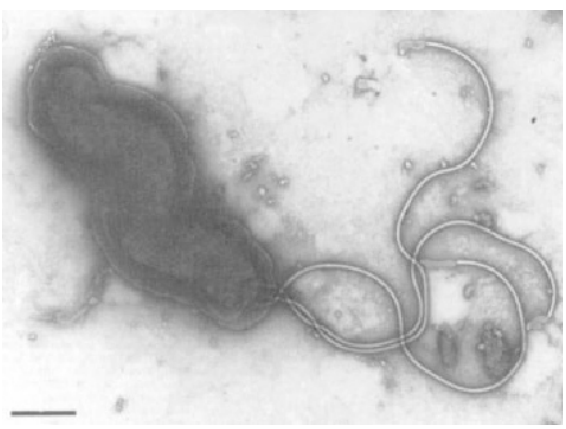

Fig. 7. Transmission electron micrograph of a negatively stained preparation of *Helicobacter acinonychis* showing its S-shaped morphology with a tuft of polar, sheathed flagella. Bar = 0.5 μm. (Reproduced with permission from Eaton et al., 1993a.)

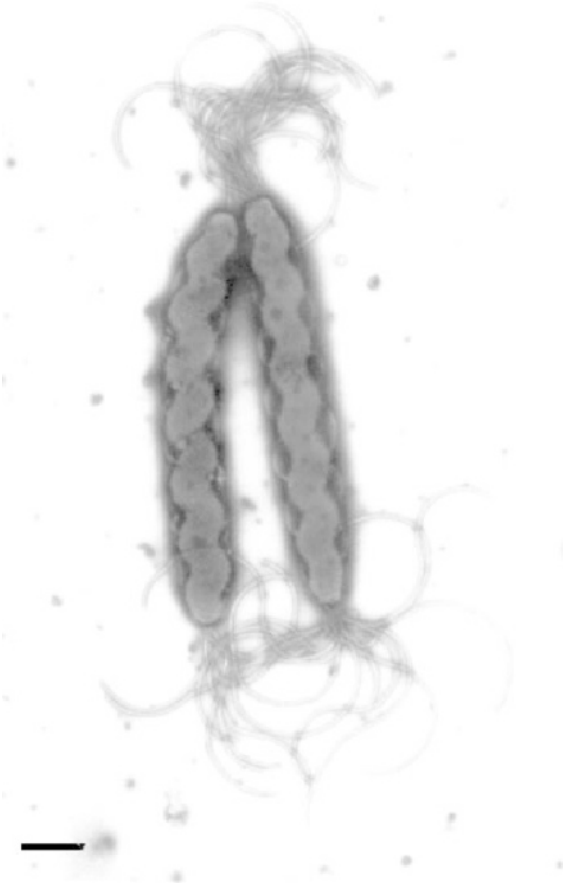

Fig. 8. Transmission electron micrograph of a negatively stained preparation of *Helicobacter bizzozeronii* showing its tight helical morphology with bipolar tufts of flagella. Bar = 0.45 μm. (Micrograph courtesy of K. Jalava, University of Finland, Helsinki.)

at 37°C, with growth also possible at 42°C. For primary isolation, 5–10 days of incubation are required, which can be reduced to 3–5 days on subsequent subculture.

All strains have been reported to be urease, catalase and oxidase positive. They are able to reduce nitrate and TTC, can hydrolyze indoxyl acetate but not hippurate, and exhibit activities for γ-glutamyl transpeptidase and alkaline phosphatase. *Helicobacter bizzozeronii* is sensitive to 1% bile, 1% glycine, 1.5% NaCl, metronidazole, cephalothin and cefoperazone and is resistant to nalidixic acid and 5-fluorouracil.

Helicobacter bizzozeronii shares detectable DNA-DNA hybridization levels with *H. felis* (2–30%) and *H. salomonis* (11–34%) but not with *H. pylori* or *H. mustelae*. It appears to be heterogenetic in nature, with different PFGE patterns obtained from 15 strains using 5 different enzymes (Hänninen and Hirvi, 1999). The genome size is 1.6–1.9 Mb.

Helicobacter salomonis

Helicobacter salomonis was isolated from the gastric mucosa of dogs (Jalava et al., 1997) and named in honor of Hugo Salomon, who initially described different morphological types of gastric bacteria in a variety of animal species (Salomon, 1896). The bacterial cells are loosely helical or wavy rods (0.8–1.2 μm × 5–7 μm), and they exhibit a slow wave-like motility by means of bipolar tuffs of sheathed flagella (Fig. 9). The bacteria grow as a thin, spreading nonhemolytic film and do not readily form discrete colonies.

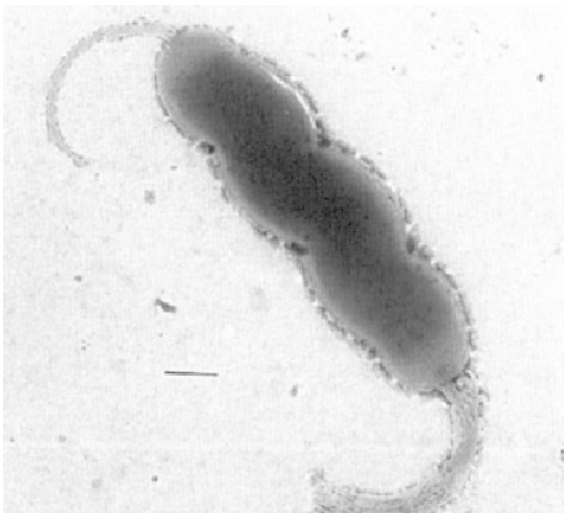

Fig. 9. Transmission electron micrograph of a negatively stained preparation of *Helicobacter salomonis* showing its undulating S-shaped morphology with bipolar tufts of flagella. Bar = 1 μm. (Reproduced with permission from Jalava et al., 1997.)

Optimum growth is obtained on fresh, moist BHI blood agar under microaerobic conditions at 37°C (no strains were able to grow at temperatures >40°C) after 3–6 days for primary isolation and 2 days for subsequent subculture.

All strains have been reported to be urease, catalase and oxidase positive. They are able to reduce nitrate and TTC, can hydrolyze indoxyl acetate but not hippurate, and exhibit activities for γ-glutamyl transpeptidase and alkaline phosphatase. *Helicobacter salomonis* is sensitive to 1% bile, 1% glycine, 1.5% NaCl, metronidazole (one strain resistant), cephalothin and cefoperazone. *Helicobacter salomonis* is resistant to nalidixic acid.

Helicobacter salomonis shares detectable DNA-DNA hybridization levels with *H. felis* (26–39%) and *H. bizzozeronii* (11–34%). In contrast to *H. felis* and *H. bizzozeronii*, *H. salomonis* appears to be relatively homogenetic. Testing of five strains by PFGE showed diversity in their patterns, although common fragments were noted with some enzymes (Hänninen and Hirvi, 1999). The genome size of *H. salomonis* is 1.7–1.8 Mb.

Helicobacter nemestrinae

An organism isolated from pig-tailed macaques (*Macaca nemestrina*) reportedly differed from *H. pylori* by virtue of growth at 42°C and its cellular fatty acid profile (Bronsdon et al., 1991). DNA-DNA hybridization and 16S rRNA studies also suggested that this was a novel species, which was designated "*H. nemestrinae*." The G+C content was reportedly only 24 mol%, markedly lower than that of all other known *Helicobacter* species, which prompted a reappraisal. Sequence analysis of seven housekeeping genes and two flagellin genes from the single strain of *H. nemestrinae* (ATCC 49396^T) showed that all sequences clustered together with those from multiple isolates of *H. pylori* (Suerbaum et al., 2002). Furthermore, repeat sequence analysis of the 16S rRNA gene from *H. nemestrinae* showed that it differed by 38 bp from that previously reported and was less than 1% different from multiple *H. pylori* strains. In addition, repeat analyses of the G+C content of *H. nemestrinae* type strain yielded a reproducible value of 39 mol% (P. Vandamme, unpublished observations). These results show that the single strain previously classified as *H. nemestrinae* is a junior heterotypic synonym for *H. pylori*.

Enterohepatic *Helicobacter* Species

Validated enterohepatic *Helicobacter* taxa are listed in Table 2, along with the type strain and GenBank 16S rRNA accession number. Charac-

teristics of cultivated and validated enterohepatic *Helicobacter* species are shown in Table 3. *Helicobacter aurati*, which has been isolated from both gastric and intestinal sites, has been included in Tables 2 and 3.

Helicobacter cinaedi

Helicobacter cinaedi has been isolated from rectal swabs and blood of homosexual men, blood, cerebrospinal fluid and feces of adults and children with and without risk factors for human immunodeficiency virus (HIV) infection, and the intestinal tract of hamsters, cats, dogs and foxes (Totten et al., 1985; Vandamme et al., 1991a; Vandamme et al., 2000; Kiehlbauch et al., 1995). Asymptomatic captive rhesus monkeys (*Macaca mulatta*) are also commonly infected (Fernandez et al., 2002), although sometimes infection may be associated with colitis (Fox et al., 2001a). The name is derived from the Latin word for "homosexuals." Recent polyphasic taxonomic studies show that "*Helicobacter westmeadii*" and *Helicobacter* sp. strain Mainz are correctly identified as *H. cinaedi*, rather than novel species as originally proposed (Vandamme et al., 2000).

Helicobacter cinaedi is an S-shaped rod (0.3–0.5 μm × 1.5–5 μm), with rapid motility by means of a single sheathed bipolar flagellum. *Helicobacter cinaedi* may form pinpoint colonies but more typically forms a translucent spreading film on moist blood agar. All strains are able to grow at 37°C under microaerobic but not aerobic or anaerobic conditions. Growth at 42°C is variable.

Strains are oxidase and catalase positive, urease negative, and reduce nitrate. They do not hydrolyze indoxyl acetate or hippurate, and there is little or no alkaline phosphatase activity. *Helicobacter cinaedi* is sensitive to 2% NaCl and is variably resistant to nalidixic acid and cephalothin. It is resistant to 1% glycine, 0.04% TTC, and trimethoprim. The G+C content is 37–38 mol%.

Helicobacter cinaedi is most closely related to *H. canis* (52% DNA:DNA hybridization; Stanley et al., 1993) and *H. fennelliae* (3–10% DNA homology; Totten et al., 1985). Ribotyping discriminates distinct patterns depending upon the host source (Kiehlbauch et al., 1995); however, high levels of similarity by phenotypic and protein-electrophoretypic traits between human and dogs strains have been reported (Vandamme et al., 2000).

A recent report indicates that the type strain of *H. cinaedi* held by the American Type Culture Collection (ATCC 35683) is mislabeled, since the 16S rRNA and *rpoB* sequences from this strain are identical to that reported for *H. fennelliae* (Kuhnert and Burnens, 2001; On and Vandamme, 2001). Until this is clarified, *H. cinaedi* should be ordered from the Culture Collection of the University of Göteborg, Dept. of Clinical Bacteriology, Göteborg, Sweden (CCUG18818^T) rather than the ATCC.

Helicobacter fennelliae

Helicobacter fennelliae was isolated from rectal swabs and blood of homosexual men, blood and feces of children with diarrhea, and the intestinal tract of hamsters and dogs. The organism was named after the technologist who first isolated it, C.L. Fennell (Totten et al., 1985; Vandamme et al., 1991a; Kiehlbauch et al., 1995). *Helicobacter fennelliae* is an S-shaped rod (0.3–0.5 μm × 1.5–5 μm), with rapid motility by means of single, sheathed bipolar flagella. Colonies are pinpoint, are translucent, may spread on moist blood agar, and have a distinctive hypochlorite odor. All strains are able to grow at 37°C under microaerobic but not aerobic or anaerobic conditions. Growth at 42°C is variable. Hydrogen is required for growth.

Strains are oxidase and catalase positive, urease negative and do not reduce nitrate. They hydrolyze indoxyl acetate but not hippurate, and there is little or no alkaline phosphatase activity. *Helicobacter fennelliae* is sensitive to 2% NaCl, metronidazole, cephalothin and nalidixic acid and is resistant to 1% glycine, 0.04% TTC, 5-fluorouracil, trimethoprim and streptomycin. The G+C content is 37–38 mol%.

Helicobacter fennelliae is most closely related to *H. canis* (30% DNA homology; Stanley et al., 1993), *H. cinaedi* (10–30% DNA homology; Totten et al., 1985), and *H. pullorum* (95.9% sequence similarity of 16S rRNA gene; Shen et al., 1997).

Helicobacter muridarum

Helicobacter muridarum was isolated from the intestinal mucosa of rats and mice (Phillips and Lee, 1983; Lee et al., 1992). It is a helical rod (0.5–0.6 μm × 3.5–5 μm), with 2–3 spirals, exhibiting a rapid, darting motility due to bipolar tufts of 10–14 sheathed flagella (Fig. 10). Ultrastructurally the cells are characterized by the presence of 9–11 periplasmic fibers coiled around the protoplasmic cylinder. Bacteria grow as a fine spreading translucent film on moist blood agar plates after 2–3 days incubation at 37°C under microaerobic conditions. There is no growth at 25 or 42°C or under aerobic or anaerobic conditions.

All strains are urease, catalase and oxidase positive. They do not reduce nitrate or hydrolyze hippurate and do exhibit activities for γ-glutamyl transpeptidase, alkaline phosphatase, arginine

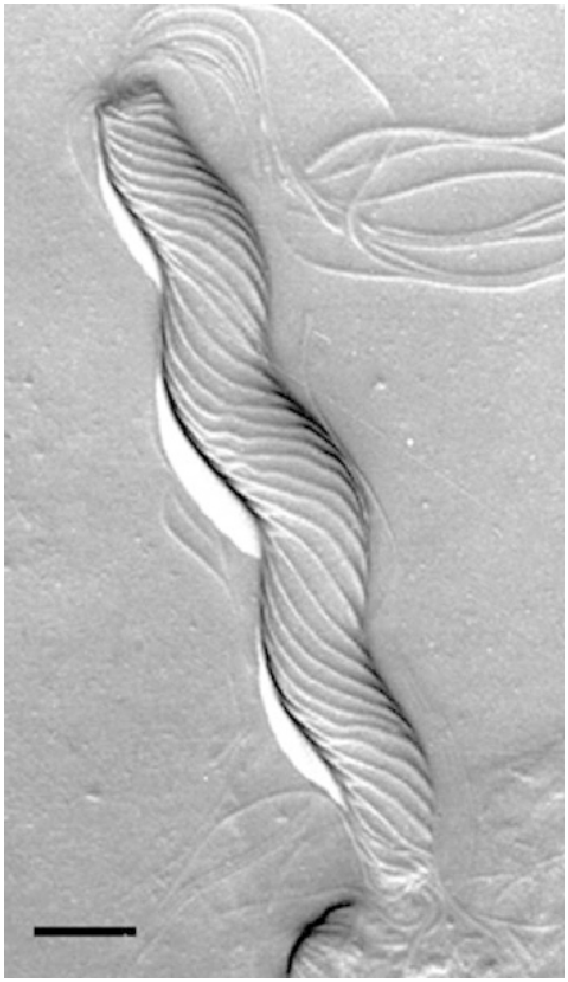

Fig. 10. Transmission electron micrograph of a freeze-dried preparation of *Helicobacter muridarum* showing its S-shaped morphology with bipolar tufts of flagella and periplasmic fibers entwining the cell. Bar = 0.5 μm. (Micrograph courtesy of M. Phillips, University of New South Wales, Sydney, Australia.)

aminopeptidase, and leucine aminopeptidase, but not histidine aminopeptidase. *Helicobacter muridarum* is sensitive to 2% bile, 1% glycine, 1.5% NaCl and is resistant to nalidixic acid and cephalothin. The G+C content is 34%. *Helicobacter muridarum* is most closely related to *H. hepaticus* (96.6% sequence similarity of 16S rRNA gene).

Helicobacter canis

Helicobacter canis was isolated from the feces of humans and dogs with diarrhea (Stanley et al., 1993) and more recently from a colony of diarrheic Bengal cats (Foley et al., 1999). The organisms are curved rods (0.25 μm × 4 μm), with occasional truncated ends (Fig. 11). Cells are motile and possess a single sheathed flagellum at each end. Colonies are pinpoint, translucent, and pigmented after 2 days growth on blood agar. Optimum growth occurs at 37°C under microaerobic conditions, with limited growth at 42°C.

All strains are oxidase positive and catalase and urease negative. They hydrolyze indoxyl acetate but do not reduce nitrate. Activity for alkaline phosphatase is detected. *Helicobacter canis* is sensitive to cephalothin and nalidixic acid but resistant to 1.5% bile and 5-fluorouracil. The G+C content is 48–49 mol%.

Insertion sequences (of 81–320 bp) have been detected in the 16S and 23S rRNA genes of some strains (Linton et al., 1994). Genetically *H. canis* shares detectable DNA-DNA hybridization levels with *H. fennelliae* and *H. cinaedi* (30 and 52%, respectively) and has approximately 98% 16S rRNA sequence similarity with *H. bilis* and "*H. rappini*."

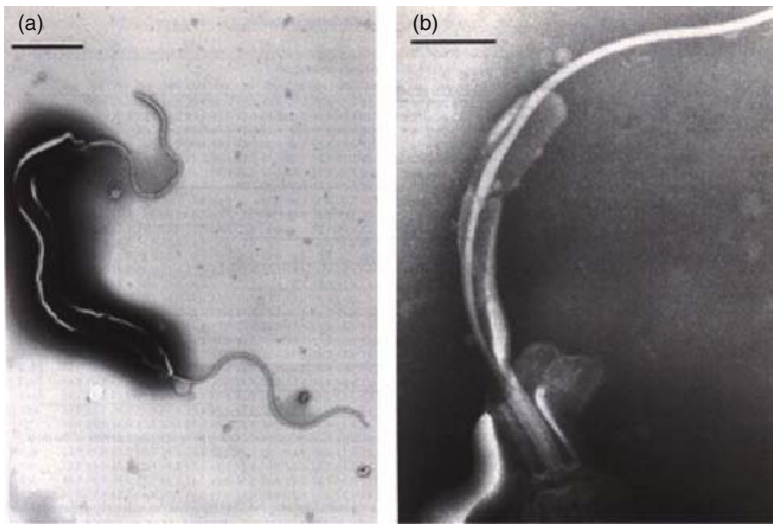

Fig. 11. Transmission electron micrograph of a negatively stained preparation of *Helicobacter canis* showing its S-shaped morphology and single, bipolar, sheathed flagella. Bar = 1 μm. (Reproduced with permission from Stanley et al., 1993.)

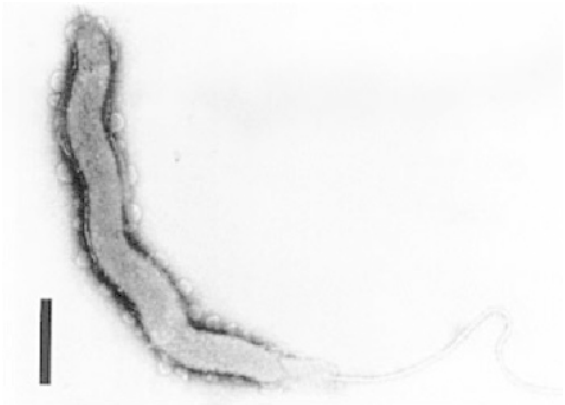

Fig. 12. Transmission electron micrograph of a negatively stained preparation of *Helicobacter hepaticus* showing its S-shaped morphology and single, bipolar, sheathed flagella. Bar = 0.5 μm. (Reproduced with permission from Fox et al., 1994.)

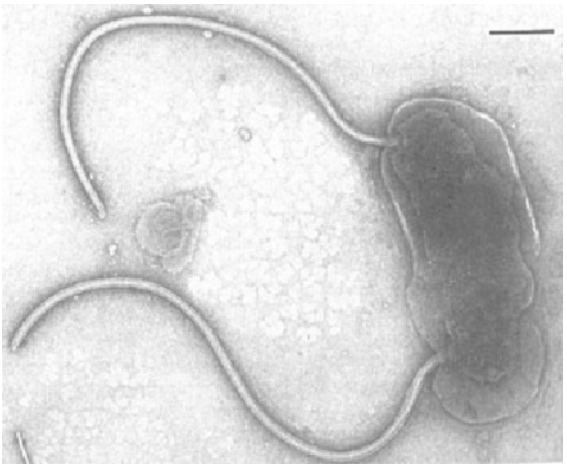

Fig. 13. Transmission electron micrograph of a negatively stained preparation of *Helicobacter pametensis* showing its curved rod morphology and single, bipolar, sheathed flagella. Bar = 0.3 μm. (Reproduced with permission from Dewhirst et al., 1994.)

Helicobacter hepaticus

Helicobacter hepaticus was isolated from the intestinal mucus and livers of mice (Fox et al., 1994). It is a Gram-negative, curved to spiral rod (0.2–0.3 μm × 1.5–5 μm; Fig. 12). Cells are motile and possess a single, bipolar, sheathed flagellum. The organism grows as a thin spreading film after 3–7 days on blood agar. Optimum growth occurs at 37°C under microaerobic conditions, with limited growth anaerobically and no growth at 42°C.

All strains are catalase, oxidase, and urease positive. They reduce nitrate and hydrolyze indoxyl acetate but not hippurate. *Helicobacter hepaticus* is sensitive to metronidazole and resistant to 1.5% NaCl, 1% glycine, cephalothin and nalidixic acid.

Helicobacter hepaticus is most closely related to *H. muridarum* by 16S rRNA sequence homology. The genomic diversity of *H. hepaticus* from four sources in the United States and three sources in Europe was determined by PFGE (Saunders et al., 1997). Three of the sites in the United States gave similar patterns. However, the fourth site and the three sites from Europe all gave different patterns, indicating significant genomic diversity among *H. hepaticus* strains. Sequencing of the complete *H. hepaticus* genome was recently reported in preliminary form (Suerbaum et al., 2001). The genome is 1.8 Mb with 1,800 open reading frames (ORFs). Notably absent were pathogenicity islands and orthologs of the *H. pylori* vacuolating cytotoxin (VacA), adhesins, and the Helicobacter outer membrane porin (HOP) family (Peck et al., 2001).

Helicobacter pametensis

Heliocbacter pametensis was isolated from tern, gull and swine feces with the species name derived from the origin of the isolates, the Pamet River in Truro, Massachusetts (Dewhirst et al., 1994). The organism is a small curved rod (0.4 μm × 1.5 μm), with rounded ends (Fig. 13). Cells are motile and possess a single sheathed flagellum at each end. The flagella are inserted subterminally, and occasional cells have a third flagellum adjacent to one of the others. Colonies are pinpoint to 0.5 mm in diameter, translucent, colorless and weakly hemolytic after 3 days growth on blood agar. Optimum growth occurs at 37°C under microaerobic conditions, with limited growth at 42°C or under anaerobic conditions.

All strains are catalase and oxidase positive and urease negative. They reduce nitrate but do not hydrolyze hippurate or indoxyl acetate. There is activity for alkaline phosphatase and arginine β-naphthylamide aminopeptidase, but not γ-glutamyl transpeptidase. *Helicobacter pametensis* is sensitive to 3.5% NaCl, nalidixic acid, and cephalothin, but resistant to 1% glycine. The G+C content is 38 mol%.

Helicobacter pullorum

Helicobacter pullorum was isolated from the ceca of asymptomatic broiler chickens, the livers and intestinal contents of hens exhibiting lesions suggestive of vibrionic hepatitis, and feces from patients with gastroenteritis (Stanley et al., 1994). The organism is a gently curved rod (0.3–

Fig. 14. Transmission electron micrograph of a negatively stained preparation of *Helicobacter pullorum* showing its curved rod morphology and single, monopolar, unsheathed flagella. Bar = 0.3 μm. (Reproduced with permission from Stanley et al., 1994.)

Fig. 15. Transmission electron micrograph of a negatively stained preparation of *Helicobacter trogontum* showing its tapered rod morphology with bipolar tufts of sheathed flagella and periplasmic fibers entwining the cell. Bar = 0.5 μm. (Reproduced with permission from Mendes et al., 1996b.)

0.5 μm × 3–4 μm), with a single unsheathed polar flagellum (Fig. 14). Colonies are pinpoint to 1 mm in diameter, translucent, watery and weakly α-hemolytic after 3 days growth on blood agar. All strains grow at 37 and 42°C under microaerobic conditions, but not anaerobically.

All strains are oxidase positive and urease negative. Most strains are catalase positive and are able to reduce nitrate. They do not hydrolyze hippurate or indoxyl acetate, and there is no alkaline phosphatase activity. *Helicobacter pullorum* is usually sensitive to 1% glycine, 2% NaCl, polymyxin B, and nalidixic acid. It is resistant to 1% bile, cefoperazone and cephalothin. The G+C content is 34–35 mol%.

Helicobacter trogontum

Helicobacter trogontum was isolated from the colonic mucosa of rats (Mendes et al., 1996b). It is a fusiform rod with tapered ends (0.6–0.7 μm × 4–6 μm; Fig. 15). It is motile by means of tufts of bipolar, sheathed flagella. Ultrastructurally the cells are characterized by the presence of periplasmic fibers coiled around the protoplasmic cylinder, giving a crisscross appearance to the bacterial surface. Bacteria initially grow as punctiform colonies and then as a fine spreading translucent film on moist blood agar plates after 3–5 days at 37 or 42°C under microaerobic conditions. No growth occurs at 25°C or under aerobic or anaerobic conditions.

All strains are urease, catalase and oxidase positive. They reduce nitrate but do not hydrolyze hippurate. Ornithine decarboxylase and γ-glutamyl transpeptidase activities are present, with variable activity for alkaline phosphatase. *Helicobacter trogontum* is resistant to nalidixic acid and cephalothin but sensitive to 1% glycine, 1.5% NaCl and metronidazole.

Helicobacter bilis

Helicobacter bilis was isolated from intestinal mucosa of mice and the bile and liver of mice with hepatitis (Fox et al., 1995). The organism has also been isolated from gastric mucosa in dogs (Eaton et al., 1996) and from the large bowel in a colony of male athymic nude rats (Haines et al., 1998). PCR evidence suggests the possibility that this bile-resistant *Helicobacter* species may also be associated with human diseases of the gallbladder (Fox et al., 1998a). *Helicobacter bilis* is a fusiform rod with tapered ends (0.5 μm × 4–5 μm; Fig. 16). It is motile by means of bipolar tufts of sheathed flagella. Ultrastructurally the cells are characterized by the presence of periplasmic fibers coiled around the protoplasmic cylinder, giving a crisscross appearance to the bacterial surface. Bacteria initially grow as punctiform colonies and then as a fine spreading translucent film on moist blood agar plates after 3–5 days at 37°C under microaerobic conditions. Microaerophilic growth occurs at 42°C but not 25°C.

All strains of *H. bilis* are urease, catalase and oxidase positive. They reduce nitrate but do not hydrolyze indoxyl acetate or hippurate. *Helicobacter bilis* is sensitive to 1.5% NaCl and metronidazole and resistant to 20% bile, 0.04% TTC, nalidixic acid and cephalothin. There is variable growth in the presence of 1% glycine.

Sequencing of the 16S rRNA gene revealed the presence of a 187-bp intervening sequence in *H. bilis*. The bacteria most closely related to *H. bilis* are *H. canis*, *H. cinaedi* and "*H. rappini*."

Helicobacter cholecystus

Helicobacter cholecystus was isolated from the gallbladders of Syrian hamsters with cholangiofibrosis and centrilobular pancreatitis (Franklin et al., 1996). It is a straight or curved rod (0.5–0.6 μm × 3–5 μm; Fig. 17). It is motile by means of a single, sheathed, polar flagellum. Colonies are pinpoint after 3 days incubation at 37°C

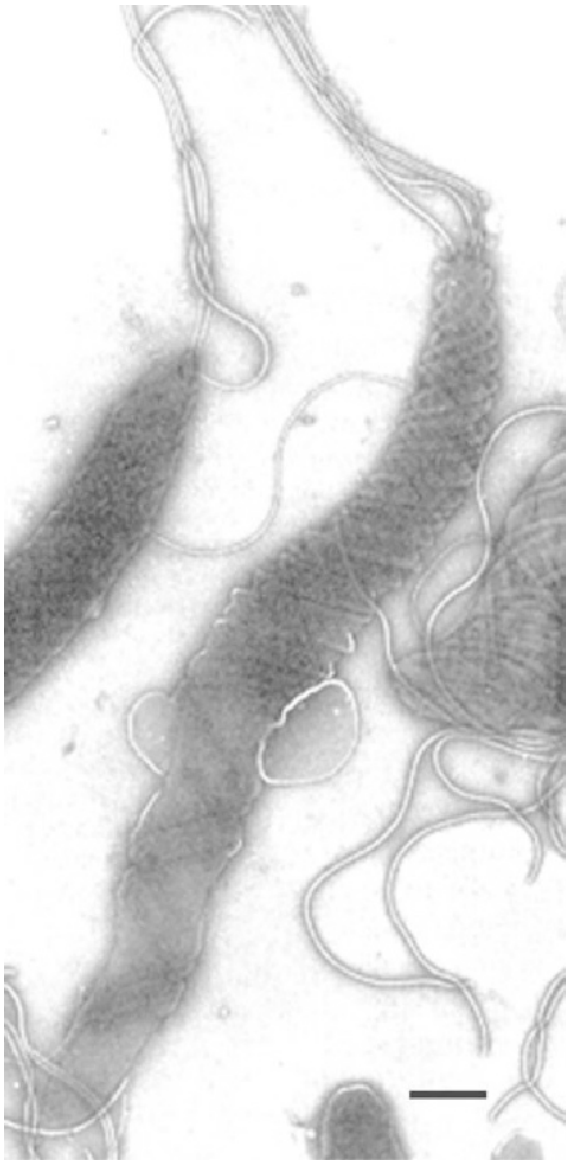

Fig. 16. Transmission electron micrograph of a negatively stained preparation of *Helicobacter bilis* showing its curved S-shaped morphology with bipolar tufts of sheathed flagella and periplasmic fibers entwining the cell. Bar = 0.35 μm. (Reproduced with permission from Fox et al., 1995.)

Fig. 17. Transmission electron micrograph of a negatively stained preparation of *Helicobacter cholecystus* showing its rod-like morphology and single, monopolar, sheathed flagellum. Bar = 1 μm. (Reproduced with permission from Franklin et al., 1996.)

under microaerobic conditions. Cells are also able to grow at 42°C and under anaerobic conditions.

All strains are catalase and oxidase positive and urease negative. They reduce nitrate but do not hydrolyze hippurate or indoxyl acetate. *Helicobacter cholecystus* exhibits activity for arginine aminopeptidase, alkaline phosphatase and L-arginine arylamidase but not γ-glutamyl transpeptidase, histidine aminopeptidase, or leucine aminopeptidase. *Helicobacter cholecystus* is sensitive to 1.5% NaCl but resistant to 5% bile and cephalothin. It exhibits intermediate sensitivity to nalidixic acid.

Helicobacter rodentium

Helicobacter rodentium was isolated from the intestinal mucosa and feces of mice (Shen et al., 1997). It is a curved or spiral rod (0.3 μm × 1.5–5 μm; Fig. 18). It is motile by means of single, bipolar, unsheathed flagella. Bacteria grow as a fine spreading translucent film on moist blood agar plates after 4–7 days incubation at 37°C under microaerobic conditions. Bacterial growth

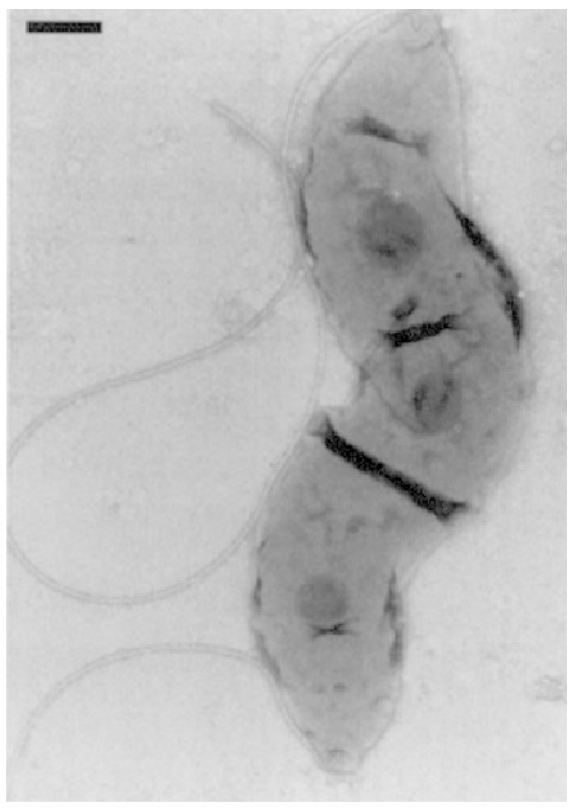

Fig. 18. Transmission electron micrograph of a negatively stained preparation of *Helicobacter rodentium* showing its S-shaped morphology and single, bipolar, unsheathed flagella. Bar = 1 μm. (Reproduced with permission from Shen et al., 1997.)

also is seen when cells are incubated at 42°C or under anaerobic conditions.

Helicobacter rodentium is urease negative and exhibits weak catalase and oxidase activities. It reduces nitrate but does not hydrolyze indoxyl acetate or hippurate, and no γ-glutamyl transferase or alkaline phosphatase activity is detected. *Helicobacter rodentium* is able to grow in the presence of 1% glycine and 1.5% NaCl, and it is resistant to nalidixic acid and cephalothin.

Helicobacter mesocricetorum

Helicobacter mesocricetorum was isolated from feces of Syrian hamsters (Simmons et al., 2000). It is a spiral, curved rod without periplasmic fibers whose average size is 0.5 μm × 2.5 μm (Fig. 19). It is motile by means of single, bipolar, unsheathed flagella. Growth occurs as pinpoint colonies or as a thin spreading film at 42°C but not at 25°C. Growth is minimal to absent under anaerobic conditions and does not occur under aerobic conditions. *Helicobacter mesocricetorum* is positive for catalase and alkaline phosphatase, and it usually expresses nitrate reduction. It is negative for urease, γ-glutamyl transferase, and hippurate hydrolysis. No growth occurs with 1% glycine or 1.5% NaCl. *Helicobacter mesocricetorum* is resistant to cephalothin and generally sensitive to nalidixic acid. Like some other enterohepatic helicobacters, *H. mesocricetorum* has a 16S rRNA gene with an intervening sequence. The closest known relative is *H. rodentium*.

Helicobacter ganmani

Helicobacter ganmani was cultured during routine screening of intestinal scrapings from conventional and specific pathogen-free mice (Robertson et al., 2001). It is a curved to spiral rod measuring 0.3 μm × 2.5 μm with two turns per cell and single, unsheathed, bipolar flagella (Fig. 20). After 3–5 days incubation at 37°C on 5% horse blood, growth appears as a thin, transparent film. No growth occurs at 42°C. Single colonies, which are not usually seen, are irregular and less than 1 mm in diameter. *Helicobacter ganmani* is anaerobic, with no growth under microaerobic or aerobic conditions.

Helicobacter ganmani is urease negative but weak catalase activity is found in some strains. There is no alkaline phosphatase or indoxyl acetate hydrolysis. No growth occurs in the presence of 1% glycine, 2–4% NaCl, or nalidixic acid, but *H. ganmani* is resistant to cephalothin. The 16S

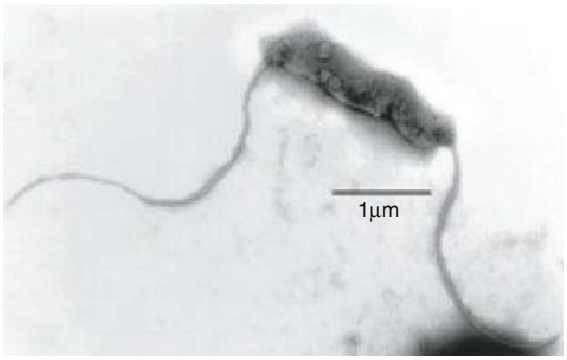

Fig. 19. Transmission electron micrograph of a negatively stained preparation of *Helicobacter mesocricetorum* showing its curved S-shaped morphology and single, bipolar, unsheathed flagella. Bar = 1 μm. (Reproduced with permission from Simmons et al., 2000.)

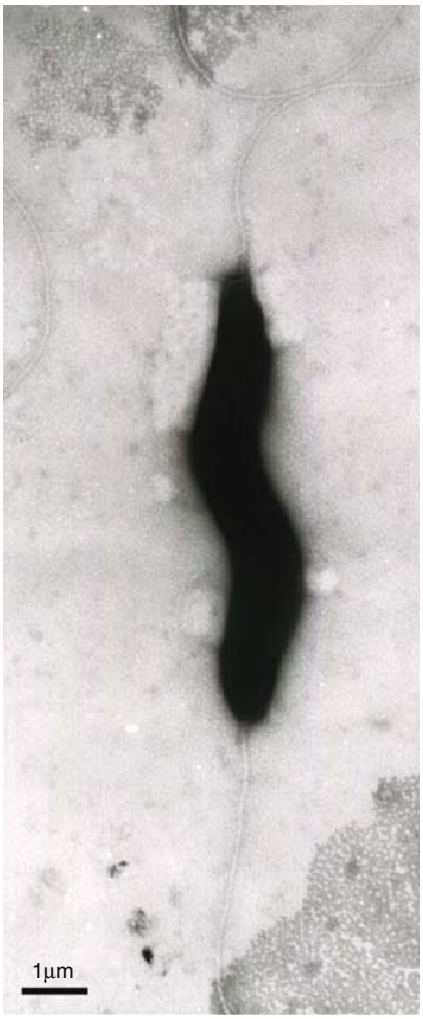

Fig. 20. Transmission electron micrograph of a negatively stained preparation of *Helicobacter ganmani* showing its S-shaped morphology and single, bipolar unsheathed flagella. Bar = 0.3 μm. (Reproduced with permission from Robertson et al., 2001.)

rRNA gene from *H. ganmani* is 98.2% similar to the type strain of *H. rodentium* and is identical to a sequence from an unnamed mouse isolate, *Helicobacter* sp. MIT 95-2011 (GenBank accession no. U96298).

Helicobacter canadensis

Some strains of *H. pullorum* identified by 16S rRNA sequence, fatty acid analysis, and restriction enzyme analysis were unexpectedly found to be indoxyl acetate positive, which had not been previously noted in *H. pullorum* (Gibson et al., 1999). Re-examination of four such isolates from patients with diarrhea showed that they differed biochemically from *H. pullorum* by their inability to hydrolyze indoxyl acetate and their resistance to nalidixic acid (Fox et al., 2000). Complete 16S rRNA sequence analysis indicated a 2% difference with *H. pullorum*, and these isolates were proposed as a novel species, designated *H. canadensis*. The ultrastructure of *H. canadensis* is very similar to that of *H. pullorum*, except that the unsheathed flagella are unipolar rather than bipolar. Growth occurs microaerobically at 37 and 42°C, but not under anaerobic or aerobic conditions. *Helicobacter canadensis* is urease, alkaline phosphatase, and γ-glutamyl transpeptidase negative but catalase and oxidase positive. Nitrate reduction is variable.

Fig. 21. Transmission electron micrograph of a negatively stained preparation of *Helicobacter aurati* showing its tapered rod morphology with bipolar tufts of sheathed flagella and periplasmic fibers entwining the cell. Bar = 0.5 μm. (Reproduced with permission from Patterson et al., 2000.)

Helicobacter aurati

Helicobacter aurati was cultivated from inflamed stomachs and ceca of adult Syrian hamsters (Patterson et al., 2000). They have a fusiform rod morphology (0.6 μm × 4–8 μm), with periplasmic fibers, and are motile by means of multiple, bipolar, sheathed flagella (Fig. 21). Growth on 5% sheep blood agar appears as a thin, spreading film without distinct colonies. Microaerobic growth occurs at 37 and 42°C. No growth occurs under anaerobic conditions or in the presence of 1–3% NaCl or 1% glycine. *Helicobacter aurati* is positive for urease, catalase, oxidase and γ-glutamyl transpeptidase activities and for indoxyl acetate hydrolysis. Nitrate reduction and alkaline phosphatase hydrolysis are absent. *Helicobacter aurati* is sensitive to nalidixic acid but resistant to cephalothin. By 16S rRNA sequence analysis, *H. aurati* is most closely related to *H. muridarum* and *H. hepaticus*.

Helicobacter typhlonius

A novel *Helicobacter* species was originally isolated from IL-10-deficient mice with colitis and typhlitis (Fox et al., 1999). An identical organism was subsequently cultivated from feces of BALB/c mice and the designation "*Helicobacter typhlonicus*" was proposed (Franklin et al., 1999). For grammatical reasons, this name was changed to *Helicobacter typhlonius* and has recently been included within the validated species (Franklin et al., 2001). It is a curved to spiral rod (0.3 μm × 2–5 μm), and it is motile by means of single, bipolar, sheathed flagella (Fig. 22). *Helicobacter typhlonius* grows as transparent, pinpoint colonies on blood agar under microaerobic conditions at 37°C, with no growth under aerobic or anaerobic conditions or at 25 or 42°C. It is oxidase and catalase positive but urease negative. *Helicobacter typhlonius* reduces nitrate but does not hydrolyze indoxyl acetate or hippurate. There is no alkaline phosphatase or γ-glutamyl transpeptidase activity. Growth occurs in the presence of 1.5% NaCl and 1% glycine. 16S rRNA sequence shows that *H. typhlonius* is most closely related to *H. hepaticus* and *H. muridarum* (97.5%). Similar to *H. bilis* and *H. canis*, there is a 164–166-bp intervening sequence in the 16S rRNA gene.

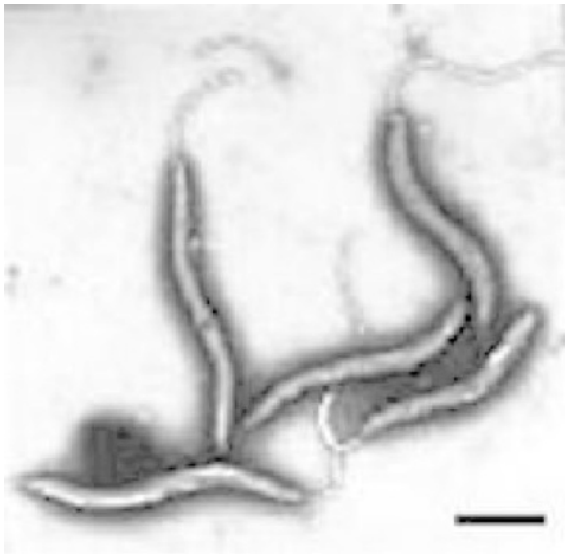

Fig. 22. Transmission electron micrograph of a negatively stained preparation of *Helicobacter typhlonius* showing its curved S-shaped morphology and single, bipolar, sheathed flagella. Bar = 1.5 μm. (Reproduced with permission from Fox et al., 1999.)

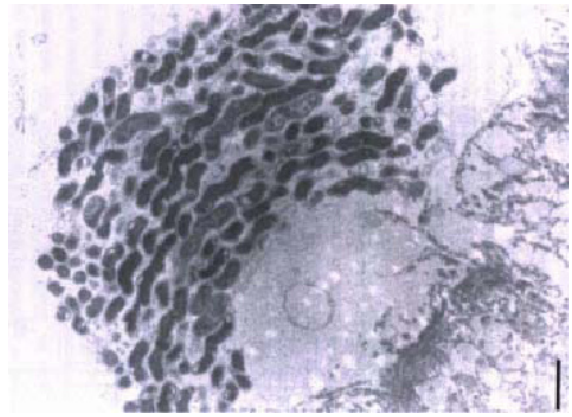

Fig. 23. Transmission electron micrograph of bovine gastric mucosa showing the S-shaped morphology of "*Candidatus Helicobacter bovis*." Bar = 1 μm. (Reproduced with permission from De Groote et al., 1999b.)

Candidate Unvalidated Species

In addition to the 22 validated *Helicobacter* species, a number of isolates currently do not fulfill criteria to be classified as novel species. In some cases the inability to culture the organisms in vitro has resulted in classification as *Candidatus* species. The International Committee on Systematic Bacteriology (Subcommittee on the Taxonomy of *Campylobacter* and Related Bacteria) has recently agreed upon the need for minimal standards for the description of new *Helicobacter* species (Dewhirst et al., 2000b). The category *Candidatus* was proposed and implemented to provide provisional status for incompletely described prokaryotes (Murray and Schleifer, 1994; Murray and Stackebrandt, 1995). In addition to DNA sequence data, a description of *Candidatus* species should include verification of localization in their natural niche by techniques such as in situ hybridization, as well as descriptive information relating to structure and metabolism so far as is available. Currently the *Helicobacter* genus includes two *Candidatus* species and numerous unvalidated species (Tables 4 and 5).

Candidatus Helicobacter bovis

Candidatus Helicobacter bovis is an uncultured bacterium found in the pyloric portion of the abomasum of calves and adult cattle (De Groote et al., 1999b). Gastric bacteria detected in the gastric crypts of the abomasal stomachs of healthy cattle crossreact with polyclonal antibody against *H. pylori*. Ultrastructural studies reveal the presence of large groups of multiple spiral bacteria with 1–3 turns (Fig. 23). The bacteria possess a Gram-negative cell wall and are 0.3 μm × 1.5–2.5 μm in size, with at least four flagella at one end. The presence or absence of a flagellar sheath was not determined. Pyloric biopsy samples were shown to be urease positive. Cloning and sequencing of the 16S rRNA gene from DNA obtained from seven different cattle showed they had a sequence homology of greater than 99%. The closest taxonomic relative was *H. bilis* (92.8%) as determined by a 16S rRNA similarity matrix. A "*Candidatus* Helicobacter bovis"-specific probe (5– AAT TGC GTT TGA AAC TAT CAT T –3) confirmed the presence of these bacteria in the cattle abomasum by in situ hybridization.

Candidatus Helicobacter suis

Candidatus Helicobacter suis is a urease-positive uncultivated bacterium identified in antral biopsies of healthy slaughterhouse pigs (De Groote et al., 1999a). Organisms (2.5–3.5 μm long × 0.6 μm wide) have a tightly coiled appearance with 1–5 bipolar flagella (Fig. 24). Polyclonal antisera against *H. pylori* crossreact with "*Candidatus Helicobacter suis*" and reveal the bacteria most commonly in superficial portions of the gastric crypts. Pairwise comparisons of 16S rDNA sequences obtained from five isolates showed a minimum of 97.7% similarity. The 16S rDNA sequence was 99.5% similar to that of "*H. heilmannii*" type 1 (Solnick et al., 1993), which has been found in humans, pigs, and nonhuman primates. Owing to precedence and nonspecific

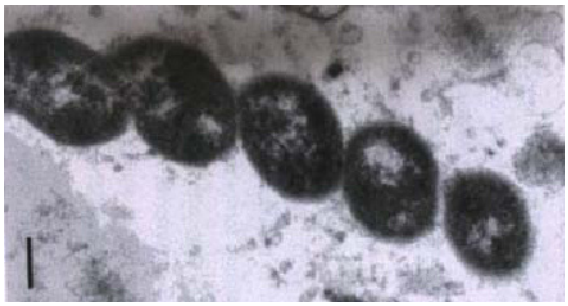

Fig. 24. Transmission electron micrograph of porcine gastric mucosa showing the tight helical morphology of "*Candidatus Helicobacter suis*." Bar = 0.3 µm. (Reproduced with permission from De Groote et al., 1999a.)

host range, it has been proposed that "*Candidatus Helicobacter suis*" be renamed "*Candidatus Helicobacter heilmannii*" (O'Rourke et al., 2001a).

Helicobacter heilmannii

Helicobacter heilmannii (Fig. 25) was initially proposed as the designation for an uncultivated urease-positive gastrospirillum identified in biopsies of human gastric mucosa (Solnick et al., 1993). The morphology is indistinguishable from that of "*Candidatus Helicobacter suis*" (Fig. 24). Since the16S rDNA sequence from the original two isolates differed by 3.5%, they were provisionally given the designation "*H. heilmannii*" type 1 and "*H. heilmannii"* type 2 (Solnick et al., 1993). Subsequent analysis based on both 16S rDNA and urease sequences confirmed that porcine, nonhuman primate, and some human isolates clustered closely together with the original "*H. heilmannii*" type 1 strain. It has been proposed that these strains be designated "*Candidatus Helicobacter heilmannii*," which would replace "*Candidatus Helicobacter suis*" (O'Rourke et al., 2001a). Another group of related strains identified in a tiger, wild dog, bobcat, and human are more closely related to the original "*H. heilmannii*" type 2 isolate and to other known species such as *H. felis* and *H. bizzozeronii*. In clinical gastroenterology, where only morphological data are available, these organisms are often collectively referred to as "*H. heilmannii*," a practice that is convenient but which obscures species differences.

Helicobacter sp. "flexispira"

A Gram-negative bacterium with a slightly spiral morphology, multiple periplasmic fibers, and bipolar tufts of sheathed flagella (Fig. 26) was originally isolated by Kirkbride from aborted

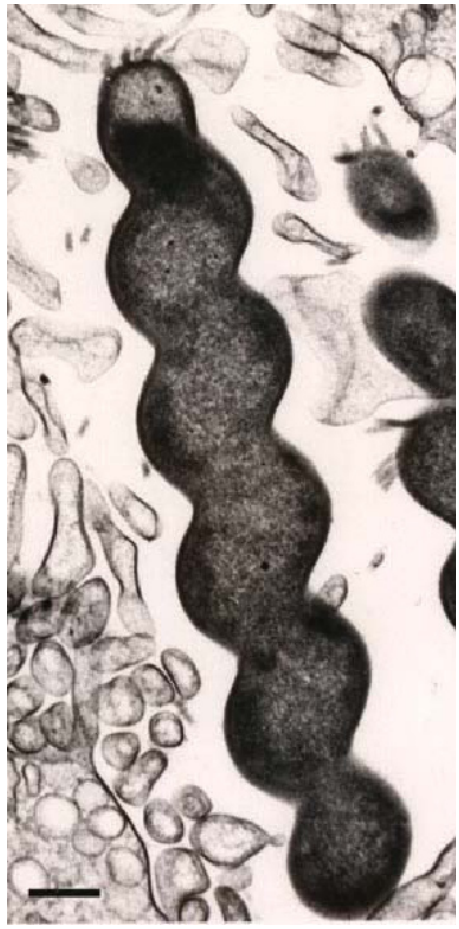

Fig. 25. Transmission electron micrograph of feline gastric mucosa showing the tight helical morphology of "*Helicobacter heilmannii*." Bar = 0.3 µm.

ovine fetuses (Kirkbride et al., 1985). Closely related organisms have subsequently been isolated from a broad range of animal hosts, as well as from normal and immunocompromised humans (Archer et al., 1988; Romero et al., 1988; Schauer et al., 1993; Tee et al., 1998). The original designation of "*Flexispira rappini*" has been abandoned in favor of *Helicobacter*, since several studies have shown that these organisms are members of this taxon (Vandamme et al., 1991a). Recent evidence suggests that this group of organisms contains at least 10 *Helicobacter* taxa, including the two named species, *H. bilis* and *H. trogontum* (Dewhirst et al., 2000a). It has been proposed that the designation "*Helicobacter rappinii*" be applied to strains referred to as *Helicobacter* sp. flexispira taxa 1, 4, and 5, which are closely related and include the original Kirkbride strain (Hänninen et al., 2001).

Helicobacter rappinii has urease, catalase, and γ-glutamyl transferase activity but does not reduce nitrate or produce alkaline phosphatase. Growth occurs as spreading colonies at 37 and

Fig. 26. Transmission electron micrograph of a negatively stained preparation of "*Helicobacter rapinni*" showing its tapered rod morphology with bipolar tufts of sheathed flagella and periplasmic fibers entwining the cell. Bar = 0.5 μm. (Reproduced with permission from Schauer et al., 1993.)

42°C. "*Helicobacter rappinii*" as represented by taxa 1, 4 and 5 grows under strictly anaerobic conditions, but other taxa (for example taxa 8, which includes the original human isolate) grow under microaerobic conditions. "*Helicobacter rappinii*" does not grow in the presence of 1% glycine, and it is resistant to nalidixic acid and cephalothin. By 16S rDNA sequence analysis, "*H. rappinnii*" is most closely related to *H. bilis*.

Helicobacter sp. strain "CLO-3"

Helicobacter sp. strain CLO-3 was one of three groups of *Campylobacter*-like organisms isolated from rectal cultures in homosexual males. The other two groups have subsequently been classified as *H. cinaedi* and *H. fennelliae* (Fennell et al., 1984; Totten et al., 1985; Vandamme et al., 1991a). *Helicobacter* sp. CLO-3 consists of only a single strain, which is a slender spiral rod (0.3–0.5 μm × 1.5–5 μm). It has rapid motility owing to the possession of single, polar, sheathed flagella. It grows as pinpoint, translucent colonies under microaerobic conditions at 37°C, with no growth aerobically or anaerobically. It is catalase positive, is urease negative, does not reduce nitrate, and weakly hydrolyzes indoxyl acetate. *Helicobacter* sp. strain "CLO-3" grows in the presence of 1% glycine and 0.04% TTC but not 2% NaCl. The G+C content is 45 mol%.

Helicobacter sp. "Bird-B" and "Bird-C"

Bacteria isolated from feces of wild terns, gulls, a house sparrow and swine were identified as three biotypes of *Helicobacter*, originally denoted *Helicobacter* sp. "Bird-A, -B and -C" (Seymour et al., 1994). The Bird-A isolate was subsequently classified as *H. pametensis* (Dewhirst et al., 1994). The other closely related organisms are not formally named and are still referred to as *Helicobacter* sp. "Bird-B and Bird-C" because they are represented by only two isolates for one species and one for the other (Dewhirst et al., 1994). Both organisms are slender Gram-negative, spiral rods (0.3–0.5 μm × 1.5–5 μm) and are motile by means of single, polar, sheathed flagella. They are distinguished from *H. pametensis* by their absence of urease activity and resistance to cephalothin. "Bird-C" is susceptible to nalidixic acid and "Bird-B" is moderately susceptible to nalidixic acid. The G+C content of "Bird-B" and "Bird-C" is 31 and 30 mol%, respectively.

Helicobacter suncus

Helicobacter suncus was isolated from the gastric mucosa of house musk shrews (*Suncus murinis*) with gastritis (Goto et al., 1998). It is a curved rod (0.5 μm × 3.55 μm) and is motile by means of bipolar, sheathed flagella (Fig. 27). Bacteria grow as transparent mucoid colonies on moist blood agar plates after incubation at 37°C under microaerobic conditions. Growth occurs under anaerobic, but not aerobic, conditions. Strains of "*H. suncus*" are catalase, oxidase and urease positive. They can reduce nitrate but do not hydrolyze indoxyl acetate or hippurate. "*Helicobacter suncus*" has alkaline phosphatase and arginine aminopeptidase activity but not γ-glutamyl transpeptidase. It is sensitive to tetracycline, erythromycin and chloramphenicol and resistant to nalidixic acid and cephalothin. Phylogenetically and biochemically "*H. suncus*" is most closely related to *H. mustelae* and the unnamed *Helicobacter* sp. "Bird-B and Bird-C."

Helicobacter colifelis

This organism was seen in fecal smears from an 8-week-old kitten with severe diarrhea (Foley et

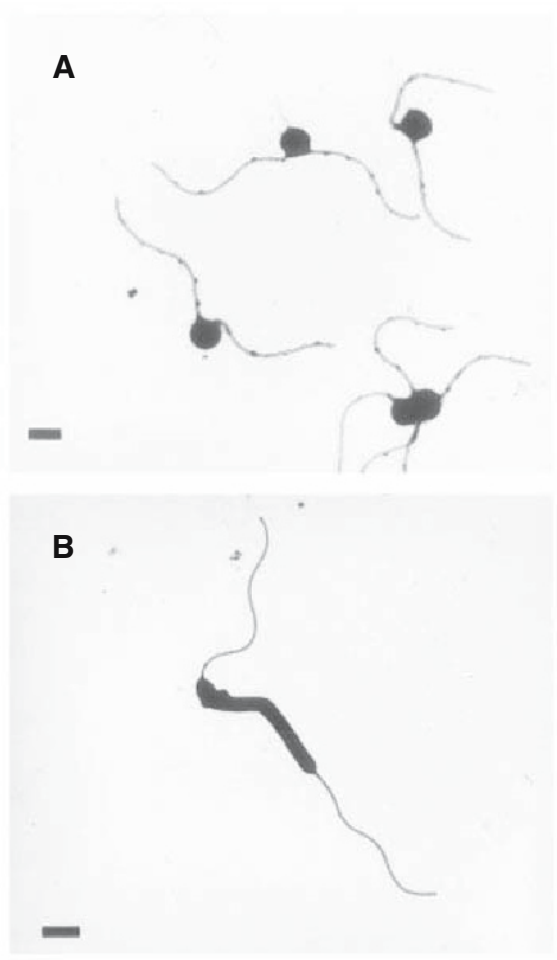

Fig. 27. Transmission electron micrograph of a negatively stained preparation of "*Helicobacter suncus*" showing its rod-like morphology and single, bipolar, sheathed flagella. Bar = 1 μm. (Reproduced with permission from Goto et al., 1998.)

al., 1998). Large numbers of spiral-shaped bacteria were seen in the crypts and surface mucus layer of the cecum and colon. The association with diarrhea was not proven since inoculation of pathogen-free cats with infected feces did not produce symptoms, although feces of the cats were positive by PCR using primers specific for the *Helicobacter* genus. Ultrastructurally "*H. colifelis*" is spiral shaped with two coils, 0.67 μm × 4–5.9 μm in size, with bipolar tufts of flagella that are connected to basal plates at the point of insertion (Fig. 28). Genetically "*H. colifelis*" is most closely related to *H. canis* (98.3%), *H. pullorum* (96.9–96.4%), *H. hepaticus* (96.7%) and *H. cinaedi* (96.5%). Since "*H. colifelis*" is currently uncultivated and was identified on the basis of 16S rRNA sequence from a single isolate, it remains unclear whether it represents a novel enterohepatic *Helicobacter* species.

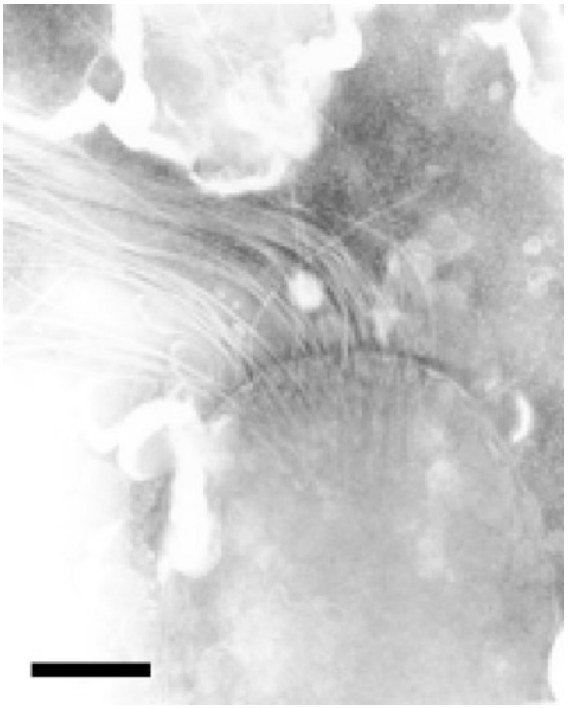

Fig. 28. Transmission electron micrograph of a negatively stained preparation of "*Helicobacter colifelis*" showing its S-shaped morphology and tufts of flagella. Bar = 1.2 μm. (Reproduced with permission from Foley et al., 1998.)

Helicobacter sp. "Cottontop tamarin" (CTT)

An unnamed *Helicobacter* was isolated from fecal samples from Cotton-top tamarins with chronic colitis (Saunders et al., 1999). The organism is a fusiform rod (0.5 μm × 4–5 μm), with periplasmic fibers (Fig. 29). It is motile by means of 6–12 bipolar, sheathed flagella. The bacteria grow as a thin spreading film after 3–5 days growth under microaerobic conditions and are capable of growth at 37 and 42°C but not at 25°C. It is urease negative but positive for catalase and oxidase. Nitrate is not reduced, indoxyl acetate is not hydrolyzed, and no alkaline phosphatase occurs. "*Helicobacter* sp. CTT" is resistant to 1% glycine, nalidixic acid, cephalothin and trimethoprim-sulfamethoxazole. The 16S rRNA gene has a 350-bp intervening sequence and is most closely related to *H. canis* and *H. fennelliae*.

Helicobacter winghamensis

Helicobacter winghamensis was isolated from stools of children and adults with gastroenteritis (Melito et al., 2001). The organism is a curved to spiral rod (0.3–0.6 μm × 2 μm) that is motile by one or two bipolar, unsheathed flagella (Fig. 30). The absence of a flagellar sheath is uncommon

Fig. 29. Transmission electron micrograph of a negatively stained preparation of "*Helicobacter tamarin*" showing its tapered rod morphology with bipolar tufts of sheathed flagella and periplasmic fibers entwining the cell. Bar = 0.5 μm. (Reproduced with permission from Saunders et al., 1999.)

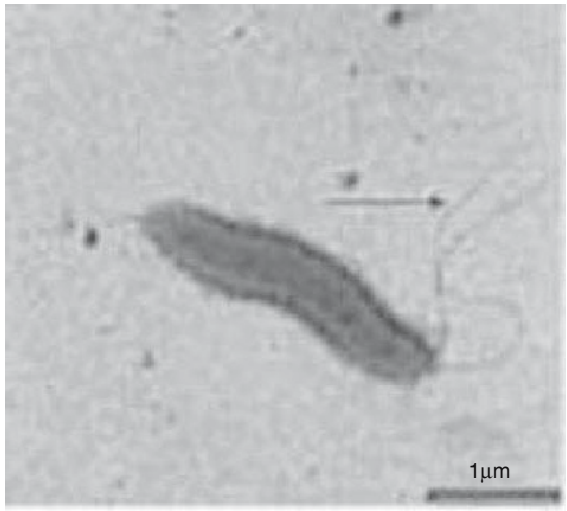

Fig. 30. Transmission electron micrograph of a negatively stained preparation of "*Helicobacter winghamensis*" showing its S-shaped morphology and unsheathed polar flagella. Bar = 1 μm. (Reproduced with permission from Melito et al., 2001.)

among helicobacters, but does occur in *H. pullorum*, *H. canadensis*, *H. mesocricetorum*, *H. ganmani*, *H. rodentium* and "*H. muricola*." Colonies on 10% sheep blood agar show a mixture of spreading and nonspreading morphology. Growth occurs under microaerobic conditions at 37°C but not at 42°C or in aerobic or anaerobic conditions. "*Helicobacter winghamensis*" is negative for urease and alkaline phosphatase activities, and it does not reduce nitrate. Catalase activity is also absent, which among helicobacters has only been described for *H. canis* and taxa 7 and 8 of *Helicobacter* sp. "*flexispira*" (Dewhirst et al., 2000a). "*Helicobacter winghamensis*" is bile tolerant, hydrolyzes indoxyl acetate, and is variably resistant to nalidixic acid and cephalothin. By 16S rRNA sequence analysis, "*H. winghamensis*" is most closely related to *Helicobacter* sp. "*flexispira*" taxon 1 and to *H. cholecystus*.

Helicobacter sp. "rhesus"

Microaerobic culture of colonic tissue from rhesus macaques revealed two novel *Helicobacter* biotypes that were isolated from clinically normal monkeys and monkeys with chronic idiopathic colitis, respectively (Fox et al., 2001b). Cells are spiral in shape, 0.2 μm × 2–3 μm in size, and motile by means of bipolar, sheathed flagella (Fig. 31). Both biotypes are oxidase positive and urease negative and grow at 37 or 42°C as pinpoint or spreading colonies. The two biotypes differ by catalase activity, ability to reduce nitrate, and sensitivity to cephalothin. By 16S rRNA sequence analysis, *Helicobacter* sp. "rhesus" type 1 is most closely related to a *Helicobacter* species isolated from a woodchuck and to *Helicobacter* sp. "Cottontop tamarin." *Helicobacter* sp. "rhesus" type 2 is most closely related to *H. fennelliae* and differs from type 1 by about 4%.

Helicobacter cetorum

Apparently novel *Helicobacter* species were identified by culture and PCR of gastric mucosa from the main stomach of stranded dolphins, *Lagenorhynchus acutus* and *Delphinus delphis* (Harper et al., 2000). Cultivated organisms had a fusiform to slightly spiral morphology and measured 4 μm by 0.6 μm, with laterally located, bipolar, sheathed flagella (Fig. 32). Organisms were positive for urease, catalase, oxidase and γ-glutamyl transpeptidase activity, but did not reduce nitrate or hydrolyze indoxyl acetate. Growth occurred at 42°C and with 1% glycine but not in the presence of nalidixic acid or cephalothin. 16S rDNA sequences from the dolphin isolates cluster with other gastric helicobacters and are most closely related to *H. pylori*. The

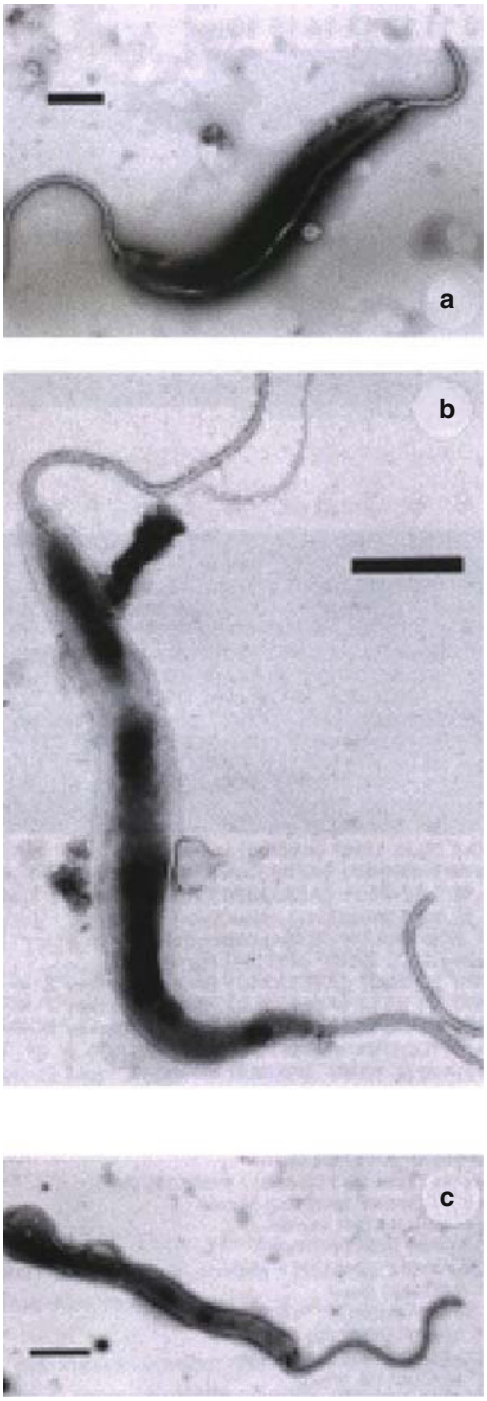

Fig. 31. Transmission electron micrograph of a negatively stained preparation of "*Helicobacter rhesus*" showing its S-shaped morphology and single, bipolar, sheathed flagella. Bar = 0.5 µm. (Reproduced with permission from Fox et al., 2001b.)

name "H. cetorum" has recently been proposed. These isolates represent the first helicobacters isolated from marine mammals. A closely related species was recently cultivated from feces of a beluga whale (Harper et al., 2002).

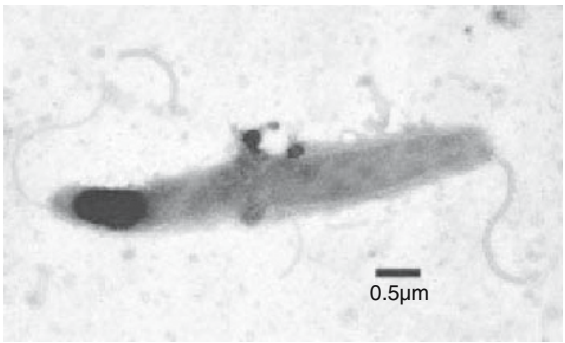

Fig. 32. Transmission electron micrograph of a negatively stained preparation of "*Helicobacter cetorum*" showing its fusiform to S-shaped morphology and bipolar, sheathed flagella. Bar = 0.5 µm. (Reproduced with permission from Harper et al., 2000.)

Helicobacter marmotae

Because woodchucks (*Marmota monax*) have a high incidence of hepatocellular carcinoma, which can be induced by *H. hepaticus* infection in mice, Fox et al. recently examined woodchuck livers to determine whether they were infected with *Helicobacter* species (Fox et al., 2002b). A novel helicobacter was isolated from a single woodchuck and also from the feces of four cats. The organism measures 0.2 by 5 µm, with bipolar, sheathed flagella similar to those of many other enterohepatic *Helicobacter* species. "*Helicobacter marmotae*" is positive for catalase and urease but negative for nitrate reduction, indoxyl acetate hydrolysis, and γ-glutamyl transpeptidase activity. Growth occurs in 1% glycine and in the presence of nalidixic acid and cephalothin, but not at 42°C. By 16S rDNA, "*H. marmotae*" clusters with *H. fennelliae* and with helicobacters isolated from rhesus monkeys and cotton-top tamarins. Whether "*H. marmotae*" promotes hepatocellular carcinoma in woodchucks or causes enterohepatic disease in cats is currently unknown.

Helicobacter muricola

Although Helicobacter species have been isolated from the intestinal tracts and hepatobiliary system of laboratory mice, helicobacters in wild mice have not been well described. Won et al. recently reported the isolation of a slowly growing species from the ceca and fecal pellets of Korean wild mice (Won et al., 2002). The organism measures 0.43–0.5 µm in width and 2.3–5.5 µm in length, with the same morphology as other enterohepatic helicobacters such as *H. typhlonius*, *H. hepaticus* and *H. rodentium*. Isolation required 7–15 days, which is longer than usual for most helicobacters. "*Helicobacter*

muricola" is catalase and urease positive but negative for alkaline phosphatase, γ-glutamyl transpeptidase activity and indoxyl acetate hydrolysis. No growth occurs in 1% glycine or at 42°C. "*Helicobacter muricola*" is sensitive to nalidixic acid but resistant to cephalothin. It is most closely related to another murine *Helicobacter* species, *H. muridarum* (96.7% similarity). Histopathologic evaluation of the gastrointestinal tract of infected mice revealed no abnormalities, so "*H. muricola*" is likely nonpathogenic in its natural host.

Helicobacter Infection and Disease

Helicobacter species are ubiquitous colonizers of the enteric mucosal surface of humans and other animals. In most cases, these bacteria do not appear to have a pathologic relationship with their host; however, there are important exceptions to this generalization, most notably *H. pylori*, which is associated with the development of peptic ulcers and gastric cancer in humans. Even with *H. pylori*, it should be remembered that the relationship between pathogen and host is complex, often commensal, and perhaps even beneficial in some cases (Blaser, 1999).

Human Medicine

H. PYLORI

Epidemiology The prevalence of *H. pylori* infection in developed countries increases with age to about 50% at age 50 years. In developing countries, the prevalence is higher and infection occurs at an earlier age, reaching 50% by age 5 years and increasing to 80% or more by adulthood (Mitchell et al., 1992; Bardhan, 1998; Brown, 2000; Everhart, 2000). *Helicobacterium pylori* infection is predominantly acquired in childhood (Mitchell et al., 1992; Parsonnet, 1995), which has led to the conclusion that the apparent association of *H. pylori* with age largely reflects a cohort effect. That is, infection is more common in older persons because they were born at a time when the prevalence was higher than it is currently. In the absence of effective antibiotic therapy, infection usually persists for decades, if not throughout the lifetime of the host, although some reports have suggested that loss of infection may be more common than previously thought (Klein et al., 1994; Malaty et al., 1999).

Transmission Helicobacter pylori is probably most often transmitted directly from person-to-person, which is suggested by case clustering in families and in institutionalized populations (Mitchell et al., 1989; Mitchell et al., 1993; Vincent et al., 1994; Rothenbacher et al., 1999) and by the fact that strains from family members are often genetically indistinguishable, while those from unrelated persons are usually unique (Wang et al., 1993). The mechanism of transmission is controversial, with evidence for and against gastro-oral, fecal-oral or oral-oral routes (Mitchell, 2001). Contaminated food and water have sometimes been implicated as a source for *H. pylori* infection, especially in developing countries (Klein et al., 1991; Goodman et al., 1996); however, the organism has never been cultivated from drinking water. Although *H. pylori* infection was identified in a single colony of laboratory cats (Handt et al., 1994) and is common among captive nonhuman primates (Dubois et al., 1995; Solnick et al., 1996), zoonotic transmission is unlikely. However, a recent report suggested that a potential zoonotic source may be sheep (Dore et al., 2001).

Associations with Disease Acute *H. pylori* infection rarely comes to clinical attention, but may be associated with self-limited epigastric pain and hypochlorhydria (Marshall et al., 1985; Morris and Nicholson, 1987). Chronic infection causes a histologic gastritis in all individuals that is usually asymptomatic, but may result in the development of duodenal or gastric ulcer. Most cases of peptic ulcer disease are now known to be caused by *H. pylori* infection (Dunn et al., 1997). This has prompted a National Institutes of Health (NIH) consensus recommendation that patients with peptic ulcer and *H. pylori* infection be treated with antibiotics in addition to the usual antisecretory agents (Anonymous, 1994). In some individuals, *H. pylori* infection leads to the development of atrophic gastritis, the histologic precursor to gastric cancer. Both epidemiological (Nomura et al., 1991; Parsonnet et al., 1991; Forman et al., 1993) and prospective studies (Uemura et al., 2001) now support the conclusion that chronic infection with *H. pylori* is associated with a significantly increased risk of developing gastric adenocarcinoma, which worldwide is the second most common cause of cancer morbidity and mortality. Chronic *H. pylori* infection may also cause gastric mucosa-associated lymphoid tissue (MALT) lymphoma, which is uncommon but of considerable interest because it is an antigen-driven tumor that, if detected early, can be cured with antibiotic therapy (Wotherspoon et al., 1991; Wotherspoon et al., 1993; Parsonnet et al., 1994; Roggero et al., 1995; Zucca et al., 1998; Isaacson et al., 1999).

Pathogenesis and Virulence A number of *H. pylori* genes are important in virulence, including those coding for urease and flagella, which are required for colonization (Dunn et al., 1997).

Nearly all strains of *H. pylori* express the VacA cytotoxin, which is an 87-kDa protein that causes vacuolization in cultured epithelial cells and is probably active in vivo (Reyrat et al., 1999). The *vacA* gene is highly polymorphic and particular alleles are more strongly associated with disease than others (Leunk et al., 1988; Atherton et al., 1995). About 60% of the United States strains have a 40-kb pathogenicity island, termed the "Cag PAI," which is more often found in strains isolated from patients with clinical disease (Censini et al., 1996). Six of the 27 genes on the Cag PAI have homology to components of the type IV secretion systems found in *E. coli*, *Agrobacterium tumefaciens*, *Bordetella pertussis* and others, which specialize in the transport of DNA and other macromolecules across the bacterial membrane or into other cells (Covacci et al., 1999). These and other genes on the Cag PAI are required for *H. pylori* to induce gastric epithelial cells to produce interleukin-8 (IL-8), which causes inflammation by recruitment of polymorphonuclear leukocytes (Segal et al., 1997; Crabtree et al., 1999; Li et al., 1999; Fischer et al., 2001). The CagA protein, itself, is also exported by the type IV apparatus and injected into the host cell, where it is tyrosine phosphorylated and likely interrupts eukaryotic signal transduction pathways (Segal et al., 1999; Odenbreit et al., 2000; Stein et al., 2000). Recent evidence suggests that at least one mechanism for this process is via formation of a physical complex between CagA and the SRC homology 2 domain (SH2)-containing tyrosine phosphatase, SHP-2 (Higashi et al., 2002).

Helicobacter pylori can survive over a pH range of 3.5–8 owing to the action of a highly adapted urease that permits maintenance of the proton motive force across the periplasmic membrane via production of ammonia, which eliminates excess hydrogen ions in the local acid milieu. Thus the energy supply to the cell is maintained (Meyer Rosberg et al., 1996). A critical gene for this acid resistance is *ureI*, which encodes a membrane protein that functions as an acid-activated urea transporter (Scott et al., 2000; Weeks et al., 2000). Variations in gastric pH caused by surgery, acid suppressive therapies, or host factors allow the bacteria to colonize different niches within the stomach environment, resulting in differing disease presentations. The location of the bacteria and its behavior at that site are a direct response to the local acid environment within the stomach. High acid output levels are associated with a predominately antral gastritis and increased risk of duodenal ulceration, whereas lower acid levels are associated with colonization through the entire stomach including the corpus mucosa, resulting in pangastritis, gastric ulceration, and gastric adenocarcinoma (Lee et al., 1995; Lee and Van Zanten, 1997; Van Zanten et al., 1999).

Diagnosis Endoscopic diagnosis of *H. pylori* infection is usually performed by histopathologic examination or rapid urease assay performed on gastric biopsies. Culture is not routinely performed in clinical practice, although in experienced laboratories, the sensitivity is probably greater than 95% and the specificity is 100% (Dunn et al., 1997). As *H. pylori* develops increasing antibiotic resistance, culture and sensitivity testing may play a greater role in diagnosis. Since the distribution of *H. pylori* infection is patchy, all endoscopic methods require examination of multiple biopsies, preferably at least three. Nonendoscopic diagnosis is most commonly performed by detection of serum IgG (Ho and Marshall, 2000), which if positive usually indicates active infection. There is currently no validated method for detection of acute *H. pylori* infection with IgM. Noninvasive diagnosis may also be performed using the urea breath test (Chey, 2000; Graham and Klein, 2000b) and stool antigen detection (Vaira et al., 2000).

Treatment Antibiotic therapy administered together with acid suppression using either a proton pump inhibitor or H2 blocker is effective in about 85% of patients, if administered for 7–14 days (Graham, 2000a). Combination of two or more antibiotics is required. Typical antibiotics commonly used are bismuth subsalicylate, tetracycline, and metronidazole administered four times daily or clarithromycin and either amoxicillin or metronidazole administered twice daily. The latter regimen is more expensive but also more convenient. Antibiotic resistance among *H. pylori* isolates is common and increasing, especially to metronidazole and clarithromycin (Meyer et al., 2002).

Prevention Currently, no vaccine is available for prevention of *H. pylori* infection. However, this is an area of active investigation (Del Giudice et al., 2001), and phase I clinical trials are currently underway.

Other *Helicobacter* Species

Gastric Infections Other than *H. pylori*, the only significant gastric *Helicobacter* seen in humans is "*Helicobacter heilmannii*," which has a prevalence of approximately 0.5% among patients undergoing endoscopy (O'Rourke et al., 2002). The low prevalence of "*H. heilmannii*" infections in humans means that a definite disease association is unlikely to be proven; however, it seems likely that this organism does cause gastritis, although the inflammation is generally less

aggressive than that seen with *H. pylori*, and may cause proportionately more cases of gastric MALT lymphoma (Stolte et al., 1997).

Enterohepatic Infections A growing number of enterohepatic *Helicobacter* species is being reported in association with gastroenteritis, hepatitis, and hepatobiliary diseases in humans (reviewed in O'Rourke et al. [2001b], Solnick and Schauer [2001], and Fox [2002a]). *Helicobacter cinaedi* and *H. fennelliae* may cause gastroenteritis, cellulitis, septic arthritis and bacteremia, most commonly but not exclusively in immunocompromised patients such as those infected with the HIV. *Helicobacter pullorum*, *H. canis*, *H. canadensis*, "*H. winghamensis*" and *Helicobacter* sp. "flexispira" may be associated with diarrhea, and *Helicobacter* sp. "flexispira" can also cause bacteremia. The association of *H. bilis* and other bile-tolerant species with hepatobiliary disease in humans is controversial but an area of active research (Fox et al., 1998a).

Veterinary Medicine

Helicobacter species are ubiquitous along the gastrointestinal mucosal surface of a broad range of domestic and feral animals. Nevertheless, there are very few well-documented instances in which infection of an immunocompetent, natural host results in clinical disease (Solnick and Schauer, 2001).

Gastric Infections

Gastric *Helicobacter* infections in animals are associated with a histologic gastritis that resembles that seen in humans, although the polymorphonuclear component is usually minimal or absent. The dominant pathology seen in cats and dogs is a mild chronic gastritis characterized by lymphocytic and lymphoplasmacytic infiltrates in the lamina propria with occasional formation of lymphoid follicles (Henry et al., 1987; Heilmann and Borchard, 1991; Geyer et al., 1993; Otto et al., 1994; Hermanns et al., 1995; Eaton et al., 1996; Happonen et al., 1996; Happonen et al., 1998; Neiger et al., 1998; Norris et al., 1999). A consensus statement by the American College of Veterinary Internal Medicine (ACVIM) concluded that most cats and dogs are infected with gastric bacteria irrespective of health status. Pathologic changes are detected but there is no simple association of bacteria with disease (Simpson et al., 2000).

Several studies have shown a significant increase in the prevalence of gastrospirilla in animals with ulceration of the *pars oesophagea* compared to animals with no ulcers (Barbosa et al., 1995; Queiroz et al., 1996; Roosendaal et al., 2000; Choi et al., 2001). It has been proposed that the bacteria may have an indirect effect on ulcer formation via induction of increased levels of acid secretion (Yeomans and Kolt, 1996). Some animal hosts are colonized by more than one *Helicobacter* species, such as *H. pylori* and *H. acinonychis* in cheetahs and *H. pylori* and "*H. heilmannii*" in nonhuman primates (Euler et al., 1990; Dubois et al., 1991; Dubois et al., 1994). In nonhuman primates, gastritis appears to be less severe in animals infected with gastrospirilla than in those infected with *H. pylori* (Dubois et al., 1991). Gastrospirilla are found intracellularly in parietal cells, which may be linked to increased acid output levels (Dubois et al., 1991; Dubois et al., 1994). Cheetahs that presented with chronic vomiting and weight loss had severe lymphocytic gastritis characterized by variable numbers of neutrophils, gland abscesses, lymphoid follicles and epithelial erosions (Eaton et al., 1991; Eaton et al., 1993b). A treatment study in cheetahs suggested that more severe pathology might be associated with *H. acinonychis* infection rather than with the other gastrospirilla (Wack et al., 1997).

Helicobacter mustelae is the natural colonizer of ferret gastric mucosa, where it is found in association with a diffuse antral chronic gastritis and gastric ulcers (Fox et al., 1986; Fox et al., 1990). The bacteria readily adhere to the epithelial surface, with evidence of endocytosis, a feature not commonly seen with other helicobacters except for *H. pylori* (O'Rourke et al., 1992). Inoculation of *H. mustelae* into uninfected ferrets results in an inflammatory response similar to that seen in naturally infected animals (Fox et al., 1991); however, an association between gastric ulcers and *H. mustelae* infection has not yet been documented.

Finally, experimental inoculation in some animal models may also reproduce pathologic evidence of malignancy. For example, experimental inoculation of *H. felis* in BALB/c mice or *H. pylori* in Mongolian gerbils can induce gastric MALT lymphoma or adenocarcinoma, respectively (Enno et al., 1995; Honda et al., 1998; Watanabe et al., 1998).

Enterohepatic Infection

Among the enterohepatic *Helicobacter* species, *H. hepaticus* infection in mice has been studied most extensively. This organism was first noted in an examination of A/JCr mice that exhibited an increased incidence of liver tumors (Fox et al., 1996a). The bacterium forms part of the normal microbiota of the lower bowel, with sporadic infection of the liver. Infection causes necrotizing hepatitis that commonly goes on to

preneoplastic hyperplasia and hepatocellular carcinoma. Infection of germ-free mice produces chronic hepatitis and hepatocellular carcinoma (Fox et al., 1996b); however, in immunocompetent mice there are no clinical signs of disease and no obvious reduction in breeding efficiency. *Helicobacter hepaticus* and other lower bowel helicobacters have been found in association with hepatitis or inflammatory bowel disease, but these were often in immunocompromised animals (Cahill et al., 1997; Shomer et al., 1997; Haines et al., 1998). "*Helicobacter rappinii*" was first isolated from aborted ovine fetuses and subsequently shown to cross the placenta in sheep, in which it induces abortion and hepatic necrosis in fetuses (Kirkbride et al., 1985; Bryner et al., 1987). These same diseases were induced in guinea pigs infected experimentally with "*H. rappinii*" (Bryner et al., 1987).

Conclusion

Members of the *Helicobacter* genus are ubiquitous colonizers of the enteric mucosal surface, which forms a critical interface between an organism and its environment. There are currently 22 formally named species (though *H. nemestrinae* is likely a junior heterotypic synonym for *H. pylori*). Many additional species have been provisionally named but not yet validated and others have been isolated but not yet named. The genus *Helicobacter* will undoubtedly continue to grow as these and other novel species are identified and characterized. Continued study of these organisms will likely yield new insights regarding the complex and dynamic host-pathogen relationship that occurs during chronic bacterial infections.

Literature Cited

Akopyanz, N., N. O. Bukanov, T. U. Westblom, S. Kresovich, and D. E. Berg. 1992a. DNA diversity among clinical isolates of Helicobacter pylori detected by PCR-based RAPD fingerprinting. Nucleic Acids Res. 20:5137–5142.

Akopyanz, N., N. O. Bukanov, T. U. Westblom, and D. E. Berg. 1992b. PCR-based RFLP analysis of DNA sequence diversity in the gastric pathogen Helicobacter pylori. Nucleic Acids Res. 20:6221–6225.

Albertson, N., I. Wenngren, and J. E. Sjöström. 1998. Growth and survival of Helicobacter pylori in defined medium and susceptibility to Brij 78. J. Clin. Microbiol. 36:1232–1235.

Alm, R. A., L. S. L. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust. 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen Helicobacter pylori. Nature 397:176–180.

Anonymous. 1994. Helicobacter pylori in peptic ulcer disease. JAMA 272:65–69.

Archer, J. R., S. Romero, A. E. Ritchie, M. E. Hamacher, B. M. Steiner, J. H. Bryner, and R. F. Schell. 1988. Characterization of an unclassified microaerophilic bacterium associated with gastroenteritis. J. Clin. Microbiol. 26:101–105.

Atherton, J. C., P. Cao, R. M. Peek, M. Tummuru, M. J. Blaser, and T. L. Cover. 1995. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori: Association of specific vacA types with cytotoxin production and peptic ulceration. J. Biol. Chem. 270:17771–17777.

Austin, J. W., P. Doig, M. Stewart, and T. J. Trust. 1992. Structural comparison of urease and a GroEL analog from Helicobacter pylori. J. Bacteriol. 174:7470–7473.

Barbosa, A. J. A., J. C. P. Silva, A. M. M. F. Nogueira, E. Paulino, and C. R. Miranda. 1995. Higher incidence of Gastrospirillum sp in swine with gastric ulcer of the pars oesophagea. Vet. Pathol. 32:134–139.

Bardhan, P. K. 1998. Epidemiological features of Helicobacter pylori in developing countries. Clin. Infect. Dis. 25:973–978.

Battles, J. K., J. C. Williamson, K. M. Pike, P. L. Gorelick, J. M. Ward, and M. A. Gonda. 1995. Diagnostic assay for Helicobacter hepaticus based on nucleotide sequence of its 16S rRNA gene. J. Clin. Microbiol. 33:1344–1347.

Bizzozero, G. 1892. Über die schlauchfoermigen Drusen des Magendarmkanals und die beziehungen ihres Epithels zu dem Oberfachenepithel der Schleimhaut. Arch. f. Mikr. Anat. 42:82–152.

Blaser, M. J., I. D. Berkowitz, F. M. LaForce, J. Cravens, L. B. Reller, and W.-L. L. Wang. 1979. Methods for isolating Campylobacter jejuni from low-turbidity water. Ann. Intern. Med. 91:179–184.

Blaser, M. J. 1999. Hypothesis: The changing relationships of Helicobacter pylori and humans—implications for health and disease. J. Infect. Dis. 179:1523–1530.

Bode, G., P. Malfertheiner, M. Nilius, G. Lehnhardt, and H. Ditschuneit. 1989. Ultrastructural localisation of urease in outer membrane and periplasm of Campylobacter pylori. J. Clin. Pathol. 42:778–779.

Bode, G., F. Mauch, H. Ditschuneit, and P. Malfertheiner. 1993. Identification of structures containing polyphosphate in Helicobacter pylori. J. Gen. Microbiol. 139:3029–3033.

Brock, F. M., and R. G. Murray. 1988. The ultrastructure and ATPase nature of polar membrane in Campylobacter jejuni. Can. J. Microbiol. 34:594–604.

Bronsdon, M. A., C. S. Goodwin, L. I. Sly, T. Chilvers, and F. D. Schoenknecht. 1991. Helicobacter nemestrinae sp. nov., a spiral bacterium found in the stomach of a pigtailed macaque (Macaca nemestrina). Int. J. Syst. Bacteriol. 41:148–153.

Brown, L. M.. 2000. Helicobacter pylori: epidemiology and routes of transmission. Epidemiol. Rev. 22:283–297.

Bryner, J. H., A. E. Ritchie, L. Pollet, C. A. Kirkbride, and J. E. Collins. 1987. Experimental infection and abortion of pregnant guinea pigs with a unique spirillum-like bacterium isolated from aborted ovine fetuses. Am. J. Vet. Res. 48:91–95.

Cahill, R. J., C. J. Foltz, J. G. Fox, C. A. Dangler, F. Powrie, and D. B. Schauer. 1997. Inflammatory bowel disease: An immunity-mediated condition triggered by bacterial

infection with Helicobacter hepaticus. Infect. Immun. 65:3126–3131.

Cellini, L., N. Allocati, D. Angelucci, T. Iezzi, E. Campli, L. Marzio, and B. Dainelli. 1994. Coccoid Helicobacter pylori not culturable in vitro reverts in mice. Microbiol. Immunol. 38:843–850.

Censini, S., C. Lange, Z. Y. Xiang, J. E. Crabtree, P. Ghiara, M. Borodovsky, R. Rappuoli, and A. Covacci. 1996. Cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors. PNAS (USA) 93:14648–14653.

Chalk, P. A., A. D. Roberts, and W. M. Blows. 1994. Metabolism of pyruvate and glucose by intact cells of Helicobacter pylori studied by C-13 NMR spectroscopy. Microbiology-UK 140:2085–2092.

Chey, W. D. 2000. Accurate diagnosis of Helicobacter pylori. 14C-urea breath test. Gastroenterol. Clin. North Am. 29:895–902.

Choi, Y. K., J. H. Han, and H. S. Joo. 2001. Identification of novel Helicobacter species in pig stomachs by PCR and partial sequencing. J. Clin. Microbiol. 39:3311–3315.

Costas, M., S. L. W. On, R. J. Owen, B. Lopez-Urquijo, and A. J. Lastovica. 1993. Differentiation of Helicobacter species by numerical analysis of their one-dimensional electrophoretic protein patterns. Syst. Appl. Microbiol. 16:396–404.

Covacci, A., J. L. Telford, G. Del Giudice, J. Parsonnet, and R. Rappuoli. 1999. Helicobacter pylori virulence and genetic geography. Science 284:1328–1333.

Cover, T. L., P. I. Hanson, and J. E. Heuser. 1997. Acid-induced dissociation of vacA, the Helicobacter pylori vacuolating cytotoxin, reveals its pattern of assembly. J. Cell Biol. 138:759–769.

Crabtree, J. E., D. Kersulyte, S. D. D. Li, I. J. D. Lindley, and D. E. Berg. 1999. Modulation of Helicobacter pylori induced interleukin-8 synthesis in gastric epithelial cells mediated by cag PAI encoded VirD4 homologue. J. Clin. Pathol. 52:653–657.

Cuccherini, B., K. Chua, V. Gill, S. Weir, B. Wray, D. Stewart, D. Nelson, I. Fuss, and W. Strober. 2000. Bacteremia and skin/bone infections in two patients with X-linked agammaglobulinemia caused by an unusual organism related to Flexispira/Helicobacter species. Clin. Immunol. 97:121–129.

De Groote, D., L.-J. van Doorn, R. Ducatelle, A. Verschuuren, F. Haesebrouck, W. G. V. Quint, K. Jalava, and P. Vandamme. 1999a. Candidatus Helicobacter suis, a gastric Helicobacter from pigs, and its phylogenetic relatedness to other gastrospiralla. Int. J. Syst. Bacteriol. 49:1769–1777.

De Groote, D., L.-J. van Doorn, R. Ducatelle, A. Verschuuren, K. Tilmant, W. G. V. Quint, F. Haesebrouck, and P. Vandamme. 1999b. Phylogenetic characterization of "Candidatus Helicobacter bovis," a new gastric helicobacter in cattle. Int. J. Syst. Bacteriol. 49:1707–1715.

De Groote, D., R. Ducatelle, L. J. van Doorn, K. Tilmant, A. Verschuuren, and F. Haesebrouck. 2000. Detection of "Candidatus Helicobacter suis" in gastric samples of pigs by PCR: comparison with other invasive diagnostic techniques. J. Clin. Microbiol. 38:1131–1135.

Del Giudice, G., A. Covacci, J. L. Telford, C. Montecucco, and R. Rappuoli. 2001. The design of vaccines against Helicobacter pylori and their development. Ann. Rev. Immunol. 19:523–563.

Dent, J. C., and C. A. McNulty. 1988. Evaluation of a new selective medium for Campylobacter pylori. Eur. J. Clin. Microbiol. Infect. Dis. 7:555–558.

Dewhirst, F., C. Seymour, G. Fraser, B. Paster, and J. Fox. 1994. Phylogeny of Helicobacter isolates from bird and swine feces and description of Helicobacter pametensis sp. nov. Int. J. Syst. Bacteriol. 44:553–560.

Dewhirst, F. E., J. G. Fox, E. N. Mendes, B. J. Paster, C. E. Gates, C. A. Kirkbride, and K. A. Eaton. 2000a. "Flexispira rapini" strains represent at least ten Helicobacter taxa. Int. J. Syst. Evol. Microbiol. 50:1781–1787.

Dewhirst, F. E., J. G. Fox, and S. L. W. On. 2000b. Proposal of minimal standards for describing new species of the genus Helicobacter. Int. J. Syst. Evol. Microbiol. 50:2231–2237.

Doenges, J. L. 1939. Spirochaetes in the gastric glands of Macacus rhesus and humans without definite history of related disease. Arch. Pathol. 27:469–477.

Doig, P., and T. J. Trust. 1994. Identification of surface-exposed outer membrane antigens of Helicobacter pylori. Infect. Immun. 62:4526–4533.

Dore, M. P., A. R. Sepulveda, H. El-Zimaity, Y. Yamaoka, M. S. Osato, K. Mototsugu, A. M. Nieddu, G. Realdi, and D. Y. Graham. 2001. Isolation of Helicobacter pylori from sheep: Implications for transmission to humans. Am. J. Gastroenterol. 96:1396–1401.

Dubois, A., A. Tarnawski, D. G. Newell, N. Fiala, W. Dabros, J. Stachura, H. Krivan, and L. M. Heman-Ackah. 1991. Gastric injury and invasion of parietal cells by spiral bacteria in rhesus monkeys: Are gastritis and hyperchlorhydria infectious diseases? Gastroenterology 100:884–891.

Dubois, A., N. Fiala, L. M. Hemanackah, E. S. Drazek, A. Tarnawski, W. N. Fishbein, G. I. Perez-Perez, and M. J. Blaser. 1994. Natural gastric infection with Helicobacter pylori in monkeys: a model for spiral bacteria infection in humans. Gastroenterology. 106:1405–1417.

Dubois, A., N. Fiala, R. H. Weichbrod, G. S. Ward, M. Nix, P. Mehlman, D. M. Taub, G. I. Perez-Perez, and M. J. Blaser. 1995. Seroepizootiology of Helicobacter pylori gastric infection in nonhuman primates housed in social environments. J. Clin. Microbiol. 33:1492–1495.

Dunn, B. E., H. Cohen, and M. J. Blaser. 1997. Helicobacter pylori. Clin. Microbiol. Rev. 10:720–741.

Eaton, K. A., M. J. Radin, L. Kramer, R. Wack, R. Sherding, S. Krakowka, and D. R. Morgan. 1991. Gastric spiral bacilli in captive cheetahs. Scand. J. Gastroenterol. 26:38–42.

Eaton, K. A., F. E. Dewhirst, M. J. Radin, J. G. Fox, B. J. Paster, S. Krakowka, and D. R. Morgan. 1993a. Helicobacter acinonyx sp.nov., isolated from cheetahs with gastritis. Int. J. Syst. Bacteriol. 43:99–106.

Eaton, K. A., M. J. Radin, L. Kramer, R. Wack, R. Sherding, S. Krakowka, J. G. Fox, and D. R. Morgan. 1993b. Epizootic gastritis associated with gastric spiral bacilli in cheetahs (Acinonyx jubatus). Vet. Pathol. 30:55–63.

Eaton, K. A., C. E. Catrenich, K. M. Makin, and S. Krakowka. 1995. Virulence of coccoid and bacillary forms of Helicobacter pylori in gnotobiotic piglets. Infect. Immun. 171:459–462.

Eaton, K. A., F. E. Dewhirst, B. J. Paster, N. Tzellas, B. E. Coleman, J. Paola, and R. Sherding. 1996. Prevalence and varieties of Helicobacter species in dogs from random sources and pet dogs—animal and public health implications. J. Clin. Microbiol. 34:3165–3170.

Enno, A., J. L. O'Rourke, C. R. Howlett, A. Jack, M. F. Dixon, and A. Lee. 1995. MALToma-like lesions in the murine gastric mucosa after long-term infection with Helicobacter felis: A mouse model of Helicobacter pylori-induced gastric lymphoma. Am. J. Pathol. 147:217–222.

Euler, A. R., G. E. Zurenko, J. B. Moe, R. G. Ulrich, and Y. Yagi. 1990. Evaluation of two monkey species (Macaca mulatta and Macaca fascicularis) as possible models for human Helicobacter pylori disease. J. Clin. Microbiol. 28:2285–2290.

Everhart, J. E. 2000. Recent developments in the epidemiology of Helicobacter pylori. Gastroenterol. Clin. North Am. 29:559–578.

Fennell, C. L., P. A. Totten, T. C. Quinn, D. L. Patton, K. K. Holmes, and W. E. Stamm. 1984. Characterization of Campylobacter-like organisms isolated from homosexual men. J. Infect. Dis. 149:58–66.

Fernandez, K. R., L. M. Hansen, P. Vandamme, B. L. Beaman, and J. V. Solnick. 2002. Captive rhesus monkeys (Macaca mulatta) are commonly infected with Helicobacter cinaedi. J. Clin. Microbiol. 40(6):1908–12.

Ferris, F. G., T. J. Beveridge, M. L. Marceau-Day, and A. D. Larson. 1984. Structure and cell envelope associations of flagellar basal complexes of Vibrio cholera and Camplyobacter fetus. Can. J. Microbiol. 30:322–333.

Fischer, W., J. Püls, R. Buhrdorf, B. Gebert, S. Odenbreit, and R. Haas. 2001. Systematic mutagenesis of the Helicobacter pylori cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. Molec. Microbiol. 42:1337–1348.

Foley, J. E., J. V. Solnick, J. M. Lapointe, S. Jang, and N. C. Pedersen. 1998. Identification of a novel enteric Helicobacter species in a kitten with severe diarrhea. J. Clin. Microbiol. 36:908–912.

Foley, J. E., S. L. Marks, L. Munson, A. Melli, F. E. Dewhirst, S. L. Yu, Z. L. Shen, and J. G. Fox. 1999. Isolation of Helicobacter canis from a colony of Bengal cats with endemic diarrhea. J. Clin. Microbiol. 37:3271–3275.

Forman, D., M. Coleman, G. Debacker, J. Eider, H. Moller, L. C. Damotta, P. Roy, L. Abid, G. Debacker, A. Tjonneland, H. Boeing, T. Haubrich, J. Wahrendorf, O. Manousos, H. Tulinius, H. Ogmundsdottir, D. Palli, F. Cipriani, A. Fukao, S. Tsugane, Y. Miyajima, W. Zatonski, J. Tyczynski, J. Calheiros, M. P. Zakelj, M. Potocnik, P. Webb, T. Knight, A. Wilson, S. Kaye, and J. Potter. 1993. An international association between Helicobacter pylori infection and gastric cancer. Lancet 341:1359–1362.

Fox, J. G., B. M. Edrise, E. B. Cabot, C. Beaucage, J. C. Murphy, and K. S. Prostak. 1986. Campylobacter-like organisms isolated from gastric mucosa of ferrets. Am. J. Vet. Res. 47:236–239.

Fox, J. G., T. Chilvers, C. S. Goodwin, N. S. Taylor, P. Edmonds, L. I. Sly, and D. Brenner. 1989. Campylobacter mustelae, a new species resulting from the elevation of Campylobacter pylori subsp. mustelae to species status. Int. J. Syst. Bacteriol. 39:301–303.

Fox, J. G., P. Correa, N. S. Taylor, A. Lee, G. Otto, J. C. Murphy, and R. Rose. 1990. Helicobacter mustelae-associated gastritis in ferrets. An animal model of Helicobacter pylori gastritis in humans. Gastroenterology 99:352–361.

Fox, J. G., G. Otto, N. S. Taylor, W. Rosenblad, and J. C. Murphy. 1991. Helicobacter mustelae-induced gastritis and elevated gastric pH in the ferret (Mustela putorius furo). Infect. Immun. 59:1875–1880.

Fox, J. G., B. J. Paster, F. E. Dewhirst, N. S. Taylor, Y. L.-L., P. J. Macuch, and L. M. Chmura. 1992. Helicobacter mustelae isolation from feces of ferrets: Evidence to support fecal-oral transmission of gastric Helicobacter. Infect. Immun. 60:606–611.

Fox, J. G., F. E. Dewhirst, J. G. Tully, B. J. Paster, L. Yan, N. S. Taylor, M. J. Collins, P. L. Gorelick, and J. M. Ward. 1994. Helicobacter hepaticus sp nov, a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. J. Clin. Microbiol. 32:1238–1245.

Fox, J. G., L. L. Yan, F. E. Dewhirst, B. J. Paster, B. Shames, J. C. Murphy, A. Hayward, J. C. Belcher, and E. N. Mendes. 1995. Helicobacter bilis sp nov, a novel Helicobacter species isolated from bile, livers, and intestines of aged, inbred mice. J. Clin. Microbiol. 33:445–454.

Fox, J. G., X. Li, L. Yan, R. J. Cahill, R. Hurley, R. Lewis, and J. C. Murphy. 1996a. Chronic proliferative hepatitis in A/JCR mice associated with persistent Helicobacter hepaticus infection: A model of Helicobacter-induced carcinogenesis. Infect. Immun. 64:1548–1558.

Fox, J. G., L. Yan, B. Shames, J. Campbell, J. C. Murphy, and X. Li. 1996b. Persistent hepatitis and enterocolitis in germfree mice infected with Helicobacter hepaticus. Infect. Immun. 64:3673–3681.

Fox, J. G., F. E. Dewhirst, Z. L. Shen, Y. Feng, N. S. Taylor, B. J. Paster, R. L. Ericson, C. N. Lau, P. Correa, J. C. Araya, and I. Roa. 1998a. Hepatic Helicobacter species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. Gastroenterology 114:755–763.

Fox, J. G., J. A. MacGregor, Z. Shen, X. Li, R. Lewis, and C. A. Dangler. 1998b. Comparison of methods of identifying Helicobacter hepaticus in B6C3F1 mice used in a carcinogenesis bioassay. J. Clin. Microbiol. 36:1382–1387.

Fox, J. G., P. L. Gorelick, M. C. Kullberg, Z. Ge, F. E. Dewhirst, and J. M. Ward. 1999. A novel urease-negative Helicobacter species associated with colitis and typhlitis in IL-10-deficient mice. Infect. Immun. 67:1757–1762.

Fox, J. G., C. C. Chien, F. E. Dewhirst, B. J. Paster, Z. L. Shen, P. L. Melito, D. L. Woodward, and F. G. Rodgers. 2000. Helicobacter canadensis sp nov isolated from humans with diarrhea as an example of an emerging pathogen. J. Clin. Microbiol. 38:2546–2549.

Fox, J. G., L. Handt, B. J. Sheppard, S. Xu, F. E. Dewhirst, S. Motzel, and I. Klein. 2001a. Isolation of Helicobacter cinaedi from the colon, liver, and mesenteric lymph node of a rhesus monkey with chronic colitis and hepatitis. J. Clin. Microbiol. 39:1580–1585.

Fox, J. G., L. Handt, S. L. Xu, Z. L. Shen, F. E. Dewhirst, B. J. Paster, C. Dangler, K. Lodge, S. Motzel, and H. Klein. 2001b. Novel Helicobacter species isolated from rhesus monkeys with chronic idiopathic colitis. J. Med. Microbiol. 50:421–429.

Fox, J. G. 2002a. The non-H pylori helicobacters: Their expanding role in gastrointestinal and systemic diseases. Gut 50:273–283.

Fox, J. G., Z. Shen, S. Xu, Y. Feng, C. A. Dangler, F. E. Dewhirst, B. J. Paster, and J. M. Cullen. 2002b. Helicobacter marmotae sp. nov. isolated from livers of woodchucks and intestines of cats. J. Clin. Microbiol. 40(7):2513–2419.

Franklin, C. L., C. S. Beckwith, R. S. Livingston, L. K. Riley, S. V. Gibson, C. L. Beschwilliford, and R. R. Hook. 1996.

Isolation of a novel helicobacter species, Helicobacter cholecystus sp. nov., from the gallbladders of syrian hamsters with cholangiofibrosis and centrilobular pancreatitis. J. Clin. Microbiol. 34:2952–2958.

Franklin, C. L., L. K. Riley, R. S. Livingston, C. S. Beckwith, R. R. Hook, C. L. Besch-Williford, R. Hunziker, and P. L. Gorelick. 1999. Enteric lesions in SCID mice infected with "Helicobacter typhlonicus," a novel urease-negative Helicobacter species. Lab. An. Sci. 49:496–505.

Franklin, C. L., P. L. Gorelick, L. K. Riley, F. E. Dewhirst, R. S. Livingston, J. M. Ward, C. S. Beckwith, and J. G. Fox. 2001. Helicobacter typhlonius sp nov., a novel murine urease-negative Helicobacter species. J. Clin. Microbiol. 39:3920–3926.

Ge, Z., D. A. White, M. T. Whary, and J. G. Fox. 2001. Fluorogenic PCR-based quantitative detection of a murine pathogen, Helicobacter hepaticus. J. Clin. Microbiol. 39:2598–2602.

Germani, Y., C. Dauga, P. Duval, M. Huerre, M. Levy, G. Pialoux, P. Sansonetti, and P. A. Grimont. 1997. Strategy for the detection of Helicobacter species by amplification of 16S rRNA genes and identification of H. felis in a human gastric biopsy. Res. Microbiol. 148:315–326.

Geyer, C., F. Colbatzky, J. Lechner, and W. Hermanns. 1993. Occurrence of spiral-shaped bacteria in gastric biopsies of dogs and cats. Vet. Record 133:18–19.

Gibson, J. R., M. A. Ferrus, D. Woodward, J. Xerry, and R. J. Owen. 1999. Genetic diversity in Helicobacter pullorum from human and poultry sources identified by an amplified fragment length polymorphism technique and pulsed-field gel electrophoresis. J. Appl. Microbiol. 87:602–610.

Go, M. F., V. Kapur, D. Y. Graham, and J. M. Musser. 1996. Population genetic analysis of Helicobacter pylori by multilocus enzyme electrophoresis: Extensive allelic diversity and recombinational population structure. J. Bacteriol. 178:3934–3938.

Goodman, K. J., P. Correa, H. J. Tenganá Aux, H. Ramírez, J. P. DeLany, O. Guerrero Pepinosa, M. López Quiñones, and T. Collazos Parra. 1996. Helicobacter pylori infection in the Colombian Andes: A population-based study of transmission pathways. Am. J. Epidemiol. 144:290–299.

Goodwin, C. S., R. K. McCulloch, J. A. Armstrong, and S. H. Wee. 1985. Unusual cellular fatty acids and distinctive ultrastructure in a new spiral bacterium (Campylobacter pyloridis) from the human gastric mucosa. J. Med. Microbiol. vol 19:257–267.

Goodwin, C. S., J. A. Armstrong, T. Chilvers, M. Peters, M. D. Collins, L. Sly, W. McConnell, and W. E. Harper. 1989a. Transfer of Campylobacter pylori and Campylobacter mustelae to Helicobacter pylori gen. nov. and Helicobacter mustelae comb. nov. respectively. Int. J. Syst. Bacteriol. 39:397–405.

Goodwin, C. S., W. McConnell, R. K. McCulloch, C. McCullough, R. Hill, M. A. Bronsdon, and G. Kasper. 1989b. Cellular fatty acid composition of Campylobacter pylori from primates and ferrets compared with those of other campylobacters. J. Clin. Microbiol. 27:938–943.

Goto, K., H. Ohashi, S. Ebukuro, K. Itoh, Y. Tohma, A. Takakura, S. Wakana, M. Ito, and T. Itoh. 1998. Isolation and characterization of Helicobacter species from the stomach of the house musk shrew (Suncus murinus) with chronic gastritis. Curr. Microbiol. 37:44–51.

Graham, D. Y., P. D. Klein, A. R. Opekun, and T. W. Boutton. 1988. Demonstration of plasmids in Campylobacter pylori. J. Infect. Dis. 157:850–851.

Graham, D. Y. 2000a. Therapy of Helicobacter pylori: Current status and issues. Gastroenterology 118:S2–S8.

Graham, D. Y., and P. D. Klein. 2000b. Accurate diagnosis of Helicobacter pylori: 13C-urea breath test. Gastroenterol. Clin. North Am. 29:885–893.

Haines, D. C., P. L. Gorelick, J. K. Battles, K. M. Pike, R. J. Anderson, J. G. Fox, N. S. Taylor, Z. Shen, F. E. Dewhirst, M. R. Anver, and J. M. Ward. 1998. Inflammatory large bowel disease in immunodeficient rats naturally and experimentally infected with Helicobacter bilis. Vet. Pathol. 35:202–208.

Handt, L. K., J. G. Fox, F. E. Dewhirst, G. J. Fraser, B. J. Paster, L. L. Yan, H. Rozmiarek, R. Rufo, and I. H. Stalis. 1994. Helicobacter pylori isolated from the domestic cat: Public health implications. Infect. Immun. 62:2367–2374.

Hänninen, M. L., I. Happonen, S. Saari, and K. Jalava. 1996. Culture and characteristics of Helicobacter bizzozeronii, a new canine gastric helicobacter sp. Int. J. Syst. Bacteriol. 46:160–166.

Hänninen, M. L., and U. Hirvi. 1999. Genetic diversity of canine gastric helicobacters, Helicobacter bizzozeronii and H. salomonis studied by pulsed-field gel electrophoresis. J. Med. Microbiol. 48:341–347.

Hänninen, M. M. L., M. Ultriainen, I. Happonen, and F. E. Dewhirst. 2001. Helicobacter rappinii: A novel Helicobacter species including the type strains Helicobacter species flexispira taxons 1, 4, and 5. Int. J. Med. Microbiol. 291 (Suppl. 31):147.

Happonen, I., S. Saari, L. Castren, O. Tyni, M. L. Hänninen, and E. Westermarck. 1996. Occurrence and topographical mapping of gastric Helicobacter-like organisms and their association with histological changes in apparently healthy dogs and cats. Zentralblatt für Veterinarmed., Reihe A. 43:305–315.

Happonen, I., J. Linden, S. Saari, M. Karjalainen, M. L. Hänninen, K. Jalava, and E. Westermarck. 1998. Detection and effects of helicobacters in healthy dogs and dogs with signs of gastritis. J. Am. Vet. Med. Assoc. 213:1767–1774.

Harper, C. M. G., C. A. Dangler, X. S., Y. Feng, Z. Shen, B. Sheppard, A. Stamper, F. E. Dewhirst, B. J. Paster, and J. G. Fox. 2000. Isolation and characterization of a Helicobacter sp. from the gastric mucosa of dolphins, Lagenorhynchus acutus and Delphinus delphis. Appl. Environ. Microbiol. 66:4751–4757.

Harper, C. M., S. Xu, Y. Feng, J. L. Dunn, N. S. Taylor, F. E. Dewhirst, and J. G. Fox. 2002. Identification of novel Helicobacter spp. from a Beluga Whale. Appl. Environ. Microbiol. 68:2040–2043.

Harrington, C. S., and S. L. On. 1999. Extensive 16S rRNA gene sequence diversity in Campylobacter hyointestinalis strains: taxonomic and applied implications. Int. J. Syst. Bacteriol. 49(3):1171–1175.

Hawtin, P. R., A. R. Stacey, and D. G. Newell. 1990. Investigation of the structure and localization of the urease of Helicobacter pylori using monoclonal antibodies. J. Gen. Microbiol. 136:1995–2000.

Hazell, S. L., and G. L. Mendz. 1997. How Helicobacter pylori works: An overview of the metabolism of Helicobacter pylori. Helicobacter 2:1–12.

Heilmann, K. L., and F. Borchard. 1991. Further observations on human spirobacteria. *In:* H. Menge, M. Gregor,

G. N. J. Tytgat, B. J. Marshall, and C. A. M. McNulty (Eds.). Helicobacter pylori 1990. Springer-Verlag. New York, NY. 63–70.

Henry, G. A., P. H. Long, J. L. Burns, and D. L. Charbonneau. 1987. Gastric spirillosis in beagles. Am. J. Vet. Res. 48:831–836.

Hermanns, W., K. Kregel, W. Breuer, and J. Lechner. 1995. Helicobacter-like organisms: Histopathological examination of gastric biopsies from dogs and cats. J. Comp. Pathol. 112:307–318.

Higashi, H., R. Tsutsumi, S. Muto, T. Sugiyama, T. Azuma, M. Asaka, and M. Hatakeyama. 2002. SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein. Science 295:683–686.

Ho, B., and B. J. Marshall. 2000. Accurate diagnosis of Helicobacter pylori. Serologic testing. Gastroenterol. Clin. North Am. 29:853–862.

Honda, S., T. Fujioka, M. Tokieda, R. Satoh, A. Nishizono, and M. Nasu. 1998. Development of Helicobacter pylori-induced gastric carcinoma in mongolian gerbils. Cancer Res. 58:4255–4259.

Hurtado, A., and R. J. Owen. 1997. A rapid identification scheme for Helicobacter pylori and other species of Helicobacter based on 23S rRNA gene polymorphisms. Syst. Appl. Microbiol. 20:222–231.

Isaacson, P. G., T. C. Diss, A. C. Wotherspoon, R. Barbazza, M. de Boni, and C. Doglioni. 1999. Long-term follow-up of gastric MALT lymphoma treated by eradication of H. pylori with antibodies. Gastroenterology 117:750–751.

Jalava, K., M. Kaartinen, M. Utriainen, I. Happonen, and M. L. Hänninen. 1997. Helicobacter salomonis sp. nov., a canine gastric Helicobacter sp. related to Helicobacter felis and Helicobacter bizzozeronii. Int. J. Syst. Bacteriol. 47:975–982.

Jalava, K., S. L. W. On, P. A. R. Vandamme, I. Happonen, A. Sukura, and M. L. Hänninen. 1998. Isolation and identification of Helicobacter spp, from canine and feline gastric mucosa. Appl. Environ. Microbiol. 64:3998–4006.

Jalava, K., M. C. De Ungria, J. O'Rourke, A. Lee, U. Hirvi, and M. L. Hänninen. 1999a. Characterization of Helicobacter felis by pulsed-field gel electrophoresis, plasmid profiling and ribotyping. Helicobacter 4:17–27.

Jalava, K., S. Hielm, U. Hirvi, and M. L. Hänninen. 1999b. Evaluation of a molecular identification scheme based on 23S rRNA gene polymorphisms for differentiating canine and feline gastric Helicobacter spp. Lett. Appl. Microbiol. 28:269–274.

Jalava, K., S. L. W. On, C. S. Harrington, L. P. Andersen, M. L. Hänninen, and P. Vandamme. 2001. A cultured strain of "Helicobacter heilmannii," a human gastric pathogen, identified as H. bizzozeronii: Evidence for zoonotic potential of Helicobacter. Emerg. Infect. Dis. 7:1036–1038.

Jones, D. M., A. Curry, and A. J. Fox. 1985. An ultrastructural study of the gastric campylobacter-like organism "Campylobacter pyloridis." J. Gen. Microbiol. 131:2335–2341.

Kiehlbauch, J. A., D. J. Brenner, D. N. Cameron, A. G. Steigerwalt, J. M. Makowski, C. N. Baker, C. M. Patton, and I. K. Wachsmuth. 1995. Genotypic and phenotypic characterization of Helicobacter cinaedi and Helicobacter fennelliae strains isolated from humans and animals. J. Clin. Microbiol. 33:2940–2947.

Kirkbride, C. A., C. E. Gates, J. E. Collins, and A. E. Ritchie. 1985. Ovine abortion associated with an anaerobic bacterium. J. Am. Vet. Med. Assoc. 186:789–791.

Klein, P. D., D. Y. Graham, A. Gaillour, A. R. Opekun, and E. O. B. Smith. 1991. Water source as risk factor for Helicobacter pylori infection in Peruvian children. Lancet i:1503–1506.

Klein, P. D., R. H. Gilman, R. Leon-Barua, F. Diaz, E. O. Smith, and D. Y. Graham. 1994. The epidemiology of Helicobacter pylori in Peruvian children between 6 and 30 months of age. Am. J. Gastroenterol. 89:2196–2200.

Krienitz, W. 1906. Über das auftreten von spirochaeten verschiedener form im magenihalt bei carcinoma ventriculi. Dtsch. Med. Wochenschr. 32:872.

Kuhnert, P., and Á. P. Burnens. 2001. Misidentifying Helicobacter cinaedi. J. Clin. Microbiol. 39:2751.

Kusters, J. G., M. M. Gerrits, J. A. Van Strijp, and C. M. Vandenbroucke-Grauls. 1997. Coccoid forms of Helicobacter pylori are the morphologic manifestation of cell death. Infect. Immun. 65:3672–3679.

Lambert, M. A., C. M. Patton, T. J. Barrett, and C. W. Moss. 1987. Differentiation of Campylobacter and Campylobacter-like organisms by cellular fatty acid composition. J. Clin. Microbiol. 25:706–713.

Lastovica, A. J., and E. le Roux. 2000a. Efficient isolation of campylobacteria from stools. J. Clin. Microbiol. 38:2798–2799.

Lastovica, A. J., and M. B. Skirrow. 2000b. Clinical significance of Campylobacter and related species other than Campylobacter jejuni and C. coli. *In:* I. Nachamkin and M. J. Blaser (Eds.). Campylobacter. ASM Press. Washington, DC. 89–120.

Lee, A., S. L. Hazell, J. O'Rourke, and S. Kouprach. 1988. Isolation of a spiral-shaped bacterium from the cat stomach. Infect. Immun. 56:2843–2850.

Lee, A., M. W. Phillips, J. L. O'Rourke, B. J. Paster, F. E. Dewhirst, G. J. Fraser, J. G. Fox, L. I. Sly, P. J. Romaniuk, T. J. Trust, and S. Kouprach. 1992. Helicobacter muridarum sp. nov., a microaerophilic helical bacterium with a novel ultrastructure isolated from the intestinal mucosa of rodents. Int. J. Syst. Bacteriol. 42:27–36.

Lee, A., and J. L. O'Rourke. 1993a. Ultrastructure of Helicobacter organisms and possible revelance for pathogenesis. *In:* C. S. Goodwin and B. W. Worsley (Eds.). Helicobacter pylori Biology and Clinical Practice. CRC Press. Boca Raton, FL. 15–35.

Lee, A., M. Chen, N. Coltro, J. O'Rourke, S. Hazell, P. Hu, and Y. Li. 1993b. Long term infection of the gastric mucosa with Helicobacter species does induce atrophic gastritis in an animal model of Helicobacter pylori infection. Zbl. Bakt. Int. J. Med. Microbiol. 280:38–50.

Lee, A., M. F. Dixon, S. J. Danon, E. Kuipers, F. Megraud, H. Larsson, and B. Mellegard. 1995. Local acid production and Helicobacter pylori: A unifying hypothesis of gastroduodenal disease. Eur. J. Gastroenterol. Hepatol. 7:461–465.

Lee, A., and S. V. Van Zanten. 1997. The aging stomach or the stomachs of the ages: Commentary. Gut 41:575–576.

Leunk, R. D., P. T. Johnson, B. C. David, W. G. Kraft, and D. R. Morgan. 1988. Cytotoxic activity in broth-culture filtrates of Campylobacter pylori. J. Med. Microbiol. 26:93–99.

Li, S. D. D., D. Kersulyte, I. J. D. Lindley, B. Neelam, D. E. Berg, and J. E. Crabtree. 1999. Multiple genes in the left half of the cag pathogenicity island of Helicobacter pylori are required for tyrosine kinase-dependent transcription of interleukin-8 in gastric epithelial cells. Infect. Immun. 67:3893–3899.

Lim, R. K. S. 1920. A parasitic spiral organism in the stomach of the cat. Parasitology 12:108–113.

Linton, D., J. P. Clewley, A. Burnens, R. J. Owen, and J. Stanley. 1994. An intervening sequence (IVS) in the 16S rRNA gene of the eubacterium Helicobacter canis. Nucleic Acids Res. 22, pp:1954–1958.

Lupetti, P., J. E. Heuser, R. Manetti, P. Massari, S. Lanzavecchia, P. L. Bellon, R. Dallai, R. Rappuoli, and J. L. Telford. 1996. Oligomeric and subunit structure of the Helicobacter pylori vacuolating cytotoxin. J. Cell Biol. 133:801–807.

Malaty, H. M., D. Y. Graham, W. A. Wattigney, S. R. Srinivasan, M. Osato, and G. S. Berenson. 1999. Natural history of Helicobacter pylori infection in childhood: 12-year follow-up cohort study in a biracial community. Clin. Infect. Dis. 28:279–282.

Mammen, M. P., Jr., N. E. Aronson, W. J. Edenfield, and T. P. Endy. 1995. Recurrent Helicobacter cinaedi bacteremia in a patient infected with human immunodeficiency virus: Case report. Clin. Infect. Dis. 21:1055.

Marshall, B. J., and J. R. Warren. 1984a. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1:1311–1315.

Marshall, B. J., H. Royce, D. I. Annear, C. S. Goodwin, J. W. Pearman, J. R. Warren, and J. A. Armstrong. 1984b. Original isolation of Campylobacter pyloridis from human gastric mucosa. Microbiol. Lett. 25:83–88.

Marshall, B. J., J. A. Armstrong, D. B. McGechie, and R. J. Glancy. 1985. Attempt to fulfil Koch's postulates for pyloric Campylobacter. Med. J. Austral. 142:436–439.

Marshall, B. J. 1989. History of the discovery of C. pylori. *In:* M. J. Blaser (Ed.). Campylobacter pylori in Gastritis and Peptic Ulcer Disease. Igaku-Shoin. New York, NY. 7–23.

Melito, P. L., C. Munro, P. R. Chipman, D. L. Woodward, T. F. Booth, and F. G. Rodgers. 2001. Helicobacter winghamensis sp nov., a novel Helicobacter sp isolated from patients with gastroenteritis. J. Clin. Microbiol. 39:2412–2417.

Mendes, E. N., D. M. Queiroz, R. S. Coimbra, S. B. Moura, A. J. Barbosa, and G. A. Rocha. 1996a. Experimental infection of Wistar rats with "Gastrospirillum suis." J. Med. Microbiol. 44:105–109.

Mendes, E. N., D. M. M. Queiroz, F. E. Dewhirst, B. J. Paster, S. B. Moura, and J. G. Fox. 1996b. Helicobacter trogontum sp nov, isolated from the rat intestine. Int. J. Syst. Bacteriol. 46:916–921.

Mendz, G. L., S. L. Hazell, and B. P. Burns. 1993. Glucose utilization and lactate production by Helicobacter pylori. J. Gen. Microbiol. 139:3023–3028.

Mendz, G. L., S. L. Hazell, and B. P. Burns. 1994. The Entner-Doudoroff pathway in Helicobacter pylori. Arch. Biochem. Biophys. 312:349–356.

Meyer, J. M., N. P. Silliman, W. Wang, N. Y. Siepman, J. E. Sugg, D. Morris, J. Zhang, H. Bhattacharyya, E. C. King, and R. J. Hopkins. 2002. Risk factors for Helicobacter pylori resistance in the United States: The Surveillance of H. pylori Antimicrobial Resistance Partnership (SHARP) Study, 1993–1999. Ann. Intern. Med. 136:13–24.

Meyer Rosberg, K., D. R. Scott, D. Rex, K. Melchers, and G. Sachs. 1996. The effect of environmental pH on the proton motive force of Helicobacter pylori. Gastroenterology 111:886–900.

Mitchell, H. M., A. Lee, and J. Carrick. 1989. Increased incidence of Campylobacter pylori infection in gastroenterologists: Further evidence to support person-to-person transmission of C. pylori. Scand. J. Gastroenterol. 24:396–400.

Mitchell, H. M., Y. Y. Li, P. J. Hu, Q. Liu, M. Chen, G. G. Du, Z. J. Wang, A. Lee, and S. L. Hazell. 1992. Epidemiology of Helicobacter pylori in Southern China: Identification of early childhood as the critical period for acquisition. J. Infect. Dis. 166:149–153.

Mitchell, H. M., T. Bohane, R. A. Hawkes, and A. Lee. 1993. Helicobacter pylori infection within families. Zbl. Bakt. Int. J. Med. Microbiol. 280:128–136.

Mitchell, H. M. 2001. Epidemiology of infection. *In:* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (Eds.). Helicobacter pylori: Physiology and Genetics. ASM Press. Washington, DC. 7–18.

Morgan, D. D., and R. J. Owen. 1990. Use of DNA restriction endonuclease digest and ribosomal RNA gene probe patterns to fingerprint Helicobacter pylori and Helicobacter mustelae isolated from human and animal hosts. Molec. Cell Probes 4:321–334.

Morris, A., and G. Nicholson. 1987. Ingestion of Campylobacter pyloridis causes gastritis and raised fasting gastric pH. Am. J. Gastroenterol. 82, pp:192–199.

Moss, C. W., M. A. Lambert Fai, M. A. Nicholson, and G. O. Guerrant. 1990. Isoprenoid quinones of Campylobacter cryaerophila, C. cinaedi, C. fennelliae, C. hyointestinalis, C. pylori, and "C. upsaliensis." J. Clin. Microbiol. 28:395–397.

Murray, R. G., and A. Birch-Andersen. 1963. Specialized structure in the region of the flagella tuft in Spirillum serpens. Can. J. Microbiol. 9:393–401.

Murray, R. G. E., and K. H. Schleifer. 1994. Taxonomic notes: A proposal for recording the properties of putative taxa of prokaryotes. Int. J. Syst. Bacteriol. 44:174–176.

Murray, R. G., and E. Stackebrandt. 1995. Taxonomic note: Implementation of the provisional status Candidatus for incompletely described procaryotes. Int. J. Syst. Bacteriol. 45:186–187.

Nedenskov, P. 1994. Nutritional requirements for growth of Helicobacter pylori. Appl. Environ. Microbiol. 60:3450–3453.

Neiger, R., C. Dieterich, A. Burnens, A. Waldvogel, I. Corthesy Theulaz, F. Halter, B. Lauterburg, and A. Schmassmann. 1998. Detection and prevalence of Helicobacter infection in pet cats. J. Clin. Microbiol. 36:634–637.

Nilsson, H. O., J. Taneera, M. Castedal, E. Glatz, R. Olsson, and T. Wadstrom. 2000. Identification of Helicobacter pylori and other Helicobacter species by PCR, hybridization, and partial DNA sequencing in human liver samples from patients with primary sclerosing cholangitis or primary biliary cirrhosis. J. Clin. Microbiol. 38:1072–1076.

Nilsson, H. O., J. Blom, W. A. Al-Soud, A. A. Ljungh, L. P. Andersen, and T. Wadstrom. 2002. Effect of cold starvation, acid stress, and nutrients on metabolic activity of Helicobacter pylori. Appl. Environ. Microbiol. 68:11–19.

Nomura, A., G. N. Stemmermann, P. H. Chyou, I. Kato, G. I. Perez Perez, and M. J. Blaser. 1991. Helicobacter pylori infection and gastric carcinoma among Japanese Americans in Hawaii. N. Engl. J. Med. 325:1132–1136.

Norris, C. R., S. L. Marks, K. A. Eaton, S. Z. Torabian, R. J. Munn, and J. V. Solnick. 1999. Healthy cats are commonly colonized with "Helicobacter heilmannii" that is associated with minimal gastritis. J. Clin. Microbiol. 37:189–194.

Odenbreit, S., J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas. 2000. Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. Science 287:1497–1500.

Olivieri, R., M. Bugnoli, D. Armellini, S. Bianciardi, R. Rappuoli, P. F. Bayeli, L. Abate, E. Esposito, L. de Gregorio, J. Aziz et al. 1993. Growth of Helicobacter pylori in media containing cyclodextrins. J. Clin. Microbiol. 31:160–162.

On, S. L. 1996. Identification methods for campylobacters, helicobacters, and related organisms. Clin. Microbiol. Rev. 9:405–422.

On, S. L. W., and P. Vandamme. 2001. Misidentifying Helicobacter cinaedi. J. Clin. Microbiol. 39:2751–2752.

On, S. L., A. Lee, J. L. O'Rourke, F. E. Dewhirst, B. J. Paster, J. G. Fox, and P. A. R. Vandamme. 2002. The genus helicobacter. *In:* G. M. Garrity, G. M. and D. J. Brenner (Eds.). Bergey's Manual of Systematic Bacteriology, 2nd ed. Bergey's Manual Trust. Springer-Verlag. New York, NY.

O'Rourke, J. L., A. Lee, and J. G. Fox. 1992. An ultrastructural study of Helicobacter mustelae and evidence of a specific association with gastric mucosa. J. Med. Microbiol. 36:420–427.

O'Rourke, J. L., D. De Groote, J. V. Solnick, B. A. Neilan, K. Seidel, R. Hayter, L. M. Hansen, P. Vandamme, and A. Lee. 2001a. "Heilmannii at last": "Candidatus Helicobacter heilmannii" as the official name for the major group of non-H. pylori gastric bacteria. Int. J. Med. Microbiol. 291 (Suppl. 31):149.

O'Rourke, J. L., M. Grehan, and A. Lee. 2001b. Non-pylori helicobacter species in humans. Gut 49:601–606.

O'Rourke, J. L., A. Lee, and J. E. Kellow. 2002. "Helicobacter heilmannii" and other gastric infections in humans. *In:* M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg, and R. L. Guerrant (Eds.). Infections of the Gastrointestinal Tract. Lippincott.Williams & Wilkins. New York, NY.

Otto, G., S. H. Hazell, J. G. Fox, C. R. Howlett, J. C. Murphy, J. L. O'Rourke, and A. Lee. 1994. Animal and public health implications of gastric colonization of cats by helicobacter-like organisms. J. Clin. Microbiol. 32:1043–1049.

Page, R. D. M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12:357–358.

Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley. 1991. Helicobacter pylori infection and the risk of gastric carcinoma. N. Engl. J. Med. 325:1127–1131.

Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. 1994. Helicobacter pylori infection and gastric lymphoma. N. Engl. J. Med. 330:1267–1271.

Parsonnet, J. 1995. The incidence of Helicobacter pylori infection. Aliment. Pharmacol. Therapeut. 9 (Suppl. 2):45–51.

Parsonnet, J., H. Shmuely, and T. Haggerty. 1999. Fecal and oral shedding of Helicobacter pylori from healthy infected adults. JAMA 282:2240–2245.

Paster, B. J., A. Lee, J. G. Fox, F. E. Dewhirst, L. A. Tordoff, G. J. Fraser, J. L. O'Rourke, N. S. Taylor, and R. Ferrero. 1991. Phylogeny of Helicobacter felis sp. nov., Helicobacter mustelae, and related bacteria. Int. J. Syst. Bacteriol. 41:31–38.

Patterson, M. M., M. D. Schrenzel, Y. Feng, S. Xu, F. E. Dewhirst, B. J. Paster, S. A. Thibodeau, J. Versalovic, and J. G. Fox. 2000. Helicobacter aurati sp. nov., a urease-positive Helicobacter species cultured from gastrointestinal tissues of Syrian hamsters. J. Clin. Microbiol. 38:3722–3728.

Peck, B., M. Ortkamp, U. Nau, M. Niederweis, E. Hundt, and B. Knapp. 2001. Characterization of four members of a multigene family encoding outer membrane proteins of Helicobacter pylori and their potential for vaccination. Microb. Infect. 3:171–179.

Penfold, S. S., A. J. Lastovica, and B. G. Elisha. 1988. Demonstration of plasmids in Campylobacter pylori. J. Infect. Dis. 157:850–851.

Phillips, M. W., and A. Lee. 1983. Isolation and characterization of a spiral bacterium from the crypts of rodent gastrointestinal tracts. Appl. Environ. Microbiol. 45:675–683.

Queiroz, D. M. D., G. A. Rocha, E. N. Mendes, S. B. de Moura, A. de Oliveira, and D. Miranda. 1996. Association between Helicobacter and gastric ulcer disease of the pars esophagea in swine. Gastroenterology 111:19–27.

Queiroz, D. M. D., and A. Santos. 2001. Isolation of a Helicobacter strain from the human liver. Gastroenterology 121:1023–1024.

Rappin, J. P. 1881. Contra l'étude de bactérium de la bouche a l'état normal. *In:* R. S. Breed, E. G. D. Murray, and A. P. Hitchens (Eds.). Bergey's Manual of Determinative Bacteriology. Williams and Wilkins. Baltimore, MD. 68.

Reynolds, D. J., and C. W. Penn. 1994. Characteristics of Helicobacter pylori growth in a defined medium and determination of its amino acid requirements. Microbiology-UK 140:2649–2656.

Reyrat, J. M., V. Pelicic, E. Papini, C. Montecucco, R. Rappuoli, and J. L. Telford. 1999. Towards deciphering the Helicobacter pylori cytotoxin. Molec. Microbiol. 34:197–204.

Riley, L. K., C. L. Franklin, R. R. Hook Jr., and C. Besch-Williford. 1996. Identification of murine helicobacters by PCR and restriction enzyme analyses. J. Clin. Microbiol. 34:942–946.

Robertson, B. R., J. L. O'Rourke, P. Vandamme, S. L. W. On, and A. Lee. 2001. Helicobacter ganmani sp nov., a urease-negative anaerobe isolated from the intestines of laboratory mice. Int. J. Syst. Evol. Microbiol. 51:1881–1889.

Roggero, E., E. Zucca, G. Pinotti, A. Pascarella, C. Capella, A. Savio, E. Pedrinis, A. Paterlini, A. Venco, and F. Cavalli. 1995. Eradication of Helicobacter pylori infection in primary low-grade gastric lymphoma of mucosa-associated lymphoid tissue. Ann. Intern. Med. 122:767–769.

Romaniuk, P. J., B. Zoltowska, T. J. Trust, D. J. Lane, G. J. Olsen, N. R. Pace, and D. A. Stahl. 1987. Campylobacter pylori, the spiral bacterium associated with human gastritis, is not a true Campylobacter sp. J. Bacteriol. 169:2137–2141.

Romero, S., J. R. Archer, M. E. Hamacher, S. M. Bologna, and R. F. Schell. 1988. Case report of an unclassified microaerophilic bacterium associated with gastroenteritis. J. Clin. Microbiol. 26:142–143.

Roosendaal, R., J. H. Vos, T. Roumen, R. van Vugt, G. Cattoli, A. Bart, H. Klaasen, E. J. Kuipers, C. Vandenbroucke-Grauls, and J. G. Kusters. 2000. Slaughter pigs are commonly infected by closely related but distinct gastric ulcerative lesion-inducing gastrospirilla. J. Clin. Microbiol. 38:2661–2664.

Rothenbacher, D., G. Bode, G. Berg, U. Knayer, T. Gonser, G. Adler, and H. Brenner. 1999. Helicobacter pylori among preschool children and their parents: Evidence of parent-child transmission. J. Infect. Dis. 179:398–402.

Salama, N., K. Guillemin, T. K. McDaniel, G. Sherlock, L. S. Tompkins, and S. Falkow. 2000. A whole-genome microarray reveals genetic diversity among Helicobacter pylori strains. PNAS (USA) 97:14668–14673.

Salomon, H. 1896. Über das Spirillum des Saugetiermagens und sein Verhalten zu den Belegzellen. Zentralbl. f. Bakt. 19:433–441.

Saunders, K. E., K. J. McGovern, and J. G. Fox. 1997. Use of pulsed-field gel electrophoresis to determine genomic diversity in strains of Helicobacter hepaticus from geographically distant locations. J. Clin. Microbiol. 35:2859–2863.

Saunders, K. E., Z. Shen, F. E. Dewhirst, B. J. Paster, C. A. Dangler, and J. G. Fox. 1999. Novel intestinal Helicobacter species isolated from cotton-top tamarins (Saguinus oedipus) with chronic colitis. J. Clin. Microbiol. 37:146–151.

Schauer, D. B., N. Ghori, and S. Falkow. 1993. Isolation and characterisation of "Flexispira rappini" from laboratory mice. J. Clin. Microbiol. 31:2709–2714.

Schroder, H. D., C. Ludwig, W. Jakob, U. Reischl, M. Stolte, and N. Lehn. 1998. Chronic gastritis in tigers associated with Helicobacter acinonyx. J. Comp. Pathol. 119:67–73.

Scott, D. R., E. A. Marcus, D. L. Weeks, A. Lee, K. Melchers, and G. Sachs. 2000. Expression of the Helicobacter pylori ureI gene is required for acidic pH activation of cytoplasmic urease. Infect. Immun. 68:470–477.

Segal, E. D., C. Lange, A. Covacci, L. S. Tompkins, and S. Falkow. 1997. Induction of host signal transduction pathways by Helicobacter pylori. PNAS (USA) 94:7595–7599.

Segal, E. D., J. Cha, J. Lo, S. Falkow, and L. S. Tompkins. 1999. Altered states: Involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. PNAS (USA). 96:14559–14564.

Seymour, C., R. G. Lewis, M. Kim, D. F. Gagnon, J. G. Fox, F. E. Dewhirst, and B. J. Paster. 1994. Isolation of helicobacter strains from wild bird and swine feces. Appl. Environ. Microbiol. 60:1025–1028.

Shen, Z., J. G. Fox, F. E. Dewhirst, B. J. Paster, C. J. Foltz, L. Yan, B. Shames, and L. Perry. 1997. Helicobacter rodentium sp. nov, a urease-negative helicobacter species isolated from laboratory mice. Int. J. Syst. Bacteriol. 47:627–634.

Shen, Z., Y. Feng, and J. G. Fox. 2000. Identification of enterohepatic Helicobacter species by restriction fragment-length polymorphism analysis of the 16S rRNA gene. Helicobacter 5:121–128.

Shen, Z., Y. Feng, F. E. Dewhirst, and J. G. Fox. 2001. Coinfection of enteric Helicobacter spp. and Campylobacter spp. in cats. J. Clin. Microbiol. 39:2166–2172.

Shomer, N. H., C. A. Dangler, M. D. Schrenzel, and J. G. Fox. 1997. Helicobacter bilis-induced inflammatory bowel disease in SCID mice with defined flora. Infect. Immun. 65:4858–4864.

Simmons, J. H., L. K. Riley, C. L. Besch-Williford, and C. L. Franklin. 2000. Helicobacter mesocricetorum sp. nov., a novel Helicobacter isolated from the feces of syrian hamsters. J. Clin. Microbiol. 38:1811–1817.

Simpson, K., R. Neiger, R. DeNovo, and R. Sherding. 2000. The relationship of Helicobacter spp. infection to gastric disease in dogs and cats. J. Vet. Int. Med. 14:223–227.

Skirrow, M. B. 1977. Campylobacter enteritis: a "new" disease. BMJ 2(6078):9–11.

Solnick, J. V., J. O'Rourke, A. Lee, B. Paster, F. E. Dewhirst, and L. S. Tompkins. 1993. An uncultured gastric spiral organism is a newly identified Helicobacter in humans. J. Infect. Dis. 168:379–385.

Solnick, J. V., D. R. Canfield, and J. Parsonnet. 1996. Seroprevalence of Helicobacter pylori infection in rhesus monkeys. Gastroenterology 110 (Suppl.):A261.

Sorlin, P., P. Vandamme, J. Nortier, B. Hoste, C. Rossi, S. Pavlof, and M. J. Struelens. 1999. Recurrent "Flexispira rappini" bacteremia in an adult patient undergoing hemodialysis: Case report. J. Clin. Microbiol. 37:1319–1323.

Solnick, J. V., and D. B. Schauer. 2001. Emergence of diverse Helicobacter species in the pathogenesis of gastric and enterohepatic diseases. Clin. Microbiol. Rev. 14:59–97.

Stanley, J., D. Linton, A. P. Burnens, F. E. Dewhirst, R. J. Owen, A. Porter, S. L. W. On, and M. Costas. 1993. Helicobacter canis sp.nov., a new species from dogs: An integrated study of phenotype and genotype. J. Gen. Microbiol. 139:2495–2504.

Stanley, J., D. Linton, A. P. Burnens, F. E. Dewhirst, S. L. W. On, A. Porter, R. J. Owen, and M. Costas. 1994. Helicobacter pullorum sp nov.: Genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. Microbiology-UK 140:3441–3449.

Stein, M., R. Rappuoli, and A. Covacci. 2000. Tyrosine phosphorylation of the Helicobacter pylori cagA antigen after cag-driven host cell translocation. PNAS (USA) 97:1263–1268.

Stolte, M., G. Kroher, A. Meining, A. Morgner, E. Bayerdorffer, and B. Bethke. 1997. Comparison of Helicobacter pylori and H. heilmannii gastritis: Matched control study involving 404 patients. Scand. J. Gastroenterol. 32:28–33.

Suerbaum, S., J. M. Smith, K. Bapumia, G. Morelli, N. H. Smith, E. Kunstmann, I. Dyrek, and M. Achtman. 1998. Free recombination within Helicobacter pylori. PNAS (USA) 95:12619–12624.

Suerbaum, S., C. Josenhans, M. Frosch, M. Bell, K. Braig, P. Brandt, B. Chevreux, G. Dietrich, B. Drescher, M. Droege, B. Fartmann, H. Fischer, Z. Ge, R. Holland, W. Mann, G. Nyakatura, O. Pfannes, D. B. Schauer, J. Weber, and J. G. Fox. 2001. Determining the whole genome sequence of Helicobacter hepaticus ATCC 51449. Int. J. Med. Microbiol. 291 (Suppl. 31):115–116.

Suerbaum, S., C. Kraft, F. E. Dewhirst, and J. G. Fox. 2002. Helicobacter nemestrinae ATCC 49396(T) is a strain of Helicobacter pylori (Marshall et al. 1985) Goodwin et al. 1989, and Helicobacter nemestrinae Brondson et al. 1991 is therefore a junior heterotypic synonym of Helicobacter pylori. Int. J. Syst. Evol. Microbiol. 52:437–439.

Taylor, D. E., M. Eaton, N. Chang, and S. M. Salama. 1992. Construction of a Helicobacter pylori genome map and demonstration of diversity at the genome level. J. Bacteriol. 174:6800–6806.

Taylor, D. E., N. Chang, N. S. Taylor, and J. G. Fox. 1994. Genome conservation in Helicobacter mustelae as determined by pulsed-field gel electrophoresis. FEMS Microbiol. Lett. 118:31–36.

Tee, W., K. Leder, E. Karroum, and M. Dyallsmith. 1998. Flexispira rappini bacteremia in a child with pneumonia. J. Clin. Microbiol. 36:1679–1682.

Testerman, T. L., D. J. McGee, and H. L. T. Mobley. 2001. Helicobacter pylori growth and urease detection in the

chemically defined medium Ham's F-12 nutrient mixture. J. Clin. Microbiol. 39:3842–3850.

Tjia, T. N., W. E. S. Harper, C. S. Goodwin, and W. B. Grubb. 1987. Plasmids in Campylobacter pyloridis. Microbios Lett. 36:7–11.

Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. X. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. Mckenney, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey et al. 1997. The complete genome sequence of the gastric pathogen Helicobacter pylori. Nature 388:539–547.

Torres, J., M. Camorlinga, G. Pérez-Peréz, G. Gonzalez, and O. Muñoz. 2001. Validation of the string test for the recovery of Helicobacter pylori from gastric secretions and correlation of its results with urea breath test results, serology, and gastric pH levels. J. Clin. Microbiol. 39:1650–1651.

Totten, P. A., C. L. Fennell, F. C. Tenover, J. M. Wezenberg, P. L. Perine, W. E. Stamm, and K. K. Homes. 1985. Campylobacter cinaedi (sp. nov.) and Campylobacter fennelliae (sp. nov.): two new Campylobacter species associated with enteric disease in homosexual men. J. Infect. Dis. 151:131–139.

Uemura, N., S. Okamoto, S. Yamamoto, N. Matsumura, S. Yamaguchi, M. Yamakido, K. Taniyama, N. Sasaki, and R. J. Schlemper. 2001. Helicobacter pylori infection and the development of gastric cancer. N. Engl. J. Med. 345:784–789.

Vaira, D., M. Menegatti, C. Ricci, L. Gatta, S. Berardi, and M. Miglioli. 2000. Accurate diagnosis of Helicobacter pylori: Stool tests. Gastroenterol. Clin. North Am. 29:917–923.

Vandamme, P., E. Falsen, B. Pot, K. Kersters, and J. De Ley. 1990. Identification of Campylobacter cinaedi isolated from blood and feces of children and adult females. J. Clin. Microbiol. 28:1016–1020.

Vandamme, P., E. Falsen, R. Rossau, B. Hoste, P. Segers, R. Tytgat, and J. De Ley. 1991a. Revision of Campylobacter, Helicobacter, and Wolinella taxonomy: Emendation of generic descriptions and proposal of Arcobacter gen. nov. Int. J. Syst. Bacteriol. 41:88–103.

Vandamme, P., B. Pot, and K. Kersters. 1991b. Differentiation of campylobacters and Campylobacter-like organisms by numerical analysis of one-dimensional electrophoretic protein patterns. Syst. Appl. Microbiol. 14:57–66.

Vandamme, P., B. Pot, M. Gillis, P. de Vos, K. Kersters, and J. Swings. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60:407–438.

Vandamme, P., C. S. Harrington, K. Jalava, and S. L. W. On. 2000. Misidentifying helicobacters: the Helicobacter cinaedi example. J. Clin. Microbiol. 38:2261–2266.

van der Ven, A. J., B. J. Kullberg, P. Vandamme, and J. F. Meis. 1996. Helicobacter cinaedi bacteremia associated with localized pain but not with cellulitis. Clin. Infect. Dis. 22:710–711.

Van Zanten, S. J. O. V., M. F. Dixon, and A. Lee. 1999. The gastric transitional zones: Neglected links between gastroduodenal pathology and Helicobacter ecology. Gastroenterology 116:1217–1229.

Vincent, P., F. Gottrand, P. Pernes, M. O. Husson, M. Lecomte-Houcke, D. Turck, and H. Leclerc. 1994. High prevalence of Helicobacter pylori infection in cohabiting children: Epidemiology of a cluster, with special emphasis on molecular typing [see comments]. Gut 35:313–316.

Wack, R. F., K. A. Eaton, and L. W. Kramer. 1997. Treatment of gastritis in cheetahs (Acinonyx jubatus). J. Zoo Wildlife Med. 28:260–266.

Wang, J. T., J. C. Sheu, J. T. Lin, T. H. Wang, and M. S. Wu. 1993. Direct DNA amplification and restriction pattern analysis of Helicobacter pylori in patients with duodenal ulcer and their families. J. Infect. Dis. 168:1544–1548.

Watanabe, T., M. Tada, H. Nagai, S. Sasaki, and M. Nakao. 1998. Helicobacter pylori infection induces gastric cancer in mongolian gerbils. Gastroenterology 115:642–648.

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, P. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37:463–464.

Weeks, D. L., S. Eskandari, D. R. Scott, and G. Sachs. 2000. A H+-gated urea channel: The link between Helicobacter pylori urease and gastric colonization. Science 287:482–485.

Weir, S. C., C. L. Gibert, F. M. Gordin, S. H. Fischer, and V. J. Gill. 1999. An uncommon Helicobacter isolate from blood: Evidence of a group of Helicobacter spp. pathogenic in AIDS patients. J. Clin. Microbiol. 37: 2729–2733.

Westblom, T. U., E. Madan, and B. R. Midkiff. 1991. Egg yolk emulsion agar, a new medium for the cultivation of Helicobacter pylori. J. Clin. Microbiol. 29:819–821.

Wotherspoon, A. C., C. Ortiz-Hidalgo, M. R. Falzon, and P. G. Isaacson. 1991. Helicobacter pylori-associated gastritis and primary B-cell gastric lymphoma. Lancet 338:1175–1176.

Wotherspoon, A. C., C. Doglioni, T. C. Diss, L. X. Pan, A. Moschini, M. Deboni, and P. G. Isaacson. 1993. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of Helicobacter pylori. Lancet 342:575–577.

Yeomans, N. D., and S. D. Kolt. 1996. Helicobacter heilmannii (formerly Gastrospirillum): Association with pig and human gastric pathology. Gastroenterology 111:244–247.

Zucca, E., F. Bertoni, E. Roggero, G. Bosshard, G. Cazzaniga, E. Pedrinis, A. Biondi, and F. Cavalli. 1998. Molecular analysis of the progression from Helicobacter pylori-associated chronic gastritis to mucosa-associated lymphoid-tissue lymphoma of the stomach. N. Engl. J. Med. 338:804–810.

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CHAPTER 3.5.3

The Genus *Wolinella*

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Introduction

The genus *Wolinella* belongs to the family Helicobacteraceae (epsilon subclass of the *Proteobacteria*). There is only one species of the genus, *W. succinogenes*. The species formerly known as *W. recta* and *W. curva* have been re-classified as *Campylobacter rectus* and *C. curvus* (see [Phylogeny] and [Taxonomy]). *W. succinogenes* was isolated from bovine rumen fluid (see [Habitat and Isolation]) and can be identified and differentiated from other species by various morphological, biochemical and genetic methods (see [Identification]). The cells grow only by anaerobic respiration and do not ferment carbohydrates (see [Cultivation] and [Physiology]). Fumarate, nitrate, nitrite, nitrous oxide (N_2O), polysulfide or dimethyl sulfoxide (DMSO) can serve as terminal electron acceptor with formate as the electron donor. Molecular hydrogen and, at least in fumarate respiration, sulfide are alternative electron donor substrates. The electron transport enzymes and the mechanism for generating the electrochemical proton potential across the membrane are described in the section [Physiology]. Most of the genes encoding the electron transport enzyme complexes are known and a system for site-directed mutagenesis in *W. succinogenes* was established for both the operons coding for the subunits of fumarate reductase and hydrogenase (see [Genetics]).

Phylogeny

The overall phylogenetic position of *Wolinella* relative to other Gram-negative bacteria was determined from 16S rRNA sequencing. This indicated that the genus *Wolinella* falls in the family Helicobacteraceae, which belongs to the epsilon subclass of the *Proteobacteria* (Fig. 1). Other closely related bacteria are grouped within the family Campylobacteraceae, including the genera *Sulfurospirillum*, *Arcobacter* and *Campylobacter*.

Taxonomy

There is only one species of the genus, the type species *Wolinella succinogenes* (previously classified as *Vibrio succinogenes* by Wolin et al., 1961). Two oral species of *Wolinella*, *W. recta* and *W. curva* (Tanner et al., 1981), are now described as members of the genus *Campylobacter*, based on 16S rRNA sequence analysis (Paster and Dewhirst, 1988; Vandamme et al., 1991).

The type strain of *W. succinogenes* is ATCC 29543^T (FDC 602W; FDC: Forsyth Dental Center, Boston, MA, USA).

Habitat

Wolinella succinogenes was isolated from bovine rumen fluid (Wolin et al., 1961). Isolates resembling *W. succinogenes* have been isolated from sewage (Yoshinari, 1980; Tanner et al., 1984) and from humans (Radcliffe et al., 1979); however, no genetic information for these strains is available.

Isolation

Wolinella succinogenes was originally isolated from an inoculum of bovine rumen fluid after serial transfer in an anaerobic methanogenic enrichment medium containing formate, sulfide, and inorganic salts. Secondary enrichment was made in a broth medium containing formate and fumarate (Wolin et al., 1961). Additional strains of *W. succinogenes* were recently isolated using the same procedure except that the primary medium contained fumarate (A. Kröger, unpublished observation). These strains were virtually indistinguishable from the original Wolin strain with respect to their 16S rRNA sequence and their growth parameters. For isolation, bovine rumen fluid was passed through filter papers, and

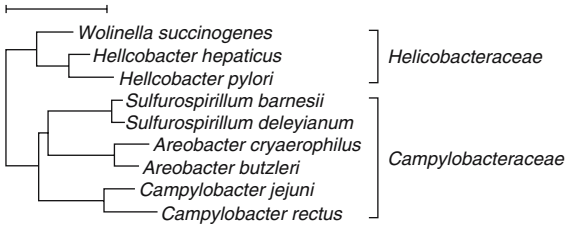

Fig. 1. Phylogeny of *Wolinella succinogenes* as derived from 16S rRNA sequences. The phylogenetic relationships of *Wolinella succinogenes*, the only member of the genus, compared to other members of the families Helicobacteraceae and Campylobacteraceae. Marker bar represents a 5% difference in nucleotide sequences.

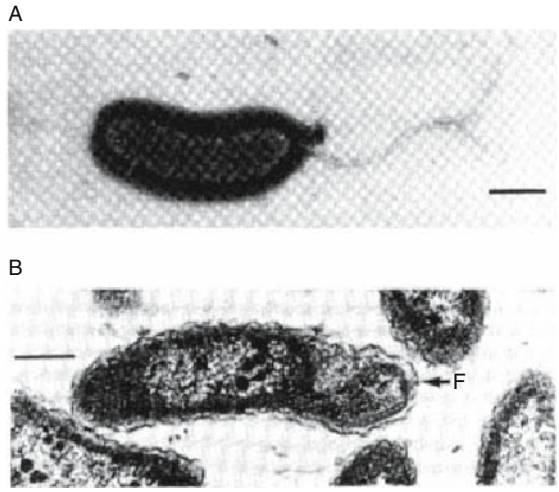

Fig. 2. Electron photomicrographs of *Wolinella succinogenes*. (a) Negatively stained cells, strain ATCC 29543 (FDC 602W): a short curved rod with a typical Gram-negative cell wall surface and a single polar flagellum. Bar = 0.5 µm. (b) Transmission electron photograph showing the flagellar insertion (F). Bar = 0.2 µm.

the filtrate (250 ml) was centrifuged for 15 min at 10,000 × *g*. The sediment was resuspended in 200 ml formate/fumarate medium (Procedure 1). Centrifugation was repeated and the sediment was suspended in 200 ml Tris-buffer (50 mM, pH 8.0, 0°C). One volume of the suspension (a 5% inoculum) was added to nineteen volumes of the "culture medium," which was designed by Wolin et al. (1961) and prepared with tap water. The culture was kept for 24 h at 37°C. Using the same growth conditions, a 5% inoculum of the bacteria was transferred to formate/fumarate medium that contained 10 mM NH_4Cl instead of $(NH_4)_2SO_4$ and lacked acetate. Additionally, 2 mM Na_2S was added before inoculation. Further cultivation made use of the formate/fumarate medium (Procedure 1). Single strains were obtained by plating and picking of colonies (see [Cultivation]).

1. Minimal medium containing formate and fumarate for isolation and cultivation of *W. succinogenes*:

Tris	6.1 g
Fumaric acid	11.6 g
Sodium formate	6.8 g
Dipotassium hydrogen phosphate	3.5 g
Ammonium sulfate	0.7 g
Sodium acetate	2.7 g
Glutamic acid	0.2 g
Calcium/magnesium stock solution (see below)	1 ml
Trace element solution (see below)	0.2 ml
Distilled water	1000.0 ml

Calcium/magnesium stock solution:

Calcium chloride	0.74 g
Magnesium chloride	5.1 g
Distilled water	100.0 ml

Trace element solution:

Na_2 EDTA	5.2 g
$FeCl_2 \cdot 4H_2O$	1.5 g
$ZnCl_2$	70 mg
$MnCl_2 \cdot 4H_2O$	0.1 g
H3BO3	62 mg
CoCl2 ·6H2O	0.19 g
CuCl2 ·2H2O	17 mg
NiCl2 ·6H2O	24 mg
Na2MoO4 ·2H2O	36 mg
Distilled water	1000.0 ml

Combine the medium components and adjust the pH to 7.6–7.8 by addition of potassium hydroxide. Make the medium anoxic by alternate evacuation and flushing with N_2 using appropriate glassware, and sterilize by autoclaving.

Identification

Morphology

Cells of *Wolinella succinogenes* are non-spore-forming Gram-negative rods with a rapid, darting motility via a single polar flagellum. The cells are helical, curved, or straight, 0.5–1.0 µm in diameter and 2–6 µm in length, with rounded or tapered ends (Fig. 2). Colonies are pale yellow opaque to gray translucent with convex, pitting and spreading variants. When grown with nitrate as terminal electron acceptor, colonies appear reddish due to an increased cytochrome *c* content.

Biochemical Properties

Common characteristics for *W. succinogenes* are listed in Table 1. Cells of *W. succinogenes* grow at 37°C, demonstrate oxidase activity, and produce hydrogen sulfide, but are asaccharolytic

Table 1. Characteristics common to *Wolinella succinogenes*[a].

Common positive characteristics:	Gram-negative rods; actively motile via a single polar flagellum; growth in broth supplemented with formate and fumarate; growth in anaerobic and microaerophilic (5% O_2) atmospheres; reduction of nitrate, nitrite, neutral red, and benzylviologen; oxidase and benzidine positive; production of hydrogen sulfide, hydrogen, carbon dioxide and succinate; growth in the presence of Evans blue (0.05 g/liter), malachite green (0.02 mg/liter), brilliant green (0.0125 g/liter), and kanamycin (1, 2, or 4 μg/ml); arginine aminopeptidase positive in API An-Ident tests.
Common negative characteristics:	No growth in unsupplemented broth media or in media supplemented with only 0.2% nitrate; no growth on agar surfaces in air or in air containing 10% CO_2; no spores formed; not haemolytic; pH is not lowered when cells are incubated in formate- and fumarate-supplemented media with 1% adonitol, 1% amygdalin, 1% arabinose, 1% dextran, 1% dulcitol, 1% fructose, 1% galactose, 1% glucose, 1% glycerol, 1% glycogen, 1% inositol, 1% inulin, 1% lactose, 1% matose, 1% mannitol, 1% mannose, 1% melezitose, 1% melibiose, 1% raffinose, 1% rhamnose, 1% ribose, 1% salicin, 1% sorbitol, 1% sucrose, 1% trehalose, 1% xylose, 0.25% esculin, or 0.5% starch; catalase negative; lysine, ornithine, and arginine not decarboxylated; starch, dextran, esculin, casein, DNA, and gelatin not hydrolyzed; hydrogen peroxide, urease, lethicinase, lipase, indole, and acetylmethylcarbinol not produced; nitrous oxide, methane, formate, acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, caproate, lactate, and pyruvate not detected; gas not formed under an agar layer over a broth culture; no growth in the presence of NaCl (20 or 40 g/liter) or penicillin (64 μg/ml); negative or weak reactions in API ZYM and API AN-Ident tests, except for arginine aminopeptidase.

[a] These characteristics are shared by *Campylobacter rectus* (formerly classified as *Wolinella recta*), *C. curvus* (formerly classified as *W. curva*), *C. gracilis*, *C. concisus*, and *C. sputorum*, except: *C. gracilis* has no flagellum and is not actively motile. Data from Tanner et al., 1981, 1984, 1985.

and are negative in many routine biochemical tests including enzyme substrate tests (Table 1). The cells contain *b*- and *c*-type cytochromes, whereas cytochromes of the *a*-, *d*-, and *o*-type are missing (Kröger and Innerhofer, 1976). Menaquinone-6 (2-methyl-3-farnesyl-farnesyl-1,4-naphthoquinone) and a methyl-substituted menaquinone-6 (2, [5 or 8]-dimethyl-3-farnesyl-farnesyl-1,4-naphthoquinone) have been reported as major respiratory quinones (Collins and Fernandez, 1984).

Differentiation of *Wolinella succinogenes* from Other Species

Cells of *W. succinogenes* have been difficult to differentiate from other species (e.g. from members of the Campylobacteraceae). *Campylobacter curvus* and *C. rectus* (formerly classified as *W. curva* and *W. recta*) share the common characteristics of *W. succinogenes* (Table 1). Characteristics that differentiate *W. succinogenes* from the *Campylobacter* species are shown in Table 2. Further differentiation is required from other non-fermentative species that either corrode agar (*Eikenella corrodens*) or demonstrate a formate- and fumarate-utilizing metabolism. *E. corrodens* (family Neisseriaceae) may be distinguished by its growth on agar surfaces in air with 10% carbon dioxide, growth in the presence of 4 μg/ml clindamycin, lack of active cell motility, lack of growth in media supplemented with formate and fumarate without nitrate, and by the presence of lysine and ornithine decarboxylases.

Nowadays, accurate species identification has become quite feasible using one or more of the following methods.

Elisa. Several antisera raised against subunits of *W. succinogenes* electron transport enzymes may be used for identification. However, the antiserum raised against the catalytic subunit (FrdA) of the fumarate reductase notably cross-reacted with the corresponding protein of *Helicobacter pylori* (Birkholz et al., 1994).

Gel Electrophoresis. Sonicated whole cell preparations of *W. succinogenes* and close relatives were run on SDS-polyacrylamide gels (Tanner, 1986). Each species demonstrated a different protein profile when gels were stained with a conventional silver stain (Fig. 3). Notably, *C. rectus* was most easily distinguished from the other species by the presence of yellow-staining bands at an approximate molecular weight of 25 kDa.

Profiling of Fatty Acids and Lipopolysaccharides. Differentiation of *W. succinogenes* from related species was accomplished by analysis of

Table 2. Characteristics that differentiate *Wolinella succinogenes* from various campylobacter species.

Characteristic	*Wolinella succinogenes*	*Campylobacter curvus*	*Campylobacter rectus*	*Campylobacter gracilis*	*Campylobacter concisus*
Source of isolation	Bovine rumen	Human clinical	Human clinical	Human clinical	Human clinical
Dominant cell shape	Helical	Curved	Straight[a]	Straight	Curved
Cells motile by flagellum	+	+	+	–	+
Double yellow bands in SDS-PAGE (20–25 kDa)	–	–	+	–	–
Growth on agar containing:					
NaF (0.5 g/liter)	+	–	–	+	+
Oxgall (10 g/liter)	+	+	–	V	+
Deoxycholate (1 g/liter)	+	+	–	+	+
Janus green (0.1 g/liter)	+	+	–	+	+
Basic fuchsin (0.032 g/liter)	+	+	–	+	+
Cystal violet (0.005 g/liter)	+	–	–	V	+
Indulin scarlet (0.5 g/liter)	–	+	–	–	–
Penicillin (4–16 µg/ml)	+	+	–	V	V
Polymixin B (2–4 µg/ml)	+	+	–	–	–

Data from Tanner et al., 1981, 1984; Tanner, 1986.
[a]*C. rectus* demonstrates an unusual ultrastructure, having hexagonal units in the cell wall.
+, positive feature; –, negative feature; V, variable feature.

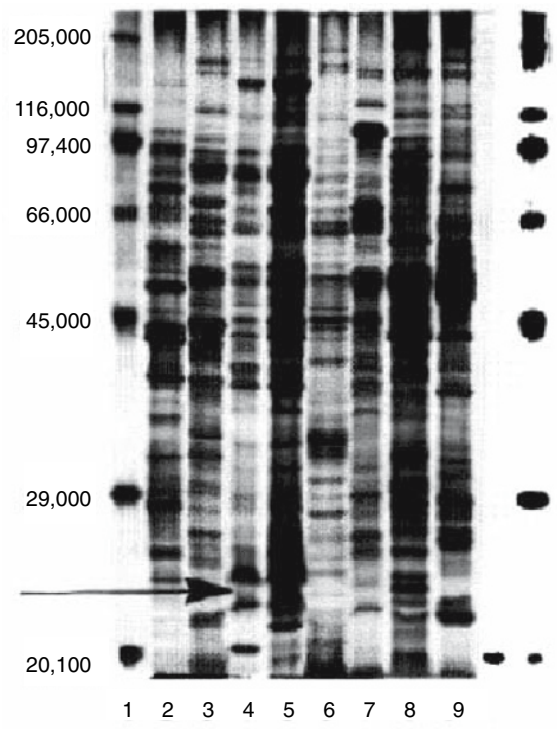

Fig. 3. Silver stained SDS-polyacrylamide gel of sonicated whole cells of species with a formate and fumarate metabolism. The running and stacking gels contained 14% and 4% acrylamide respectively. The current was run at 15 mA per 0.75 mm thick gel. The arrow indicates the level of yellow-staining bands of *Campylobacter rectus* isolates in lanes 4 and 5. Lane 1, high-molecular-weight standard mixture; lane 2, *Wolinella succinogenes* ATCC 29543^T; lane 3, *C. curvus* ATCC 35244^T; lanes 4 and 5, *C. rectus* strains ATCC 33238^T and FDC 267; lane 6, *C. concisus* ATCC 33237^T; lane 7, *C. sputorum* ATCC 35980^T (VPI S-17); lane 8, *C. gracilis* ATCC 33236^T; and lane 9, *Bacteroides ureolyticus* VPI 7815.

either cellular fatty acids (Brondz and Olsen, 1991) or lipopolysaccharides (Kokeguchi et al., 1991; Firoozkoohi et al., 1997). Fatty acids were identified and quantified by gas chromatography and gas chromatography-mass spectrometry. Lipopolysaccharides were extracted from cells by a hot phenol-water method and further purified by nuclease treatment and by repeated ultracentrifugation. Purified lipopolysaccharides were examined by SDS-PAGE and subsequent staining of the gel or by quantification of fatty acid and sugar composition.

Genetic Methods. *W. succinogenes* was discriminated from *Campylobacter* species by restriction fragment length polymorphism (RFLP) analysis of a PCR-amplified fragment of the gene encoding 16S rRNA (Cardarelli-Leite et al., 1996). Furthermore, specific DNA probes generated by PCR using nucleotide sequences of genes encoding electron transport enzymes may be applied for identification of *W. succinogenes* (see [Genetics]).

Cultivation

W. succinogenes can be cultivated using minimal media containing appropriate electron donor and electron acceptor substrates according to the various modes of anaerobic respiration (Table 3 and [Physiology]). The medium for growth with formate and fumarate is presented in the section [Isolation]. In this section, media are shown in which fumarate is replaced by either nitrate or polysulfide. For growth with H_2, formate is left out of the medium and the cultures are stirred

under an atmosphere of H_2 (150 kPa). Further media are described in the literature for growth with sulfide and fumarate (Macy et al., 1986), formate and DMSO (Lorenzen et al., 1994), formate and nitrite (Lorenzen et al., 1993), or formate and N_2O (Yoshinari, 1980). All media may be supplemented with 0.5% (w/v) yeast extract or 0.5% (w/v) brain-heart-infusion broth; this addition raises slightly the growth rate and the growth yield. The addition of brain-heart-infusion broth is recommended when the cells are cultivated on agar plates. The cells may be spread on an agar plate or, alternatively, may be mixed with medium containing 2.6% (w/v) brain-heart-infusion agar and the mixture poured into a Petri dish.

2. Minimal medium for growth with formate and nitrate.

Tris	6.1 g
Potassium nitrate	2.0 g
Sodium formate	5.4 g
Dipotassium hydrogen phosphate	0.2 g
Potassium sulfate	0.9 g
Disodium succinate	0.8 g
Glutamic acid	0.1 g
Cysteine	69 mg
Calcium/magnesium stock solution (see [Isolation])	4.0 ml
Trace element solution (see [Isolation])	0.2 ml
Distilled water	1000.0 ml

Combine the medium components and adjust the pH to 7.5. Make the medium anoxic by alternate evacuation and flushing with N_2 using appropriate glassware, and sterilize by autoclaving.

3. Minimal medium for growth with formate and polysulfide.

Bicine	9.0 g
Sodium acetate	2.4 g
Sodium formate	3.4 g
Dipotassium hydrogen phosphate	4.0 g
Ammonium chloride	0.6 g
Sodium sulfide	0.6 g
Glutamic acid	0.1 ml
Calcium/magnesium stock solution (see below)	1.0 ml
Trace element solution (see below)	0.4 ml
Distilled water	1000.0 ml

Sodium tetrathionate dihydrate stock solution:

Tetrathionate	30.6 g
Distilled water	100.0 ml

Sodium hydroxide stock solution:

Sodium hydroxide	8.0 g
Distilled water	100.0 ml

Combine the medium components and adjust the pH to 8.2. Sterilize the medium by autoclaving and make it anoxic by flushing with N_2 while stirring. After inoculation add 1 ml of the sodium tetrathionate stock solution and 1 ml of the sodium hydroxide stock solution. These additions are repeated after decolorization of the medium due to polysulfide reduction.

Preservation

W. succinogenes cells may be maintained in the laboratory by weekly transfer on the media described in the section [Cultivation]. Cells may be stored frozen either in liquid nitrogen or at –70°C. For longterm maintenance, cells are centrifuged and resuspended in 5% of the volume in the same medium. The suspension is mixed with the same volume of sterile glycerol (86%, v/v), incubated for 5 min at 37°C and stored in 1-ml portions in reaction tubes at –70°C. See The Anaerobic Way of Life in the second edition; anaerobism; ATP, electron acceptor; proton translocation; menaquinone; dicarboxylic acid.

Physiology

Energy Metabolism

W. succinogenes can grow only by anaerobic respiration and does not ferment carbohydrates (Wolin et al., 1961). Growth is sustained by the reduction of fumarate, nitrate, nitrite, N_2O, polysulfide ([S]), or dimethylsulfoxide (DMSO) as terminal electron acceptor and formate as electron donor (reactions a–f in Table 3). The stoichiometry of reactions (a), (b), (c), and (e) was confirmed experimentally with growing cultures, and cell formation was shown to be proportional to substrate consumption and product formation. Polysulfide is the actual terminal electron acceptor of sulfur respiration with *W. succinogenes*. Polysulfide is formed abiotically from elemental sulfur in sulfide solutions. Sulfate, thiosulfate, organic disulfides (R-S-S-R) and trimethylamine-N-oxide (TMAO) are not used as terminal electron acceptors. Growth is observed with fumarate, nitrate, polysulfide or DMSO when formate is replaced by H_2. *W. succinogenes* also can grow by fumarate respiration with sulfide as electron donor (reaction g; Macy et al., 1986).

The ATP required for growth is synthesized according to the mechanism of oxidative phosphorylation. Reactions (a)–(g) are envisaged to be coupled to the generation of an electrochemical proton potential (Δp) across the cytoplasmic membrane. In the second step, the free energy stored in the Δp is used for ATP synthesis from ADP and inorganic phosphate. The latter reaction is catalyzed by an ATP synthase. The ATP synthase of *W. succinogenes* is similar to those of aerobic or phototrophic bacteria (Bokranz et al., 1985a and b). When incorporated into liposomes, the isolated enzyme catalyzes ATP synthesis at the expense of an artificial Δp across the liposomal membrane. The measured Δp generated by reactions (a), (b), (c), (e) or (g) was 160–180 mV,

Table 3. Catabolic reactions sustaining growth of *W. succinogenes*.

Growth reaction	t_d (h)	Y (g dry cells/mol formate)	Reference
(a) $HCO^-_2 + Fumarate + H^+ \rightarrow CO_2 + Succinate$	1.5	7.0	Bronder et al., 1982
(b) $HCO^-_2 + NO^-_3 + H^+ \rightarrow CO_2 + NO^-_2 + H_2O$	1.4	5.2	Bokranz et al., 1983
(c) $3HCO^-_2 + NO^-_2 + 5H^+ \rightarrow 3CO_2 + NH^-_4 + 2H_2O$	1.4	5.2	Bokranz et al., 1983
(d) $HCO^-_2 + N_2O + H^+ \rightarrow CO_2 + N_2 + H_2O$	≤2.7[a]	5.7[a]	Yoshinari, 1980
(e) $HCO^-_2 + [S] \rightarrow CO_2 + HS^-$	2.3	3.2	Klimmek et al., 1991
(f) $HCO^-_2 + (CH_3)_2SO + H^+ \rightarrow CO_2 + (CH_3)_2S + H_2O$	14	6.7	Lorenzen et al., 1994
(g) $HS^- + Fumarate + H^+ \rightarrow [S] + Succinate$	3.6	2.4[b]	Simon et al., 1998

[a]evaluated from the data of Yoshinari (1980); [b]g dry cells/mol fumarate.

which is sufficient to drive ATP synthesis at a H^+/ATP ratio of 3 (Mell et al., 1986; Brune et al., 1987; Wloczyk et al., 1989; Hedderich et al., 1999; Simon et al., 2000).

Biosynthetic Metabolism

Fumarate can be used as the sole source of carbon during growth of *W. succinogenes* by fumarate respiration in a minimal medium (Bronder et al., 1982). Fumarate can be replaced by L-malate, L-aspartate, or L-asparagine (Wolin et al., 1961; Kafkewitz, 1975). Both the growth rate and yield of *W. succinogenes* cultivated in fumarate-containing medium nearly double when glutamate is present. The growth parameters in medium with yeast extract are slightly higher than they are in medium with glutamate. When grown in a minimal medium with nitrate, *W. succinogenes* required succinate as carbon source (Bokranz et al., 1983). Succinate could be substituted by malate, fumarate or L-asparagine, whereas oxaloacetate, pyruvate, bicarbonate, acetate, propionate, butyrate, L-glutamate, D-aspartate, or δ-aminolevulinic acid could not replace succinate (Niederman and Wolin, 1972). When polysulfide serves as terminal electron acceptor, acetate and glutamate are used as carbon sources in a minimal medium (Macy et al., 1986). While sulfate is used as the source of sulfur during growth with fumarate, either sulfide or cysteine has to be supplied for growth by nitrate respiration (Bokranz et al., 1983).

The biosynthetic metabolism of fumarate was elucidated using labeled fumarate (Fig. 4; Bronder et al., 1982). In addition, the specific activities of the key anabolic enzymes were measured. The synthesis of each aspartate and alanine residue of bacterial protein was found to be from one fumarate molecule, whereas the synthesis of each glutamate was found to be from two fumarate molecules, suggesting that glutamate is formed via citrate. In agreement with this view, *W. succinogenes* was found to contain citrate synthase; however, the specific activity was very low. During growth with glutamate and fumarate, *W. succinogenes* specifically incorporated the carbon of glutamate into the residues of the glutamate family (glutamate, glutamine, proline and arginine) of the bacterial protein. This suggests that growth in the absence of glutamate would be limited by glutamate biosynthesis. While approximately half of the added glutamate was incorporated into bacterial protein, the residual glutamate was oxidized to succinate. The reducing equivalents liberated in the latter process are used in biosynthesis. Hence, the catabolic electron donors H_2 or formate are not involved in biosynthesis, in agreement with the absence of enzymes catalyzing the reduction of NAD, NADP or ferredoxin by H_2 or formate. As an important consequence, *W. succinogenes* can be grown at limiting energy supply in continuous culture by making the concentration of formate in the medium growth limiting. Under these conditions, the growth yield was measured to be 43% higher than that during growth at limiting fumarate concentration (Mell et al., 1982).

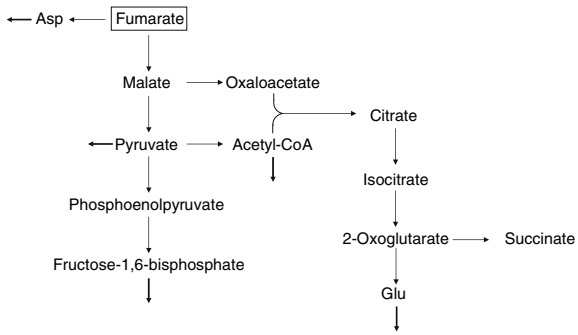

Fig. 4. Biosynthetic pathways of *W. succinogenes* growing with fumarate (boxed) as sole carbon source (Bronder et al., 1982). For details see subsection [Biosynthetic metabolism].

Electron Transport Enzymes

Fumarate reductase, hydrogenase, formate dehydrogenase, nitrite reductase, and polysulfide reductase are the key enzymes of anaerobic

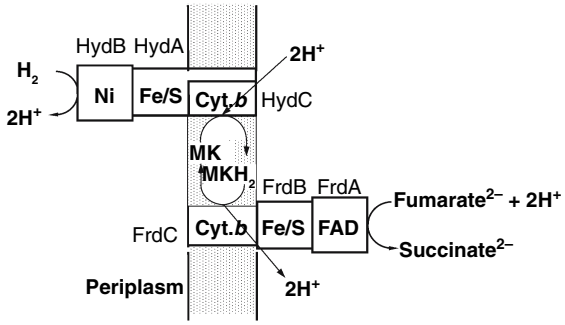

Fig. 5. Composition and orientation of hydrogenase (HydA, B, C) and of fumarate reductase (FrdA, B, C) in the membrane of *W. succinogenes*, and hypothetical mechanism of Δp generation by fumarate respiration with molecular hydrogen as electron donor. Hydrogenase is anchored in the membrane by its HydC subunit and the C-terminal hydrophobic region of HydA (Gross et al., 1998a). Ni, nickel ion; Fe/S, iron-sulfur centers; Cyt. *b*, diheme cytochrome *b*; MK, menaquinone.

fumarate respiration, nitrite respiration and polysulfide respiration, with molecular hydrogen or formate serving as electron donor (Table 3). These five enzymes are discussed below in more detail. Other electron transport enzymes have not been isolated, i.e., the nitrate reductase, the dimethylsulfoxide (DMSO) reductase, or the sulfide dehydrogenase that serves in fumarate respiration with sulfide as electron donor. The nitrous oxide reductase was isolated as a dimer with a molecular weight of 162,000 (Terguchi and Hollocher, 1989).

Fumarate Reductase Fumarate reductase catalyzes the reduction of fumarate by menaquinol (MKH_2; Unden et al., 1980; Unden and Kröger, 1982; Kröger et al., 1992). The enzyme consists of three different subunits and is anchored in the membrane by FrdC, a diheme cytochrome *b* that interacts with the quinol (Fig. 5; Unden et al., 1980; Körtner et al., 1990; Lauterbach et al., 1990). The crystal structure of *W. succinogenes* fumarate reductase has been solved at 2.2 Å resolution (Lancaster et al., 1999). The subunit FrdB carries three iron-sulfur centers and lies between FrdC and the catalytic subunit FrdA to which FAD is covalently linked. The fumarate catalytic site protrudes 40 Å into the cytoplasm. Fumarate is bound close to the flavin and is probably reduced by hydride transfer from N5 of reduced flavin. There is a pathway for single electron transfer extending from the heme groups via the iron-sulfur centers to the flavin. The [3Fe-4S] iron-sulfur center is closest to the heme group situated next to the cytoplasmic membrane surface, and the [2Fe-2S] center is closest to the flavin. Electron transfer between these two centers is mediated by the [4Fe-4S] center. When viewed along the membrane normal, the heme groups are on top of each other with their planes perpendicular to the membrane surface and with an interplanar angle of 95°. The centers of the heme groups are only 15.6 Å apart, suggesting a role in electron transfer for both heme groups. The site of quinol oxidation on FrdC is not seen. The structure of *E. coli* fumarate reductase is similar to that of *W. succinogenes* with respect to the hydrophilic subunits FrdA and FrdB (Iverson et al., 1999). The enzymes differ in their membrane anchor domains. The subunits FrdC and FrdD, which carry two menaquinone (MK) molecules but no heme, anchor the *E. coli* enzyme.

Hydrogenase Hydrogenase is bound to the membrane of *W. succinogenes* and catalyzes the reduction of menaquinone (MK) by H_2 (Fig. 5; Dross et al., 1992). The enzyme consists of three different subunits, HydA, B and C. The two hydrophilic subunits HydA and HydB are similar to the two polypeptides making up the periplasmic Ni (nickel)-hydrogenases with known crystal structures from two *Desulfovibrio* species (Volbeda et al., 1995; Higuchi et al., 1997). The larger subunit HydB carries the Ni/Fe catalytic center, and HydA carries three iron-sulfur centers. The structures suggest that H_2 is split into electrons and protons at the catalytic center. The protons are released on the surface of HydB through a proton pathway, while the electrons are guided by the three iron-sulfur centers to the binding site of the electron acceptor on the surface of HydA. Because nearly all the relevant residues are conserved in the membrane-bound Ni-hydrogenases, the same catalytic mechanism likely applies to these enzymes. In the enzyme of *W. succinogenes*, the electrons are transferred from HydA to the third subunit (HydC), a diheme cytochrome *b* that is integrated into the membrane and reacts with MK (Gross et al., 1998a; and 1998b). The catalytic subunit of HydB is oriented to the periplasmic side of the membrane. The subunit HydA carries a C-terminal hydrophobic helix that is integrated into the membrane. Such a hydrophobic C-terminus with a conserved histidine residue is predicted to occur in the HydA subunits of all membrane-bound Ni-hydrogenases and is missing in the periplasmic enzymes.

Formate Dehydrogenase Formate dehydrogenase catalyzes the reduction of menaquinone (MK) by formate (Unden and Kröger, 1982; Bokranz et al., 1991). The enzyme consists of two hydrophilic subunits, FdhA and FdhB, and of FdhC, a cytochrome *b* that is integrated into the membrane and reacts with MK (Fig. 6). The sub-

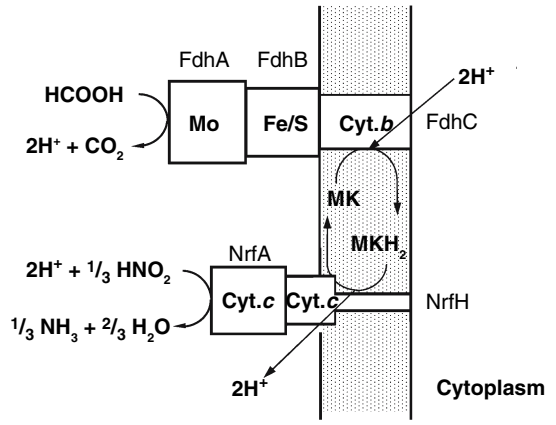

Fig. 6. Composition and orientation of formate dehydrogenase (FdhA, B, C) and of nitrite reductase (NrfA, H) in the membrane of *W. succinogenes*, and hypothetical mechanism of Δp generation by nitrite respiration with formate as electron donor. The substrates and products of nitrite reduction are drawn in their neutral forms for simplicity. Mo, molybdenum linked to molybdopterin guanine dinucleotide; Fe/S, iron-sulfur centers; Cyt. *b*, cytochrome *b*; Cyt. *c*, cytochrome *c*. (See The Dissimilatory Sulfate- and Sulfur-Reducing Prokaryotes in the second edition; elemental sulfur).

unit FdhC is similar to HydC of *W. succinogenes* hydrogenase and carries probably two heme B groups (Gross et al., 1998b). *W. succinogenes* contains a single formate dehydrogenase, but two gene loci code for the subunits of the enzyme (Lenger et al., 1997). These *fdh* operons differ in their promoter regions, but are nearly identical in their gene sequences. Deletion mutants lacking one of the operons still grow with formate as electron donor. The formate dehydrogenases of the mutants appear to be identical.

The catalytic subunit FdhA contains molybdenum ligated by molybdopterin guanine dinucleotide and is predicted to carry a tetranuclear iron sulfur center (Bokranz et al., 1991; Jankielewicz et al., 1994). The subunit FdhB is predicted to carry four tetranuclear or one trinuclear and three tetranuclear iron-sulfur centers. The sequence of FdhA is similar to those of several other molybdo-oxidoreductases whose crystal structures are known, including formate dehydrogenase H of *Escherichia coli* (Boyington et al., 1997). The structures of these enzymes share a deep crevice extending from the surface of the subunit to the molybdenum, the site of substrate conversion. In *E. coli* formate dehydrogenase H, an iron-sulfur center is localized at an appropriate distance to allow rapid electron transfer from the molybdenum site. This suggests that formate is split at the molybdenum site to yield two electrons, CO_2 and a proton. While the electrons are transferred to the iron-sulfur center, CO_2 and the protons probably leave the catalytic site via the substrate crevice. Therefore, the protons produced from formate are thought to be liberated at the surface of FdhA whereas the electron transfer from FdhA to FdhC is thought to be mediated by the iron-sulfur centers of FdhB (Fig. 6), similar to the mechanism proposed for hydrogenase (Fig. 5). The formate catalytic site of *W. succinogenes* formate dehydrogenase is exposed to the periplasm (Kröger et al., 1980).

Nitrite Reductase The nitrite reductase of *W. succinogenes* catalyzes the reduction of nitrite to NH_4^+ by menaquinol (MKH_2; Fig. 6; Simon et al., 2000). The enzyme consists of the hydrophilic catalytic subunit NrfA and the quinone reactive subunit NrfH that probably anchors the enzyme in the membrane by a single hydrophobic helix at its N-terminus. The subunit NrfH belongs to the *c*-type cytochromes of the NapC/NirT family (Roldán et al., 1998). Subunits NrfA and NrfH are predicted to carry five and four heme C groups, respectively. The subunit NrfA catalyzes nitrite reduction by reduced benzyl viologen, but NrfA does not react with quinols. NrfA-like nitrite reductases have been isolated from various bacteria, including *W. succinogenes*, *Escherichia coli* and *Sulfurospirillum deleyianum* (Liu et al., 1983; Schröder et al., 1985; Schumacher et al., 1994). The subunit NrfA of *W. succinogenes* is similar to NrfA of *S. deleyianum* (75% identity), whose crystal structure is known (Einsle et al., 1999). Four heme groups of this enzyme are bound to conventional CxxCH motifs. The fifth heme group is bound to a CxxCK motif and is localized near the substrate site of the enzyme.

Polysulfide Reductase Polysulfide reductase catalyzes the reduction of polysulfide by BH_4^- as well as sulfide oxidation to polysulfide by 2,3-dimethyl-1,4-naphthoquinone (Hedderich et al., 1999). The enzyme consists of two hydrophilic subunits (PsrA and PsrB) and a hydrophobic subunit (PsrC) that anchors the enzyme in the membrane (Fig. 7). The catalytic subunit (PsrA) is exposed to the periplasm (Krafft et al., 1995). Polysulfide reductase contains molybdenum and molybdopterin guanine dinucleotide (Jankielewicz et al., 1994). The sequences of PsrA and the catalytic subunits of the formate dehydrogenases (FdhA) of *W. succinogenes* and *Escherichia coli* are similar and have the cysteine residues that are typical for a tetranuclear iron-sulfur center (Krafft et al., 1992). The similarity of PsrA to the catalytic subunits of the formate dehydrogenases suggests similar catalytic mechanisms. The electrons for polysulfide reduction at the molybdenum site are probably provided via the iron-sulfur centers of PsrA and of PsrB (both are iron-sulfur proteins), while the protons

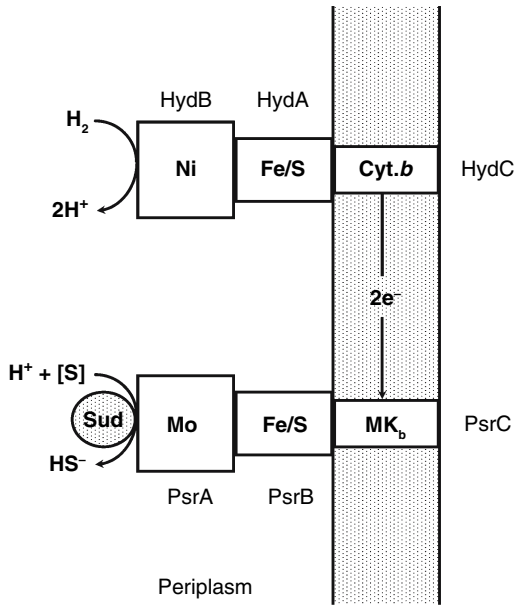

Fig. 7. Composition and orientation of Ni/Fe hydrogenase (HydA, B, C) and of polysulfide reductase (PsrA, B, C) in the membrane of *W. succinogenes*; the two enzymes make up the electron transport chain catalyzing polysulfide respiration with molecular hydrogen in *W. succinogenes*. Polysulfide-sulfur transferase (Sud) is described in subsection [Polysulfide respiration]. Mo, molybdenum linked to molybdopterin guanine dinucleotide; Ni, nickel ion; Fe/S, iron-sulfur centers; Cyt. *b*, cytochrome *b*; MK_b, menaquinone bound to PsrC.

required for HS^- formation enter the catalytic site via a channel in PsrA (Fig. 7). Bound menaquinone is assumed to serve as the prosthetic group of PsrC that accepts the electrons delivered by hydrogenase or formate dehydrogenase (Hedderich et al., 1999). The subunit PsrC does not carry heme.

In contrast to the wild-type strain, a mutant (Δ*psrABC*) lacking the *psrABC* operon does not catalyze polysulfide reduction by H_2 or formate when grown with fumarate as terminal electron acceptor (Krafft et al., 1995). Surprisingly, the mutant does grow with polysulfide, then forming a membrane-integrated enzyme that replaces polysulfide reductase. This enzyme has not been isolated as yet. Its properties appear to differ considerably from those of the polysulfide reductase enzyme. The enzyme does not cross-react with an antiserum raised against PsrA. Like polysulfide reductase, the enzyme catalyzes sulfide oxidation by 2,3-dimethyl-1,4-naphthoquinone. However, the apparent K_M values for sulfide differ drastically. The value measured with polysulfide reductase is 25 mM and that of the enzyme induced in the Δ*psrABC* mutant is approximately 1 mM (Klimmek, 1996). The enzyme present in the mutant is apparently absent from the wild-type strain, as suggested by this difference in the apparent K_M values.

Fumarate Respiration and Dicarboxylate Transport

The electron transport chain catalyzing fumarate respiration with H_2 consists of hydrogenase, menaquinone (MK) and fumarate reductase (Fig. 5). This was shown by restoring the activity of electron transport in liposomes containing the isolated enzymes (Graf et al., 1985; Kröger et al., 1992). Formate dehydrogenase replaces hydrogenase in the chain using formate as electron donor (Unden and Kröger, 1982). The electrochemical proton potential (Δp) is thought to be generated by the reduction of MK with H_2 (Fig. 5) or formate (according to Fig. 6; Kröger, 1978; Mell et al., 1986; Kröger et al., 1992; Geisler et al., 1994; Gross et al., 1998a). The protons generated from H_2 at the substrate site of the hydrogenase are released on the periplasmic side of the membrane, while the protons consumed by MK reduction are taken up from the cytoplasmic side. The oxidation of MKH_2 by fumarate does not contribute to Δp generation. The protons generated by MKH_2 oxidation via fumarate reductase are liberated on the cytoplasmic side, and the same number of protons are consumed by the reduction of fumarate, which also occurs on the cytoplasmic side of the membrane. Thus the Δp in fumarate respiration of *W. succinogenes* is generated by transmembrane electron transport without proton translocation.

The H^+/electron ratio postulated by the mechanism of Fig. 5 is consistent with the experimentally determined ratio of approximately one (Mell et al., 1986). The mechanism of Fig. 5 was confirmed by the observation that the reduction by H_2 of 2,3-dimethyl-1,4-naphthoquinone (DMN), a water-soluble MK analogue, is coupled to the generation of a Δp, whereas fumarate reduction by $DMNH_2$ is not (Geisler et al., 1994). Furthermore, coupled fumarate respiration was observed with liposomes containing hydrogenase, MK and fumarate reductase (A. Kröger, unpublished observation). Fumarate reduction by H_2 generated a Δp of approximately 100 mV across the liposomal membrane (positive outside). Only fumarate reductase molecules exposed to the outside of the liposomes are expected to participate in the electron transport because the permeation of fumarate across the liposomal membrane should be very slow. Thus the direction of the Δp appears to be independent of the orientation of fumarate reductase, in agreement with the mechanism of Fig. 5.

Because the substrate site of fumarate reductase faces the bacterial cytoplasm, fumarate and succinate have to be transported across the

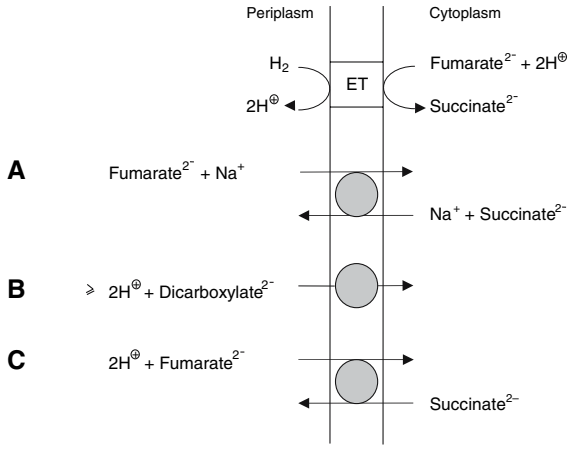

Fig. 8. Transport of fumarate and succinate across the membrane of *W. succinogenes*. (A) Na^+ dependent electroneutral antiport; (B) electrogenic uniport; (C) electrogenic antiport. ET, electron transport chain of fumarate respiration with molecular hydrogen as electron donor (see Fig. 5 for more detail).

membrane of *W. succinogenes* (Fig. 8). The fumarate that is converted to succinate in growing bacteria is taken up in exchange for succinate (Fig. 8A). This antiport process was found to be electroneutral in the presence of Na^+ (Ullmann et al., 2000). In the absence of Na^+, the cells take up fumarate in an electrogenic process according to the uniport (Fig. 8B) and the antiport (Fig. 8C) mechanism. The uniport process probably serves in the uptake of fumarate as a carbon source. In the absence of Na^+, the antiport process was found to dissipate the Δp generated by electron transport. This is probably the reason why *W. succinogenes* does not grow without Na^+ in the medium. In the absence of Na^+, resting cells catalyze fumarate reduction by H_2 or formate, but a Δp is not generated. In contrast, inverted membrane vesicles catalyze fumarate reduction with H_2 but generate a Δp in the presence or absence of Na^+. The transporters DcuA and DcuB of *W. succinogenes*, which are similar to the Dcu transporters of *Escherichia coli*, catalyze the transport of fumarate, succinate and other C_4-dicarboxylates (Six et al., 1994). A mutant of *W. succinogenes* that lacks both Dcu transporters does not grow by fumarate respiration, but the mutant still takes up dicarboxylates as a carbon source (Ullmann et al., 2000). (See The Dissimilatory Sulfate- and Sulfur-Reducing Prokaryotes in the second edition; elemental sulfur).

Nitrite Respiration

Liposomes containing formate dehydrogenase, menaquinone (MK) and nitrite reductase catalyze electron transport from formate to nitrite. This is coupled to the generation of an electrochemical proton potential (Δp) across the liposomal membrane (positive outside; Simon et al., 2000). The mechanism of Δp generation by nitrite respiration (Fig. 6) is envisaged to be similar to that of fumarate respiration (Fig. 5). The mechanisms differ in the orientation of the substrate sites of the reductases, nitrite reductase being exposed to the outside. The MK reduction with formate is thought to generate Δp, while MKH_2 oxidation by nitrite is assumed to be electroneutral (see [Fumarate respiration and dicarboxylate transport]). The protons produced by MKH_2 oxidation are thought to be liberated on the outside and to balance the protons consumed in nitrite reduction.

Polysulfide Respiration

The electron transport chain catalyzing polysulfide respiration with H_2 consists of hydrogenase and of polysulfide reductase (Fig. 7; Hedderich et al., 1999). Hydrogenase is replaced by formate dehydrogenase in the chain when formate is used as the electron donor. The two dehydrogenases are identical with those involved in fumarate respiration of *W. succinogenes*. The mechanism of electron transfer from the dehydrogenases to polysulfide reductase is not known. Electron transfer by menaquinone (MK) diffusion, as in fumarate respiration, appears to be unlikely because the standard redox potential of MK at pH 7.0 is more than 200 mV more electropositive than that of polysulfide. Experimental evidence suggests that electron transfer from the dehydrogenases to polysulfide reductase may require diffusion and collision of the enzymes within the membrane. The MK involved in polysulfide respiration is thought to be bound to PsrC (MK_b in Fig. 7). Bound MK probably serves as the prosthetic group of PsrC and as primary acceptor of the electrons delivered by HydC or FdhC. The mechanism of Δp generation in polysulfide respiration is not known. Possibly, protons are translocated by the redox reactions of the bound MK.

The K_M for polysulfide measured with intact cells of *W. succinogenes* grown with polysulfide (7 μM) is seven times lower than that measured with fumarate-grown bacteria or with the membrane fraction derived from bacteria grown under either condition (Klimmek et al., 1998). The lower K_M is caused by a polysulfide-sulfur transferase (Sud) that is induced during growth with polysulfide (Fig. 7). The Sud protein is localized in the periplasm; it consists of two identical subunits (14.3 kDa each) and does not contain prosthetic groups or heavy metal ions. The Sud protein binds sulfur from aqueous polysulfide solutions and probably transfers it to the cata-

lytic site of polysulfide reductase. Sulfur is covalently bound to the single cysteine residue of the Sud protein (Klimmek et al., 1999).

Genetics

Genetic Information

The G+C content of the DNA of *W. succinogenes* is 47 mol% (T_m); this number agrees well with the G+C contents of sequenced regions deposited in the data banks (47.6–50.0 mol%). A notable exception is the DNA of an insertion sequence (IS*1302*) whose G+C content is only 33.8 mol% (Simon and Kröger, 1998). IS*1302* is 1,306 bp in size, with 36-bp imperfect terminal repeats, and belongs to the IS*3* family of insertion sequences. As suggested by Southern-blot analysis, at least 13 copies are present in the genome. Insertion of IS*1302* resulted in a duplication of 3 bp of the target DNA. IS*1302* encodes a putative transposase whose predicted amino acid sequence is similar to those of transposases of various IS*3* family elements. IS*1302* may have been acquired from another organism inasmuch as the G+C contents of IS elements are usually in line with the G+C contents of the host DNA.

In recent years, a growing number of genes from *W. succinogenes* have been cloned and sequenced, including the operons encoding the subunits of fumarate reductase (*frdCAB*), of hydrogenase (*hydABC*), of polysulfide reductase (*psrABC*) and of nitrite reductase (*nrfHA*; Lauterbach et al., 1990; Körtner et al., 1990; Dross et al., 1993; Krafft et al., 1992; Simon et al., 2000). Lenger et al. (1997) detected two operons encoding the subunits of formate dehydrogenase (*fdhABC*); the operons differ in their promoter regions, but are nearly identical in their gene sequences. Other sequenced genes encode C_4-dicarboxylate transporters (*dcuA*, *dcuB*, *dctPQM*), L-asparaginase (*ansA*), L-aspartase (*aspA*), the two subunits of fumarase (*fumBα* and *fumBβ*), and the polysulfide-sulfur transferase (*sud*).

Genetic Manipulation

Transformation of *W. succinogenes* cells with plasmid DNA was achieved by electroporation under anaerobic conditions (Krafft et al., 1995; Simon et al., 1998). Cells were grown in media containing either fumarate or nitrate as terminal electron acceptor (see [Cultivation]) and were harvested in the exponential growth phase. The cells were washed with 0.3 M sucrose and suspended (10 g protein/liter) in growth medium. After addition of plasmid DNA (5–10 µg to 50 µl bacterial suspension), the mixture was subjected to electroporation in a 0.2-cm pathlength cuvette (25 µF capacity, 1.5 kV, 800 ω resistance). Anoxic growth medium (1 ml) was added; the bacteria were incubated for 2 h at 37°C and plated on agar medium containing an appropriate antibiotic.

Selection of transformants was accomplished using antibiotic resistance genes. The kanamycin resistance gene (*kan*) derived from the *Escherichia coli* plasmid pUC4K (Oka et al., 1981; Vieira and Messing, 1982) and the chloramphenicol resistance gene (*cat*) were obtained from the *E. coli* /*Helicobacter pylori* shuttle vector pDF4a (Haas et al., 1993). For selection, 25 µg/ml kanamycin or 12.5 µg/ml chloramphenicol is suitable.

Up to now, no plasmid replication was observed in *W. succinogenes*. Therefore, the generation of colonies on agar plates containing an antibiotic is dependent on recombination of the plasmid and the genome. Fragments of genomic DNA were replaced with a resistance gene by double homologous recombination. Using this method, various deletion mutants, each lacking, for example, an operon encoding an electron transport enzyme, were constructed. These strains comprise *W. succinogenes* Δ*frdCAB* (Simon et al., 1998), *W. succinogenes* Δ*hydABC* (Gross et al., 1998a), *W. succinogenes* Δ*psrABC* (Krafft et al., 1995), and mutants lacking either one of the two *fdh* operons (Lenger et al., 1997). The mutants *W. succinogenes* Δ*frdCAB* and *W. succinogenes* Δ*hydABC* were complemented by single crossover events of the mutant genome and plasmids carrying the entire *frdCAB* or *hydABC* operon (Simon et al., 1998; Gross et al., 1998a). This approach enabled the performance of site-directed mutagenesis of the two operons in *W. succinogenes*.

Applications

L-Asparaginase has been isolated from *W. succinogenes* (Kafkewitz and Goodman, 1974; Albanese and Kafkewitz, 1978). This enzyme inhibited growth of an in vitro culture of human pancreatic carcinoma cells (Wu et al., 1978) and was thus of particular interest for its potential antitumor activity. The crystal structure of *W. succinogenes* L-asparaginase, a homotetrameric enzyme, was determined (Lubkowski et al., 1996).

Literature Cited

Albanese, E., and D. Kafkewitz. 1978. Effect of medium composition on the growth and asparaginase production of Vibrio *succinogenes*. Appl. Environ. Microbiol. 36:25–30.

Birkholz, S., U. Knipp, E. Lemma, A. Kröger, and W. Opferkuch. 1994. Fumarate reductase of Helicobacter pylori—an immunogenic protein. J. Med. Microbiol. 41:56–62.

Bokranz, M., E. Mörschel, and A. Kröger. 1985a. Structural and ATP hydrolyzing properties of the ATP synthase isolated from *Wolinella succinogenes*. Biochim. Biophys. Acta 810:84–93.

Bokranz, M., E. Mörschel, and A. Kröger. 1985b. Phosphorylation and phosphate-ATP exchange catalyzed by the ATP synthase isolated from *Wolinella succinogenes*. Biochim. Biophys. Acta 810:332–339.

Bokranz, M., J. Katz, I. Schröder, A.M. Roberton, and A. Kröger. 1983. Energy metabolism and biosynthesis of Vibrio *succinogenes* growing with nitrate or nitrite as terminal electron acceptor. Arch. Microbiol. 135:36–41.

Bokranz, M., M. Gutmann, C. Körtner, E. Kojro, F. Fahrenholz, F. Lauterbach, and A. Kröger. 1991. Cloning and nucleotide sequence of the structural genes encoding the formate dehydrogenase of *Wolinella succinogenes*. Arch. Microbiol. 156:119–128.

Boyington, J.C., V.N. Gladyshev, S.V. Khangulov, T.C. Stadtman, and P.D. Sun. 1997. Crystal structure of formate dehydrogenase H: catalysis involving Mo, molybdopterin, selenocysteine, and an Fe4S4 cluster. Science 275:1305–1308.

Bronder, M., H. Mell, E. Stupperich, and A. Kröger. 1982. Biosynthetic pathways of Vibrio *succinogenes* growing with fumarate as terminal electron acceptor and sole carbon source. Arch. Microbiol. 131:216–223.

Brondz, I., and I. Olsen. 1991. Multivariate analyses of cellular fatty acids in Bacteroides, Prevotella, Porphyromonas, Wolinella, and Campylobacter ssp. J. Clin. Microbiol. 29:183–189.

Brune, A., J. Spillecke, and A. Kröger. 1987. Correlation of the turnover number of the ATP synthase in liposomes with the proton flux and the proton potential across the membrane. Biochim. Biophys. Acta 893:499–507.

Cardarelli-Leite, P., K. Blom, C.M. Patton, M.A. Nicholson, A.G. Steigerwalt, S.B. Hunter, D.J. Brenner, T.J. Barrett, and B. Swaminathan. 1996. Rapid identification of Campylobacter species by restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA. J. Clin. Microbiol. 34:62–67.

Collins, M.D., and F. Fernandez. 1984. Menaquinone-6 and thermoplasmaquinone-6 in *Wolinella succinogenes*. FEMS Microbiol. Lett. 22:273–276.

Dross, F., V. Geisler, R. Lenger, F. Theis, T. Krafft, F. Fahrenholz, E. Kojro, A. Duchêne, D. Tripier, K. Juvenal, and A. Kröger. 1992. The quinone-reactive Ni/Fe-hydrogenase of Wolinella succinogenes. [author's correction: 214:949–950]. Eur. J. Biochem. 206:93–102.

Einsle, O., A. Messerschmidt, P. Stach, G.P. Bourenkov, H.D. Bartunik, R. Huber, and P.M.H. Kroneck. 1999. Structure of cytochrome c nitrite reductase. Nature 400:476–480.

Firoozkoohi, J., H. Zandi, and I. Olsen. 1997. Comparison of lipopolysaccharides from Bacteroides, Porphyromonas, Prevotella, Campylobacter and Wolinella ssp. by tricine-SDS-PAGE. Endod. Dent. Traumatol. 13:13–18.

Geisler, V., Ullmann, R., and A. Kröger. 1994. The direction of the proton exchange associated with the redox reactions of menaquinone during the electron transport in *Wolinella succinogenes*. Biochim. Biophys. Acta 1184:219–226.

Graf, M., M. Bokranz, R. Böcher, P. Friedl, and A. Kröger. 1985. Electron transport driven phosphorylation catalyzed by proteoliposomes containing hydrogenase, fumarate reductase and ATP synthase. FEBS Lett. 184:100–103.

Gross, R., J. Simon, C.R.D. Lancaster, and A. Kröger. 1998b. Identification of histidine residues in *Wolinella succinogenes* hydrogenase that are essential for menaquinone reduction by H2. Mol. Microbiol. 30:639–646.

Gross, R., J. Simon, F. Theis, and A. Kröger. 1998a. Two membrane anchors of *Wolinella succinogenes* hydrogenase and their function in fumarate and polysulfide respiration. Arch. Microbiol. 170:50–58.

Haas, R., A.F. Kahrs, D. Facius, H. Allmeier, R. Schmitt, and T.F. Meyer. 1993. TnMax—a versatile mini-transposon for the analysis of cloned genes and shuttle mutagenesis. Gene 130:23–31.

Hedderich, R., O. Klimmek, A. Kröger, R. Dirmeier, M. Keller, and K.O. Stetter. 1999. Anaerobic respiration with sulfur and with organic disulfides. FEMS Microbiol. Rev. 22:353–381.

Higuchi, Y., T. Yagi, and N. Yasuoka. 1997. Unusual ligand structure in Ni-Fe active center and an additional Mg site in hydrogenase revealed by high resolution X-ray structure analysis. Structure 5:1671–1680.

Iverson, T.M., C. Luna-Chavez, G. Cecchini, and D.C. Rees. 1999. Structure of the Escherichia coli fumarate reductase respiratory complex. Science 284:1961–1966.

Jankielewicz, A., R.A. Schmitz, O. Klimmek, and A. Kröger. 1994. Polysulphide reductase and formate dehydrogenase from *Wolinella succinogenes* contain molybdopterin guanine dinucleotide. Arch. Microbiol. 162:238–242.

Kafkewitz, D., and D. Goodman. 1974. L-asparaginase production by the rumen anaerobe Vibrio *succinogenes*. Appl. Environ. Microbiol. 27:206–209.

Kafkewitz, D. 1975. Improved growth media for Vibrio *succinogenes*. Appl. Environ. Microbiol. 29:121–122.

Klimmek, O., A. Kröger, R. Steudel, and G. Holdt. 1991. Growth of *Wolinella succinogenes* with polysulphide as terminal acceptor of phosphorylative electron transport. Arch. Microbiol. 155:177–182.

Klimmek, O. 1996. Thesis, J.W. Goethe-Universität Frankfurt am Main, FB Biologie.

Klimmek, O., T. Stein, R. Pisa, J. Simon, and A. Kröger. 1999. The single cysteine residue of the Sud protein is required for its function as a polysulphide-sulphur transferase in *Wolinella succinogenes*. Eur. J. Biochem. 263:79–84.

Klimmek, O., V. Kreis, C. Klein, J. Simon, A. Wittershagen, and A. Kröger. 1998. The function of the periplasmic Sud protein in polysulfide respiration of *Wolinella succinogenes*. Eur. J. Biochem. 253:263–269.

Kokeguchi, S., O. Tsutsui, K. Kato, and T. Matsumura. 1991. Comparative study of lipopolysaccharides from Wolinella recta, W. curva, W. succinogenes and Campylobacter sputorum ssp. sputorum. FEMS Microbiol. Lett. 65:291–297.

Körtner, C., F. Lauterbach, D. Tripier, G. Unden, and A. Kröger. 1990. *Wolinella succinogenes* fumarate reductase contains a diheme cytochrome b. Mol. Microbiol. 4:855–860.

Krafft, T., M. Bokranz, O. Klimmek, I. Schröder, F. Fahrenholz, E. Kojro, and A. Kröger. 1992. Cloning and nucleotide sequence of the psrA gene of *Wolinella succinogenes* polysulphide reductase. Eur. J. Biochem. 206:503–510.

Krafft, T., R. Groß and A. Kröger. 1995. The function of *Wolinella succinogenes* psr genes in electron transport with polysulphide as the terminal electron acceptor. Eur. J. Biochem. 230:601–606.

Kröger, A., and A. Innerhofer. 1976. The function of the b cytochromes in the electron transport from formate to fumarate of Vibrio *succinogenes*. Eur. J. Biochem. 69:497–506.

Kröger, A., E. Dorrer, and E. Winkler. 1980. The orientation of the substrate sites of formate dehydrogenase and fumarate reductase in the membrane of Vibrio *succinogenes*. Biochim. Biophys. Acta 589:118–136.

Kröger, A., V. Geisler, E. Lemma, F. Theis, and R. Lenger. 1992. Bacterial fumarate respiration. Arch. Microbiol. 158:311–314.

Kröger, A. 1978. Fumarate as terminal acceptor of phosphorylative electron transport. Biochim. Biophys. Acta 505:129–145.

Lancaster, C.R.D., A. Kröger, M. Auer, and H. Michel. 1999. Structure at 2.2 âresolution of the compolex II-like fumarate reductase from *Wolinella succinogenes*. Nature 402:377–385.

Lauterbach, F., C. Körtner, S.P.J. Albracht, G. Unden, and A. Kröger. 1990. The fumarate reductase operon of *Wolinella succinogenes*: sequence and expression of the frdA and frdB genes. Arch. Microbiol. 154:386–393.

Lenger, R., U. Herrmann, R. GroßJ. Simon, and A. Kröger. 1997. Structure and function of a second gene cluster encoding the formate dehydrogenase of *Wolinella succinogenes*. Eur. J. Biochem. 246:646–651.

Liu, M.-C., M.-Y. Liu, W.J. Payne, H.D. Peck Jr., and J. LeGall. 1983. *Wolinella succinogenes* nitrite reductase: purification and properties. FEMS Microbiol. Lett. 19:201–206.

Lorenzen, J.P., A. Kröger, and G. Unden. 1993. Regulation of anaerobic pathways in *Wolinella succinogenes* by the presence of electron acceptors. Arch. Microbiol. 159: 477–483.

Lorenzen, J.P., S. Steinwachs, and G. Unden. 1994. DMSO respiration by the anaerobic rumen bacterium *Wolinella succinogenes*. Arch. Microbiol. 162:277–281.

Lubkowski, J., G.J. Palm, G.L. Gilliland, C. Derst, K.H. Röhm, and A. Wlodawer. 1996. Crystal structure and amino acid sequence of *Wolinella succinogenes* L-asparaginase. Eur. J. Biochem. 241:201–207.

Macy, J.M., I. Schröder, R.K. Thauer, and A. Kröger. 1986. Growth of *Wolinella succinogenes* on H2S plus fumarate and on formate plus sulfur as energy sources. Arch. Microbiol. 144:147–150.

Mell, H., C. Wellnitz, and A. Kröger. 1986. The electrochemical proton potential and the proton/electron ratio of the electron transport with fumarate in *Wolinella succinogenes*. Biochim. Biophys. Acta 852:212–221.

Mell, H., M. Bronder, and A. Kröger. 1982. Cell yields of Vibrio *succinogenes* growing with formate and fumarate as sole carbon and energy sources in chemostat culture. Arch. Microbiol. 131:224–228.

Niederman, R.A., and M.J. Wolin. 1972. Requirement of succinate for the growth of Vibrio *succinogenes*. J. Bacteriol. 109:546–549.

Oka, A., H. Sugisaki, and M. Takanami. 1981. Nucleotide sequence of the kanamycin resistance transposon Tn903. J. Mol. Biol. 147:217–226.

Paster, B.J., and F.E. Dewhirst. 1988. Phylogeny of campylobacters, Wolinella, Bacteroides gracilis and Bacteroides ureolyticus by 16S ribosomal ribonucleic acid sequencing. Int. J. Syst. Bacteriol. 38:56–62.

Radcliffe, C.W., D. Kafkewitz, and S. Abouchowski. 1979. Asparaginase production by human clinical isolates of Vibrio *succinogenes*. Appl. Environ. Microbiol. 38:761–762.

Roldán, M.D., H.J. Sears, M.R. Cheesman, S.J. Ferguson, A.J. Thomason, B.C. Berks, and D.J. Richardson. 1998. Spectroscopic characterization of a novel multiheme c-type cytochrome widely implicated in bacterial electron transport. J. Biol. Chem. 273:28785–28790.

Schröder, I., A.M. Roberton, M. Bokranz, G. Unden, R. Böcher, and A. Kröger. 1985. The membraneous nitrite reductase involve in the electron transport of *Wolinella succinogenes*. Arch. Microbiol. 140:380–386.

Schumacher, W., U. Hole, and P.M.H. Kroneck. 1994. Ammonia-forming cytochrome c nitrite reductase from Sulfurospirillum deleyianum is a tetraheme protein: New aspects of the molecular composition and spectroscopic properties. Biochem. Biophys. Res. 205:911–916.

Simon, J., and A. Kröger. 1998a. Identification and characterization of IS1302, a novel insertion element from *Wolinella succinogenes* belonging to the IS3 family. Arch. Microbiol. 170:43–49.

Simon, J., R. Gross, M. Ringel, E. Schmidt, and A. Kröger. 1998b. Deletion and site-directed mutagenesis of the *Wolinella succinogenes* fumarate reductase operon. Eur. J. Biochem. 251:418–426.

Simon, J., R. Gross, O. Einsle, P.M.H. Kroneck, A. Kröger, and O. Klimmek. 2000. A NapC/NirT-type cytochrome c (NrfH) is the mediator between the quinone pool and the cytochrome c nitrite reductase of *Wolinella succinogenes*. Mol. Microbiol. 35:686–696.

Six, S., S.C. Andrews, G. Unden, and J.R. Guest. 1994. Escherichia coli possesses two homologous anaerobic C4-dicarboxylate membrane transporters (DcuA and DcuB) distinct from the aerobic dicarboxylate transport system (Dct). J. Bacteriol. 176:6470–6478.

Tanner, A.C.R., M.A. Listgarten, and J.L. Ebersole. 1984. Wolinella curva sp. nov.: Vibrio succinogenes of human origin. Int. J. Syst. Bacteriol. 34:275–282.

Tanner, A.C.R., M.N. Strzempko, C.A. Belsky, and G.A. McKinley. 1985. API ZYM and API An-Ident reactions of fastidious oral Gram-negative species. J. Clin. Microbiol. 22:333–335.

Tanner, A.C.R., S. Badger, C.-H. Lai, M.A. Listgarten, R.A. Visconti, and S.S. Socransky. 1981. Wolinella gen. nov., Wolinella succinogenes (Vibrio succinogenes Wolin et al.) comb. nov., and description of Bacteroides gracilis sp. nov., Wolinella recta sp. nov., Campylobacter concisus sp. nov., and Eikenella corrodens from with periodontal disease. Int. J. Syst. Bacteriol. 31:432–445.

Tanner, A.C.R. 1986. Characterization of Wolinella species, Campylobacter concisus, Bacteroides gracilis and Eikenella corrodens using polyacrylamide gel electrophoresis. J. Clin. Microbiol. 24:562–565.

Terguchi, S., and T.C. Hollocher. 1989. Purification and some characteristics of a cytochrome C-containing nitrous oxide reductase from *Wolinella succinogenes*. J. Biol. Chem. 264:1972–1979.

Ullmann, R., R. Gross, J. Simon, G. Unden, and A. Kröger. 2000. Transport of C4-dicarboxylates in Wolinella succinogenes. J. Bacteriol. 182:5757–5764.

Unden, G., and A. Kröger. 1982. Reconstitution in liposomes of the electron transport chain catalyzing fumarate

reduction by formate. Biochim. Biophys. Acta 682:258–263.

Unden, G., H. Hackenberg, and A. Kröger. 1980. Isolation and functional aspects of the fumarate reductase involved in the phosphorylative electron transport of Vibrio *succinogenes*. Biochim. Biophys. Acta 591:275–288.

Vandamme, P., E. Falsen, R. Rossau, B. Hoste, P. Segers, R. Tytgat, and J. De Ley. 1991. Revision of Campylobacter, Helicobacter, and Wolinella taxonomy: emendation of generic descriptions and proposal of Arcobacter gen. nov. Int. J. Syst. Bacteriol. 41:88–103.

Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259–268.

Volbeda, A., M.-H. Charon, C. Piras, E.C. Hatchikian, M. Frey, and J.C. Fontecilla-Camps. 1995. Crystal structure of the nickel-iron hydrogenase from Desulfovibrio gigas. Nature 373:580–587.

Wloczyk, C., A. Kröger, T. Göbel, G. Holdt, and R. Steudel. 1989. The electrochemical proton potential generated by the sulphur respiration of *Wolinella succinogenes*. Arch. Microbiol. 152:600–605.

Wolin, M.J., E.A. Wolin, and N.J. Jacobs. 1961. Cytochrome-producing anaerobic vibrio, Vibrio *succinogenes* sp. n. J. Bacteriol. 81:911–917.

Wu, M.-C., G.K. Arimura, and A.A. Yunis. 1978. Mechanism of sensitivity of cultured pancreatic carcinoma to asparaginase. Int. J. Cancer 22:728–733.

Yoshinari, T. 1980. N_2O reduction by *Vibrio succinogenes*. Appl. Environ. Microbiol. 39:81–84.

PART 4

Spirochetes

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CHAPTER 4.1

Free-Living Saccharolytic Spirochetes: The Genus *Spirochaeta*

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Introduction

The genus *Spirochaeta* includes anaerobic and facultatively anaerobic spirochetes that are indigenous to aquatic environments such as the mud and water of ponds and marshes. These spirochetes occur in nature as free-living forms; that is, their existence does not depend on physical associations with other organisms (Canale-Parola, 1984a).

Spirochaeta cells are helically shaped (Fig. 1–12) and possess the typical ultrastructural features of spirochetes (Canale-Parola, 1984b; Fig. 13). The outermost structure of the cells is an "outer membrane," or "outer sheath," which encloses the coiled cell body ("protoplasmic cylinder") consisting of the cytoplasm, the nuclear region, and the peptidoglycan-cytoplasmic membrane complex (Fig. 13). Organelles ultrastructurally similar to bacterial flagella are located in the area between the outer membrane and the protoplasmic cylinder (Fig. 13). These organelles are essential components of the motility apparatus of spirochetes (Paster and Canale-Parola, 1980) and are usually called "periplasmic flagella." Other names used to designate these motility organelles are "periplasmic fibrils," "axial fibrils," "axial filaments," and "endoflagella."

One end of each periplasmic flagellum is inserted near a pole of the protoplasmic cylinder, while the other end is not inserted (Fig. 13A). Individual periplasmic flagella extend for most of the length of *Spirochaeta* cells so that those inserted near opposite ends overlap in the central region of the organism (Fig. 13A). The *Spirochaeta* cell illustrated in Figure 13 has two overlapping periplasmic flagella, each inserted near a cell pole in a "1-2-1" arrangement. With one exception, all the known *Spirochaeta* species have two periplasmic flagella per cell. The exception is the large spirochete *Spirochaeta plicatilis*, which has as many as 18–20 periplasmic flagella inserted near each end of the protoplasmic cylinder (Blakemore and Canale-Parola, 1973).

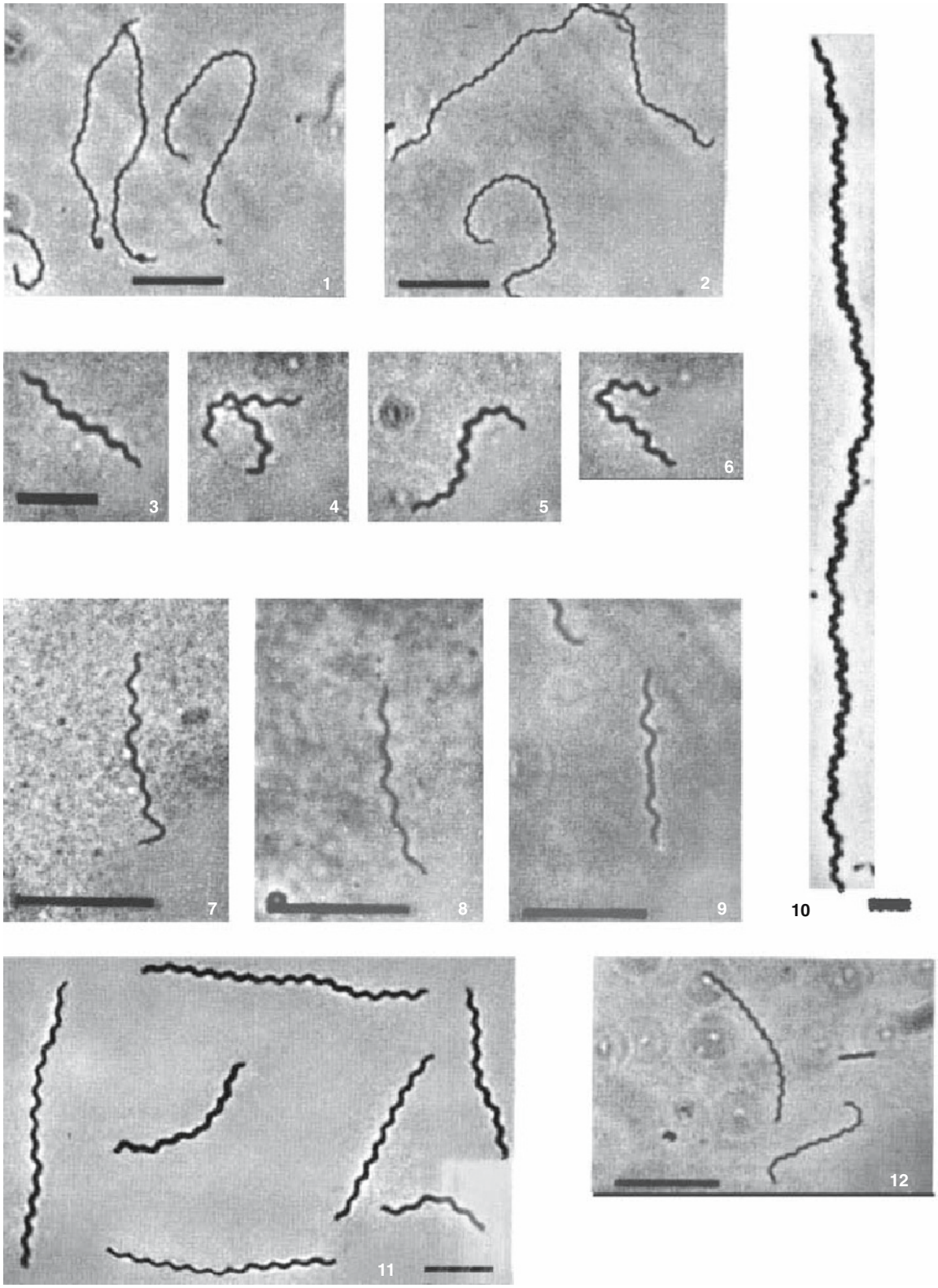
In contrast to flagella of other bacteria, the periplasmic flagella of spirochetes are permanently wound around the cell body and are entirely endocellular (Fig. 13B). Thus, the motility mechanism of spirochetes is different from that of other bacteria, which have flagella that function in direct contact with the external environment and are not wound around the cell body.

Phylogeny

Based on 16S rRNA gene sequence comparisons, most members of the genus *Spirochaeta* form one of the nine phylogenetic clusters of the spirochetes (Fig. 14). All spirochetes are presently classified in the class or phylum Spirochaetes in the order Spirochetales and are divided into three families. The first family, Spirochaetaceae, contains species of the genera *Borrelia*, *Brevinema*, *Cristispira*, *Spirochaeta*, "*Spironema*" and *Treponema*. The second proposed family, Serpulinaceae, contains the genus *Brachyspira* (*Serpulina*). The third family, Leptospiraceae, contains species of the genera *Leptonema* and *Leptospira*.

Novel *Spirochaeta* species, or phylotypes, which have not yet been cultivated in vitro, have been identified. For example, several phylotypes from sulfide-rich mud (Tanner et al., 2000) and one associated with the gutless marine oligochete *Olavius loisae* (Dubilier et al, 1999) clearly fall within the genus *Spirochaeta* (Fig. 14). Based on microscopic observations of aquatic and marine sediments, it is apparent that a significant population of additional "uncultivable" spirochetes, including the type species of the genus, *Spirochaeta plicatilis*, remains to be identified.

As shown in Fig. 14, the free-living, obligately anaerobic spirochetes from freshwater environments, *Spirochaeta stenostrepta*, *Spirochaeta zuelzerae* and *Spirochaeta caldaria*, are more closely related to members of the genus *Treponema* than to members of the genus *Spirochaeta*. The phylogenetic clustering of these species has been confirmed by single-base signature analysis, i.e., the sequences of these species possess more



Figs. 1–12. Phase contrast photomicrographs of living *Spirochaeta* cells. Wet mount preparations. Figs. 1 and 2, *Spirochaeta stenostrepta* strain Z1 (DSM 2028, ATCC 25083). Bars = 10 μm. (From Canale-Parola et al., 1968); Figs. 3–6, *Spirochaeta litoralis*, strain R1 (DSM 2029, ATCC 27000). Bar = 5 μm. (From Hespell and Canale-Parola, 1970b); Figs. 7–9, *Spirochaeta aurantia* subsp. *aurantia*, strain J1 (DSM 1902, ATCC 25082), irregularly coiled (Figs. 7 and 9) and regularly coiled (Fig. 8) cells. Bars = 10 mm. (From Breznak and Canale-Parola, 1969); Fig. 10, *Spirochaeta plicatilis*. Bar = 10 μm. (From Blakemore and Canale-Parola, 1973); Fig. 11, *Spirochaeta halophila*, strain RS1 (ATCC 29478). Bar = 5 μm. (From Greenberg and Canale-Parola, 1976); Fig. 12, *Spirochaeta zuelzerae*, strain ATCC 19044. Bar = 10 μm. (From Canale-Parola et al., 1968.)

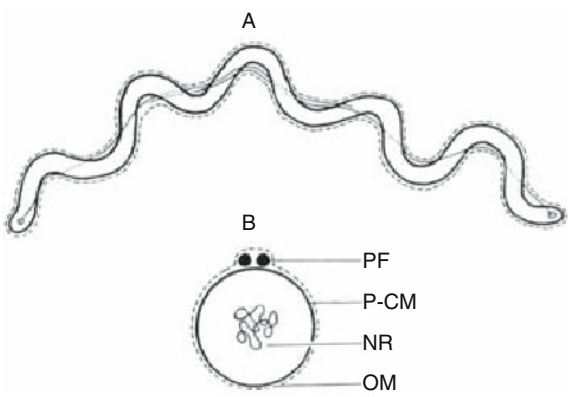


Fig. 13. (A) schematic representation of a *Spirochaeta* cell. The outermost broken line indicates the outer membrane (outer sheath). The protoplasmic cylinder is represented by the area delimited by the solid line adjacent to the outermost broken line. The cell has two periplasmic flagella indicated by the solid-dotted thin lines wound around the protoplasmic cylinder. The insertion points of the periplasmic flagella are represented by the small circles near the ends of the cell. (B) schematic representation of a cross section through a *Spirochaeta* cell. PF, periplasmic flagella; P-CM, peptidoglycan-cytoplasmic membrane complex; NR, nuclear region; OM, outer membrane (outer sheath).

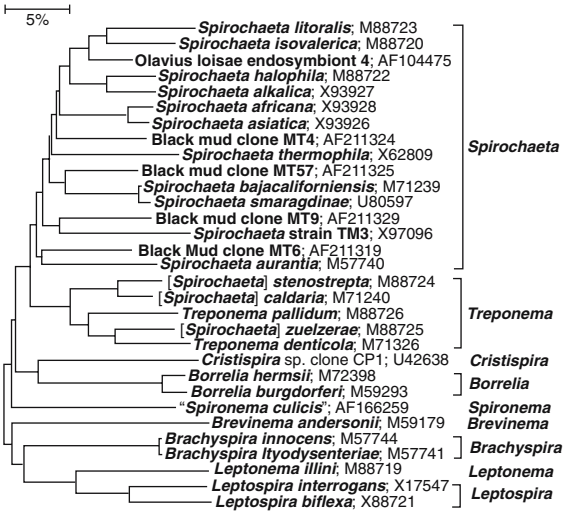


Fig. 14. Phylogeny of the genus *Spirochaeta*. The phylogenetic position of known species and phylotypes of *Spirochaeta* is shown relative to other genera of spirochetes as based on 16S rRNA gene sequence comparisons. GenBank accession numbers for the 16S rRNA sequences of the species tested are shown. Similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor (1969). The neighbor-joining method (Saitou and Nei, 1987) was used for phylogenetic tree construction. TREECON,™a software package for the Microsoft Windows environment, was used for the construction and drawing of evolutionary trees (Van de Peer et al., 1994). The scale bar represents a 5% difference in nucleotide sequence determined by taking the sum of all of the horizontal lines connecting two species. Vertical distance has no meaning.

bases found in the sequences of the treponemes than in the sequences of *Spirochaeta* species (Paster et al., 1991). It was speculated that these "*Treponema*-like" free-living spirochetes might represent transitional species, i.e., descendants of the precursors to host-associated treponemes (Paster et al., 1991). However, it is possible that the source of these species may be hosts that release them with their feces. Consequently, it would be worthwhile to screen host environments for these species using specific DNA probes or oligonucleotide primers in polymerase chain reactions (PCRs).

Taxonomy

Fourteen species of *Spirochaeta* are presently known (Table 1). One of these, *Spirochaeta plicatilis*, has not been grown in pure culture, but its ultrastructure and some of its ecological characteristics have been described (Blakemore and Canale-Parola, 1973). Nine species (*Spirochaeta stenostrepta*, *Spirochaeta litoralis*, *Spirochaeta zuelzerae*, *Spirochaeta isovalerica*, *Spirochaeta bajacaliforniensis*, *Spirochaeta thermophila*, *Spirochaeta caldaria*, *Spirochaeta smaragdinae* and *Spirochaeta asiatica*) are obligate anaerobes, and two species (*Spirochaeta alkalica* and *Spirochaeta africana*) are aerotolerant anaerobes. Two other species, *Spirochaeta aurantia* and *Spirochaeta halophila*, are facultative anaerobes and characteristically produce carotenoid pigments when growing aerobically (see Identification). Most species of *Spirochaeta* are mesophilic, growing at optimum temperatures ranging from 15 to 40°C. However, the thermophilic species, *Spirochaeta thermophila* and *Spirochaeta caldaria*, both from thermal springs, have optimum growth temperatures of 66–68 and 48–52°C, respectively (Table 1).

Two subspecies of *Spirochaeta aurantia* are known (Table 1). One of these (subsp. *stricta*) is characterized by significantly narrower coils than the other (subsp. *aurantia*), and its DNA possesses a slightly lower G+C content (Breznak and Canale-Parola, 1975; Canale-Parola, 1984a).

Spirochaeta stenostrepta, *Spirochaeta zuelzerae*, *Spirochaeta caldaria* and *Spirochaeta aurantia* are freshwater species, whereas *Spirochaeta litoralis*, *Spirochaeta isovalerica*, *Spirochaeta bajacaliforniensis* and *Spirochaeta thermophila* are marine species (Table 1) and require sodium ion (Na^+) concentrations ranging from 200 to 480 mM for optimal growth (Aksenova et al., 1992; Fracek and Stolz, 1985; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970b). *Spirochaeta halophila* was isolated from a high-salinity pond and grows optimally when 750 mM NaCl, 200 mM $MgSO_4$

Table 1. Characteristics of the fourteen species of the genus *Spirochaeta*.

Species	Size[a] (μm)	Relationship to O_2	Optimum growth temperatures (°C)	Habitat	G+C content DNA (mol%)[b]
1. *Spirochaeta stenostrepta*	0.2–0.3 × 15–45	OA	30–37	Freshwater	60.2
2. *Spirochaeta litoralis*	0.4–0.5 × 5–7	OA	Near 30	Marine	50.5
3. *Spirochaeta zuelzerae*	0.2–0.4 × 8–16	OA	37–39	Freshwater	56.1
4. *Spirochaeta isovalerica*	0.4 × 10–15	OA	15–35	Marine	63.6–65.6
5. *Spirochaeta bajacaliforniensis*	0.1–0.2 × 15–45	OA	36	Marine (laminated sediment)	50.1
6. *Spirochaeta thermophila*	0.2–0.25 × 16–50	OA	66–68	Marine (hot springs)	52.0
7. *Spirochaeta caldaria*	0.2–0.3 × 15–45	OA	48–52	Freshwater (hot springs)	45.0
8. *Spirochaeta smaragdinae*	0.3–0.5 × 5–30	OA	37	Oil field, injection water	50.0
9. *Spirochaeta asiatica*	0.2–0.25 × 15–22.5	OA	35	Alkaline lake	49.2
10. *Spirochaeta alkalica*	0.4–0.5 × 9–18	AT	33–37	Alkaline lake	57.1
11. *Spirochaeta africana*	0.25–0.3 × 15–30	AT	30–37	Alkaline lake	56.1
12. *Spirochaeta aurantia*[c]					
subsp. *aurantia*	0.3 × 10–20	FA	25–30	Freshwater	62.2–65.3
subsp. *stricta*	0.3 × 10–20	FA	25–30	Freshwater	61.2
13. *Spirochaeta halophila*	0.4 × 15–30	FA	35–40	High salinity	62
14. *Spirochaeta plicatilis*[T]	0.75 × 80–250	Unknown	Unknown	Unknown	Unknown

Abbreviations: O_2, molecular oxygen; OA, obligate anaerobic; AT, aerotolerant; FA, facultative anaerobe; and [T], type species.
[a]Lengths indicated are of the majority of cells. Shorter and longer cells are also present in cultures.
[b]Buoyant density determinations, except for species 4, 6, 7, and 9–11 (determined by thermal denaturation method) and species 8 (determined using HPLC).
[c]Wavelength of the cell: subsp. *aurantia* cells, 2.0–2.8 mm; subsp. *stricta* cells, 1.1–1.5 mm.

and 10 mM $CaCl_2$ are present in the medium (Greenberg and Canale-Parola, 1976). Other halophilic species include *Spirochaeta asiatica*, *Spirochaeta alkalica* and *Spirochaeta africana*, which were isolated from the sediments of alkaline lakes and require Na^+ concentrations ranging from 850 to 1,200 mM for optimal growth (Zhilina et al., 1996). The latter three species are also alkaliphilic and growth does not occur below pH 8. *Spirochaeta smaragdinae*, isolated from a production water sample collected from an offshore oil field, requires at least 170 mM NaCl and grows optimally with 850 mM NaCl (Magot et al., 1997).

Habitat

Species of *Spirochaeta* occur, grow, and persist as free-living organisms in a variety of aquatic environments, such as the water, sediments, and muds of ponds, marshes, lakes, rivers, and oceans. Numerous strains of *Spirochaeta* have been isolated from various freshwater environments (Canale-Parola, 1984a; Pohlschroeder et al., 1994) and from marine muds collected in Pacific and Atlantic coastal regions (Aksenova et al., 1992; Fracek and Stolz, 1985; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970b). Using 16S rRNA phylogenetic analyses to investigate the microbial diversity of sulfur-rich black mud marine sediments, Tanner et al. (2000) reported the presence of several novel spirochete phylotypes in samples from hypersaline and brackish marshes. Moreover, Weber and Greenberg (1981) determined that *Spirochaeta* cells are present in salt marshes at densities ranging from 104 to 106 per gram (wet weight) of the top 1 cm of sediment.

Selective isolation procedures did not yield spirochetes from deep-sea sediments and water from the Sargasso Sea (3,630 m) and the Puerto Rico Trench (8,140 m; Harwood et al., 1982b). These results indicate that although anaerobic and facultatively anaerobic free-living spirochetes are common in marine coastal environments, they are not widely distributed in deep-sea regions. However, Harwood et al. (1982b) isolated an obligately anaerobic spirochete from a sample collected near a deep-sea (2,550 m) hydrothermal vent at the Galapagos Rift tectonic spreading center, and they observed bacteria with morphologies typical of spirochetes in surface scrapings from mussels present near a vent. The occurrence of spirochetes in this deep-sea region may be ascribed to vent area environmental conditions, which are favorable to the growth of various bacteria. Magot et al. (1997) isolated *Spirochaeta smaragdinae* from a production water sample collected from an offshore oil field of Congo, Africa, and speculated that this

spirochete might be indigenous to the deep subsurface waters of oil fields.

Three alkaliphilic *Spirochaeta* species have been isolated from the sediments of alkaline lakes, including *Spirochaeta alkalica* and *Spirochaeta africana* from Lake Magadi, East African Rift, Kenya, and *Spirochaeta asiatica* from Lake Khatyn in Tuva, Central Asia (Zhilina et al., 1996). These spirochetes grow at pHs of >9, and growth does not occur below pH 8. Moreover, these species are halophilic, requiring Na^+ concentrations ranging from 850 to 1,200 mM for optimal growth (Zhilina et al., 1996). Another halophilic species, *Spirochaeta halophila*, has been isolated from a high-salinity pond located on the Sinai shore of the Gulf of Aqaba (Greenberg and Canale-Parola, 1976).

Most species of *Spirochaeta* are mesophilic, growing at optimum temperatures ranging from 15 to 40°C (Canale-Parola, 1984a). However, thermophilic spirochetes have been isolated from various hot springs in geographically widely separated locations. Obligately anaerobic thermophilic spirochetes isolated from New Zealand thermal springs grow optimally at 45 to 50°C (Patel et al., 1985). Aksenova et al. (Aksenova et al., 1990; Aksenova et al., 1992), and Rainey et al. (1991) isolated (from a marine hot spring on the beach of Shiashkotan Island, Kuril Islands, Kamchatka, Russia, and a brackish hot spring on Raoul Island, Kermadec Archipelago, New Zealand) two strains of thermophilic cellulolytic spirochetes, which they named *Spirochaeta thermophila* (Aksenova et al., 1992). *Spirochaeta thermophila* type strain grew between 40 and 73°C with a growth optimum of 66 to 68°C. Strains of *Spirochaeta caldaria*, which were isolated from hot springs in Oregon and Utah, USA, grew optimally between 48 and 52°C (Pohlschroeder et al., 1994). In addition, based on 16S rRNA gene sequence analyses, spirochete phylotypes have been identified in samples from a hot spring (Octopus Spring) in Yellowstone National Park, USA (Weller et al., 1992). Thus, it is likely that thermophilic spirochetes are widely distributed in thermal spring waters.

Isolation

Selective Procedures

Anaerobic and facultatively anaerobic spirochetes are readily isolated from natural environments by means of selective procedures and usually grow abundantly in ordinary laboratory media. Anaerobic growth yields of the isolates range from 2×10^8 to approximately 10^{10} cells/ml, but commonly are 6×10^8 to 8×10^8 cells/ml (Breznak and Canale-Parola, 1975; Canale-Parola, 1973; Greenberg and Canale-Parola, 1976; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970a; Hespell and Canale-Parola, 1970b). Cell population doubling times in anaerobic cultures vary from 2.2 to 12 h, depending on the species and the growth conditions. Aerobically grown cultures yield from 0.7×10^9 to 1.2×10^9 spirochetes/ml, with doubling times of 2 to 4 h (Breznak and Canale-Parola, 1975; Canale-Parola, 1973; Greenberg and Canale-Parola, 1976).

A procedure in which the antibiotic rifampin (rifampicin) serves as a selective agent is quite effective for the isolation of free-living spirochetes (genus *Spirochaeta*) from natural environments (Harwood et al., 1982b; Patel et al., 1985; Stanton and Canale-Parola, 1979; Weber and Greenberg, 1981). This procedure, which is described below, is based on the observation that spirochetes in general are naturally resistant to rifampin (Leschine and Canale-Parola, 1986; Stanton and Canale-Parola, 1979). Thus, spirochetes such as *Spirochaeta stenostrepta* and *Spirochaeta aurantia* grow in the presence of as much as 100 to 200 μg of rifampin per ml of medium (Leschine and Canale-Parola, 1986), whereas the growth of many other bacteria is inhibited. The resistance of spirochetes to rifampin is probably due to the low affinity of their RNA polymerase for the antibiotic (Allan et al., 1986; Leschine and Canale-Parola, 1986).

Other enrichment procedures used in the isolation of *Spirochaeta* species are based either on one or both of the following selective factors: 1) the ability of spirochetes to pass through filters that retain most other bacteria, and 2) the migratory movement of spirochetes through agar media (Canale-Parola, 1973; Canale-Parola, 1984a). These selective procedures enrich for species of *Spirochaeta* measuring less than 0.5 μm in diameter (Table 1).

In the enrichment-by-filtration procedure, which is described in detail below, separation of *Spirochaeta* species from most of the microorganisms present in mud or water is achieved by techniques involving filtration through cellulose ester filter discs (e.g., Millipore filters) having an average pore diameter of 0.3 or 0.45 μm. Spirochetes pass through these filter discs because of their relatively small cell diameter (Table 1) and probably also because their motility apparatus enables them to swim freely in liquids as well as to move in contact with solid surfaces.

The enrichment-by-migration procedure uses the ability of spirochetes to move through agar gels or media containing as much as 1 to 2% (w/v) agar. This movement or migration occurs primarily within the agar gel, i.e., below the surface of the agar medium. In contrast, flagellated bacteria usually cannot carry out translational

movement through gels or media containing agar at the above-mentioned concentrations, although several exceptions have been reported (Greenberg and Canale-Parola, 1977b). Apparently, the cell coiling of spirochetes is important for their translational motion through agar gels, inasmuch as this type of movement is impaired in mutant spirochetes lacking the cell-coiling characteristic of the parental strain (Greenberg and Canale-Parola, 1977c).

Migration of spirochetes through agar media results from the unique motility mechanism of these bacteria (Canale-Parola, 1977; Canale-Parola, 1978), as well as from chemotaxis toward the energy and carbon source (Breznak and Canale-Parola, 1975; Greenberg and Canale-Parola, 1977a). The role of chemotaxis in the migration of saccharolytic spirochetes through agar media has been studied (Breznak and Canale-Parola, 1975). When these spirochetes are inoculated in the center of glucose-containing agar medium plates, they grow using this sugar as their energy source. Utilization of the sugar by the spirochetes gives rise to a glucose concentration gradient that moves away from the center of the plate as more of this carbohydrate is metabolized by the spirochetes. Because the spirochetes exhibit chemotaxis toward glucose and are able to move through the agar gel, they migrate into the areas of higher glucose concentration within the gradient. Thus, the spirochetal population follows the outward movement of the gradient and migrates toward the periphery of the plate. This behavior results in the formation of a growth "veil" or "ring" of spirochetes for which glucose serves both as the energy source for growth and as the chemoattractant (Breznak and Canale-Parola, 1975; Canale-Parola, 1973). The veil or ring increases continuously in diameter during incubation and may reach the outer edge of the plate. The migration rate of the spirochetal population is greatest in agar media containing low substrate concentrations (e.g., 0.02% glucose). In these media the substrate becomes rapidly depleted in the region where spirochetes are growing, and the spirochetal population moves toward the outer zone of higher substrate concentrations at a relatively fast rate (Breznak and Canale-Parola, 1975).

In procedures for the isolation of *Spirochaeta* species from natural environments, the chemotactic behavior and the ability of these bacteria to move through agar gels have important selective functions. In a typical isolation procedure, a small, shallow cylindrical hole is made through the surface of an agar medium containing a low concentration of carbohydrate. Rifampin may be included in the medium as an additional selective agent for spirochetes. The medium may be in a Petri dish or a small bottle. A tiny drop of pond water, or of any other material in which spirochetes have been observed, is placed within the hole. The chemotactic, saccharolytic spirochetes in the inoculum multiply and form a growth veil that extends outwardly through the agar medium. Thus, the spirochetes in the veil move away from contaminants, which remain mainly in the vicinity of the inoculation site. Spirochetal cells from the outermost edge of the veil are used to obtain pure cultures by conventional methods, such as streaking on agar medium plates. Isolation procedures involving chemotaxis and movement through agar gels are described below.

Selective isolation techniques have not been developed for the large *Spirochaeta* species, such as *Spirochaeta plicatilis*.

Enrichment by Migration

When other bacteria vastly outnumber spirochetes in the inoculum, it is advisable to begin the isolation procedure with an enrichment-by-migration step (the principle has been discussed above). A suitable medium (EBM agar medium) for this enrichment has the following composition in g/100 ml distilled water: yeast extract (Difco), 0.1; Trypticase (BBL Microbiology Systems), 0.1; L-cysteine, HCl, 0.05; resazurin, 10^{-4}; and agar (Difco), 1. For the enrichment and isolation of marine spirochetes, a mixture of seawater (70 ml) and distilled water (30 ml) is used instead of plain distilled water. The pH of the medium is adjusted to 7.2. After autoclaving, cellobiose is added as a sterile solution to a final concentration of 0.01 g/100 ml medium. Rifampin (filter-sterilized solution) may be used as a selective agent (0.5 mg per 100 ml of medium).

The medium is pre-reduced (Hungate, 1969) and is dispensed into narrow-necked 60-ml glass bottles. During this step and the steps that follow, the medium is maintained in an anaerobic atmosphere by delivering a stream of oxygen (O_2)-free nitrogen (N_2) into the bottles. The bottles containing the medium are sealed with neoprene rubber stoppers and are placed in a press to hold the stoppers in place during autoclaving. The sterilized medium is allowed to cool to approximately 50°C, and cellobiose and, if desired, rifampin are added. The complete medium is solidified at a 45° angle so that bottle slants are obtained. Then, a small (5-mm wide, 6-mm deep), cylindrical well is melted halfway down each slant by heating the tip of a thin metal rod and touching the agar with it. Alternatively, the well can be made by aspirating some of the agar medium with a sterile Pasteur pipette connected to a vacuum apparatus. All further manipulations are made either while maintaining an N_2 atmosphere within the bottles (Hungate, 1969)

or with the bottles placed within an anaerobic chamber and the rubber stoppers replaced by cotton stoppers.

Before inoculation, any liquid that oozed from the agar medium is removed by suction from the well and the lower part of each slant. The bottle slants are inoculated by carefully placing a small volume of inoculum (e.g., mud) into the well, and they are incubated at 30°C. In successful enrichments, the spirochetes form a characteristic, semitransparent growth veil that extends down into the agar medium and diffuses out toward the periphery of the slant, away from contaminating organisms growing in and near the well. The veil usually is visible after 4 to 7 days of incubation. Cells from the edges of the growth veil are used to obtain pure cultures of the spirochetes by means of a procedure involving serial dilutions in a rifampin-containing medium (see below).

Isolation in Rifampin-Containing Media

Serial dilutions in melted, rifampin-containing agar (RIM) medium can be used to isolate spirochetes from materials in which they are present in relatively large numbers (e.g., directly from mud or from the outer edges of growth veils in the bottle slants mentioned above). The spirochete-containing inoculum is serially diluted in tubes of melted (45°C), pre-reduced RIM medium. This medium is identical to the EBM agar medium described above except that the final concentration of cellobiose is 0.2% (w/v), rifampin (0.5 mg/100 ml of medium) is added, and the agar (Bacto, Difco) concentration is 0.8% (w/v). The medium is dispensed and sealed in anaerobic culture tubes containing an N_2 atmosphere (Hungate, 1969).

Spirochete colonies within the RIM agar medium are recognizable inasmuch as they are spherical and because, as a result of cell migration through the agar medium, they appear as "transparent bubbles," "veil-like growth with a dense center," or "cotton ball-like growth" (Paster and Canale-Parola, 1982; Stanton and Canale-Parola, 1979; Weber and Greenberg, 1981). To obtain pure cultures, the serial dilution step is repeated at least twice, using cells from spirochete colonies that developed within the RIM agar medium.

Procedures involving serial dilutions in rifampin-containing agar media have been used by Weber and Greenberg (1981) and by Patel et al. (1985) to isolate spirochetes from salt marsh sediments and hot springs, respectively.

Selective Enrichment by Filtration

A filtration technique has been used in the isolation of *Spirochaeta stenostrepta* (Canale-Parola et al., 1967; Canale-Parola et al., 1968), *Spirochaeta litoralis* (Hespell and Canale-Parola, 1970b), and the facultatively anaerobic *Spirochaeta halophila* (Greenberg and Canale-Parola, 1976). In all cases, the source of the spirochetes was black mud that had the characteristic smell of hydrogen sulfide (H_2S). *Spirochaeta stenostrepta* was isolated from a mud sample collected from a fresh-water pond, *Spirochaeta litoralis* from marine mud, and *Spirochaeta halophila* from the mud of Solar Lake, a high-salinity pond located on the Sinai shore of the Gulf of Aqaba. A filtration technique also was used in the isolation of the alkaliphiles, *Spirochaeta asiatica*, *Spirochaeta alkalica* and *Spirochaeta africana*, from the sediments of alkaline lakes located in the East African Rift region and central Asia (Zhilina et al., 1996).

Medium for Isolating *Spirochaeta stenostrepta*

Distilled water	875 ml
Glucose	5.0 g
Peptone	2.0 g
Yeast extract	0.3 g
Vitamin B_{12}	10^{-5} g
Phosphate solution	15 ml
Salts solution	100 ml
Sulfide solution	10 ml

The phosphate solution is prepared by dissolving 30 g of KH_2PO_4 and 70 g of K_2HPO_4 in 1,000 ml of distilled water.

To prepare the salts solution, 0.2 g of ethylenediamine tetraacetic acid are dissolved in 800 ml of distilled water by heating. The pH of the resulting solution is adjusted to 7.0 with 2.5% KOH. Then the following additions are made: $MgSO_4 7H_2O$, 2.0 g; $CaCl_2 2H_2O$, 0.75 g; $FeSO_4 7H_2O$, 0.1 g; trace elements solution (below), 5.0 ml. Finally, the volume of the salts solution is adjusted to 1,000 ml with distilled water. The sulfide solution (2 g of $Na_2S 9H_2O$/100 ml distilled water) is autoclaved separately and added shortly before inoculating the (precooled to 30–35°C) sterile medium. After sterilization by autoclaving, a precipitate is present in the medium, but it disappears as the medium cools. The final pH of the complete medium is 6.9–7.0.

Trace elements solution: a separate solution of each salt listed below, in the amount indicated. Heating may be required to dissolve some of the salts. The pH of the sodium molybdate and sodium vanadate (Na_2MoO_4 and $NaVO_3$) solutions is adjusted to a value below 7. The potassium iodide (KI) solution is added to the aluminum chloride ($AlCl_3$) solution and mixed by stirring. Then the other solutions are added, one at a time, to this mixture, with stirring, in the order in which they are listed below.

$AlCl_3 \cdot 6H_2O$	0.50 g
KI	0.25 g
KBr	0.25 g
LiCl	0.25 g
$MnCl_2 \cdot 4H_2O$	3.50 g
H_3BO_3	5.50 g
$ZnCl_2$	0.50 g
$CuCl_2 \cdot 2H_2O$	0.50 g
$NiCl_2 \cdot 6H_2O$	0.50 g
$CoCl_2 \cdot 6H_2O$	0.50 g
$SnCl_2 \cdot 2H_2O$	0.15 g
$BaCl_2 \cdot 2H_2O$	0.15 g
$Na_2MoO_4 \cdot 2H_2O$	0.25 g
$NaVO_3$	0.05 g

The volume of the final mixture is adjusted to 1,800 ml by adding distilled water and to pH 3–4 with HCl. A fine, white precipitate replaces a yellow precipitate after a few days. The solution should be mixed thoroughly immediately before it is used and may be stored at room temperature. This trace element solution is a modification of a solution described by Pfennig (1965).

Isolation of *Spirochaeta stenostrepta* (Canale-Parola et al., 1967) Black mud, from which a strong odor of H_2S could be detected, was suspended in aqueous sodium sulfide (0.02% Na_2S9H_2O). The slurry was filtered through Whatman No. 40 filter paper, and the filtrate was subjected to filtration through sterile cellulose ester filter discs (Millipore, pore diameter 0.45 μm). Each 1-ml aliquot of the resulting filtrate was added aseptically to a 60-ml glass stoppered bottle, half filled with sterile isolation medium. The bottles were then completely filled with medium, stoppered without trapping air bubbles, and incubated at 30°C (each bottle was covered with a sterile 50-ml beaker). After 5 to 7 d of incubation, the microbial population in many of the bottles consisted predominantly of thin spirochetes. Pure cultures were obtained by use of dilution shake cultures (the medium was covered with sterile paraffin) or by plating serial dilutions and incubating the plates in the absence of O_2 (Bray dishes). The isolation medium solidified with 1.5% agar was used. Spirochetal colonies appeared after 5 to 6 d. After isolation, the organisms were also grown in Florence flasks filled with a medium GYPT containing (g/100 ml distilled water): glucose, 0.5; yeast extract and peptone, 0.2 each; and sodium thioglycolate, 0.05. The pH of this medium was adjusted to 7.0–7.3 before sterilization. The spirochetes were maintained in paraffin-layered stab cultures of medium GYPT containing 1.5% agar at 5°C and transferred monthly. Subsurface colonies of *Spirochaeta stenostrepta* in agar media are white, spherical, and characteristically fluffy in appearance.

Spirochaeta litoralis and *Spirochaeta halophila* were isolated by a procedure similar to that used for *Spirochaeta stenostrepta*, except that different isolation media were used and the incubation temperatures were 22–23°C for *Spirochaeta litoralis* and 37°C for *Spirochaeta halophila* (Hespell and Canale-Parola, 1970b; Greenberg and Canale-Parola, 1976). Furthermore, one volume of the mud used as the source of *Spirochaeta litoralis* was suspended in five volumes of isolation medium before filtration through filter paper.

Isolation of *Spirochaeta litoralis* (Hespell and Canale-Parola, 1970b) The isolation medium for the marine spirochete included (g/100 ml of distilled water): tryptone (Difco), 0.3; yeast extract (Difco), 0.05; and NaCl, 2.0. To this mixture 2 ml of 1 M potassium phosphate buffer (pH 7.4) and 0.2 ml of a salt solution (see below) were added. The pH of the medium was adjusted to 7.3 with KOH before sterilization. Immediately before inoculation, the medium was supplemented with 2 ml of a sterile glucose solution (25 g per 100 ml distilled water) and with 0.5 ml of a sterile Na_2S9H_2O solution (10 g per 100 ml of distilled water). The final pH of the medium ranged from 7.4 to 7.5.

The salts solution contained (g/75 ml of distilled water): tetrasodium ethylenediamine tetraacetate, 1; $CaCl_22H_2O$, 3.75; $MgCl_26H_2O$, 12.5; and $FeSO_47H_2O$, 0.5. To 75 ml of this salt mixture, 25 ml of a trace elements solution (see Medium for Isolating *Spirochaeta stenostrepta*) was added.

After cloning, *Spirochaeta litoralis* was grown routinely in the isolation medium. Subsurface colonies of *Spirochaeta litoralis* in agar media are white to cream-colored and resemble those of *Spirochaeta stenostrepta*.

Maintenance procedures: Broth cultures of *Spirochaeta litoralis* in test tubes remained viable for 3 d at 30°C. Agar stabs grown at 30°C and then kept either at 5 or 30°C were no longer viable after approximately one week, whether they were layered with paraffin or not. Viable cells were not recovered from lyophilized preparations.

Spirochaeta litoralis was satisfactorily maintained by using "depression" cultures (Canale-Parola and Wolfe, 1960) prepared by using 1-liter Erlenmeyer flasks, each containing 800 ml of isolation medium to which 2 g of agar per 100 ml were added. These cultures, when incubated for 2 d at room temperature and then at 15°C, remained viable for at least three months.

Isolation of *Spirochaeta halophila* (Greenberg and Canale-Parola, 1976) The isolation medium contained 0.2 g of peptone (Difco) and 0.4 g of yeast extract (BBL) per 97 ml of an inorganic salt solution which had the

following composition: $CaCl_2 \cdot 2H_2O$, 0.04 M; NaCl, 0.85 M; and $MgSO_4 7H_2O$, 0.2 M. In preparing the salt solution, salts were added in the order in which they are listed to avoid formation of a precipitate. After adjusting the pH to 7.5 with KOH, the medium was sterilized by autoclaving. The volume of the medium was brought to 100 ml by adding separately sterilized solutions of glucose and $Na_2S 9H_2O$ to final concentrations of 0.5% and 0.05%, respectively.

Spirochaeta halophila was grown routinely at 37°C in ISM broth (Greenberg and Canale-Parola, 1975), which differed from the isolation medium because maltose replaced glucose, the $Na_2S 9H_2O$ was omitted, and the composition of the salt solution was changed (to $CaCl_2$, 0.01 M; NaCl, 0.75 M; and $MgSO_4$, 0.2 M). Furthermore, BBL yeast extract was replaced by Difco yeast extract, which supported higher growth yields of our isolate.

Spirochaeta halophila was maintained by storing ISM agar (ISM broth containing 0.75 g agar per 100 ml) plate cultures at 5°C. The cultures were transferred monthly.

Colonies of *Spirochaeta halophila* are pigmented when growing aerobically but lack pigmentation under anaerobic growth conditions. Colonies grown in air on ISM agar plates were red and appeared circular when viewed from above. A portion of each colony grew above the surface of the medium and part of the colony extended into the agar. Areas of diffuse growth were present at the periphery of the colonies. Generally, colonies measured 2–6 mm in diameter after 5 days at 35°C but were smaller or larger depending on the number of colonies on the plate and the length of incubation. Cells streaked on ISM agar plates and grown anaerobically in Bray dishes formed colonies that developed below the surface of the agar medium and were white, spherical and diffuse.

Cultivation of most of the anaerobic species of *Spirochaeta* described here does not require the use of stringent anaerobic procedures (e.g., the Hungate technique). Reducing agents, such as sodium thioglycolate or L-cysteine, are commonly added to the growth media, and other conventional techniques for the growth of anaerobes are used, as discussed elsewhere (Canale-Parola, 1973).

Enrichment by Filtration and Migration

Many strains of *Spirochaeta aurantia* have been isolated by means of a selective technique that combines filtration of the inoculum through a cellulose ester filter disk with migration of the spirochetes in agar media (Breznak and Canale-Parola, 1969; Canale-Parola, 1973; Breznak and Canale-Parola, 1975).

Isolation of *Spirochaeta aurantia* by Filtration and Migration

Ingredient	Medium HE	Medium PEP
Distilled water	50 ml	100 ml
Peptone	0.1 g	0.5 g
Yeast extract	0.1 g	0.05 g
K_2HPO_4	—	0.01 g
Hay extract	50 ml	—
Agar	1 g	1 g

The pH of medium HE is adjusted to 6.5 before sterilization. To prepare the hay extract, 0.5 g of dried barn hay are boiled in 100 ml of distilled water for 10 min. The boiled mixture is filtered using Whatman No. 40 filter paper. The filtrate is the hay extract.

The inoculum, consisting either of pond or marsh water or of a water-mud slurry, was prefiltered through Whatman No. 40 filter paper to remove large particles. Then, the enrichment cultures were prepared by depositing one or two drops of the filtrate near the center of each of a number of sterile cellulose ester filter disks (47-mm disk diameter, 0.3- or 0.45-μm pore diameter, Millipore) previously placed on the surface of isolation medium plates. One filter disk had been placed on each plate, approximately in the center. The cultures were incubated at 30°C for 12–24 h to allow spirochetes in the inoculum to move through the filter disk onto the surface of the medium. Then the filter disks were removed aseptically from the plates, and incubation of the plate cultures was continued. Spirochetes that had passed through the filter disks grew and migrated through the agar medium, forming semitransparent growth veils that diffused toward the edge of the plates. Spirochetal growth veils usually developed in 5–10 days.

Generally, 10–20% of the plate enrichments were successful (i.e., yielded growth veils) when spirochetes were present in the inoculum, as determined by light microscopy. Analyses showed (Breznak and Canale-Parola, 1975) that the total carbohydrate content of a batch of isolation medium HE was 40 mg/100 ml and the glucose content 5 mg/100 ml. Medium PEP contained 6 mg total carbohydrate and less than 1 mg glucose per 100 ml. As discussed previously, low carbohydrate concentrations, such as those in isolation media HE and PEP, are used to enhance the rate of spreading of the spirochetal growth veil through the agar medium.

It should be noted that bacteria other than spirochetes may form subsurface spreading growth veils in the enrichment plates. Most common among these veil-forming bacteria are *Aquaspirillum* (*Spirillum*) *gracile* (Canale-Parola et al., 1966) and *Serpens flexibilis* (Hespell, 1977). *Serpens flexibilis* cells are flexible, Gram-negative rods that have bipolar as well as lateral flagella.

Pure cultures of *Spirochaeta aurantia* are obtained by streaking cells from the outer edge of the growth veil onto isolation medium plates or growth medium plates.

A suitable growth medium for *Spirochaeta aurantia* contains (g/99 ml distilled water): glucose, 0.2; yeast extract, 0.2; and Trypticase (BBL), 0.5. The pH is adjusted to 7.5 before sterilization. When desired, agar (1 g) is added. After autoclaving and allowing the medium to cool, 1 ml of sterile 1 M potassium phosphate buffer (pH 7) is added.

Growth of some *Spirochaeta aurantia* strains is either partially or totally inhibited in media containing agar (Difco) concentrations higher than 1% (w/v). Thus, viable cell counts usually are higher when performed using plates of media containing 0.75 or 1% agar than when the cells are grown in media including agar at higher concentrations. *Spirochaeta aurantia* cells grown in media containing agar at concentrations higher than 1% frequently are aberrant in morphology. Many usually long, poorly coiled, filamentous cells are present, as well as an abundance of spherical bodies (Breznak and Canale-Parola, 1975).

Surface, aerobic colonies of *Spirochaeta aurantia* in growth medium (agar, 1%) are light orange to orange, round or nearly round, with slightly irregular edges, and measure 1–4 mm in diameter after 4–7 days at 30°C. The colonies grow mostly within the agar medium, just under the surface, but many have a slightly raised central portion. One strain (Vinzent strain) was found to produce both this type of colony and a "pinpoint" type of colony, measuring approximately 0.5 mm in diameter and growing primarily on the surface of the agar medium (Breznak and Canale-Parola, 1975).

Surface, anaerobic colonies of *Spirochaeta aurantia* (growth medium, 1% agar) are similar in morphology to the aerobic ones, but are not pigmented. Subsurface anaerobic colonies are white and spherical.

Spirochaeta aurantia may be maintained on slants of growth medium at 5°C. These stock cultures are transferred monthly.

Identification

At present, free-living, anaerobic and facultatively anaerobic, helical bacteria that possess the ultrastructural features typical of spirochetes (e.g., periplasmic flagella and outer sheath) are classified in the genus Spirochaeta. However, phylogenetic analyses have clearly indicated that some species identified as *Spirochaeta* using these criteria—namely, the free-living, obligately anaerobic spirochetes from freshwater environments, *Spirochaeta stenostrepta*, *Spirochaeta zuelzerae* and *Spirochaeta caldaria*—are more closely related to members of the genus *Treponema* than to other members of the genus *Spirochaeta* (see Phylogeny).

Differentiation among the obligately anaerobic species (Table 1, species 1–9; see Taxonomy) may be based, in part, on determinations of carbohydrate fermentation end products. *Spirochaeta stenostrepta*, *Spirochaeta litoralis*, *Spirochaeta isovalerica* and *Spirochaeta bajacaliforniensis* form acetate, ethanol, carbon dioxide (CO_2) and molecular hydrogen (H_2) as major products of glucose fermentation, whereas *Spirochaeta zuelzerae*, *Spirochaeta thermophila* and *Spirochaeta caldaria* produce acetate, lactate, CO_2, H_2, and, in some cases, small amounts of succinate (Aksenova et al., 1992; Canale-Parola, 1984a; Fracek and Stolz, 1985; Pohlschroeder et al., 1994). *Spirochaeta asiatica* produces acetate, ethanol, and lactate, but not H_2, during glucose fermentation (Zhilina et al., 1996). In addition, *Spirochaeta isovalerica* forms small amounts of isovalerate, 2-methylbutyrate and isobutyrate as fermentation end products when growing in media containing L-leucine, L-isoleucine and L-valine, as well as a fermentable carbohydrate (see Physiology; Harwood and Canale-Parola, 1983). *Spirochaeta smaragdinae* is the only species of *Spirochaeta* known to reduce thiosulfate or sulfur (but not sulfate) to H_2S and produces acetate, lactate, CO_2, and H_2S in the presence of thiosulfate and ethanol, lactate, CO_2, and H_2 in its absence (Magot et al., 1997).

Spirochaeta stenostrepta, *Spirochaeta zuelzerae* and *Spirochaeta caldaria* are freshwater anaerobes, whereas *Spirochaeta litoralis*, *Spirochaeta isovalerica*, *Spirochaeta bajacaliforniensis* and *Spirochaeta thermophila* were isolated from seawater environments and have salt requirements typical of marine bacteria (Aksenova et al., 1992; Fracek and Stolz, 1985; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970b; Pohlschroeder et al., 1994). Another anaerobic species, *Spirochaeta asiatica*, was isolated from the sediments of an alkaline lake and requires Na^+ concentrations ranging from 500 to 1,000 mM for optimal growth (Zhilina et al., 1996).

This latter species is also alkaliphilic, and growth does not occur below pH 8. *Spirochaeta smaragdinae*, an obligate anaerobe isolated from a production water sample collected from an offshore oil field, requires at least 170 mM NaCl and grows optimally with 850 mM NaCl (Magot et al., 1997). *Spirochaeta thermophila* and *Spirochaeta caldaria*, both isolated from hot springs, are thermophilic anaerobes with optimum growth temperature of 66–68 and 48–52°C, respectively (Aksenova et al., 1992; Pohlschroeder et al., 1994). Further characteriza-

tion of the obligate anaerobes should take into account interspecific differences in G+C content of the DNA and in cell size (Table 1).

Spirochaeta alkalica and *Spirochaeta africana* (Table 1, species 10 and 11) are aerotolerant species that survive two or three transfers in a glucose-containing medium incubated in air; however, aerobic growth is weak as compared to growth under anaerobic conditions (Zhilina et al., 1996).

These species may be distinguished by their ion requirements. *Spirochaeta africana* requires NaCl for growth, whereas *Spirochaeta alkalica* will grow in media supplemented with equimolar sodium carbonate and bicarbonate (Na_2CO_3 + $NaHCO_3$) in place of NaCl (Zhilina et al., 1996).

Salt requirements constitute an important basis of distinction between the facultative anaerobes *Spirochaeta aurantia* and *Spirochaeta halophila* (Table 1, species 12 and 13). The latter species is halophilic and has an absolute requirement for relatively high concentrations of Na^+, Cl^-, Ca^{++} and Mg^{++} (Greenberg and Canale-Parola, 1976), whereas *Spirochaeta aurantia* strains have been isolated only from freshwater environments and do not exhibit special salt requirements (Breznak and Canale-Parola, 1969; Breznak and Canale-Parola, 1975).

Pigment production is a characteristic of the aerotolerant and facultatively anaerobic species (Table 1, species 10–13). Zhilina et al. (1996) reported that the cell mass of the aerotolerant species *Spirochaeta alkalica* and *Spirochaeta africana* is orange. When growing aerobically, the facultative anaerobes *Spirochaeta halophila* and *Spirochaeta aurantia* produce carotenoid pigments. The major pigment of *Spirochaeta halophila* (strain RS1) is 4-keto-1′,2′-dihydro-1′-hydroxytorulene, whereas *Spirochaeta aurantia* (strain J1) produces mainly 1′, 2′-dihydro-1′-hydroxytorulene (Greenberg and Canale-Parola, 1975). Nonpigmented mutants of *Spirochaeta halophila* (Greenberg and Canale-Parola, 1976) and of *Spirochaeta aurantia* (B. J. Paster and E. Canale-Parola, unpublished data) have been isolated.

Cells of *Spirochaeta aurantia* subspecies *stricta* are more tightly coiled than those of *Spirochaeta aurantia* subspecies *aurantia* (Table 1, footnote c).

Criteria used for the identification of *Spirochaeta plicatilis* (Table 1, species 7) are its large size (Table 1) and its characteristic morphology and motility (Blakemore and Canale-Parola, 1973).

Preservation

Cells of *Spirochaeta* species can be preserved in a viable condition for several years by maintaining them at the temperature of liquid nitrogen. Conventional methods are used to prepare liquid nitrogen stock cultures of spirochetes (Canale-Parola, 1973).

Physiology

Motility and Chemotaxis

Three main types of motion are observed in species of *Spirochaeta*: 1) translational motion; 2) rotation of the cell around its longitudinal axis; and 3) flexing motion (Canale-Parola, 1978). In addition to swimming freely in liquid environments, some *Spirochaeta* are able to "creep" or "crawl" on solid surfaces, a movement resembling that of the gliding bacteria (Blakemore and Canale-Parola, 1973). Species of *Spirochaeta* retain their translational motion in environments of relatively high viscosity, e.g., 500 centipoise (Greenberg and Canale-Parola, 1977c). In comparison, flagellated bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Aquaspirillum serpens*, are immobilized at viscosities of or above 60 centipoise (Greenberg and Canale-Parola, 1977b).

The motility of a strain of *Spirochaeta aurantia* in liquid environments has been described as follows (Greenberg and Canale-Parola, 1977a; Greenberg et al., 1985): The spirochete usually swims in nearly straight lines (runs) and appears to spin about its longitudinal axis as it progresses through the liquid. From time to time the cell reverses swimming direction, the anterior end of the cell becoming the posterior end. Occasionally, the spirochete stops running, flexes, and then resumes its translational motion. However, upon resuming its translational motion, the spirochete usually alters the direction of its movement, and the previously leading cell end may or may not become the trailing cell end. Thus, *Spirochaeta aurantia* performs three kinds of behavior (runs, flexes, and reversals), in contrast to *Escherichia coli*, which performs two kinds (runs and tumbles). During runs, *Spirochaeta aurantia* cells have an average linear speed of approximately 16 μm/s (Fosnaugh and Greenberg, 1988). Flexes last from a fraction of a second to several seconds. The average frequency of reversals in cell populations is approximately 0.31 reversals/5 s (Fosnaugh and Greenberg, 1988).

A model that interprets the motile behavior of *Spirochaeta aurantia* has been proposed (Berg, 1976; Greenberg et al., 1985). According to this model, the two periplasmic flagella of *Spirochaeta aurantia* rotate, each driven by a motor at the insertion end. Rotation of the flagella in one direction (looking at the cell head on; see Fig. 13B, Introduction) causes the periplasmic cylin-

der and the outer membrane to move in directions opposite to each other. Owing to its helical shape, the cell rotates about its longitudinal axis and moves along it. Runs occur when the flagellar motor at one cell end rotates clockwise (CW) while the motor at the other cell end rotates counterclockwise (CCW). When both motors switch direction of rotation at the same time, a reversal takes place. A flex is generated during asynchronous switching, i.e., when only one motor switches direction so that both motors are rotating CW or CCW. Asynchronous switching causes the cell ends to twist in opposition to each other, and a flex occurs.

Spirochaeta aurantia exhibits chemotaxis toward D-glucose, D-xylose, cellobiose, and various other sugars, but not toward amino acids (Greenberg and Canale-Parola, 1977a). Many of the chemoattractants also serve as carbon and energy sources for growth of *Spirochaeta aurantia*. *Spirochaeta aurantia* cells grown in a chemostat at very low concentrations of an attractant that serves as a carbon and energy source exhibit an enhanced chemotactic response toward that attractant and are able to sense concentrations of the attractant much lower than those sensed by cells grown in the presence of excess attractant (Terracciano and Canale-Parola, 1984). Most likely, the ability to regulate its chemosensory system provides *Spirochaeta aurantia* with competitive advantages in natural environments deficient in nutrients.

Fosnaugh and Greenberg (1988) carried out an analysis of the motility and chemotaxis behavior of *Spirochaeta aurantia*. They observed that a population of *Spirochaeta aurantia* cells spent, on average, 66% of the time swimming smoothly (runs), 33% of the time flexing, and 1% of the time in reversals. After addition of an attractant (D-xylose, 10 mM final concentration), there was an increase in smooth swimming, a decrease in flexing, and a complete suppression of reversals. From 1.5 to 2 min after addition of the attractant, the population resumed its unmodified behavior. On the basis of their observations and of the above-mentioned motility model for *Spirochaeta aurantia*, Fosnaugh and Greenberg (1988) postulated that a mechanism for communication between the two flagellar motors is present in this spirochete and that a motor-switch-synchronizing device is also operating.

Fermentation Products and ATP-Yielding Pathways

Under anaerobic conditions, several species of *Spirochaeta* that have been cultured (Table 1, see Taxonomy) ferment carbohydrates with formation of acetate, ethanol, CO_2, and H_2 as major end products (Canale-Parola, 1984a; Fracek and Stolz, 1985; Harwood and Canale-Parola, 1983). *Spirochaeta zuelzerae*, *Spirochaeta thermophila* and *Spirochaeta caldaria* produce acetate, lactate, CO_2 and H_2 from carbohydrates (Aksenova et al., 1992; Canale-Parola, 1984a; Pohlschroeder et al., 1994), and *Spirochaeta zuelzerae* also produces small amounts of succinate (Canale-Parola, 1977; Canale-Parola, 1984a; Veldkamp, 1960). *Spirochaeta alkalica* and *Spirochaeta africana* produce acetate, ethanol, lactate and H_2, and *Spirochaeta asiatica* produces acetate, ethanol and lactate, but not H_2, during glucose fermentation (Zhilina et al., 1996). *Spirochaeta smaragdinae* is the only species of *Spirochaeta* that has been shown to reduce thiosulfate and sulfur to H_2S, producing acetate, lactate, CO_2 and H_2S in the presence of thiosulfate and producing ethanol, lactate, CO_2 and H_2 in its absence (Magot et al., 1997).

Spirochaeta isovalerica (Table 1) ferments (in addition to carbohydrates) L-leucine, L-isoleucine and L-valine, forming isovaleric, 2-methylbutyric and isobutyric acids, respectively, as end products (Harwood and Canale-Parola, 1983). However, *Spirochaeta isovalerica* requires a fermentable carbohydrate for growth. When *Spirochaeta isovalerica* is grown in medium containing both glucose and the three above-mentioned amino acids, only a relatively small fraction of the total amount of available amino acids is fermented (Harwood and Canale-Parola, 1981a; Harwood and Canale-Parola, 1981b). Under these growth conditions, the ATP derived from amino acid catabolism is estimated to be 4 to 5% of the total ATP formed. Fermentation of the amino acids in the absence of glucose does not support measurable growth of *Spirochaeta isovalerica*, but serves to generate ATP, which is utilized as a source of maintenance energy by the spirochete when fermentable carbohydrates are not available (Harwood and Canale-Parola, 1981a; Harwood and Canale-Parola, 1983). In addition to the branched-chain fatty acids, amino acid catabolism by *Spirochaeta isovalerica* yields small quantities of isobutanol and isoamyl alcohol (Harwood and Canale-Parola, 1981b; 1983).

All *Spirochaeta* species whose metabolic pathways have been studied (species 1, 2, 4, 6, 12 and 13 in Table 1; see Taxonomy) catabolize glucose to pyruvate via the Embden-Meyerhof pathway (Aksenova et al., 1992; Canale-Parola, 1984a; Harwood and Canale-Parola, 1983). Anaerobic metabolism of pyruvate yields acetyl-CoA, CO_2 and H_2 via a clostridial-type clastic reaction (Canale-Parola, 1977; Greenberg and Canale-Parola, 1976). Acetate is formed from acetyl CoA in reactions catalyzed by phosphotransacetylase and acetate kinase. A double reduction involving aldehyde and alcohol dehydrogenases

is responsible for ethanol production from acetyl CoA (Canale-Parola, 1977).

When growing aerobically, the two facultatively aerobic species (*Spirochaeta aurantia* and *Spirochaeta halophila*) oxidize glucose incompletely, with formation of CO_2 and acetate as major end products. Aerobically, both oxidative phosphorylation and substrate-level phosphorylation are utilized by the two species to generate ATP (Breznak and Canale-Parola, 1972b; Greenberg and Canale-Parola, 1976). The tricarboxylic acid cycle either is absent or plays a minor catabolic role in these two species.

Rubredoxin was detected in cell extracts of the obligate anaerobes *Spirochaeta stenostrepta* and *Spirochaeta litoralis* and of the facultative anaerobe *Spirochaeta aurantia* (Breznak and Canale-Parola, 1972a; Hespell and Canale-Parola, 1973; Johnson and Canale-Parola, 1973). Rubredoxin was isolated from extracts of both aerobically and anaerobically grown cells of *Spirochaeta aurantia*. Ferredoxin was present in cell extracts of anaerobically grown *Spirochaeta aurantia*, but was not found in aerobically grown cells of this bacterium (Johnson and Canale-Parola, 1973).

Ecology

Species of *Spirochaeta* are free-living bacteria indigenous to a variety of aquatic environments, such as the water, sediments and muds of ponds, marshes, lakes, rivers and oceans (Canale-Parola, 1984a). In these environments, *Spirochaeta* cells display a wide range of motility behaviors. When *Spirochaeta* cells swim through liquid environments, they may perform rotatory, locomotory and flexing movements (Canale-Parola, 1978). Also, spirochetes may move through environments of viscosity high enough to impede the progress of most flagellated bacteria (Harwood and Canale-Parola, 1984). Moreover, some *Spirochaeta* are able to move on solid surfaces by "creeping" or "crawling," a movement resembling that of the gliding bacteria (Blakemore and Canale-Parola, 1973). Complex physiological and behavioral adaptations, many of which involve one or more types of spirochaetal motility behavior, enable *Spirochaeta* species to persist in their environments and to compete successfully with other organisms for available nutrients (Harwood and Canale-Parola, 1984).

All species of *Spirochaeta* that have been cultivated are saccharolytic, and they usually lack the ability to utilize compounds other than carbohydrates as oxidizable substrates for growth. Various pentoses, hexoses, disaccharides and polysaccharides, such as starch, are used as carbon and energy sources (Aksenova et al., 1992; Canale-Parola, 1984a; Fracek and Stolz, 1985; Greenberg and Canale-Parola, 1976; Magot et al., 1997; Pohlschroeder et al., 1994; Zhilina et al., 1996). Even though free-living spirochetes are commonly observed in natural anaerobic environments in which plant material, containing cellulose and hemicelluloses, is biodegraded (Canale-Parola, 1978; Harwood and Canale-Parola, 1984; Leschine, 1995), only *Spirochaeta thermophila* has been reported to ferment cellulose and xylan (Aksenova et al., 1992). It may be inferred that, in their habitats, which frequently are rich in decaying plant material, *Spirochaeta* species usually ferment soluble sugars released into the environment by the enzymatic activities of other microorganisms that depolymerize plant polysaccharides. Pohlschroeder et al. (1994) reported that *Spirochaeta caldaria*, a thermophilic spirochete from a freshwater hot spring, forms stable, cellulose-degrading cocultures with the cellulolytic bacterium, *Clostridium thermocellum*. Cellulose is degraded more rapidly in cocultures than in monocultures of *Clostridium thermocellum*, suggesting that species of *Spirochaeta* may enhance cellulose breakdown in natural environments (Leschine, 1995).

Both facultatively and obligately anaerobic species of *Spirochaeta* grow abundantly when the energy source available to them is cellobiose, a major product of cellulose depolymerization, which is produced by the extracellular cellulase systems of some microorganisms (Breznak and Canale-Parola, 1969; Breznak and Canale-Parola, 1975; Greenberg and Canale-Parola, 1976; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970a; Hespell and Canale-Parola, 1970b; Leschine, 1995; Pohlschroeder et al., 1994). Furthermore, free-living spirochetes, such as *Spirochaeta aurantia*, exhibit a strong tactic response toward very low cellobiose concentrations (Greenberg and Canale-Parola, 1977a) and possess regulatory systems that enable them to enhance their chemotactic responses when attractants that serve as energy sources are present at very low concentrations in the environment (Terracciano and Canale-Parola, 1984). Inasmuch as cellobiose levels in environments in which cellulose is degraded are likely to be quite low (Smith et al., 1973), a strong tactic response to very low concentrations of cellobiose may confer an important selective advantage on the spirochetes among cellobiose-utilizing microorganisms.

In addition to their ability to regulate their chemosensory apparatus in response to low substrate concentrations, some species of *Spirochaeta* have developed other strategies to survive in environments lacking or nearly depleted of energy sources. One of these survival strategies

has been studied in *Spirochaeta isovalerica*, a saccharolytic anaerobe that does not utilize amino acids as fermentable substrates for growth, but catabolizes small amounts of L-leucine, L-isoleucine and L-valine with the formation of isovalerate, 2-methylbutyrate and isobutyrate, respectively, as end products (Harwood and Canale-Parola, 1981b; Harwood and Canale-Parola, 1983; Harwood and Canale-Parola, 1984). Although these amino acids are not used by *Spirochaeta isovalerica* as fermentable substrates for growth, their fermentation serves to generate ATP, which is utilized by the spirochetes as a source of maintenance energy. This process allows cells to survive during periods of growth substrate starvation (Harwood and Canale-Parola, 1981a; Harwood and Canale-Parola, 1981b; Harwood and Canale-Parola, 1982a). Other starvation-survival strategies utilized by *Spirochaeta* species may involve ATP generation through metabolism of endogenous RNA (Canale-Parola and Kidder, 1982; Harwood and Canale-Parola, 1984) or metabolism of intracellular polyglucose storage granules (Kropinski et al., 1988).

The large spirochete, *Spirochaeta plicatilis*, has been observed within masses of *Beggiatoa* trichomes in samples of black, sulfide-rich marsh mud covered with a layer of marsh water (Blakemore and Canale-Parola, 1973). In this study, the spirochetes were seen to swim freely among the tangled *Beggiatoa* trichomes, and they crept on or otherwise moved in contact with the surface of the trichomes. The close association with *Beggiatoa* suggested a chemotactic response by the large spirochete toward metabolites produced by the sulfur bacteria. As the level of sulfide generated by biological activities in the mud became low, gradual lysis of the *Beggiatoa* trichomes was observed. This lytic process coincided with a dramatic increase in the number of large spirochetes (Blakemore and Canale-Parola, 1973). Possibly, substances released by the lysing *Beggiatoa* were used as growth substrates for *Spirochaeta plicatilis*.

Applications

Thus far, few applications have been suggested for species of *Spirochaeta*. Pohlschroeder et al. (1994) reported that cellulose degradation by *Clostridium thermocellum* is enhanced in the presence of *Spirochaeta caldaria*, a thermophilic spirochete from a freshwater hot spring. Possibly, species of *Spirochaeta* might be employed in processes for the direct bioconversion of cellulose-containing wastes to fuels such as H_2 or ethanol.

Literature Cited

Aksenova, E. Y., V. A. Svetlichnyi, and G. A. Zavarzin. 1990. Spirochaeta thermophila sp. nov.: A thermophilic marine spirochete isolated from a littoral hydrotherm of Shiashkotan island. Mikrobiologiya 59:1050–1057.

Aksenova, H. Y., F. A. Rainey, P. H. Janssen, G. A. Zavarzin, and H. W. Morgan. 1992. Spirochaeta thermophila sp. nov., an obligate anaerobic, polysaccharolytic, extremely thermophilic bacterium. Int. J. Syst. Bacteriol. 42:175–177.

Allan, B., E. P. Greenberg, and A. Kropinski. 1986. DNA-dependent RNA polymerase from Spirochaeta aurantia. FEMS Microbiol. Lett. 35:205–210.

Berg, H. C. 1976. How spirochetes may swim. J. Theor. Biol. 56:269–273.

Blakemore, R. P., and E. Canale-Parola. 1973. Morphological and ecological characteristics of Spirochaeta plicatilis. Arch. Mikrobiol. 89:273–289.

Breznak, J. A., and E. Canale-Parola. 1969. Spirochaeta aurantia, a pigmented, facultatively anaerobic spirochete. J. Bacteriol. 97:386–395.

Breznak, J. A., and E. Canale-Parola. 1972a. Metabolism of Spirochaeta aurantia. I: Anaerobic energy-yielding pathways. Arch. Mikrobiol. 83:261–277.

Breznak, J. A., and E. Canale-Parola. 1972b. Metabolism of Spirochaeta aurantia. II: Aerobic oxidation of carbohydrates. Arch. Mikrobiol. 83:278–292.

Breznak, J. A., and E. Canale-Parola. 1975. Morphology and physiology of Spirochaeta aurantia strains isolated from aquatic habitats. Arch. Mikrobiol. 105:1–12.

Canale-Parola, E., and R. S. Wolfe. 1960. Studies on Sarcina ventriculi. I: Stock culture method. J. Bacteriol. 79:857–859.

Canale-Parola, E., S. L. Rosenthal, and D. G. Kupfer. 1966. Morphological and physiological characteristics of Spirillum gracile sp. n. Ant. v. Leeuwenhoek J. Microbiol. Serol. 32:113–124.

Canale-Parola, E., S. C. Holt, and Z. Udris. 1967. Isolation of free-living, anaerobic spirochetes. Arch. Mikrobiol. 59:41–48.

Canale-Parola, E., Z. Udris, and M. Mandel. 1968. The classification of free-living spirochetes. Arch. Mikrobiol. 63:385–397.

Canale-Parola, E. 1973. Isolation, growth and maintenance of anaerobic free-living spirochetes. *In:* J. R. Norris and D. W. Ribbons (Eds.). Methods in Microbiology. Academic Press. New York, NY. 8:61–73.

Canale-Parola, E. 1977. Physiology and evolution of spirochetes. Bacteriol. Rev. 41:181–204.

Canale-Parola, E. 1978. Motility and chemotaxis of spirochetes. Ann. Rev. Microbiol. 32:69–99.

Canale-Parola, E., and G. W. Kidder. 1982. Enzymatic activities for interconversion of purines in spirochetes. J. Bacteriol. 152:1105–1110.

Canale-Parola, E. 1984a. Genus I: Spirochaeta. *In:* N. R. Krieg (Ed.). Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 1:39–46.

Canale-Parola, E. 1984b. Order I: Spirochaetales. *In:* N. R. Krieg (Ed.). Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 1:38–39.

Dubilier, N., R. Amann, C. Erseus, G. Muyzer, S. Y. Park, O. Giere, and C. M. Cavanaugh. 1999. Phylogenetic diversity of bacterial endosymbionts in the gutless marine

oligochete Olavius loisae (Annelida). Mar. Ecol. Prog. Ser. 178:271–280.

Fosnaugh, K., and E. P. Greenberg. 1988. Motility and chemotaxis of Spirochaeta aurantia: Computer-assisted motion analysis. J. Bacteriol. 170:1768–1774.

Fracek Jr., S. P., and J. F. Stolz. 1985. Spirochaeta bajacaliforniensis sp. n. from a microbial mat community at Laguna Figueroa, Baja California Norte, Mexico. Arch. Microbiol. 142:317–325.

Greenberg, E. P., and E. Canale-Parola. 1975. Carotenoid pigments of facultatively anaerobic spirochetes. J. Bacteriol. 123:1006–1012.

Greenberg, E. P., and E. Canale-Parola. 1976. Spirochaeta halophila sp. n., a facultative anaerobe from a high-salinity pond. Arch. Microbiol. 110:185–194.

Greenberg, E. P., and E. Canale-Parola. 1977a. Chemotaxis in Spirochaeta aurantia. J. Bacteriol. 130:485–494.

Greenberg, E. P., and E. Canale-Parola. 1977b. Motility of flagellated bacteria in viscous environments. J. Bacteriol. 132:356–358.

Greenberg, E. P., and E. Canale-Parola. 1977c. Relationship between cell coiling and motility of spirochetes in viscous environments. J. Bacteriol. 131:960–969.

Greenberg, E. P., B. Brahamsha, and K. Fosnaugh. 1985. The motile behavior of Spirochaeta aurantia: A twist to chemosensory transduction in bacteria. *In:* M. Eisenbach and M. Balaban (Eds.). Sensing and Response in Microorganisms. Elsevier Science Publications. New York, NY. 107–118.

Harwood, C. S., and E. Canale-Parola. 1981a. Adenosine 5′-triphosphate-yielding pathways of branched-chain amino acid fermentation by a marine spirochete. J. Bacteriol. 148:117–123.

Harwood, C. S., and E. Canale-Parola. 1981b. Branched-chain amino acid fermentation by a marine spirochete: Strategy for starvation survival. J. Bacteriol. 148:109–116.

Harwood, C. S., and E. Canale-Parola. 1982. Properties of acetate kinase isozymes and a branched-chain fatty acid kinase from a spirochete. J. Bacteriol. 152:246–254.

Harwood, C. S., H. W. Jannasch, and E. Canale-Parola. 1982b. Anaerobic spirochete from a deep-sea hydrothermal vent. Appl. Environ. Microbiol. 44:234–237.

Harwood, C. S., and E. Canale-Parola. 1983. Spirochaeta isovalerica sp. nov., a marine anaerobe that forms branched-chain fatty acids as fermentation products. Int. J. Syst. Bacteriol. 33:573–579.

Harwood, C. S., and E. Canale-Parola. 1984. Ecology of spirochetes. Ann. Rev. Microbiol. 38:161–192.

Hespell, R. B., and E. Canale-Parola. 1970a. Carbohydrate metabolism in Spirochaeta stenostrepta. J. Bacteriol. 103:216–226.

Hespell, R. B., and E. Canale-Parola. 1970b. Spirochaeta litoralis sp. n., a strictly anaerobic marine spirochete. Arch. Mikrobiol. 74:1–18.

Hespell, R. B., and E. Canale-Parola. 1973. Glucose and pyruvate metabolism of Spirochaeta litoralis, an anaerobic marine spirochete. J. Bacteriol. 116:931–937.

Hespell, R. B. 1977. Serpens flexibilis gen. nov., sp. nov., an unusually flexible, lactate-oxidizing bacterium. Int. J. Syst. Bacteriol. 27:371–381.

Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes. *In:* J. R. Norris and D. W. Ribbons (Eds.). Methods in Microbiology. Academic Press. New York, NY. 3B:117–132.

Johnson, P. W., and E. Canale-Parola. 1973. Properties of rubredoxin and ferredoxin isolated from spirochetes. Arch. Mikrobiol. 89:341–353.

Jukes, T.H., and C.R. Cantor. 1969. Evolution of protein molecules. *In:* H.N. Munro (Ed.). Mammalian Protein Metabolism. Academic Press. New York, NY. 3:21–132.

Kropinski, A. M., W. C. Ghiorse, and E. P. Greenberg. 1988. The intracellular polyglucose storage granules of Spirochaeta aurantia. Arch. Microbiol. 150:289–295.

Leschine, S. B., and E. Canale-Parola. 1986. Rifampin-resistant RNA polymerase in spirochetes. FEMS Microbiol. Lett. 35:199–204.

Leschine, S. B. 1995. Cellulose degradation in anaerobic environments. Ann. Rev. Microbiol. 49:399–426.

Magot, M., M.-L. Fardeau, O. Arnauld, C. Lanau, B. Ollivier, P. Thomas, and B. K. C. Patel. 1997. Spirochaeta smaragdinae sp. nov., a new mesophilic strictly anaerobic spirochete from an oil field. FEMS Microbiol. Lett. 155:185–191.

Paster, B. J., and E. Canale-Parola. 1980. Involvement of periplasmic fibrils in motility of spirochetes. J. Bacteriol. 141:359–364.

Paster, B. J., and E. Canale-Parola. 1982. Physiological diversity of rumen spirochetes. Appl. Environ. Microbiol. 43:686–693.

Paster, B. J., F. E. Dewhirst, W. G. Weisburg, L. A. Tordoff, G. J. Fraser, R. B. Hespell, T. B. Stanton, L. Zablen, L. Mandelco, and C. R. Woese. 1991. Phylogenetic analysis of the spirochetes. J. Bacteriol. 173:6101–6109.

Patel, B. K. C., H. W. Morgan, and R. M. Daniel. 1985. Thermophilic anaerobic spirochetes in New Zealand hot springs. FEMS Microbiol. Lett. 26:101–106.

Pfennig, N. 1965. Anreicherungskulturen fa rote und gra Schwefelbakterien. Zentr. Bakteriol. Parasitenk. Infekt. Hyg., Abt. 1 Orig., Reihe A, Suppl. 1:179–189.

Pohlschroeder, M., S. Leschine, and E. Canale-Parola. 1994. Spirochaeta caldaria sp. nov., a thermophilic bacterium that enhances cellulose degradation by Clostridium thermocellum. Arch. Microbiol. 161:17–24.

Rainey, F. A., P. H. Janssen, D. J. C. Wild, and H. W. Morgan. 1991. Isolation and characterization of an obligately anaerobic, polysaccharolytic, extremely thermophilic member of the genus Spirochaeta. Arch. Microbiol. 155:396–401.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molec. Biol. Evol. 4:406–425.

Smith, W. R., I. Yu, and R. E. Hungate. 1973. Factors affecting cellulolysis by Ruminococcus albus. J. Bacteriol. 114:727–737.

Stanton, T. B., and E. Canale-Parola. 1979. Enumeration and selective isolation of rumen spirochetes. Appl. Environ. Microbiol. 38:965–973.

Tanner, M. A., C. L. Everett, W. J. Coleman, M. M. Yang, and D. C. Youvan. 2000. Complex microbial communities inhabiting sulfide-rich black mud from marine coastal environments. Biotechnology et alia (http://www.et-al.com) 8:1–16.

Terracciano, J. S., and E. Canale-Parola. 1984. Enhancement of chemotaxis in Spirochaeta aurantia grown under conditions of nutrient limitation. J. Bacteriol. 159:173–178.

Van de Peer, Y., and R. De Wachter. 1994. TREECON for Windows: A software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Applic. Biosci. 10:569–570.

Veldkamp, H. 1960. Isolation and characteristics of Treponema zuelzerae nov. sp., an anaerobic, free-living spirochete. Ant. v. Leeuwenhoek J. Microbiol. Serol. 26:103–125.

Weber, F. H., and E. P. Greenberg. 1981. Rifampin as a selective agent for the enumeration and isolation of spirochetes from salt marsh habitats. Curr. Microbiol. 5:303–306.

Weller, R., M. M. Bateson, B. K. Heimbuch, E. D. Kopczynski, and D. M. Ward. 1992. Uncultivated cyanobacteria, Chloroflexus-like inhabitants, and spirochete-like inhabitants of a hot spring microbial mat. Appl. Environ. Microbiol. 58:3964–3969.

Zhilina, T. N., G. A. Zavarzin, F. Rainey, V. V. Kevbrin, N. A. Kostrikina, and A. M. Lysenko. 1996. Spirochaeta alkalica sp. nov., Spirochaeta africana sp. nov., and Spirochaeta asiatica sp. nov., alkaliphilic anaerobes from the continental soda lakes in central Asia and the East African Rift. Int. J. Syst. Bacteriol. 46:305–312.

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CHAPTER 4.2

The Genus *Treponema*

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Introduction and Habitats

The genus *Treponema* (phylum Spirochaetes, order Spirochetales, family Spirochaetaceae) is composed of both pathogenic and nonpathogenic species indigenous to humans and animals. They are helical, tightly coiled, motile bacteria, ranging from 5–20 μm in length and 0.1–0.4 μm in diameter, and are best observed by dark-field microscopy (Fig. 1). The organisms stain poorly with the usual aniline dyes; however, those that are capable of aniline-dye uptake are Gram-negative. Staining can best be accomplished by the use of silver impregnation or immunofluorescent methods. The sequence of the genome of *Treponema pallidum* subsp. *pallidum*, the type species of the genus, was determined in 1998 (Fraser et al., 1998), and the sequence of the *Treponema denticola* genome is nearly completed. Information based on these genome sequences has been incorporated into sections of this chapter.

Several topics covered in this chapter have been reviewed previously, including procedures for propagation of pathogenic treponemes in laboratory animals (Turner and Hollander, 1957; Miller, 1971), clinical aspects and diagnosis of treponemal infections (United States Public Health Service, 1968; Larsen, et al., 1998, 1999), the endemic treponematoses (Antal et al., 2002), the phylogeny of spirochetes (Paster and Dewhirst, 2000), *T. pallidum* pathogenesis and immunology (Norris, 1988a; Radolf, 1994; Blanco et al., 1997; Radolf et al., 1999), and the biology and in vitro culture of *T. pallidum* (Cox, 1994; Norris et al., 2001). In addition, a book focused on current topics in spirochete biology was published in 2001 (Saier and Garcia-Lara, 2001). Portions of this chapter were modified from the previous version by Miller, Smibert, and Norris (Miller et al., 1990). The reader is referred to these previous reviews where they are applicable, and individual articles are cited where they are particularly pertinent.

The known frank pathogens of the genus *Treponema* are virtually identical organisms that are obligate parasites of humans or (in the case of one species) rabbits (Table 1). These organisms are morphologically indistinguishable and share extensive DNA homology and proteome similarity; in addition, they have not been cultured continuously in vitro. The four human pathogens and the diseases they cause are: *Treponema pallidum* subsp. *pallidum* (syphilis), *Treponema pallidum* subsp. *pertenue* (yaws), *Treponema pallidum* subsp. *endemicum* (nonvenereal endemic syphilis), and *Treponema carateum* (pinta). The reclassification of *T. pertenue* and *T. endemicum* as subspecies of *T. pallidum* (Smibert, 1984a) is supported by >95% DNA homology by hybridization (Fieldsteel, 1983) and the virtual identity of known sequences from this group of organisms. *Treponema carateum* cannot be propagated in small laboratory animals or in culture and is still classified separately owing to the lack of genetic information. *Treponema paraluiscuniculi* (venereal rabbit spirochetosis) is the only treponeme that has been established unequivocally as an animal pathogen (Bayon, 1913) and is also closely related to the *T. pallidum* subspecies (Baker-Zander and Lukehart, 1984; Centurion-Lara et al., 1997). Limited multiplication of *Treponema pallidum* subsp. *pallidum* has been accomplished in a tissue culture monolayer system (Cox, 1994).

The largest group of *Treponema* species identified thus far consists of oral spirochetes (Table 2). These include nine classified species and a large number of unnamed genospecies that have been identified by polymerase chain reaction (PCR) amplification of 16S rRNA sequences of gingival crevice specimens from patients with periodontal disease (Dewhirst et al., 2000). Periodontal disease is an important dental problem, resulting in inflammation of the gingival tissue, bone resorption, and tooth loss. A high proportion of oral treponemes are found in the flora of specimens from patients with acute necrotizing ulcerative gingivitis and other forms of periodontal disease, providing a strong association between these organisms and the disease process. In addition, oral *Treponema* species have

Table 1. Characteristics of pathogenic *Treponema* species.

Organism	Disease	Distribution	Predominant age of onset	Transmission	Congenital infection
T. pallidum subsp. *pallidum*	Venereal syphilis	Worldwide	Adolescents and adults	Sexual contact	Yes
T. pallidum subsp. *pertenue*	Yaws (frambesia, pian)	Tropical areas, Africa, South America, Caribbean, Indonesia	Children	Skin contact	No
T. pallidum subsp. *endemicum*	Endemic syphilis (bejel, dichuchwa)	Arid areas, Africa, Middle East	Children, adolescents and adults	Mucous membrane	Rarely
T. carateum	Pinta (carate, cute)	Semi-arid, warm areas, Central and South America	Children and adolescents	Skin contact	No
T. paraluis-cuniculi	Venereal spirochetosis of rabbits	Not applicable	Not applicable	Sexual contact	No

From Antal et al. (2002) and Larsen et al. (1999).

Fig. 1. Appearance of *Treponema pallidum* subsp. *pallidum* Nichols by darkfield microscopy.

been implicated in endodontic (tooth root) infection (Newman, 1984; Dahle et al., 1993a; Dahle et al., 1993b; Jung et al., 2000; Rupf et al., 2000; Siqueira et al., 2000a; Siqueira et al., 2000b; Sunde et al., 2000; Rocas et al., 2001) and atherosclerosis (Okuda et al., 2001), although additional studies are needed to determine whether the spirochetes play a causal role in these conditions. A recent report provides evidence against a previously proposed association between periodontal disease and diabetes (Yuan et al., 2001).

Skin-associated commensal spirochetes include *Treponema phagedenis*, *Treponema refringens*, and *Treponema minutum*. These organisms colonize smegma (desquamated epithelial cells and secretions) under the prepuce and other skin folds of the genitalia of humans, chimpanzees, and other animals. These skin-associated *Treponema*, some of which were initially thought to be avirulent forms of *T. pallidum* subsp. *pallidum*, are morphologically and genetically distinct from *T. pallidum* (Miao and Fieldsteel, 1978; Fieldsteel, 1983; Smibert, 1984a) and are not known to cause disease.

Recently, the anaerobic spirochetes that colonize termite guts were shown on the basis of 16S rRNA homology to be related to the genus *Treponema* (Paster et al., 1996; Lilburn et al., 1999). These organisms, which have not been classified as yet, are the subject of the chapter Termite Gut Spirochetes in this Volume.

Some organisms of the genus *Brachyspira*, including *B. hyodysenteriae* and *B. innocens*, were initially characterized as *Treponema* species; however, these intestinal spirochetes were reclassified, first as *Serpulina* then as *Brachyspira* (Ochiai et al., 1997), on the basis of dissimilar DNA homology and %G+C with *Treponema* species, as well as relatedness to *Brachyspira aalborgii* (Hovind-Hougen et al., 1982). The genus Brachyspira in this Volume is described in another chapter.

Well-defined, cultivable species of treponemes have been found in the oral cavity, intestinal tract, and/or genitalia of chimpanzees, pigs and dogs, as well as in the rumen of cattle (Pindak et al., 1965; Socransky et al., 1969; Zymet, 1969; Hanson, 1970; Leach et al., 1973; Holdemann et al., 1977; Cwyk and Canale-Parola, 1979; Stanton and Canale-Parola, 1979; Wojciechowicz and Ziolecki, 1979; Ziolecki, 1979; Stanton and Canale-Parola, 1980; Ziolecki and Wojciechowicz, 1980; Paster and Canale-Parola, 1982; Paster and Canale-Parola, 1985). In addition, spirochetes resembling treponemes have been observed in the intestinal tract of horses, mice, rats, guinea pigs, opossums, insects and termites and in the tissues of brine shrimp (Osborne and Bain, 1961; Breznak, 1973; McLeod et al., 1977;

Table 2. Other *Treponema* species associated with mammalian hosts.

Type	Habitat	Species and groups	References
Oral spirochetes	Dental plaque in gingival crevices	*Treponema denticola*	Miller et al., 1990 Moore et al., 1987 Riviere et al., 1995 Smibert, 1984
		Treponema socranskii	Moore et al., 1987 Riviere et al., 1995 Smibert et al., 1984
		Treponema vincentii	Miller et al., 1990
		Treponema skoliodontum	Miller et al., 1990 Smibert, 1984
		Treponema maltophilum	Wyss et al., 1996
		Treponema medium	Umemoto et al., 1997
		Treponema amylovorum	Wyss et al., 1997
		Treponema lecithinolyticum	Wyss et al., 1999
		Treponema parvum	Wyss et al., 2001
Skin-associated *Treponema*	Sebaceous secretions in genital region	*Treponema phagedenis*	Miller et al., 1990 Smibert, 1984
		Treponema refringens	
		Treponema minutum	
Digital dermatitis *Treponema*	Hooves of affected ungulates	*Treponema brennaborense*	Schrank et al., 1999
		Unspeciated *Treponema*	Choi et al., 1997 Demirkan et al., 2001
Intestinal *Treponema*	Colon of swine	*Treponema succinifaciens*	Cwyk and Canale-Parola, 1979
	Rumen fluid of cattle	*Treponema bryantti*	Stanton and Canale-Parola, 1980
		Treponema saccharophilium	Paster and Canale-Parola, 1985

Lee and Philips, 1978). Although many of these anaerobic and putative microaerophilic host-associated treponemes have been cultured in vitro and subsequently characterized, microscopic and PCR analyses of animal and human tissues, exudates, and normal flora suggest that other species have yet to be isolated (Dewhirst et al., 2000). Whether these spirochetes, alone or in combination with other organisms, can be implicated as the cause of oral, genital, or gastrointestinal disease remains uncertain.

In humans, as in animals, treponemes are found as part of the normal oral, intestinal, and genital flora (Parr, 1923; Rosebury and Foley, 1941; Shera, 1962; Hanson and Cannefax, 1964; Harland and Lee, 1967; Lee et al., 1971; Takeuchi et al., 1974; Ruane et al., 1989). Most of the organisms associated with human intestinal spirochetosis are *Brachyspira* species (Duhamel, 2001), although *Treponema* species are part of the normal flora of the human gastrointestinal tract (Table 2). *Treponema* is the predominant genus of spirochetes in the oral cavity. Although these organisms are part of the normal flora in the gingival crevices, *Treponema* are more prevalent in persons with periodontal disease and gingivitis than in people with a healthy gingiva (Moore and Moore, 1994; Moter et al., 1998b; Dewhirst et al., 2000). It is estimated that approximately 80 different phylotypes of *Treponema* are present in the oral cavity, only 10 of which have been speciated (Dewhirst et al., 2000).

Phylogeny/Taxonomy

On the basis of comparative analysis of 16S rRNA gene sequences, members of the genus *Treponema* form one of the nine phylogenetic clusters (genera) of spirochetes (Fig. 2). According to the most recent taxonomic outline of the prokaryotic genera in *Bergeys Manual of Systematic Bacteriology*, 2nd edition (Garrity, 2001), all spirochetes are classified in the phylum Spirochaetes in the order Spirochetales and are divided into three families. The first family, Spirochaetaceae, contains species of the genera *Borrelia*, *Brevinema*, *Cristispira*, *Spirochaeta*, "*Spironema*," and *Treponema*. Other spirochaetal genera, such as *Pillotina*, *Clevelandina*, *Hollandina* and *Diplocalyx*, are included in this family, but 16S rRNA sequences of these not-yet-cultivated species from the termite hindgut are not available to determine their true phylogenetic position. The second proposed family, "Serpulinaceae," or "Brachyspiraceae," contains the species of the genus *Brachyspira* (*Serpulina*). The third family, Leptospiraceae, contains free-living or pathogenic species of the genera *Leptonema* and *Leptospira*.

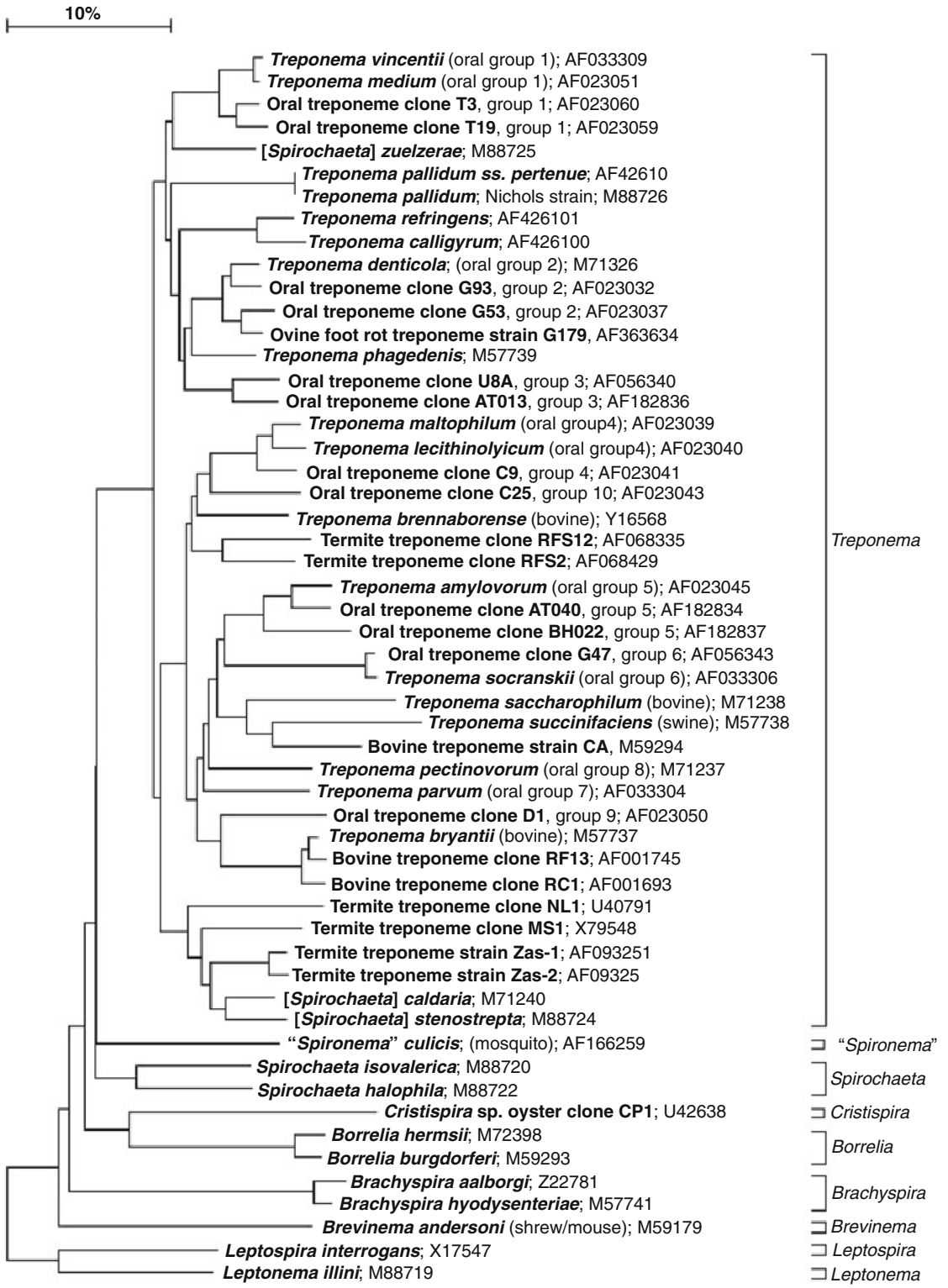

Fig. 2. Phylogeny based on 16S rRNA gene sequence comparisons of members of the genus *Treponema*. The phylogenetic position of known species and phylotypes of *Treponema* is shown relative to other genera of spirochetes. GenBank accession numbers for the 16S rRNA sequences of the species tested are shown. Previously unpublished 16S rRNA gene sequences of *T. refringens*, *T. calligyrum* and *T. pallidum* subsp. *pertenue* are reported here for the first time. Similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor (1969). The neighbor-joining method (Saitou and Nei, 1987) was used for phylogenetic tree construction. TREECON, a software package for the Microsoft Windows environment, was used for the construction and drawing of evolutionary trees (Van de Peer and De Wachter, 1994). The scale bar represents a 10% difference in nucleotide sequence determined by taking the sum of all of the horizontal lines connecting two species. Vertical distance has no meaning.

Members of the genus *Treponema* and three species of the free-living *Spirochaeta*, namely *S. stenostrepta*, *S. zuelzerae* and *S. caldaria*, form a coherent phylogenetic cluster, referred to as the treponemes (Fig. 2). In addition to known species of *Treponema*, novel phylotypes, i.e., species that have not yet been cultivated in vitro, have been identified by analysis of 16S rRNA genes of DNA isolated from host-associated sources, such as the human oral cavity (Choi et al., 1994; Dewhirst et al., 2000; Paster et al., 2001), the hindgut of the termite (Lilburn et al., 1999), and the bovine rumen (Tajima et al., 1999). Representatives of each of these phylotypes are included in Fig. 2. In the human oral cavity, the diversity of treponemes is extensive. There are approximately 60 oral species of *Treponema*, of which 50 are presently represented only as phylotypes (see expanded phylogenetic trees in Dewhirst et al. [2000] and Paster et al. [2001]). These oral treponemes have been divided into 10 phylogenetic groups (Dewhirst et al., 2000; Paster et al., 2001). An equally impressive diversity of treponemes has been observed in the termite hindgut (Lilburn et al., 1999; Ohkuma et al., 1999) and is described in detail in a separate chapter.

Three species of the free-living *Spirochaeta*, namely *S. stenostrepta*, *S. zuelzerae* and *S. caldaria*, fall within the *Treponema* cluster rather than within the cluster of other free-living *Spirochaeta* (Fig. 2). The phylogenetic clustering of these species has been confirmed by single-base signature analysis, i.e., the sequences of these species possess more characteristic bases found in the sequences of the treponemes than in the sequences of *Spirochaeta* species (Paster et al., 1991). It has been speculated that these free-living spirochetes might represent transitional species, i.e., descendants of the precursors to host-associated treponemes (Paster et al., 1991). However, it is possible that these species may have originated from the host and were disseminated via fecal contamination.

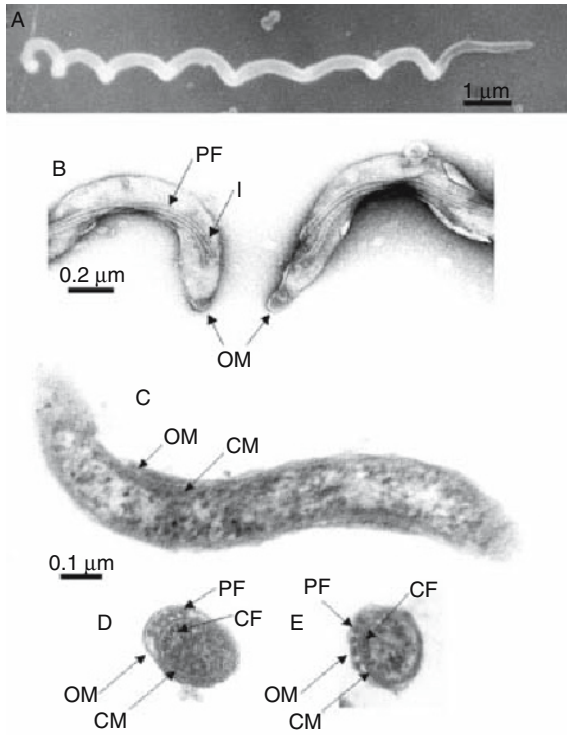

Fig. 3. Ultrastructure of *Treponema pallidum* subsp. *pallidum* Nichols. A) Scanning electron micrograph of an intact spirochete. B) Negatively stained, intact organisms examined by transmission electron microscopy, showing the periplasmic flagella (PF) and the flagellar insertion points near the end of the cell (I). The outer membrane can be seen extruding from the ends of the cells. C-E) Transmission electron microscopy of ultrathin longitudinal and cross sections. The locations of the PF, cytoplasmic membrane (CM), outer membrane (OM), and cytoplasmic filaments (CF) are indicated by arrows. The PF are located in the periplasmic space and extend from the ends of the cell toward the middle. The CF are ribbon-like structures (composed of an 80-kDa protein) found only in *Treponema* or related spirochetes (e.g., *Spirochaeta zuelzerae*) and run parallel to the PF in the cytoplasm of the cell; they are seen here in cross-section.

Morphology and Structure-Function Relationships

Selected General Characteristics

Treponemes, like all spirochetes, are composed of an outer membrane (OM) that delimits the outer cellular surface of the organisms, a cytoplasmic membrane (CM) that encloses the protoplasmic cylinder, and flagella that lie in between the two membranes (Johnson et al., 1973; Hovind-Hougen, 1976; Canale-Parola, 1977; Holt, 1978; Hovind-Hougen, 1983; Walker et al., 1989; Fig. 3). Unlike the flagella of members of other bacterial genera, spirochetal flagellar organelles lie entirely within the periplasmic space between the inner membrane (IM) and CM and are commonly referred to as periplasmic flagella (PF); other names for PF are endoflagella and axial filaments. The PF attach at either pole of the organism through basal body structures and wrap around the helical protoplasmic cylinder (Hovind-Hougen, 1976; Canale-Parola, 1977; Holt, 1978). The number of PF and the wavelength of the helix differ among members of this diverse group of organisms. *Treponema pallidum* and several other treponemes have been shown to possess cytoplasmic filaments, thin filaments which run the length of the organism just under the cytoplasmic membrane

(Hovind-Hougen and Birch-Andersen, 1971; Eipert and Black, 1979; Hovind-Hougen, 1983; You et al., 1996; Izard et al., 1999).

Outer Membrane Structure

The OM of *Treponema pallidum* subsp. *pallidum* and *pertenue* differs significantly from that of other organisms in its strikingly low number of intramembranous membrane particles when examined by freeze fracture microscopy (Radolf et al., 1989; Walker et al., 1989; Walker et al., 1991). In contrast, the free-living *Spirochaeta aurantia* has a number of OM intramembranous particles similar to that of *Escherichia coli*, and *T. phagedenis* Reiter and *T. denticola* have intermediate quantities (Radolf et al., 1989; Walker et al., 1989). This observation indicates a relatively low abundance of integral membrane proteins in the OM of *T. pallidum*.

Concerted efforts to identify the OM proteins of *T. pallidum* have been inconclusive. Treponemal rare outer-membrane proteins (TROMP) 1 and 2 were identified on the basis of their presence in preparations enriched for OM by Blanco et al. (1994). TROMP1 has been characterized as an OM porin that forms a trimeric structure (Blanco et al., 1995; Blanco et al., 1996; Blanco et al., 1997; Blanco et al., 1999; Zhang et al., 1999). However, sequence analysis and three dimensional structure determinations indicate that this protein (also called TroA) is the periplasmic protein of an ATP-binding cassette (ABC) transporter involved in Zn^{++} transport (Hardham et al., 1997; Lee et al., 1999; Lee et al., 2002). TROMP2 has sequence similarity to the *T. pallidum* flagellar sheath protein FlaA, but the recombinant form is associated with the OM of *E. coli* following heterologous expression (Champion et al., 1997). Other proteins, including glycerophosphodiester phosphodiesterase (GlpQ) and the *T. pallidum* repeat (Tpr) protein, TprK, have been identified as OM protein candidates, but have yielded conflicting data (Shevchenko et al., 1997; Shevchenko et al., 1999; Cameron et al., 1998; Centurion-Lara et al., 1999; Hazlett et al., 2001). It has been hypothesized that the relatively low abundance of OM proteins in *T. pallidum* may provide an antigenically inert surface and thus promote evasion of the immune response (Penn et al., 1985b; Stamm et al., 1987; Radolf, 1994; Blanco et al., 1997).

Another difference between the OMs of Gram-negative bacteria and *T. pallidum* is the apparent lack of lipopolysaccharide (LPS) in *T. pallidum* subsp. *pallidum* (Penn et al., 1985a; Radolf and Norgard, 1988a). Both *T. phagedenis* and *T. denticola* have been shown to contain material extractable with phenol-chloroform that has properties similar to those of LPS (Cockayne et al., 1989; Yotis et al., 1995; Dahle et al., 1996; Schultz et al., 1998). A recent analysis of phenol/water extracts from *T. maltophilum* and *T. brennaborense* cells revealed novel glycolipids consisting of a diacylglycerol-lipid anchor, a core region, and carbohydrate repeating units that interact with LPS-binding protein (LBP), CD14, and toll-like receptors TLR2 or TLR4, respectively (Schröder et al., 2000).

Subsurface Structures

Treponemal subsurface structures are defined as those molecules that lie beneath the OM. The most prominent structures are the PF, which mediate the striking translational and axial motility common to all spirochetes (Charon et al., 1992; Li et al., 2000). Members of the genus *Treponema* contain two to nine PF inserted into each end of the cell (Smibert, 1984a). An unusual feature of treponemal PF is that they are composed of multiple polypeptide subunits, as reviewed previously (Norris and Treponema pallidum Polypeptide Research Group, 1993; Li et al., 2000). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis has shown that *Treponema pallidum* subsp. *pallidum* PF is composed of at least four polypeptides with molecular weights of 37, 34.5, 33, and 31 kDa, while the PF of *Treponema phagedenis* biotypes Reiter and Kazan 5 are made up of four polypeptides with similar molecular weights (Sand-Peterson et al., 1981; Limberger and Charon, 1986; Radolf et al., 1986; Blanco et al., 1988; Norris et al., 1988b). FlaB1 (34.5 kDa), FlaB2 (33 kDa), and FlaB3 (31 kDa) are closely related to one another and to the flagellins of other bacteria (Pallesen and Hindersson, 1989; Champion et al., 1990) and comprise the central core of the flagella (Cockayne et al., 1987). Interestingly, the predicted products of the three encoding genes are all ~31,000 Da; the differences in electrophoretic mobility may be due to glycosylation (Norris and Treponema pallidum Polypeptide Research Group, 1993; Wyss, 1998). FlaA forms a sheath on the outer surface of the flagellar shaft and is expressed as a precursor protein with a signal sequence (Cockayne et al., 1987; Isaacs et al., 1989; Isaacs and Radolf, 1990). The flagella of the other members of the genus *Treponema* examined thus far have a similar protein content and structure (Limberger and Charon, 1986; Norris et al., 1988b; Ruby et al., 1997).

Additional motility-related operons encoding basal body and accessory proteins have been characterized in *T. pallidum* (Limberger et al., 1996; Fraser et al., 1998), *T. denticola* (Heinzerling et al., 1997; Stamm and Bergen, 1999; Stamm

and Bergen, 2001), and *T. phagedenis* (Limberger et al., 1996). These loci are highly conserved relative to the motility operons of Gram-positive and Gram-negative bacteria.

In addition to the proteins that make up the PF subunit structures, several antigenic polypeptides from *Treponema pallidum* subsp. *pallidum* and *pertenue*, ranging in molecular weights from 12 to 190 kDa, have been identified by immunoprecipitation, crossed immunoelectrophoresis, and western blot analysis (reviewed by Norris et al. [1987] and Norris and the Treponema pallidum Polypeptide Research Group [1993]). Characterization of *Treponema pallidum* subsp. *pallidum* by two-dimensional electrophoresis has revealed at least 78 antigenic polypeptides (Norris and Sell, 1984; Norris et al., 1987; Norris and Treponema pallidum Polypeptide Research Group, 1993). Phase partitioning with the nonionic detergent Triton X-114 has resulted in identification of several *Treponema pallidum* subsp. *pallidum* antigenic polypeptides in the hydrophobic phase with molecular weights of 14–57 kDa (Cunningham et al., 1988; Radolf et al., 1988b; Norris and Treponema pallidum Polypeptide Research Group, 1993). The majority of these antigens are, in fact, lipoproteins, which anchor to the CM or OM by means of their covalently attached fatty acids (Chamberlain et al., 1989a; Chamberlain et al., 1989b; Swancutt et al., 1990). Three of these lipoproteins, the pathogen-specific 47-, 34-, and 15-kDa antigens (Radolf and Norgard, 1988a), stimulate immobilizing antibody (47 and 34 kDa), treponemicidal antibody (47 kDa), and lymphocyte proliferative responses (15 kDa; Robertson et al., 1982; Jones et al., 1984; Baker-Zander et al., 1988). Immunofluorescent studies indicate that the major lipoproteins are anchored to the outer leaflet of the CM, and little if any of these antigens is expressed on the OM surface (Cox et al., 1995). Many genes expressing *T. pallidum* subsp. *pallidum* antigens were cloned, expressed in *E. coli* and sequenced in the late 1900s (reviewed by Norris and the Treponema pallidum Polypeptide Research Group, 1993), and several of these have been identified as useful serodiagnostic antigens (Larsen et al., 1999).

Masuda and Kawata (1989) initially isolated and characterized the cytoplasmic filaments (CF) of *T. phagedenis* and other treponemes; they found that these structures were comprised of a single major 80-kDa polypeptide. The genes encoding the 80-kDa subunit called "CfpA" of *T. pallidum* subsp. *pallidum* and *pertenue*, *T. phagedenis*, *T. denticola* and *T. vincentii* have since been cloned and characterized (You et al., 1996; Izard et al., 1999). Izard et al. (2001) constructed a targeted disruption mutant of *cfpA* in *T. denticola*, which resulted in loss of the cytoplasmic filaments. They found that the mutation had pleiotropic effects, including an abnormal condensation of chromosomal DNA and incomplete cell division. These effects suggest that the cytoplasmic filaments are involved in DNA segregation and/or cell division (Izard et al., 2001).

The major 60-kDa polypeptide of *Treponema pallidum* subsp. *pallidum* has been shown to be the subunit of a multimeric protein homologous to the *groEL* chaperonin protein of *E. coli* (Houston et al., 1990). These chaperonins are found in all prokaryotes and eukaryotes and apparently are involved in protein folding and assembly. Similar proteins exist in the other treponemes.

Isolation, Identification, and Characterization

Human Treponemal Pathogens

Methods of Isolation and Cultivation Limited multiplication of *Treponema pallidum* subsp. *pallidum* has been obtained in a tissue culture system (Fieldsteel et al., 1981), as reviewed previously (Cox, 1994; Norris et al., 2001). However, continuous in vitro culture has not been achieved, thus precluding the use of this procedure for isolation and identification. *Treponema pallidum* subsp. *pallidum*, *pertenue* and *endemicum*, along with the rabbit pathogen *Treponema paraluis-cuniculi* can be isolated by inoculation of infectious tissue or exudates into adult male rabbits by either the intratesticular or intradermal routes. *Treponema carateum* is isolated by the intradermal inoculation of chimpanzees or other primates (see Table 1 for animal susceptibility to cutaneous lesions). Detailed procedures for the preparation of suspensions and methods of inoculation, and accepted criteria for determining infectivity, and identification by dark-field microscopy have been described by Turner and Hollander (1957) and Miller (1971). The development of lesions in these animals characteristic for each species and subspecies of *Treponema* (Turner and Hollander, 1957; Miller, 1971), as well as the pattern of lesion development in human disease (see Table 1 and Miller, 1987), are useful criteria for identification. Several isolates of *Treponema pallidum* subspecies continue to be propagated by intratesticular passage in rabbits. The Nichols strain of *Treponema pallidum* subsp. *pallidum* has been passaged continuously by this route since 1912. It has been studied more extensively than any other isolate and is the strain used in the preparation of treponemal antigens for immunologically specific serodiagnostic assays.

The in vitro multiplication of *T. pallidum* subsp. *pallidum* in a tissue culture system was first described by Fieldsteel et al. (1981). Treponemes extracted from infected rabbit tests are inoculated into cultures of Sf1Ep rabbit epithelial cells (American Type Culture Collection [ATCC], Rockville, MD, USA). The medium used is a modification of Eagle's minimal essential medium with Earl's salts, containing 13.9 mM D-glucose, 0.63 mM dithiothreitol, 31.3 mM HEPES (*N*-2-hydroxyethylpiperizine-*N*′2-ethane sulfonic acid) buffer (pH 7.4), resazurin (0.126 mg/ml), and 20% fetal bovine serum or calf serum inactivated at 56°C for 30 min. Optimal conditions for growth consist of incubation at 33–35°C in an atmosphere containing 1.5–5% O_2, 5% CO_2, and the balance N_2 (Fieldsteel et al., 1982). *Treponema pallidum* subsp. *pallidum* attaches to the Sf1Ep cells and multiplies on the mammalian cell surface (Fieldsteel et al., 1981; Konishi et al., 1986). Under these conditions, the Nichols strain of *Treponema pallidum* subsp. *pallidum* can increase in numbers up to 100-fold and maintain viability and infectivity for rabbits for 12–15 days. Although several strains of *T. pallidum* subsp. *pallidum* multiply in this system, isolates of *T. pallidum* subsp. *pertenue* and *endemicum* fail to grow (Cox et al., 1984). Attempts to extend multiplication by subculture have thus far been unsuccessful (Norris and Edmondson, 1986a; Norris et al., 2001). Enhanced multiplication of *T. pallidum* subsp. *pallidum* can be achieved in a modified medium (*T. pallidum* Culture Medium or TPCM) that contains the additives cobalt chloride, cocarboxylase, mannitol, histidine, superoxide dismutase and catalase (Cox et al., 1990; Cox, 1994). These agents help to scavange reactive oxygen intermediates and hence reduce oxygen toxicity.

Little is known about the growth requirements of *Treponema pallidum*. Treponemal multiplication does not occur in the absence of mammalian cells; the reason for this dependence has not been determined. The requirement for serum is stringent, and the required components are associated with the protein fraction (Norris and Edmondson, 1986b). Inasmuch as only certain lots of fetal bovine or calf sera are effective in maintaining growth, serum lots must be prescreened for this activity. Human serum inactivated at 56°C for 30 min can also fulfill the serum requirement (Norris and Edmondson, 1986b). Optimal multiplication at 1.5–5% O_2 (Fieldsteel et al., 1982; Cox et al., 1990) and metabolic and survival studies (reviewed by Cox [1983], Jenkin and Sandok [1983], Sell and Norris [1983], and Norris et al. [2001]) provide evidence that *Treponema pallidum* subsp. *pallidum* is microaerophilic. Optimal growth of *Treponema pallidum* subsp. *pallidum* (Nichols) at 33–35°C (Fieldsteel et al., 1982) in vitro mirrors its in vivo growth, as seen in rabbits where infection is enhanced in cooler regions of the body, such as the testes and exposed areas of the skin (Turner and Hollander, 1957; Miller, 1971).

Identification in Exudates and Tissues The restrictions of in vitro cultivation, together with the impracticability of animal inoculation, preclude the use of these isolation procedures in the diagnosis of treponemal disease. At present, the identification of treponemes in lesion exudates from patients with early-acquired treponemal disease or early congenital syphilis is usually attempted by dark-field microscopy and is based upon the demonstration of characteristic morphology and motility. Unfortunately, the use of dark-field microscopy for the identification of *T. pallidum* and diagnosis of syphilis and other treponemal infections has several limitations:

1. *Treponema pallidum* identification can be difficult because of the potential presence of host indigenous, nonpathogenic treponemes with similar morphology and motility characteristics in the oral cavity, genitalia, and gastrointestinal tract, precluding an unequivocal identification from these sites. Indeed, dark-field examination of lesions within the oral cavity and the anal region is most often contraindicated due to the potential presence of nonpathogenic spirochetes.

2. Prepared slides must be examined rapidly. The relatively rapid loss of motility in the presence of atmospheric O_2 necessitates that slide specimens be examined within a short period of time after preparation (15–30 min) to ensure the observation of characteristic movement. This restriction not only reduces the chances for accurately identifying organisms in the exudate but also reduces the probability of observing the treponemes that might be present.

3. Sensitivity is relatively poor. Dark-field examination of lesion exudates from patients with early treponemal disease may be negative, despite the presence of *Treponema pallidum*. This lack of sensitivity is not surprising. Dark-field examination of *Treponema pallidum* suspensions containing 10^6 organisms per ml would present as 2 organisms per high dry field (HDF) and 10^3 treponemes per ml of lesion exudate (a significant number of organisms) would present as 2 organisms per 1,000 HDFs and would not be detectable.

4. A specially equipped microscope is required, as well as personnel with considerable training, experience, and expertise. This combination is generally not available at most health care facilities. As a result, dark-field examination may not be done or may be performed incorrectly. Again, because antibody may be lacking

or its presence is inconclusive, this creates a serious problem for both physicians and health care officers responsible for controlling spread of the disease.

The limitations associated with dark-field examination have been recognized since the procedure was first described, and as might be expected, attempts have been made to develop a more efficient method. One such assay utilizes direct immunofluorescence and is referred to as the "Direct Fluorescent Antibody Test for *T. pallidum*" (DFA-TP; Larsen et al., 1998, 1999). With this procedure, lesion material or tissue sections are fixed to a microscopic slide and combined with fluorescein isothiocyanate-labeled rabbit, anti-*Treponema pallidum* immunoglobulin conjugate previously absorbed with crossreacting nonpathogenic treponemes to render it specific for *T. pallidum*. Following a 30-min incubation and washing, the slide is examined for the presence or absence of treponemes by fluorescence microscopy. This immunological procedure provides the opportunity to examine accurately not only smears from oral and anal lesions, but also tissue sections for the presence of treponemes. In addition, the organisms need not be motile, thus eliminating the need for immediate examination. The use of adequate controls with this assay is essential because conjugates of poor specificity due to incomplete absorbance of crossreacting antibodies may give misleading results.

A modification of this procedure (DFAT-TP) can be used for identification of *T. pallidum* in tissue sections (Larsen et al., 1998).

Silver impregnation methods have proven valuable in the identification of treponemes in tissues. However, their technical difficulty, poor reproducibility, and presence of artifacts resembling treponemes often make identification difficult. The modified Krajian procedure is one of many available silver impregnation methods and is described in detail by Miller (1971), along with techniques for tissue fixation, embedding and sectioning. A method in which the tissue sections are heated in a microwave oven (Swisher, 1987) provides more rapid and reproducible staining.

Another method that has been developed for the detection of *T. pallidum* in recent years has been PCR (reviewed by Larsen et al., 1999). New procedures permit not only the detection of *T. pallidum*, but also the identification of subspecies (Centurion-Lara et al., 1998) or even the direct detection and delineation of *T. pallidum* subsp. *pallidum* strains during syphilis outbreaks, using lesion exudates or blood (Pillay et al., 1998; Sutton et al., 2001).

A discussion of nontreponemal and treponemal tests in the serological diagnosis of treponemal infection is beyond the scope of this chapter. However, detailed presentations of the principles, procedures, interpretations of results, precautions, and potential problems associated with these serological assays are available (Larsen et al., 1998, 1999).

Oral Spirochetes

Isolation and Preservation

General Comments Owing to their fastidious nature, most oral treponemes cannot be cultured on a routine basis. Their isolation requires complex media and microaerophilic or strictly anaerobic growth conditions rendering their cultivation laborious, time-consuming, and often frustrating. Oral treponemes were first cultured by Noguchi in 1912 using a medium containing sheep serum and kidney tissue or rabbit or sheep testicle. A routine method for culturing small oral treponemes was published by Hampp and Bethesda (1943), and since then, media have been supplemented with a variety of components such as serum, volatile fatty acids, vitamins, carbohydrates or sterilized body fluids. However, because nutritional needs of oral treponeme species vary as much as their diversity, there is no single medium optimal for the recovery of all oral treponemes. Addition of up to 10% inactivated animal serum has been used successfully for cultivation of many species. However, some fermentative treponemes, e.g., *T. maltophilum* and *T. lecithinolyticum*, were found to be inhibited by serum (Koseki et al., 1996b; Wyss et al., 1996, 1999). Systematic studies on essential nutrients and environmental growth factors are scarce. Fiehn investigated nine oral, small-sized spirochete strains (Fiehn, 1989). These strains grew in the absence of rabbit serum. Human globulin and certain carbohydrates stimulated growth, while bovine albumin and long-chain fatty acids inhibited growth. The pH optimum was 7.5. Changes by only one half of a pH unit resulted in approximately 50% growth reduction.

Atmospheric Requirements In general, oral treponemes are sensitive to oxygen. In the study mentioned above, spirochetes tolerated 3% oxygen, but strictly anaerobic culturing conditions gave the highest average cell yields. Handling of cultures under aerobic conditions decreased cell counts (Fiehn, 1989). Therefore culturing can be accomplished with an anaerobic chamber, anaerobic jars, or roll tubes. The gas mixture used in an anaerobic jar with palladium catalyst can be either a hydrogen-generating system, such as GasPac (BBL) envelope, or a tank gas mixture containing H_2 and CO_2, e.g., 95% H_2-5% CO_2. Handling of cultures in an anaerobic glove

box is of help, and prereduction of growth media prior to inoculation of the sample in anaerobic jars is advantageous. The plate-in-bottle method (Umeda et al., 1990) has been developed to produce strict anaerobic conditions. Autoclaved steel wool (1.0 g) was activated by an acidic copper sulfate solution and placed quickly in a stainless steel pan inside a glass bottle that contained growth medium. All steps were carried out under anaerobic conditions by flushing with 100% CO_2 gas. This technique yielded a higher recovery of oral treponemes from subgingival plaque than the anaerobic chamber yielded (Koseki et al., 1996a).

Growth Media and Microscope Observation Treponemes can be cultured in semisolid or liquid media either in roll tubes or on agar plates. Observations of growth, size, purity of strains, multiplication and motility should be conducted by dark-field microscopy every 3–8 days. The media most commonly used are as follows.

GM-1 and NOS Medium The GM-1 and new oral spirochete (NOS) medium supplemented with rifampicin as selective agent both resulted in high recovery of oral spirochetes (Blakemore and Canale-Parola, 1976; Leschine and Canale-Parola, 1980). Gingival crevice material was collected with sterile toothpicks and subsequently placed in tubes with prereduced medium. Tubes were gently shaken for 2 hours and serially diluted into tubes of melted GM-1 or NOS agar. After incubation at 37°C, spirochete colonies appeared as "cotton balls" or "transparent bubbles" within the agar. They were removed with sterile Pasteur pipettes for successive transfer in agar medium. NOS-medium has been used to isolate various species, e.g., *T. denticola*, *T. vincentii*, *T. socranskii* and *T. phagedenis* and, supplemented with pectin, the treponemes from the Smibert collection (Chan et al., 1993a; Paster et al., 1998). For enumeration of colony forming units, NOS medium was solidified by the addition of agarose (NOS-A), gelatin/Noble Agar (NOS-GN) or gelatin/Bacto agar (NOS-GB; Chan et al., 1993b; Chan et al., 1997; Qiu et al., 1994). NOS medium was used also to study the growth requirements of nine small-sized oral spirochetes by varying oxygen content, pH or nutrient factors (Fiehn, 1989). For details of the formula, see ATCC medium (http://www.atcc.org/) no. 1357 and no. 1494.

OTI Medium The oral treponeme isolation medium (OTI) contains pectin, clarified rumen fluid, yeast autolysate and serum. It was first used to culture treponemes from young human adults with experimental gingivitis (Moore et al., 1982). For isolation of treponemes, authors used the membrane technique described below. This way *T. pectinovorum*, *T. socranskii* subsp. *socranskii*, *T. socranskii* subsp. *buccale* and *T. socranskii* subsp. *paredis* were isolated from periodontitis patients for the first time (Smibert and Burmeister, 1983; Smibert et al., 1984b). For the formula, see ATCC medium (http://www.atcc.org/) no. 1367.

(M)TYGVS Medium TYGVS is modified Laughon medium (Laughon et al., 1982) and has been used for enzyme analyses of *T. denticola* (Otha et al., 1986) and quantitative recovery of spirochetes from subgingival plaque (MTYGVS; Salvador et al., 1987). Combined with the filter technique, this medium allowed the isolation of treponemes from different clinical sources, including the novel species *T. medium* (DiRienzo et al., 1991; Nakazawa et al., 1997; Umemoto et al., 1997; Sato and Kuramitsu, 1999).

Medium 10 Medium 10 (M10), a medium without serum and rumen fluid, was originally designed for nonselective enumeration and cultivation of rumen bacteria (Caldwell and Bryant, 1966). It was later successfully used to isolate treponemes from plaque samples of periodontitis patients. While well suited for primary isolation of treponemes, it could not be used for maintaining the strains. The authors later developed enriched medium10, containing trypticase peptone, yeast extract, glucose and volatile fatty acids (Koseki et al., 1996b).

OMIZ-Pat To develop a chemically defined medium for culturing a wide range of oral anaerobes, Wyss (1992) described the OMIZ-W1, containing more than 80 components. This medium is laborious to prepare. However, using this mixture supplemented with asialofetuin, a methanol soluble fraction of yeast extract, neopeptone and some carbohydrates, a number of new oral treponeme species were cultivated (Wyss et al., 1996). This complex medium was called "OMIZ-Pat." For isolation of new strains, antibiotics rifampicin and phosphomycin were added and a limiting dilution technique in 96-well plates was performed. This technique succeeded in the isolation of *T. maltophilum* (Wyss et al., 1996); supplemented with human serum, *T. amylovorum* (Wyss et al., 1997); prepared without dipalmitoyl phosphatidylcholine, *T. lecithinolyticum* (Wyss et al., 1999); and supplemented with serum, polymyxin and nalidixic acid, *T. parvum* (Wyss et al., 2001). For the formula, see ATCC medium (http://www.atcc.org/) no. 2131.

Systematic comparisons of different growth media are rare. The detection frequency and recovery of oral treponemes from subgingival plaque in different cultivation media were inves-

tigated (Koseki et al., 1995; Koseki et al., 1996a). The authors used M10, M10 supplemented with rumen fluid, serum or rifampicin, NOS, NOS with rifampicin, altered oral treponeme isolation medium (aOTI) and enriched trypticase soy agar. NOS yielded the highest detection frequency of treponemes in all samples tested, whereas M10 with 10% rabbit serum showed the highest number of colony-forming units of treponemes in the positive samples. Therefore, the authors recommended M10 combined with the plate-in-bottle technique for high recovery of oral treponemes from subgingival plaque. However, cultivation bias may be significant as serum-free M10 selected mainly saccharolytic strains such as *T. socranskii* or related species (Koseki et al., 1995; Koseki et al., 1996a).

Techniques for Isolation of Treponemes from Clinical Samples It is difficult to obtain pure subcultures of treponemes from highly contaminated primary cultures. Because of bacterial coaggregation in subgingival plaque, it is necessary to disrupt the biofilm to obtain single species colonies; however, care should be taken not to harm the fragile treponemes by the procedures used. This issue has been addressed by Salvador et al. (1987) in studying the quantitative recovery of oral spirochetes after sonification, mechanical mixing, and homogenization of plaque samples, the latter technique being the most suitable one.

Though most media for isolation of treponemes are highly enriched, long cultivation periods up to two weeks may be required. Most isolation techniques rely on treponeme motility. In solid or semisolid media, treponeme colonies appear hazy, like cotton balls or nearly transparent bubbles within the agar (Paster and Canale-Parola, 1982). Colonies can be removed using sterile Pasteur pipettes and can be cloned by several rounds of subcultivation to obtain isogenic colonies.

Well-Plate Method This method uses agar plates containing a thick layer of solid medium with a well cut into the center of the plate (Rosebury and Foley, 1941; Hampp and Bethesda, 1943). Clinical samples are placed into agar wells and incubated anaerobically. Treponemes appear as a white haze growing out from the well into the agar and away from contaminants. Agar plugs of the leading haze are cut with sterile glass pipettes and transferred into agar wells of fresh agar plates. This procedure was used successfully to isolate *Treponema medium* (Umemoto et al., 1997).

Membrane-Filter Methods These techniques rely on the ability of treponemes to pass through pores of membrane filters that hold back most other bacteria. These methods are limited, therefore, to the isolation of treponemes smaller than other bacteria. Samples are inoculated onto membrane filters placed on top of agar media with a pore size ranging from 1.5–100 μm. A sterile O-ring sealed to the filter with 3% agar can help to keep the sample in place. This prevents other bacteria from swarming or being washed off and growing on the agar medium (Loesche and Socransky, 1962; Moore et al., 1982). Motile treponemes migrate through the pores of the filter and grow into the agar medium. After 2 days to 2 weeks, filters are removed and agar plates are further incubated and observed for the development of colonies that can be picked and subcultured. This technique allowed the isolation of *T. denticola*, *T. pectinovorum* and *T. socranskii* spp. (Smibert and Burmeister, 1983; Smibert et al., 1984b; Chan et al., 1993a).

Serial Dilutions Treponemes can be isolated using the limiting dilution assay in liquid media (Wyss et al., 1996). *Treponema maltophilum* was isolated by ten-fold dilutions of selective medium with subgingival plaque in 96-well plates. After anaerobic incubation of plates, dilutions of 10^{-5} to 10^{-7} resulted in growth in about 50% of the wells. After 10 days, wells were examined for growth of spirochetes, and respective samples were subcultured on solid media for further isolation.

U-tube Method Patient samples also can be incubated in U-tubes containing pectin medium (Dahle et al., 1995). In these U-tubes, the "bacterial sample side" and the "sterile medium side" are separated by a membrane filter and an agar plug at the caudal part of the tube. The bacterial sample is placed on the agar-plug side of the tube, whereas fresh medium is added on the other side. The U-tube is then incubated for 4 days. The sterile medium side is examined daily for viable treponemal cells by aspirating a drop of culture with a needle and observation in a dark-field microscope. Motile treponemes start to migrate through the agar-plug and the membrane immediately after being transferred to the U-tube. Using this technique, the authors managed to isolate treponemes from all 51 clinical samples investigated.

Selective Media While most conventional isolation techniques depend upon the migration of treponemes, therefore selecting for highly motile or slender treponemes, selective media allow isolation independent of motility or size. Treponemes are resistant to a number of antimicrobial agents (Abramson and Smibert, 1971). Rifampicin, phosphomycin and polymyxin have

been used to suppress contaminating oral flora, allowing the isolation of slowly growing spirochetes. Addition of rifampicin significantly increased the numerical ratio of spirochete colonies to other oral species (Leschine and Canale-Parola, 1980; Koseki et al., 1996a), thus facilitating the isolation and enumeration of spirochetes. On the other hand, addition of antibiotics strongly reduced the absolute number of spirochete colony forming units (Koseki et al., 1996a). Antibiotics have successfully been used in combination with various media: NOS was supplemented with rifampicin (4 mg/liter) (Chan et al., 1993a) or with rifampicin (1 mg/liter) plus phosphomycin (100 mg/liter; Chan et al., 1997); M10 was supplemented with rifampicin (5 mg/liter; Koseki et al., 1996a); OTI with rifampicin (2 mg/liter) plus polymyxin (800 U/ml; Smibert et al., 1984b); and OMIZ-Pat with rifampicin (1 mg/liter) plus phosphomycin (100 mg/liter; Wyss et al., 1996).

Subcultivation and Preservation of Cultures

The isolation procedures described above select treponemes from other bacteria, but not from different species of treponemes. Isolated colonies from mixed treponemal cultures must, therefore, be streaked and subcultured several times. Fiehn et al. used a dilution technique for subcultures. An agar plug with colony material was transferred to liquid medium. After 4 days of growth, a 10-fold serial dilution of the culture was re-streaked on solid media and incubated for a 14-day period again to obtain single-species colonies (Fiehn et al., 1995). Pure-culture isolates must be identified to the species level and must be fully characterized prior to conduct of further studies. As treponemes do not typically form colonies on the surface, but rather migrate into solid media, the agar should be softer than usual to allow treponemes to migrate. Concentrations of 1.25–1.3% Bacto-agar or Noble agar are therefore recommended. All cultures must be examined by dark-field microscopy (magnification at least 1000×) to determine purity.

Depending on the species, treponemes are transferred to a suitable medium every 3–7 days for experimental use. For preservation of treponeme cultures over several months or years, cryoprotective agents such as 10–15% glycerol or 3% dimethylsulfoxide (DMSO) are added to the liquid cultures. Aliquots are prepared in sterile cryotubes that are gassed with N_2 until the top is closed and stored in liquid nitrogen or a freezer at –70 or –80°C. To recover the cultures, aliquots are removed from storage, thawed to room temperature, transferred to fresh, pre-reduced medium, and incubated at 37°C until growth is observed.

Digital Dermatitis Spirochetes

Digital dermatitis is a condition occurring in ungulates, including cattle and sheep, in which ulcers or papillomatous lesions occur either in the pastern region or between the hooves (Cheli and Mortellaro, 1974; Blowey and Sharp, 1988; el-Ghoul and Shaheed, 2001). Although the lesions represent a polymicrobial infection, treponemes are prominent constituents (Blowey et al., 1994; Walker et al., 1995; Borgmann et al., 1996; Dopfer et al., 1997; Demirkan et al., 1999; Mumba et al., 1999b; Collighan et al., 2000). At least five different phylotypes of *Treponema* can be present (Choi et al., 1997), including organisms with rDNA sequences resembling those of *T. denticola*, *T. vincentii* and *T. maltophilum*. Fluorescence in situ hybridization with rDNA probes (Fig. 4) indicates that, as in periodontal plaque (Moter et al., 1998a), the microbial flora in bovine digital dermatitis are stratified with different treponemal phylotypes occupying different layers of the biomass (Moter et al., 1998b). On the basis of PCR results, topical antibiotic treatment reduces, but does not appear to eradicate, the treponemal infection (Mumba et al., 1999a). Thus far, only one isolate has been speciated and was provided the name *Treponema brennaborense* (Schrank et al., 1999); this organism was isolated using the OMIZ-Pat medium described above. Two treponemal isolates have been obtained from a condition called "severe virulent ovine foot rot" (SVOFR; Naylor et al., 1998; Demirkan et al., 2001), which appears to be distinct from the interdigital infection in which *Dichelobacter nodulans* and other bacteria are the primary causative agents. In one of these studies, immunomagnetic separation using magnetic beads coated with anti-treponemal antibodies was employed as a novel approach to enriching for spirochetes prior to culture. The spirochetes from SVOFR cases were cultured using Oral Treponema Enrichment Broth (OTEB; Anaerobe Systems, Morgan Hill, CA, USA) supplemented with rifampicin (5 mg/liter) and nalidixic acid (500 mg/liter) to inhibit growth of other bacteria.

Skin Commensal Spirochetes

Treponema phagedenis, *T. refringens* and *T. minutum* are spirochetes that can be isolated from human sebaceous secretions and desquamated epithelial cells in skin folds of the genital region. Specimens can be collected using moistened sterile swabs, and the organisms can be cultured readily under anaerobic conditions at 37°C in thioglycollate medium with 10% heat-inactivated rabbit serum (or a variety of other serum-containing media). Rifampin (2 μg/ml)

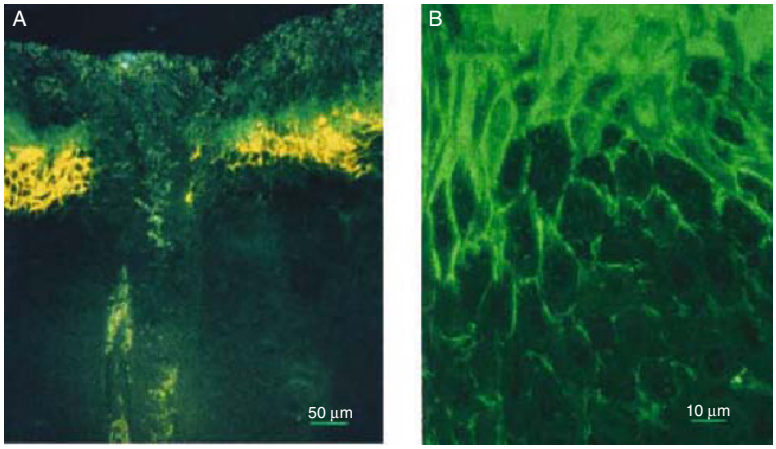

Fig. 4. Treponemes in a bovine digital dermatitis biopsy, as shown by fluorescence in situ hybridization (FISH). A) Simultaneous hybridization with EUB338FITC and DDK4Cy3, a probe specific for treponemes associated with digital dermatitis. Note the stratification with EUB338-positive bacteria (green) predominantly on the outer surface and DDK4-positive treponemes (yellow) within the stratum corneum and the stratum spinosum. B) High-power view. Single spirochetes seem to invade the tissue through the intercellular spaces in the stratum corneum (top) and the stratum spinosum (bottom). Epidermal cells exhibiting weak autofluorescence with black nuclei allow orientation within the tissue. From Moter et al. (1998b), with permission.

and polymyxin (800 µg/ml) can be added to inhibit the growth of other bacteria.

Genetics

The genome of *Treponema pallidum* subsp. *pallidum* Nichols was sequenced in 1998 (Fraser et al., 1998) and consists of a single circular chromosome of 1,138,006 bp. The G+C content of 52.8 mol% is considerably higher than that of *T. denticola* (~37%), *T. phagedenis* (38–39%), and *T. refringens* (41.5%; Miao and Fieldsteel, 1978), consistent with the evolutionary distance between the noncultivated, pathogenic treponemes and the cultivable *Treponema*. The pathogenic *Treponema* (Table 1) are essentially a clonal population. By all indications, this group of organisms have essentially identical genomes that differ by only a few base pairs concentrated in a relatively small number of repetitive genes or sequences (Miao and Fieldsteel, 1980; Fieldsteel, 1983; E. Sodergren, unpublished data). The regions flanking *tpp15*, encoding a 15-kDa lipoprotein, contain heterogeneous sequences and have been utilized to devise PCR, sequencing, and restriction fragment length polymorphism (RFLP) techniques for distinguishing *T. pallidum* subspecies and strains (Centurion-Lara et al., 1998). A set of 12 related *T. pallidum* repeat (*tpr*) genes constitute "hot spots" of recombination in *T. pallidum*, and the resulting heterogeneity represents a major portion of the genetic differences between strains (Centurion-Lara et al., 2000a; Centurion-Lara et al., 2000b; Stamm and Bergen, 2000; E. Sodergren, unpublished data). Recently, Pillay et al. devised a system utilizing a combination of PCR and RFLP of *tpr* genes and the acidic repeat protein gene (*arp*) to distinguish between *T. pallidum* subsp. *pallidum* strains in skin lesions or blood from syphilis patients (Pillay et al., 1998; Sutton et al., 2001). It should be emphasized, however, that nearly all other sequences examined have been identical in *T. pallidum* strains, and the overall genome arrangement appears to be invariant among the *T. pallidum* strains and subspecies.

The *T. pallidum* subsp. *pallidum* Nichols genome encodes ~1,040 open reading frames, which have been evaluated in some detail in other publications (Fraser et al., 1998; Radolf et al., 1999; Norris et al., 2001). The resulting information has been incorporated in the Physiology section and elsewhere in this chapter.

The *T. denticola* ATCC 35405 genome is also being subjected to sequence analysis, and at the time of this writing, the sequence is nearly completed. The genome is a single circular chromosome approximately 2.8 million base pairs (Mb) in size; a similar size of 3.0 Mb was determined previously by physical mapping of *T. denticola* ATCC 33520 (MacDougall and Saint Girons, 1995). In addition, *T. denticola* and other oral treponemes have been found to contain circular plasmids ranging in size from 2.0 kb to 4.2 kb (MacDougall et al., 1992; Chan et al., 1996). The 4.2-kb plasmid, pTS1, was present in several different periodontal isolates, including clones of *T. denticola* and *T. socranskii* (Chan et

al., 1996); in addition, transfer of a plasmid from *T. denticola* to *Streptococcus gordonii* has been demonstrated in broth and artificial biofilm cultures (Wang et al., 2002). These results suggest that genetic transfer can occur both among *Treponema* strains and between *Treponema* and other bacteria in the oral microbiological environment.

Treponema pallidum is not amenable to genetic manipulation because it has not been cultured continuously in vitro. *Treponema denticola* has become the model for gene disruption and other forms of genetic manipulation of the genus *Treponema*. Several genes of *T. denticola* have been inactivated by targeted insertional mutation with suicide vectors containing an erythromycin resistance gene (Li et al., 1996; Kataoka et al., 1997; Ruby et al., 1997; Fenno et al., 1998; Li et al., 1999). In addition, heterologous expression and complementation have been achieved using a shuttle vector with a coumermycin A1-resistance marker (Chi et al., 1999; Chi et al., 2002; St. Girons et al., 2000). Thus the combination of the availability of the *T. denticola* genomic sequence and genetic manipulation tools promises to be of value in determining the functions of *Treponema*-specific genes.

Physiology

The physiology of *T. pallidum* has been discussed in other articles (Cox, 1983; Fraser et al., 1998; Radolf et al., 1999; Subramanian et al., 2000; Norris et al., 2001) and will not be described in detail here. Briefly, the *T. pallidum* genome sequence has confirmed previous indications that the organism has extremely limited metabolic capabilities. Genes corresponding to biosynthetic pathways are virtually nonexistent, indicating a profound dependence on the human host for provision of amino acids, cofactors, fatty acids, and nucleic acid precursors. In addition, the glycolytic pathway is the only form of ATP production identified thus far, in that the tricarboxylic acid cycle and elements of electron transport systems are absent. As discussed above, *T. pallidum* is microaerophilic and requires small quantities of oxygen for optimal survival and multiplication in vitro; however, the mechanism of oxygen utilization has not been determined. Genes for reduced nicotinamide adenine dinucleotide (NADH) oxidase (*nox*), alkyl hydroperoxidase (*ahpC*), and neelaredoxin (TP0823) are present in the *T. pallidum* subsp. *pallidum* genome (Fraser et al., 1998); the catalysis of superoxide anion-hydrogen peroxide conversion has been demonstrated with recombinant *T. pallidum* neelaredoxin (Jovanovi'c et al., 2000). These enzymes may provide some measure of protective activity against reactive oxygen intermediates. Overall, the apparent loss of genes encoding activities not required in the mammalian tissue environment explains the highly fastidious nature of *T. pallidum* and indicates an extreme adaptation to obligate parasitism.

All members of the genus *Treponema* appear to require fatty acids in some form. Albumin serves as a detoxifying carrier for fatty acids in cultures of *T. denticola* and *T. vincentii* (Van Horn and Smibert, 1982; Van Horn and Smibert, 1983). The albumin fraction of serum also replaces the serum requirement of *T. pallidum* in culture, suggesting that fatty acids associated with albumin are required for multiplication in this system (Norris and Edmondson, 1986b). Fatty acids are incorporated in an unmodified form into membrane lipids by *T. pallidum* (Schiller and Cox, 1977) and may be assimilated into the outer membrane directly from the host milieu (Cox and Radolf, 2001). Fermentative treponemes isolated from subgingival plaque require *iso*-butyric acid, 2-methylbutyric acids and/or *iso*-caproic acid for optimal growth in M10 medium, substituted for the rumen fluid commonly used in culture media (Livermore and Johnson, 1975; Koseki et al., 1996b). Although most *Treponema* species appear to lack LPS, recent studies have indicated that the outer membrane of *T. denticola* contains an LPS-like substance (Dahle et al., 1996; Schultz et al., 1998; Walker et al., 1999).

Epidemiology

Syphilis is a sexually transmitted disease, whereas yaws, endemic syphilis, and pinta are all transmitted by direct skin-to-skin contact, usually during childhood and adolescence. Syphilis is still a common disease in many areas of the world, with an estimated 12 million new cases occurring per year (World Health Organization, 2001). In the United States, the annual reported incidence of syphilis was at its highest point in 1946 (963,647 reported cases), just after World War II and prior to the widespread availability of penicillin. During the 1950s, syphilis cases dropped dramatically, leading to hopes of eradication of the disease. However, the rates again increased in the 1960s and 1970s. Following an epidemic that peaked in 1990, the reported incidence of syphilis has dropped to historic lows (Fig. 5); a total of 31,575 cases was reported in 2000. The reasons for this decrease are unclear, in that incidence of other sexually transmitted diseases (e.g., chlamydia infections) have remained high. The Centers of Disease Control

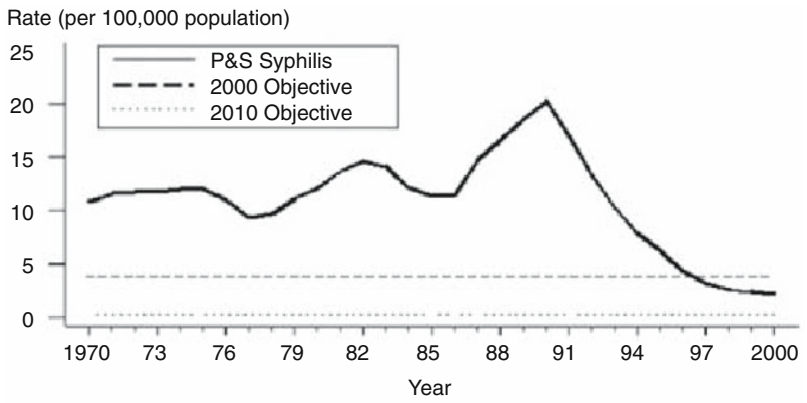

Fig. 5. Annual reported incidence per 100,000 population of primary and secondary syphilis in the United States, 1980–2000. Target objectives for 2000 and 2010 are shown. From United States Centers for Disease Control and Prevention.

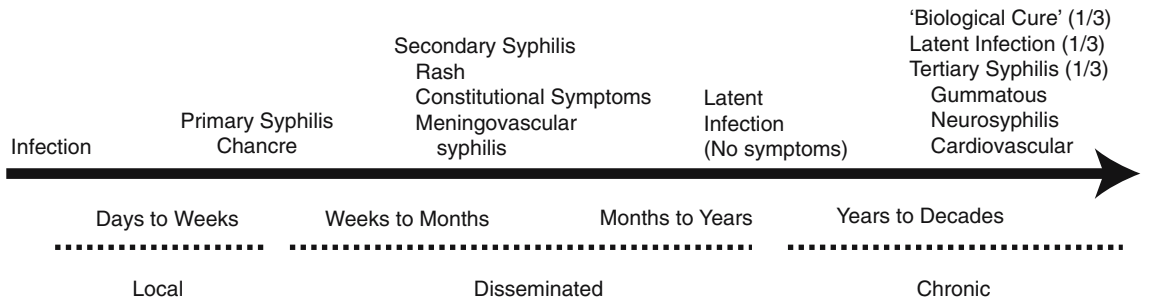

Fig. 6. Clinical course of syphilis.

and Prevention and the National Institutes of Health have established a program for the elimination of Syphilis in the United States (http://www.cdc.gov/stopsyphilis/).

The endemic treponematoses (yaws, endemic syphilis, and pinta) are restricted to certain geographic regions, and incidence is related to both socioeconomic conditions and climate (Table 1). Yaws and pinta are found predominantly in rural, tropical rain forest areas, whereas endemic syphilis may occur also in arid regions and some temperate areas (Antal et al., 2002). In 1950, the worldwide incidence of yaws alone was estimated to be 50 million cases. A global endemic treponematoses control program was initiated by the World Health Organization in 1949, and approximately 160 million people were treated between 1952 and 1964 (Antal et al., 2002). The collective incidences of these diseases was estimated to be 2.5 million cases in 1998 (World Health Organization, 1998). Foci of yaws are still found in Africa, Central and South America, Southeast Asia, and some Pacific Islands, and endemic syphilis is present in the Near East. Pinta is restricted to regions in or near the Amazon basin (Antal et al., 2002).

Disease

Thorough descriptions of syphilis and the other treponematoses can be found in infectious disease textbooks and other sources (United States Public Health Service, 1968; Larsen et al., 1999) and will not be covered in detail here. Briefly, syphilis is a multistage, invasive infection with local, disseminated, and chronic manifestations (Fig. 6). Infection occurs when viable organisms from an infectious lesion enter through a fissure in the epidermis or a mucous membrane. *Treponema pallidum* multiplies locally and causes a characteristic painless, ulcerative lesion called a "chancre." At the same time, treponemes disseminate through the bloodstream or lymphatics and can infect almost any organ or tissue. Secondary syphilis lesions may occur weeks to months after infection and signify the disseminated stage of the disease. They consist of multiple maculopapular lesions that may occur preferentially on the palms of the hands and soles of the feet or appear throughout the torso and extremities. Both primary and secondary lesions contain large numbers of *T. pallidum* and represent the infectious stage of the disease. All

untreated patients enter a period of latent infection, in which manifestations of the disease are not evident; the only indications of infection are positive serologic tests. Disease of approximately two-thirds of patients is diagnosed during this stage, and diagnosis is based solely on patient history and reactive serologies. Infection with *T. pallidum* may last the lifetime of the individual in untreated cases and can result in debilitating or life-threatening disease years to decades after the initial infection. Late syphilis manifestations occur in roughly one-third of untreated patients, according to records from the early 1900s (Gjestland, 1955). These consist of granulomatous lesions called "gummas," cardiovascular manifestations such as aortic aneurysm, or neurosyphilis, including infection of the brain (paresis) or of the sensory ganglia and spinal cord (tabes dorsalis). Some forms of neurosyphilis, such as meningovascular syphilis, can occur at earlier stages. Congenital syphilis results from transplacental transmission of *T. pallidum* from the mother during the infectious stage, usually in the first year of maternal infection; it can cause abortion, stillbirth, fulminant infection and destructive lesions at the time of birth or latent infection that results in manifestations later in childhood, including bone and tooth deformation, interstitial keratitis (clouding of the cornea), deafness, and intellectual deficits. Late syphilis has become virtually nonexistent in the United States (owing in part to coincidental treatment of other infections), but continued surveillance is needed to minimize the occurrence of congenital syphilis.

Yaws, endemic syphilis, and pinta each have cutaneous manifestations, but have progressively less internal organ involvement (Antal et al., 2002). The initial lesion of yaws ("mother yaw") usually occurs on the legs, feet or buttocks and consists of a raised papule containing large numbers of *T. pallidum* subsp. *pertenue*. Secondary lesions may occur as long as two years after infection and are also highly infectious. Late manifestations are typically restricted to the skin, subcutaneous tissue, cartilage and bone. Hypopigmented areas and gummas may occur in the skin, and painful bone and cartilage deformations result in curvature of the tibia, destruction of the nose and palate (gangosa), or nodules in the facial bones or near joints. In endemic syphilis, the primary lesion is usually not evident and may involve the oral mucosa. Secondary lesions consist of papules, typically in the mouth or warm, moist areas of the skin. Gummas again occur in later stages in skin and bone, causing deformities similar to those seen in yaws. Pinta ("spot") is a skin disease that results in hyper- or hypopigmented lesions, desquamation, or erythematous rashes over a period of several years; it does not involve internal organs. Neurologic involvement is nonexistent in the endemic treponematoses; congenital transmission may occur rarely in yaws, but has not been observed in endemic syphilis or pinta. Penicillin remains the treatment of choice for *T. pallidum* infections.

Periodontal disease represents a spectrum of polymicrobial infections of the gingival crevice, which in its various forms can cause gum inflammation, expansion of the gingival pocket, bone resorption, and tooth loss (Moore and Moore, 1994). It is estimated that over 80% of adults in the United States have experienced some form of periodontal disease. Dental plaque is essentially a complex, stratified biofilm (Fig. 7). If it is not removed by brushing and flossing, gum inflammation and the progressive changes associated with periodontal disease will result from both preferential outgrowth of subsets of anaerobic organisms (including oral spirochetes) within the deep layers of the biofilm and prolonged exposure to the bacteria and their products. At least part of this effect appears to be due to the production of inflammatory cytokines by host cells in response to bacterial products. The lack of effective animal models and the poly-

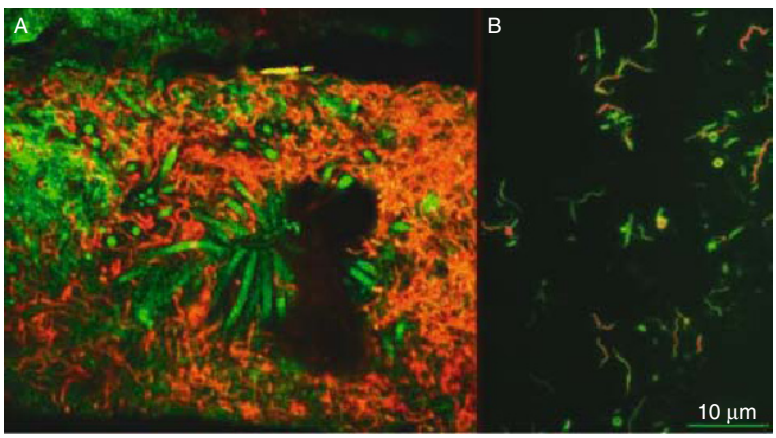

Fig. 7. Fluorescent in situ hybridization (FISH) on subgingival biofilm (plaque) from a human subject. A section of a fixed and sectioned region of the biofilm A) and a smear B) are shown. Probes used were the general eubacterial 16S rDNA oligonucleotide EUB338FluoX (green) and the 16S rDNA probe TRE ICy3 (red) for the detection of phylogenetic group I treponemes, most of which are yet uncultured. The results show the high proportion of treponemes involved in biofilm formation. A) is from Wecke et al. (2000), with permission, and B) was provided by A. Moter.

microbial nature of periodontal disease have complicated the assessment of the role of oral spirochetes and other bacteria in its pathogenesis.

Literature Cited

Abramson, I. J., and R. M. Smibert. 1971. Inhibition of growth of treponemes by antimicrobial agents. Br. J. Ven. Dis. 47:407–412.

Antal, G. M., S. A. Lukehart, and A. Z. Meheus. 2002. The endemic treponematoses. Microb. Infect. 4:83–94.

Baker-Zander, S. A., and S. A. Lukehart. 1984. Antigenic cross-reactivity between Treponema pallidum and other pathogenic members of the family Spirochaetaceae. Infect. Immun. 46:116–121.

Baker-Zander, S. A., M. J. Fohn, and S. A. Lukehart. 1988. Development of cellular immunity to individual soluble antigens of Treponema pallidum during experimental syphilis. J. Immunol. 141:4363–4369.

Bayon, H. 1913. A new species of Treponema found in the genital sores of rabbits. Br. Med. J. 2:1159.

Blakemore, R. P., and E. Canale-Parola. 1976. Arginine catabolism by Treponema denticola. J. Bacteriol. 128:616–622.

Blanco, D. R., C. I. Champion, J. N. Miller, and M. A. Lovett. 1988. Antigenic and structural characterization of Treponema pallidum (Nichols strain) endoflagella. Infect. Immun. 56:168–175.

Blanco, D. R., K. Reimann, J. Skare, C. I. Champion, D. Foley, M. M. Exner, R. E. Hancock, J. N. Miller, and M. A. Lovett. 1994. Isolation of the outer membranes from Treponema pallidum and Treponema vincentii. J. Bacteriol. 176:6088–6099.

Blanco, D. R., C. I. Champion, M. M. Exner, H. Erdjument-Bromage, R. E. Hancock, P. Tempst, J. N. Miller, and M. A. Lovett. 1995. Porin activity and sequence analysis of a 31-kilodalton Treponema pallidum subsp. pallidum rare outer membrane protein (Tromp1). J. Bacteriol. 177:3556–3562.

Blanco, D. R., C. I. Champion, M. M. Exner, E. S. Shang, J. T. Skare, R. E. Hancock, J. N. Miller, and M. A. Lovett. 1996. Recombinant Treponema pallidum rare outer membrane protein 1 (Tromp1) expressed in Escherichia coli has porin activity and surface antigenic exposure. J. Bacteriol. 178:6685–6692.

Blanco, D. R., J. N. Miller, and M. A. Lovett. 1997. Surface antigens of the syphilis spirochete and their potential as virulence determinants. Emerg. Infect. Dis. 3:11–20.

Blanco, D. R., J. P. Whitelegge, J. N. Miller, and M. A. Lovett. 1999. Demonstration by mass spectrometry that purified native Treponema pallidum rare outer membrane protein 1 (Tromp1) has a cleaved signal peptide. J. Bacteriol. 181:5094–5098.

Blowey, R. W., and M. W. Sharp. 1988. Digital dermatitis in dairy cattle. Vet. Record 122:505–508.

Blowey, R. W., S. H. Done, and W. Cooley. 1994. Observations on the pathogenesis of digital dermatitis in cattle. Vet. Record 135:115–117.

Borgmann, I. E., J. Bailey, and E. G. Clark. 1996. Spirochete-associated bovine digital dermatitis. Can. Vet. J. 37:35–37.

Breznak, J. A. 1973. Biology of nonpathogenic, host-associated spirochetes. CRC Rev. Microbiol. 2:457–489.

Caldwell, D. R., and M. P. Bryant. 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. 14:794–801.

Cameron, C. E., C. Castro, S. A. Lukehart, and W. C. Van Voorhis. 1998. Function and protective capacity of Treponema pallidum subsp. pallidum glycerophosphodiester phosphodiesterase. Infect. Immun. 66:5763–5770.

Canale-Parola, E. 1977. Physiology and evolution of spirochetes. Bacteriol. Rev. 41:181–204.

Centurion-Lara, A., and T. Arroll, R. Castillo, J. M. Shaffer, C. Castro, W. C. Van Voorhis, and S. A. Lukehart. 1997. Conservation of the 15-kilodalton lipoprotein among Treponema pallidum subspecies and strains and other pathogenic treponemes: genetic and antigenic analyses. Infect. Immun. 65:1440–1444.

Centurion-Lara, A., C. Castro, R. Castillo, J. M. Shaffer, W. C. Van Voorhis, and S. A. Lukehart. 1998. The flanking region sequences of the 15-kDa lipoprotein gene differentiate pathogenic treponemes. J. Infect. Dis. 177:1036–1040.

Centurion-Lara, A., C. Castro, L. Barrett, C. Cameron, M. Mostowfi, W. C. Van Voorhis, and S. A. Lukehart. 1999. Treponema pallidum major sheath protein homologue Tpr K is a target of opsonic antibody and the protective immune response. J. Exp. Med. 189:647–656.

Centurion-Lara, A., C. Godornes, C. Castro, W. C. Van Voorhis, and S. A. Lukehart. 2000a. The tprK gene is heterogeneous among Treponema pallidum strains and has multiple alleles. Infect. Immun. 68:824–831.

Centurion-Lara, A., E. S. Sun, L. K. Barrett, C. Castro, S. A. Lukehart, and W. C. Van Voorhis. 2000b. Multiple alleles of Treponema pallidum repeat gene D in Treponema pallidum isolates. J. Bacteriol. 182:2332–2335.

Chamberlain, N. R., M. E. Brandt, A. L. Erwin, J. D. Radolf, and M. V. Norgard. 1989a. Major integral membrane protein immunogens of Treponema pallidum are proteolipids. Infect. Immun. 57:2872–2877.

Chamberlain, N. R., L. DeOgny, C. Slaughter, J. D. Radolf, and M. V. Norgard. 1989b. Acylation of the 47-kilodalton major membrane immunogen of Treponema pallidum determines its hydrophobicity. Infect. Immun. 57:2878–2885.

Champion, C. I., J. N. Miller, M. A. Lovett, and D. R. Blanco. 1990. Cloning, sequencing, and expression of two class B endoflagellar genes of Treponema pallidum subsp. pallidum encoding the 34.5- and 31.0-kilodalton proteins. Infect. Immun. 58:1697–1704.

Champion, C. I., D. R. Blanco, M. M. Exner, H. Erdjument-Bromage, R. E. Hancock, P. Tempst, J. N. Miller, and M. A. Lovett. 1997. Sequence analysis and recombinant expression of a 28-kilodalton Treponema pallidum subsp. pallidum rare outer membrane protein (Tromp2). J. Bacteriol. 179:1230–1238.

Chan, E. C. S., R. Siboo, T. Keng, N. Psarra, R. Hurley, S.-L. Cheng, and I. Iugovaz. 1993a. Treponema denticola (ex Brumpt 1925) sp. nov., nom. rev., and identification of new spirochete isolates from periodontal pockets. Int. J. Syst. Bacteriol. 43:196–203.

Chan, E. C. S., R. Siboo, L. Z. G. Touyz, Y. Qiu, and A. Klitorinos. 1993b. A successful method for quantifying viable oral anaerobic spirochetes. Oral Microbiol. Immunol. 8:80–83.

Chan, E. C., A. Klitorinos, S. Gharbia, S. D. Caudry, M. D. Rahal, and R. Siboo. 1996. Characterization of a 4.2-kb plasmid isolated from periodontopathic spirochetes. Oral Microbiol. Immunol. 11:365–368.

Chan, E. C. S., A. De Ciccio, R. McLaughlin, A. Klitorinos, and R. Siboo. 1997. An inexpensive solid medium for obtaining colony-forming units of oral spirochetes. Oral Microbiol. Immunol. 12:372–376.

Charon, N. W., E. P. Greenberg, M. B. Koopman, R. J. Limberger. 1992. Spirochete chemotaxis, motility, and the structure of the spirochetal periplasmic flagella. Res. Microbiol. 143:597–603.

Cheli, R., and C. Mortellaro. 1974. *In:* Presented at the Proceedings of the 8th International Conference on Diseases of Cattle. Milan, Italy.

Chi, B., S. Chauhan, and H. Kuramitsu. 1999. Development of a system for expressing heterologous genes in the oral spirochete Treponema denticola and its use in expression of the Treponema pallidum flaA gene. Infect Immun. 67:3653–3656.

Chi, B., R. J. Limberger, and H. K. Kuramitsu. 2002. Complementation of a Treponema denticola flgE mutant with a novel coumermycin A1-resistant T. denticola shuttle vector system. Infect. Immun. 70:2233–2237.

Choi, B. K., B. J. Paster, F. E. Dewhirst, and U. B. Göbel. 1994. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect. Immun. 62:1889–1895.

Choi, B. K., H. Nattermann, S. Grund, W. Haider, and U. B. Göbel. 1997. Spirochetes from digital dermatitis lesions in cattle are closely related to treponemes associated with human periodontitis. Int. J. Syst. Bacteriol. 47:175–181.

Cockayne, A., M. J. Bailey, and C. W. Penn. 1987. Analysis of sheath and core structures of the axial filament of Treponema pallidum. J. Gen. Microbiol. 133:1397–1407.

Cockayne, A., R. Sanger, A. Ivic, R. A. Strugnell, J. H. MacDougall, R. R. Russell, and C. W. Penn. 1989. Antigenic and structural analysis of Treponema denticola. J. Gen. Microbiol. 135:3209–3218.

Collighan, R. J., R. D. Naylor, P. K. Martin, B. A. Cooley, N. Buller, and M. J. Woodward. 2000. A spirochete isolated from a case of severe virulent ovine foot disease is closely related to a Treponeme isolated from human periodontitis and bovine digital dermatitis. Vet. Microbiol. 74:249–257.

Cox, C. D. 1983. Metabolic activities. *In:* R. F. Schell and D. M. Musher (Eds.). Pathogenesis and Immunology of Treponemal Infection. Marcel Dekker. New York, NY. 57–70.

Cox, D. L., R. A. Moeckli, and A. H. Fieldsteel. 1984. Cultivation of pathogenic Treponema in tissue cultures of Sf1Ep cells. In Vitro 20:879–883.

Cox, D. L., B. Riley, P. Chang, S. Sayahtaheri, S. Tassell, and J. Hevelone. 1990. Effects of molecular oxygen, oxidation-reduction potential, and antioxidants upon in vitro replication of Treponema pallidum subsp. pallidum. Appl. Environ. Microbiol. 56:3063–3072.

Cox, D. L. 1994. Culture of Treponema pallidum. Meth. Enzymol. 236:390–405.

Cox, D. L., D. R. Akins, S. F. Porcella, M. V. Norgard, and J. D. Radolf. 1995. Treponema pallidum in gel microdroplets: a novel strategy for investigation of treponemal molecular architecture. Molec. Microbiol. 15:1151–1164.

Cox, D. L., and J. D. Radolf. 2001. Insertion of fluorescent fatty acid probes into the outer membranes of the pathogenic spirochaetes Treponema pallidum and Borrelia burgdorferi. Microbiology 147:1161–1169.

Cunningham, T. M., E. M. Walker, J. N. Miller, and M. A. Lovett. 1988. Selective release of the Treponema pallidum outer membrane and associated polypeptides with Triton X-114. J. Bacteriol. 170:5789–5796.

Cwyk, W. M., and E. Canale-Parola. 1979. Treponema succinifaciens sp. nov., an anaerobic spirochete from the swine intestine. Arch. Microbiol. 122:231–239.

Dahle, U. R., L. Tronstad, and I. Olsen. 1993a. Observation of an unusually large spirochete in endodontic infection. Oral Microbiol. Immunol. 8:251–253.

Dahle, U. R., L. Tronstad, and I. Olsen. 1993b. Spirochaetes in oral infections. Endo. Dent. Traumatol. 9:87–94.

Dahle, U. R., I. Olsen, L. Tronstad, and D. A. Caugant. 1995. Population genetic analysis of oral treponemes by multilocus enzyme electrophoresis. Oral Microbiol. Immunol. 10:265–270.

Dahle, U. R., L. Tronstad, and I. Olsen. 1996. 3-hydroxy fatty acids in a lipopolysaccharide-like material from Treponema denticola strain FM. Endo. Dent. Traumatol. 12:202–205.

Demirkan, I., S. D. Carter, C. A. Hart, and M. J. Woodward. 1999. Isolation and cultivation of a spirochaete from bovine digital dermatitis. Vet. Record 145:497–498.

Demirkan, I., S. D. Carter, C. Winstanley, K. D. Bruce, N. M. McNair, M. Woodside, and C. A. Hart. 2001. Isolation and characterisation of a novel spirochaete from severe virulent ovine foot rot. J. Med. Microbiol. 50:1061–1068.

Dewhirst, F. E., M. A. Tamer, R. E. Ericson, C. N. Lau, V. A. Levanos, S. K. Boches, J. L. Galvin, and B. J. Paster. 2000. The diversity of periodontal spirochetes by 16S rRNA analysis. Oral Microbiol. Immunol. 15:196–202.

DiRienzo, J. M., S. Cornell, and H. Boehringer. 1991. Use of randomly cloned DNA fragments for the identification of oral spirochetes. Oral Microbiol. Immunol. 6:88–96.

Dopfer, D., A. Koopmans, F. A. Meijer, I. Szakall, Y. H. Schukken, W. Klee, R. B. Bosma, J. L. Cornelisse, A. J. van Asten, and A. A. ter Huurne. 1997. Histological and bacteriological evaluation of digital dermatitis in cattle, with special reference to spirochaetes and Campylobacter faecalis. Vet. Record 140:620–623.

Duhamel, G. E. 2001. Comparative pathology and pathogenesis of naturally acquired and experimentally induced colonic spirochetosis. Anim. Health Res. Rev. 2:3–17.

Eipert, S. R., and S. H. Black. 1979. Characterization of the cytoplasmic fibrils of Treponema refringens (Nichols). Arch. Microbiol. 120:205–214.

el-Ghoul, W., and B. I. Shaheed. 2001. Ulcerative and papillomatous digital dermatitis of the pastern region in dairy cattle: clinical and histopathological studies. Dtsch. Tierarztl. Wochenschr. 108:216–222.

Fenno, J. C., G. W. Wong, P. M. Hannam, and B. C. McBride. 1998. Mutagenesis of outer membrane virulence determinants of the oral spirochete Treponema denticola. FEMS Microbiol. Lett. 163:209–215.

Fiehn, N.-E. 1989. Nutrient and environmental growth factors for nine oral small-sized spirochete strains containing one endoflagellum from each end. APMIS 97:287–296.

Fiehn, N.-E., J. M. Bangsborg, and H. Colding. 1995. Ribotyping on small-sized spirochetes isolated from subgingival plaque. Oral Microbiol. Immunol. 10:13–18.

Fieldsteel, A. H., D. L. Cox, and R. A. Moeckli. 1981. Cultivation of virulent Treponema pallidum in tissue culture. Infect. Immun. 32:908–915.

Fieldsteel, A. H., D. L. Cox, and R. A. Moeckli. 1982. Further studies on replication of virulent Treponema pallidum in tissue cultures of Sf1Ep cells. Infect. Immun. 35:449–455.

Fieldsteel, A. H. 1983. Genetics. *In:* R. F. Schell and D. M. Musher (Ed.). Pathogenesis and Immunology of Treponemal Infection. Marcel Dekker. New York, NY. 39–54.

Fraser, C. M., S. J. Norris, G. M. Weinstock, O. White, G. G. Sutton, R. Dodson, M. Gwinn, E. K. Hickey, R. Clayton, K. A. Ketchum, E. Sodergren, J. M. Hardham, M. P. McLeod, S. Salzberg, J. Peterson, H. Khalak, D. Richardson, J. K. Howell, M. Chidambaram, T. Utterback, L. McDonald, P. Artiach, C. Bowman, M. D. Cotton, and J. C. Venter. 1998. Complete genome sequence of Treponema pallidum, the syphilis spirochete. Science 281:375–388.

Garrity, G. M. (Ed.). 2001. Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY.

Gjestland, T. 1955. The Oslo study of untreated syphilis: An epidemiologic investigation of the natural course of syphilis infection based upon a re-study of the Boeck-Bruusgaard material. Acta Dermato. Vener. 35:1–368.

Hampp, E. G., and M. S. Bethesda. 1943. Method for routine isolation and cultivation of smaller oral treponemes. Am. J. Dent. Assoc. 30:1066–1075.

Hanson, A. W., and G. R. Cannefax. 1964. Isolation of Borrelia refringens in pure culture from patients with condylomata acuminata. J. Bacteriol. 88:111–113.

Hanson, A. W. 1970. Isolation of spirochetes from primates and other mammalian species. Br. J. Ven. Dis. 46:303–306.

Hardham, J. M., L. V. Stamm, S. F. Porcella, J. G. Frye, N. Y. Barnes, J. K. Howell, S. L. Mueller, J. D. Radolf, G. M. Weinstock, and S. J. Norris. 1997. Identification and transcriptional analysis of a Treponema pallidum operon encoding a putative ABC transport system, an iron-activated repressor protein homolog, and a glycolytic pathway enzyme homolog. Gene 197:47–64.

Harland, W. A., and F. D. Lee. 1967. Intestinal spirochaetosis. Br. Med. J. 3:718–719.

Hazlett, K. R., T. J. Sellati, T. T. Nguyen, D. L. Cox, M. L. Clawson, M. J. Caimano, and J. D. Radolf. 2001. The TprK protein of Treponema pallidum is periplasmic and is not a target of opsonic antibody or protective immunity. J. Exp. Med. 193:1015–1026.

Heinzerling, H. F., M. Olivares, and R. A. Burne. 1997. Genetic and transcriptional analysis of flgB flagellar operon constituents in the oral spirochete Treponema denticola and their heterologous expression in enteric bacteria. Infect. Immun. 65:2041–2051.

Holdemann, L. V., L. P. Cato, and W. L. C. Moore. 1977. Anaerobe Laboratory Manual, 4th ed.. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Holt, S. C. 1978. Anatomy and chemistry of spirochetes. Microbiol. Rev. 42:114–160.

Houston, L. S., R. G. Cook, and S. J. Norris. 1990. Isolation and characterization of a Treponema pallidum major 60-kilodalton protein resembling the GroEL protein of Escherichia coli. J. Bacteriol. 172:2862–2870.

Hovind-Hougen, K., and A. Birch-Andersen. 1971. Electron microscopy of endoflagella and microtubules in Treponema Reiter. Acta Pathol. Microbiol. Scand. Sec. B 79:37–50.

Hovind-Hougen, K. 1976. Determination by means of electron microscopy of morphological criteria of value for classification of some spirochetes, in particular treponemes. Acta. Path. Microbiol. Scand. Sect. B., Supp. 255:1–41.

Hovind-Hougen, K., A. Birch-Andersen, R. Henrik-Nielsen, M. Orholm, J. O. Pedersen, P. S. Teglbjaerg, and E. H. Thaysen. 1982. Intestinal spirochetosis: morphological characterization and cultivation of the spirochete Brachyspira aalborgi gen. nov., sp. nov. J. Clin. Microbiol. 16:1127–1136.

Hovind-Hougen, K. 1983. Pathogenesis and Immunology of Treponemal Infection. Marcel Dekker. New York, NY.

Isaacs, R. D., J. H. Hanke, L. M. Guzman-Verduzco, G. Newport, N. Agabian, M. V. Norgard, S. A. Lukehart, and J. D. Radolf. 1989. Molecular cloning and DNA sequence analysis of the 37-kilodalton endoflagellar sheath protein gene of Treponema pallidum. Infect Immun. 57:3403–3411.

Isaacs, R. D., and J. D. Radolf. 1990. Expression in Escherichia coli of the 37-kilodalton endoflagellar sheath protein of Treponema pallidum by use of the polymerase chain reaction and a T7 expression system. Infect Immun. 58:2025–2034.

Izard, J., W. A. Samsonoff, M. B. Kinoshita, and R. J. Limberger. 1999. Genetic and structural analyses of cytoplasmic filaments of wild-type Treponema phagedenis and a flagellar filament-deficient mutant. J. Bacteriol. 181:6739–6746.

Izard, J., W. A. Samsonoff, and R. J. Limberger. 2001. Cytoplasmic filament-deficient mutant of Treponema denticola has pleiotropic defects. J. Bacteriol. 183:1078–1084.

Jenkin, H. M., and P. L. Sandok. 1983. In vitro cultivation of Treponema pallidum. *In:* R. F. Schell and D. M. Musher (Eds.). Pathogenesis and Immunology of Treponemal Infection. Marcel Dekker. New York, NY. 71–98.

Johnson, R. C., D. M. Ritzi, and B. P. Livermore. 1973. Outer envelope of virulent Treponema pallidum. Infect. Immun. 8:291–295.

Jones, S. A., K. S. Marchitto, J. N. Miller, and M. V. Norgard. 1984. Monoclonal antibody with hemagglutination, immunobilization, and neutralization activities defines an immunodominant, 47,000 mol wt, surface-exposed immunogen of Treponema pallidum (Nichols). J. Exp. Med. 160:1404–1420.

Jovanovi'c, T., C. Ascenso, K. R. Hazlett, R. Sikkink, C. Krebs, R. Litwiller, L. M. Benson, I. Moura, J. J. Moura, J. D. Radolf, B. H. Huynh, S. Naylor, and F. Rusnak. 2000. Neelaredoxin, an iron-binding protein from the syphilis spirochete, Treponema pallidum, is a superoxide reductase. J. Biol. Chem. 275:28439–28448.

Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules. *In:* H. N. Munro (Ed.). Mammalian Protein Metabolism. . 3:21–132.

Jung, I. Y., B. K. Choi, K. Y. Kum, B. D. Roh, S. J. Lee, C. Y. Lee, and D. S. Park. 2000. Molecular epidemiology and association of putative pathogens in root canal infection. J. Endod. 26:599–604.

Kataoka, M., H. Li, S. Arakawa, and H. Kuramitsu. 1997. Characterization of a methyl-accepting chemotaxis protein gene, dmcA, from the oral spirochete Treponema denticola. Infect. Immun. 65:4011–4016.

Konishi, H., Z. Yoshii, and D. L. Cox. 1986. Electron microscopy of Treponema pallidum (Nichols) cultivated in tissue cultures of Sf1Ep cells. Infect. Immun. 53:32–37.

Koseki, T., I. Ishikawa, M. Umeda, and Y. Benno. 1995. Characterization of oral treponemes isolated from human periodontal pockets. Oral Microbiol. Immunol. 10:271–277.

Koseki, T., Y. Benno, Y. J. Zhang-Koseki, M. Umeda, and I. Ishikawa. 1996a. Detection frequencies and the colony-

forming unit recovery of oral treponemes by different cultivation methods. Oral Microbiol. Immunol. 11:203–208.

Koseki, T., I. Ishikawa, E. Boutsi, T. He, and Y. Benno. 1996b. Nutritional analysis and an enriched medium for fermentative treponemes isolated from subgingival plaque. Oral Microbiol. Immunol. 11:166–171.

Larsen, S. A., V. Pope, R. E. Johnson, and E. J. Kennedy Jr. 1998. A Manual of Tests for Syphilis, 9th ed. American Public Health Association. Washington, DC.

Larsen, S. A., S. J. Norris, and V. Pope. 1999. Treponema and other host-associated spirochetes. *In:* P. R. Murray (Ed.). Manual of Clinical Microbiology. ASM Press. Washington, DC. 759–776.

Laughon, B., S. A. Syed, and W. J. Loesche. 1982. API ZYM system for identification of Bacteroides spp., Capnocytophaga spp., and spirochetes of oral origin. J. Clin. Microbiol. 15:97–102.

Leach, W. D., A. Lee, and R. P. Stubbs. 1973. Localization of bacteria in the gastrointestinal tract: a possible explanation of intestinal spirochetosis. Infect. Immun. 7:961–972.

Lee, F. D., A. Krazewski, J. Gordon, J. G. R. Howie, D. McSeveney, and W. A. Harland. 1971. Intestinal spirochetosis. J. Br. Soc. 12:126–133.

Lee, A., and M. Philips. 1978. Isolation and cultivation of spirochetes and other spiral shaped bacteria associated with the cecal mucosa of rats and mice. Appl. Environ. Microbiol. 35:610–613.

Lee, Y. H., R. K. Deka, M. V. Norgard, J. D. Radolf, and C. A. Hasemann. 1999. Treponema pallidum TroA is a periplasmic zinc-binding protein with a helical backbone. Nat. Struct. Biol. 6:628–633.

Lee, Y. H., M. R. Dorwart, K. R. Hazlett, R. K. Deka, M. V. Norgard, J. D. Radolf, and C. A. Hasemann. 2002. The crystal structure of Zn(II)-free Treponema pallidum TroA, a periplasmic metal-binding protein, reveals a closed conformation. J. Bacteriol. 184:2300–2304.

Leschine, S. B., and E. Canale-Parola. 1980. Rifampicin as a selective agent for isolation of oral spirochetes. J. Clin. Microbiol. 12:792–795.

Li, H., J. Ruby, N. Charon, and H. Kuramitsu. 1996. Gene inactivation in the oral spirochete Treponema denticola: construction of an flgE mutant. J. Bacteriol. 178:3664–3667.

Li, H., S. Arakawa, Q. D. Deng, and H. Kuramitsu. 1999. Characterization of a novel methyl-accepting chemotaxis gene, dmcB, from the oral spirochete Treponema denticola. Infect Immun. 67:694–699.

Li, C., A. Motaleb, M. Sal, S. F. Goldstein, and N. W. Charon. 2000. Spirochete periplasmic flagella and motility. J. Molec. Microbiol. Biotechnol. 2:345–354.

Lilburn, T. G., T. M. Schmidt, and J. A. Breznak. 1999. Phylogenetic diversity of termite gut spirochaetes. Environ. Microbiol. 1:331–345.

Limberger, R. J., and N. W. Charon. 1986. Treponema phagedenis has at least two proteins residing together on its periplasmic flagella. J. Bacteriol. 166:105–112.

Limberger, R. J., L. L. Slivienski, M. C. El-Afandi, and L. A. Dantuono. 1996. Organization, transcription, and expression of the 5' region of the fla operon of Treponema phagedenis and Treponema pallidum. J. Bacteriol. 178:4628–4634.

Livermore, B. P., and R. C. Johnson. 1975. The lipids of four unusual non-pathogenic host-associated spirochetes. Can. J. Microbiol. 21:1877–1880.

Loesche, W. J., and S. S. Socransky. 1962. Defect in small millipore filters disclosed by new technique for isolating oral treponemes. Science 138:139–140.

MacDougall, J., D. Margarita, and I. Saint Girons. 1992. Homology of a plasmid from the spirochete Treponema denticola with the single-stranded DNA plasmids. J. Bacteriol. 174:2724–2728.

MacDougall, J., and I. Saint Girons. 1995. Physical map of the Treponema denticola circular chromosome. J. Bacteriol. 177:1805–1811.

Masuda, K., and T. Kawata. 1989. Isolation and characterization of cytoplasmic fibrils from treponemes. Microbiol. Immunol. 33:619–630.

McLeod, C. G., J. L. Stookey, D. G. Harrington, and J. D. White. 1977. Intestinal Tyzer's disease and spirochetosis in a guinea pig. Vet. Pathol. 14:229–235.

Miao, R., and A. H. Fieldsteel. 1978. Genetics of Treponema: relationship between Treponema pallidum and five cultivable treponemes. J. Bacteriol. 133:101–107.

Miao, R. M., and A. H. Fieldsteel. 1980. Genetic relationship between Treponema pallidum and Treponema pertenue, two noncultivable human pathogens. J. Bacteriol. 141:427–429.

Miller, J. N. 1971. Spirochetes in Body Fluids and Tissues: Manual of Investigative Methods. Charles C. Thomas. Springfield, IL.

Miller, J. N. 1987. Nonvenereal treponematoses. *In:* R. D. Feigin and J. D. Cherry (Eds.). Textbook of Pediatric Infectious Diseases. 587–595.

Miller, J. N., R. M. Smibert, and S. J. Norris. 1990. The genus treponema. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.). The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 3537–3559.

Moore, W. E. C., L. V. Holdemann, R. M. Smibert, I. J. Good, J. A. Burmeister, K. G. Palcanis, and R. R. Ranney. 1982. Bacteriology of experimental gingivitis in young adult humans. Infect. Immun. 38:651–667.

Moore, L. V. H., W. E. C. Moore, E. P. Cato, R. M. Smibert, J. A. Burmeister, A. M. Best, and R. R. Ranney. 1987. Bacteriology of human gingivitis. J. Dent. Res. 66:989–995.

Moore, W. E., and L. V. Moore. 1994. The bacteria of periodontal diseases. Periodontology 2000 5:66–77.

Moter, A., C. Hoenig, B. Choi, B. Riep, and U. Göbel. 1998a. Molecular epidemiology of oral treponemes associated with periodontal disease. J. Clin. Microbiol. 36:1399–1403.

Moter, A., G. Leist, R. Rudolph, K. Schrank, B. K. Choi, M. Wagner, and U. B. Göbel. 1998b. Fluorescence in situ hybridization shows spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions. Microbiology 144:2459–2467.

Mumba, T., D. Dopfer, C. Kruitwagen, M. Dreher, W. Gaastra, and B. A. van der Zeijst. 1999a. Detection of spirochetes by polymerase chain reaction and its relation to the course of digital dermatitis after local antibiotic treatment in dairy cattle. Zentralbl. Veterinarmed. [B] 46:117–126.

Mumba, T., D. Dopfer, C. Kruitwagen, M. Dreher, W. Gaastra, and B. A. van der Zeijst. 1999b. Detection of spirochetes by polymerase chain reaction and its relation to the course of digital dermatitis after local antibiotic treatment in dairy cattle. Zentralbl Veterinarmed [B] 46:117–126.

Nakazawa, F., T. Umemoto, I. Namikawa, and E. Hoshino. 1997. Genetic characterization of an oral treponeme iso-

lated from human subgingival plaque. Oral Microbiol. Immunol. 12:189–192.

Naylor, R. D., P. K. Martin, J. R. Jones, and M. C. Burnell. 1998. Isolation of spirochaetes from an incident of severe virulent ovine footrot. Vet. Record 143:690–691.

Newman, M. G. 1984. Anaerobic oral and dental infection. Rev. Infect. Dis. 6, Suppl. 1:S107–S114.

Norris, S. J., and S. Sell. 1984. Antigenic complexity of Treponema pallidum: antigenicity and surface localization of major polypeptides. J. Immunol. 133:2686–2692.

Norris, S. J., and D. G. Edmondson. 1986a. Factors affecting the multiplication and subculture of Treponema pallidum subsp. pallidum in a tissue culture system. Infect. Immun. 53:534–539.

Norris, S. J., and D. G. Edmondson. 1986b. Serum requirement for the multiplication of Treponema pallidum in a tissue-culture system: Association of growth-promoting activity with the protein fraction. Sex. Transm. Dis. 13:207–213.

Norris, S. J., J. F. Alderete, N. H. Axelsen, M. J. Bailey, S. A. Baker-Zander, J. B. Baseman, P. J. Bassford, R. E. Baughn, A. Cockayne, P. A. Hanff, P. Hindersson, S. A. Larsen, M. A. Lovett, S. A. Lukehart, J. N. Miller, M. A. Moskiphidis, F. Miller, M. V. Norgard, C. W. Penn, L. V. Stamm, J. D. van Embden, and K. Wicher. 1987. Identity of Treponema pallidum susp. pallidum polypeptides: Correlation of sodium dodecyl sulfate-polyacylamide gel electrophoresis results from different laboratories. Electrophoresis 8:77–92.

Norris, S. J. 1988a. Syphilis. *In:* D. M. Wright (Ed.). Immunology of Sexually Transmitted Diseases. MTP Press. Lancaster, UK. 1–31.

Norris, S. J., N. W. Charon, R. G. Cook, M. D. Fuentes, and R. J. Limberger. 1988b. Antigenic relatedness and N-terminal sequence homology define two classes of periplasmic flagellar proteins of Treponema pallidum subsp, pallidum and Treponema phagedenis. J. Bacteriol. 170:4072–4082.

Norris, S. J., and Treponema pallidum Polypeptide Research Group. 1993. Polypeptides of Treponema pallidum: Progress toward understanding their structural, functional, and immunologic roles. Microbiol. Rev. 57:750–779.

Norris, S. J., D. L. Cox, and G. M. Weinstock. 2001. Biology of Treponema pallidum: correlation of functional activities with genome sequence data. J. Molec. Microbiol. Biotechnol. 3:37–62.

Ochiai, S., Y. Adachi, and K. Mori. 1997. Unification of the genera Serpulina and Brachyspira, and proposals of Brachyspira hyodysenteriae Comb. Nov., Brachyspira innocens comb. nov. and Brachyspira pilosicoli comb. nov. Microbiol. Immunol. 41:445–452.

Ohkuma, M., T. Iida, and T. Kudo. 1999. Phylogenetic relationships of symbiotic spirochetes in the gut of diverse termites. FEMS Microbiol. Lett. 181:123–129.

Okuda, K., K. Ishihara, T. Nakagawa, A. Hirayama, and Y. Inayama. 2001. Detection of Treponema denticola in atherosclerotic lesions. J. Clin. Microbiol. 39:1114–1117.

Osborne, V. E., and R. V. S. Bain. 1961. Genital infection of a horse with spirochetes. Austral. Vet. J. 37:190–191.

Otha, K., K. K. Makinen, and W. Loesche. 1986. Purification and characterization of an enzyme produced by Treponema denticola capable of hydrolysing synthetic trypsin substrates. Infect. Immun. 53:213–220.

Pallesen, L., and P. Hindersson. 1989. Cloning and sequencing of a Treponema pallidum gene encoding a 31.3-kilodalton endoflagellar subunit (FlaB2). Infect. Immun. 57:2166–2172.

Parr, L. W. 1923. Intestinal spirochetes. J. Infect. Dis. 33:369–383.

Paster, B. J., and E. Canale-Parola. 1982. Physiological diversity of rumen spirochetes. Appl. Environ. Microbiol. 43:686–693.

Paster, B. J., and E. Canale-Parola. 1985. Treponema saccharophilum sp. nov., a large pectinolytic spirochete from the bovine rumen. Appl. Environ. Microbiol. 50:212–219.

Paster, B. J., F. E. Dewhirst, W. G. Weisburg, L. A. Tordoff, G. J. Fraser, R. B. Hespell, T. B. Stanton, L. Zablen, L. Mandelco, and C. R. Woese. 1991. Phylogenetic analysis of spirochetes. J. Bacteriol. 173:6101–6109.

Paster, B. J., F. E. Dewhirst, S. M. Cooke, V. Fussing, L. K. Poulsen, and J. A. Breznak. 1996. Phylogeny of not-yet-cultured spirochetes from termite guts. Appl. Environ. Microbiol. 62:347–352.

Paster, B. J., F. E. Dewhirst, B. C. Coleman, C. N. Lau, and R. L. Ericson. 1998. Phylogenetic analysis of cultivable oral treponemes from the Smibert collection. Int. J. Syst. Bacteriol. 48:713–722.

Paster, B., and F. Dewhirst. 2000. Phylogenetic foundation of spirochetes. J. Molec. Microbiol. Biotechnol. 2:341–344.

Paster, B., S. Boches, J. Galvin, R. Ericson, C. Lau, V. Levanos, A. Sahasrabudhe, and F. Dewhirst. 2001. Bacterial diversity in subgingival plaque. J. Bacteriol. 183:3770–3783.

Penn, C. W., M. J. Bailey, and A. Cockayne. 1985a. The outer membrane of Treponema pallidum: biological significance and biochemical properties. J. Gen. Microbiol. 131:2349–2357.

Penn, C. W., A. Cockayne, and M. J. Bailey. 1985b. The outer membrane of Treponema pallidum: biological significance and biochemical properties. J. Gen. Microbiol. 131:2349–2357.

Pillay, A., H. Liu, C. Y. Chen, B. Holloway, W. Sturm, B. Steiner, and S. A. Morse. 1998. Molecular subtyping of Treponema pallidum subspecies pallidum. Sex. Transm. Dis. 25:408–414.

Pindak, F. F., W. E. Clapper, and J. H. Sherod. 1965. Incidence and distribution of spirochetes in the digestive tract of dogs. Am. J. Vet. Res. 26:1391–1402.

Qiu, Y. S., A. Klitorinos, M. D. Rahal, R. Siboo, and E. C. S. Chan. 1994. Enumeration of viable oral spirochetes from peridontal pockets. Oral Microbiol. Immunol. 9:301–304.

Radolf, J. D., D. R. Blanco, J. N. Miller, and M. A. Lovett. 1986. Antigenic interrelationship between endoflagella and Treponema phagedenis biotype Reiter and Treponema pallidum (Nichols): Molecular characterization of endoflagellar proteins. Infect. Immun. 54:626–634.

Radolf, J. D., and M. V. Norgard. 1988a. Pathogen specificity of Treponema pallidum subsp. pallidum integral membrane proteins identified by phase partitioning with Triton X-114. Infect. Immun. 56:1825–1828.

Radolf, J. D., N. R. Chamberlain, A. Clausell, and M. V. Norgard. 1988b. Identification and localization of integral membrane proteins of virulent Treponema pallidum identified by phase partitioning with the nonionic detergent Triton X-114. Infect. Immun. 56:490–498.

Radolf, J. D., M. V. Norgard, and W. W. Schulz. 1989. Outer membrane ultrastructure explains the limited antigenicity of virulent Treponema pallidum. Proc. Natl. Acad. Sci. USA 86:2051–2055.

Radolf, J. D. 1994. Role of outer membrane architecture in immune evasion by Treponema pallidum and Borrelia burgdorferi. Trends Microbiol. 2:307–311.

Radolf, J. D., B. Steiner, and D. Shevchenko. 1999. Treponema pallidum: doing a remarkable job with what it's got. Trends Microbiol. 7:7–9.

Riviere, G. R., K. S. Smith, N. Carranza Jr., E. Tzagaroulaki, S. L. Kay, and M. Dock. 1995. Subgingival distribution of Treponema denticola, Treponema socranskii, and pathogen-related oral spirochetes: Prevalence and relationship to periodontal status of sampled sites. J. Periodontol. 66:829–837.

Robertson, S. M., J. R. Kettman, J. N. Miller, and M. V. Norgard. 1982. Murine monoclonal antibodies specific for virulent Treponema pallidum (Nichols). Infect. Immun. 36:1076–1085.

Rocas, I. N., J. F. Siqueira, Jr., K. R. Santos, and A. M. Coelho. 2001. "Red complex" (Bacteroides forsythus, Porphyromonas gingivalis, and Treponema denticola) in endodontic infections: a molecular approach. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 91:468–471.

Rosebury, T., and G. Foley. 1941. Isolation and pure cultivation of the smaller mouth spirochetes by an improved method. Proc. Soc. Exp. Biol. Med. 47:368–374.

Ruane, P. J., M. M. Nakata, J. F. Reinhardt, and W. L. George. 1989. Spirochete-like organisms in the human gastrointestinal tract. Rev. Infect. Dis. 11:184–196.

Ruby, J. D., H. Li, H. Kuramitsu, S. J. Norris, S. F. Goldstein, K. F. Buttle, and N. W. Charon. 1997. Relationship of Treponema denticola periplasmic flagella to irregular cell morphology. J. Bacteriol. 179:1628–1635.

Rupf, S., S. Kannengiesser, K. Merte, W. Pfister, B. Sigusch, and K. Eschrich. 2000. Comparison of profiles of key periodontal pathogens in periodontium and endodontium. Endo. Dent. Traumatol. 16:269–275.

Saier, J., M. H., and J. Garcia-Lara. 2001. The Spirochetes: Molecular and Cellular Biology. Horizon Press. Wymondham, UK.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molec. Biol. Evol. 4:406–425.

Salvador, S. L., S. A. Syed, and W. J. Loesche. 1987. Comparison of three dispersion procedures for quantitative recovery of cultivable species of subgingival spirochetes. J. Clin. Microbiol. 25:2230–2232.

Sand-Peterson, C., N. S. Pedersen, and N. H. Axelsen. 1981. A simple method for the isolation of flagella from Treponema Reiter. Acta. Path. Microbiol. Scand., Sect. C. 89:379–385.

Sato, T., and H. K. Kuramitsu. 1999. Restriction fragment-length polymorphism analysis of 16S ribosomal RNA genes amplified by polymerase chain reaction for rapid identification of cultivable oral treponemes. Oral Microbiol. Immunol. 14:117–121.

Schiller, N. L., and C. D. Cox. 1977. Catabolism of glucose and fatty acids by virulent Treponema pallidum. Infect. Immun. 16:60–68.

Schrank, K., B. K. Choi, S. Grund, A. Moter, K. Heuner, H. Nattermann, and U. B. Göbel. 1999. Treponema brennaborense sp. nov., a novel spirochaete isolated from a dairy cow suffering from digital dermatitis. Int. J. Syst. Bacteriol. 49:43–50.

Schröder, N. W., B. Opitz, N. Lamping, K. S. Michelsen, U. Zahringer, U. B. Göbel, and R. R. Schumann. 2000. Involvement of lipopolysaccharide binding protein, CD14, and Toll-like receptors in the initiation of innate immune responses by Treponema glycolipids. J. Immunol. 165:2683–2693.

Schultz, C. P., V. Wolf, R. Lange, E. Mertens, J. Wecke, D. Naumann, and U. Zahringer. 1998. Evidence for a new type of outer membrane lipid in oral spirochete Treponema denticola. Functioning permeation barrier without lipopolysaccharides. Am. J. Phys. Anthropol. 106:229–248.

Sell, S., and S. J. Norris. 1983. The biology, pathology, and immunology of syphilis. Int. Rev. Exp. Pathol. 24:203–276.

Shera, A. G. 1962. Specific granular lesions associated with intestinal spirochetosis. Br. J. Surg. 50:68–77.

Shevchenko, D. V., D. R. Akins, E. J. Robinson, M. Li, O. V. Shevchenko, and J. D. Radolf. 1997. Identification of homologs for thioredoxin, peptidyl prolyl cis-trans isomerase, and glycerophosphodiester phosphodiesterase in outer membrane fractions from Treponema pallidum, the syphilis spirochete. Infect. Immun. 65:4179–4189.

Shevchenko, D. V., T. J. Sellati, D. L. Cox, O. V. Shevchenko, E. J. Robinson, and J. D. Radolf. 1999. Membrane topology and cellular location of the Treponema pallidum glycerophosphodiester phosphodiesterase (GlpQ) ortholog. Infect. Immun. 67:2266–2276.

Siqueira Jr., J. F., I. N. Rocas, A. Favieri, and K. R. Santos. 2000a. Detection of Treponema denticola in endodontic infections by 16S rRNA gene-directed polymerase chain reaction. Oral Microbiol. Immunol. 15:335–337.

Siqueira Jr., J. F., I. N. Rocas, R. Souto, M. de Uzeda, and A. P. Colombo. 2000b. Checkerboard DNA-DNA hybridization analysis of endodontic infections. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 89:744–748.

Smibert, R. M., and J. A. Burmeister. 1983. Treponema pectinovorum sp. nov. isolated from humans with periodontitis. Int. J. Syst. Bacteriol. 33:852–856.

Smibert, R. M. 1984a. Genus III: Treponema Schaudinn 1905, 1728^{AL}. *In:* N. R. Kreig and J. G. Holt (Eds.). Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:49–57.

Smibert, R. M., J. L. Johnson, and R. R. Ranney. 1984b. Treponema socranskii sp. nov., Treponema socranskii subsp. socranskii subsp. nov., Treponema socranskii subsp. buccale subsp. nov., and Treponema socranskii subsp. paredis subsp. nov. isolated from the human periodontia. Int. J. Syst. Bacteriol. 34:457–462.

Socransky, S. S., M. Listgarten, C. Hubersak, J. Cotmore, and A. Clark. 1969. Morphological and biochemical differentiation of three types of small oral spirochetes. J. Bacteriol. 98:878–882.

Stamm, L. V., R. L. Hodinka, P. B. Wyrick, and P. J. Bassford Jr. 1987. Changes in the cell surface properties of Treponema pallidum that occur during in vitro incubation of freshly extracted organisms. Infect. Immun. 55:2255–2261.

Stamm, L. V., and H. L. Bergen. 1999. Molecular characterization of a flagellar (fla) operon in the oral spirochete Treponema denticola ATCC 35405. FEMS Microbiol. Lett. 179:31–36.

Stamm, L. V., and H. L. Bergen. 2000. The sequence-variable, single-copy tprK gene of Treponema pallidum Nichols strain UNC and Street strain 14 encodes heterogeneous TprK proteins. Infect Immun. 68:6482–6486.

Stamm, L. V., and H. L. Bergen. 2001. Molecular characterization of the Treponema denticola fliQ region. DNA Seq. 12:463–467.

Stanton, T. B., and E. Canale-Parola. 1979. Enumeration and selective isolation of rumen spirochetes. Appl. Environ. Microbiol. 38:965–973.

Stanton, T. B., and E. Canale-Parola. 1980. Treponema bryantii sp. nov., a rumen spirochete that interacts with cellulolytic bacteria. Arch. Microbiol. 127:145–156.

St. Girons, I., B. Chi, and H. Kuramitsu. 2000. Development of shuttle vectors for spirochetes. J. Molec. Microbiol. Biotechnol. 2:443–445.

Subramanian, G., E. V. Koonin, and L. Aravind. 2000. Comparative genome analysis of the pathogenic spirochetes Borrelia burgdorferi and Treponema pallidum. Infect. Immun. 68:1633–1648.

Sunde, P. T., L. Tronstad, E. R. Eribe, P. O. Lind, and I. Olsen. 2000. Assessment of periradicular microbiota by DNA-DNA hybridization. Endo. Dent. Traumatol. 16:191–196.

Sutton, M. Y., H. Liu, B. Steiner, A. Pillay, T. Mickey, L. Finelli, S. Morse, L. E. Markowitz, and M. E. St. Louis. 2001. Molecular subtyping of Treponema pallidum in an Arizona County with increasing syphilis morbidity: use of specimens from ulcers and blood. J. Infect. Dis. 183:1601–1606.

Swancutt, M. A., J. D. Radolf, and M. V. Norgard. 1990. The 34-kilodalton membrane immunogen of Treponema pallidum is a lipoprotein. Infect. Immun. 58:384–392.

Swisher, B. L. 1987. Modified Steiner procedure for microwave staining of spirochetes and nonfilamentous bacteria. J. Histotechnol. 10:241–243.

Tajima, K., R. I. Aminov, T. Nagamine, K. Ogata, M. Nakamura, H. Matsui, and Y. Benno. 1999. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. FEMS Microbiol. Ecol. 29:159–169.

Takeuchi, A., H. R. Jervis, H. Nakazawa, and D. M. Robinson. 1974. Spiral-shaped organisms on the surface of colonic epithelium of the monkey and man. Am. J. Clin. Nutrit. 27:1287–1295.

Turner, T. B., and D. H. Hollander. 1957. *In:* Biology of the Treponematoses. World Health Organization. Geneva, Switzerland.

Umeda, M., I. Ishikawa, Y. Benno, and T. Mitsuoka. 1990. Improved detection of oral spirochetes with an anaerobic culture method. Oral Microbiol. Immunol. 5:90–94.

Umemoto, T., F. Nakazawa, E. Hoshino, K. Okada, M. Fukunaga, and I. Namikawa. 1997. Treponema medium sp. nov., isolated from human subgingival dental plaque. Int. J. Syst. Bacteriol. 47:67–72.

United States Public Health Service. 1968. Syphilis: A Synopsis. US Government Printing Office. Washington, DC.

Van de Peer, Y., and R. De Wachter. 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Applic. Biosci. 10:569–570.

Van Horn, K. G., and R. M. Smibert. 1982. Fatty acid requirement of Treponema denticola and Treponema vincentii. Can. J. Microbiol. 28:344–350.

Van Horn, K. G., and R. M. Smibert. 1983. Albumin requirement of Treponema denticola and Treponema vincentii. Can. J. Microbiol. 29:1141–1148.

Walker, E. M., G. A. Zampighi, D. R. Blanco, J. N. Miller, and M. A. Lovett. 1989. Demonstration of rare protein in the outer membrane of Treponema pallidum subsp. pallidum by freeze-fracture analysis. J. Bacteriol. 171:5005–5011.

Walker, E. M., L. A. Borenstein, D. R. Blanco, J. N. Miller, and M. A. Lovett. 1991. Analysis of outer membrane ultrastructure of pathogenic Treponema and Borrelia species by freeze-fracture electron microscopy. J. Bacteriol. 173:5585–5588.

Walker, R. L., D. H. Read, K. J. Loretz, and R. W. Nordhausen. 1995. Spirochetes isolated from dairy cattle with papillomatous digital dermatitis and interdigital dermatitis. Vet. Microbiol. 47:343–355.

Walker, S. G., X. Xu, E. Altman, K. J. Davis, J. L. Ebersole, and S. C. Holt. 1999. Isolation and chemical analysis of a lipopolysaccharide from the outer membrane of the oral anaerobic spirochete Treponema pectinovorum. Oral Microbiol. Immunol. 14:304–308.

Wang, B. Y., B. Chi, and H. K. Kuramitsu. 2002. Genetic exchange between Treponema denticola and Streptococcus gordonii in biofilms. Oral Microbiol. Immunol. 17:108–112.

Wecke, J., T. Kersten, K. Madela, A. Moter, U. B. Göbel, A. Friedmann, and J. Bernimoulin. 2000. A novel technique for monitoring the development of bacterial biofilms in human periodontal pockets. FEMS Microbiol. Lett. 191:95–101.

World Health Organization. 1998. The Health Report 1998: Life in the 21st Century—A Vision for All. World Health Organization.

World Health Organization. 2001. *In:* Global Prevalence and Incidence of Selected Curable Sexually Transmitted Diseases: Overview and Estimates. World Health Organization. New York, NY. WHO/HIV_AIDS/2001.02.

Wojciechowicz, M., and A. Ziolecki. 1979. Pectinolytic enzymes of large rumen treponemes. Appl. Environ. Microbiol. 37:136–142.

Wyss, C. 1992. Growth of Porphyromonas gingivalis, Treponema denticola, T. pectinovorum, T. socranskii, and T. vincentii in a chemically defined medium. J. Clin. Microbiol. 30:2225–2229.

Wyss, C., B. K. Choi, P. Schüpbach, B. Guggenheim, and U. B. Göbel. 1996. Treponema maltophilum sp. nov., a small oral spirochete isolated from human periodontal lesions. Int. J. Syst. Bacteriol. 46:745–752.

Wyss, C., B. K. Choi, P. Schüpbach, B. Guggenheim, and U. B. Göbel. 1997. Treponema amylovorum sp. nov., a saccharolytic spirochete of medium size isolated from an advanced human periodontal lesion. Int. J. Syst. Bacteriol. 47:842–845.

Wyss, C. 1998. Flagellins, but not endoflagellar sheath proteins, of Treponema pallidum and of pathogen-related oral spirochetes are glycosylated. Infect. Immun. 66:5751–5754.

Wyss, C., B. K. Choi, P. Schupbach, A. Moter, B. Guggenheim, and U. B. Göbel. 1999. Treponema lecithinolyticum sp. nov., a small saccharolytic spirochaete with phospholipase A and C activities associated with periodontal diseases. Int. J. Syst. Bacteriol. 49:1329–1339.

Wyss, C., F. Dewhirst, R. Gmür, T. Thurnheer, Y. Xue, P. Schüpbach, B. Guggenheim, and B. Paster. 2001. Treponema parvum sp. nov., a small, glucuronic or galacturonic acid-dependent oral spirochaete from lesions of human periodontitis and acute necrotizing ulcerative gingivitis. Int. J. Syst. Evol. Biol. 51:955–962.

Yotis, W. W., F. Macaluso, and C. Gopalsami. 1995. Immunochemical features of a macromolecule of Treponema denticola. J. Basic Microbiol. 35:255–268.

You, Y., S. Elmore, L. L. Colton, C. Mackenzie, J. K. Stoops, G. M. Weinstock, and S. J. Norris. 1996. Characterization

of the cytoplasmic filament protein gene (cfpA) of Treponema pallidum subsp. pallidum. J. Bacteriol. 178:3177–3187.

Yuan, K., C. J. Chang, P. C. Hsu, H. S. Sun, C. C. Tseng, and J. R. Wang. 2001. Detection of putative periodontal pathogens in non-insulin-dependent diabetes mellitus and non-diabetes mellitus by polymerase chain reaction. J. Periodont. Res. 36:18–24.

Zhang, H. H., D. R. Blanco, M. M. Exner, E. S. Shang, C. I. Champion, M. L. Phillips, J. N. Miller, and M. A. Lovett. 1999. Renaturation of recombinant Treponema pallidum rare outer membrane protein 1 into a trimeric, hydrophobic, and porin-active conformation. J. Bacteriol. 181:7168–7175.

Ziolecki, A. 1979. Isolation and characterization of large treponemes from the bovine rumen. Appl. Environ. Microbiol. 39:131–135.

Ziolecki, A., and M. Wojciechowicz. 1980. Small pectinolytic spirochetes from the rumen. Appl. Environ. Microbiol. 39:919–922.

Zymet, C. L. 1969. Canine spirochetosis and its association with diarrhea. VM/SAC 64:883–887.

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CHAPTER 4.3

The Genus *Borrelia*

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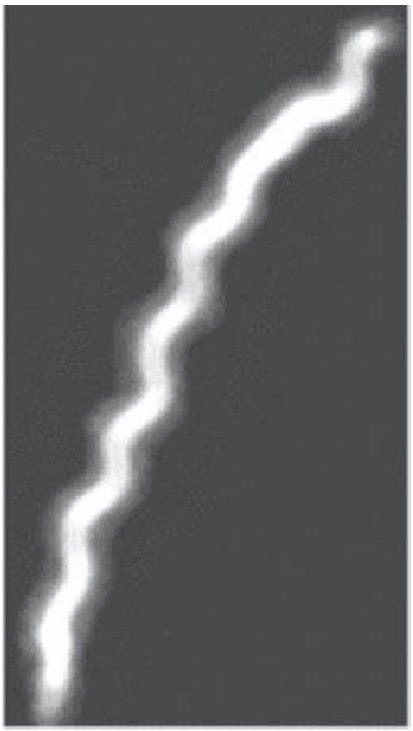
Phylogeny

The inability to cultivate many spirochetes, including the borreliae, at one time significantly hindered the placement of these organisms into a taxonomic scheme based on traditional biochemical methodologies. As a result, these organisms were initially grouped based primarily on their common helical or spiral shape (Fig. 1). The advent of contemporary ribosomal RNA cataloging has facilitated the division of the spirochetes into three phylogenetic families, the Spirochaetaceae (including the genera *Borrelia*, *Brevinema*, *Cristispira*, *Spirochaeta*, *Spironema* and *Treponema*), the Brachyspiraceae (including the genus *Brachyspira* [*Serpulina*]), and the Leptospiraceae (including the genera *Leptospira* and *Leptonema*; Thomas et al., 2001). All species in the genus *Borrelia* are transmitted to vertebrates by hematophagous (blood-feeding) arthropods. In most cases, the differentiation of borreliae species was based on 1) identification of the specific vector that transmits the spirochete, 2) the vertebrate host (humans, animals or birds), and 3) the variable infectivity of isolated borreliae for different species of laboratory animals (Davis, 1956; Felsenfeld, 1971; Burgdorfer, 1976a; Table 1). To date, thirty species of *Borrelia* have been recognized (Thomas et al., 2001). The principal species of *Borrelia* and their primary vectors are listed in Table 1. Additional information on *B. burgdorferi* sensu lato strains is also available: (http://www.pasteur.fr/recherche/borrelia/Bb_ strains_alphabetic.html.) With the exception of louse-borne relapsing fever, all of the borrelioses are zoonoses.

Borreliae, like most spirochetes, were historically considered to be Gram negative because of their double-membrane structure (Fig. 6), but as stated above, genetic analyses have placed them, along with the other spirochetes, into a separate eubacterial phylum (Paster et al., 1991). Ultrastructural (*Borrelia* Molecular Architecture), molecular, and biochemical studies also have emphasized the wide taxonomic gap between spirochetes and Gram-negative bacteria. Most notably, *Borrelia burgdorferi*, and presumably all *Borrelia* spp., does not contain lipopolysaccharide (LPS; Takayama et al., 1987). In addition to lacking LPS, borrelial outer membranes differ from those of Gram-negative bacteria in that they 1) exhibit considerably greater fluidity, 2) are more susceptible to physical manipulations (i.e., centrifugation, washing and resuspension; Brusca and Radolf, 1994; Cox et al., 1994; Cox et al., 1996), 3) are more easily solubilized in very dilute detergents than are the membranes of Gram-negative bacteria (Barbour and Hayes, 1986b; Brusca and Radolf, 1994; Cox et al., 1994; Cox et al., 1996), and 4) have a much lower density of transmembrane proteins (Walker et al., 1991; Radolf et al., 1994b; Radolf et al., 1995b). (For excellent reviews of *Borrelia* morphology and ultrastructure, see Hovind-Hougen, 1976, Barbour, 1989, Hayes and Burgdorfer, 1993, and Radolf, 1994a.)

Habitat

All known *Borrelia* species have an inherent requirement for a competent (often genospecies-specific) arthropod vector and vector-suitable mammalian host(s) to be maintained in their natural enzootic cycles (Table 1). Three types of arthropod vectors have been associated with transmission of *Borrelia*: the human body louse (Fig. 2), the argasid or soft tick (Fig. 3), and the ixodid or hard tick (Fig. 4). Numerous mammalian hosts appear to be involved in maintaining *Borrelia* within a population, with the primary reservoir usually being a small mammal. The coincident geographic distribution of both of these elements is thought to be foremost in establishing and perpetuating a given *Borrelia* species within a locale. This point is clearly demonstrated by Lyme borreliosis; the geographic distribution of its two arthropod vectors (Fig. 15), *Ixodes scapularis* and *I. pacificus*, closely mirrors that of the disease (Orloski et al., 2000; Fig. 16).



Darkfield Image of Spirochete

Fig. 1. *Borrelia burgdorferi* strain 297, a cerebrospinal isolate (Steere et al., 1983) visualized by darkfield microscopy. Micrograph image kindly provided by Ken Bourell, University of Connecticut Health Center, Center for Microbial Pathogenesis (cmp.uchc.edu).

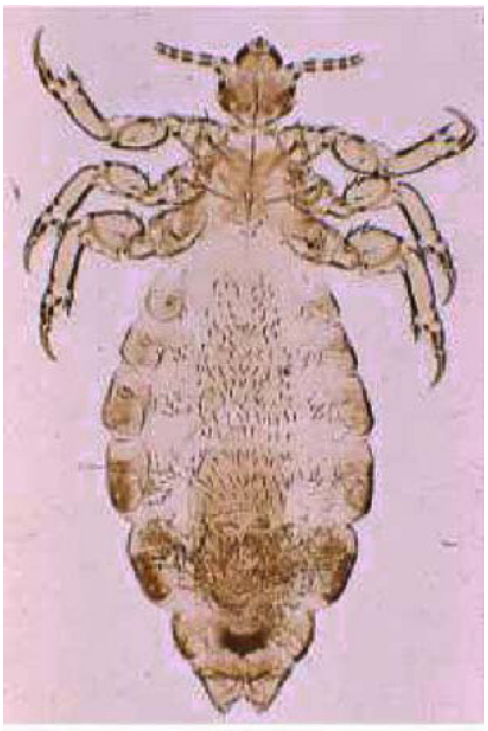


Image of a body louse

Fig. 2. Human body louse (*Pediculus humanus*). Image kindly provided by Stephen Wikel, Ph.D., University of Connecticut Health Center, Center for Microbial Pathogenesis (cmp.uchc.edu).

Presence in Lice

Borrelia recurrentis is the etiological agent of louse-borne (epidemic) relapsing fever and is the only species of borreliae that is louse-transmitted (Table 1). The human is the only known reservoir of this spirochete. The human body louse, *Pediculus humanus* subsp. *humanus* (Fig. 2), becomes infected after feeding on a spirochetemic individual and remains infected during its life span of approximately 10–61 days. The ingested borreliae enter the louse's midgut where, within several days, they penetrate the gut epithelium to gain entrance to the hemolymph where they multiply. Infection of the mammalian host is thought to occur when spirochetes, liberated when the lice are crushed on the skin by scratching, enter abraded skin or mucous membranes. Since body lice prefer normal body temperature to the higher temperatures of patients with acute relapsing fever, they are predisposed to seek an afebrile person after feeding on a febrile individual. This behavior results in the rapid transmission of infection during epidemics.

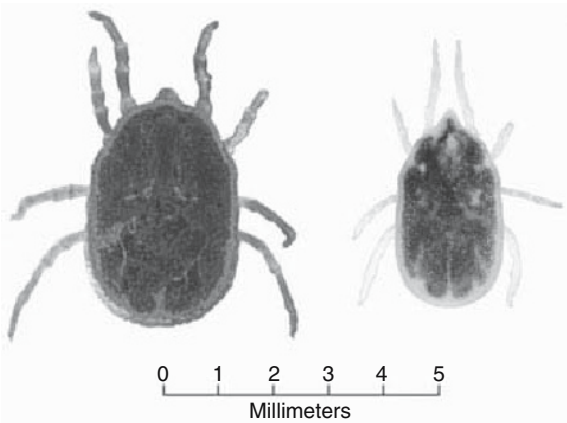


Fig. 3. Soft tick (*Ornithodoros* spp.). Image kindly provided by Julie Rawlings, MPH, Texas Department of Health (http://www.tdh.state.tx.us/yellow/r.htm).

Presence in Soft Ticks

All of the other pathogenic borreliae are parasites of either soft or hard ticks (Table 1). All tick-borne borrelial infections, with the possible exception of infections by *Borrelia duttonii*,

Table 1. Characteristics of *Borrelia* species of medical and veterinary significance.

Species	Vector	Animal Reservoir	Disease	Geographic distribution
B. recurrentis	*Pediculus humanus* subsp. *humanus*	Humans	Louse-borne relapsing fever	Worldwide
B. duttonii	*Ornithodoros moubata*	Humans	Old World tick-borne relapsing fever	Central, eastern and southern Africa
B. hispanica	*O. marocanus*	Rodents	Old World tick-borne relapsing fever	Spain, Portugal, Morocco, Algeria, Tunisia
B. crocidurae, *B. merionesi*, *B. microti*, *B. dipodilli*	*O. erraticus*	Rodents	Old World tick-borne relapsing fever	Northern and eastern Africa, Near and Middle East, southeastern Europe
B. persica	*O. tholozani*	Rodents	Old World tick-borne relapsing fever	Middle East, Greece, Central Asia, Russia, western China
B. caucasica	*O. verrucosus*	Rodents	Old World tick-borne relapsing fever	Iraq, eastern Europe
B. latyschewii	*O. tartakowskyi*	Rodents	Old World tick-borne relapsing fever	Iraq, Iran, Afghanistan, Russia
B. coriaceae	*O. coriaceus*	Rodents	Epizootic bovine abortion	California
	Alectorobius sonrai		Tick-borne relapsing fever	Western Africa
B. hermsii	*O. hermsi*	Rodents, chipmunks, tree squirrels	New World tick-borne relapsing fever	Canada, western USA
B. turicatae	*O. turicata*	Rodents	New World tick-borne relapsing fever	Southwestern USA
B. parkeri	*O. parkeri*	Rodents	New World tick-borne relapsing fever	Western USA
B. mazziottii	*O. talaje (O. dugesi)*	Rodents	New World tick-borne relapsing fever	Southwestern USA, Mexico, Central and South America
B. venezuelensis	*O. rudis*	Rodents	New World tick-borne relapsing fever	Central and South America
B. venezuelensis	*O. rudis*	Rodents	New World tick-borne relapsing fever	Central and South America
B. burgdorferi sensu lato	*Ixodes scapularis*	Rodents, Lagomorpha, Deer, Fowl	Lyme (borreliosis) disease	Midwestern and eastern USA
	I. pacificus			Western USA
	I. ricinus			Europe
	I. persulcanis			Asiatic Europe, China, Japan
B. lonestari	*Amblyomma americanum*	Rodents	Lyme disease-like illness	Southern USA
B. theileri	*Rhipicephalus evertsi*, *Boophilus microplus*, *B. annulatus*, *B. decoloratus*	Cattle, Horses, Sheep	Bovine borreliosis	South Africa, Australia, Brazil, Mexico
B. anserina	*Argas persicus* and other *Argas* spp.	Fowl	Avian borreliosis	Worldwide

Adapted from Barbour (1986), used with permission.

Image of Hard Ticks

Fig. 4. From left to right: The deer tick (*Ixodes scapularis*) adult female, adult male, nymph, and larva on a centimeter scale. Image taken from the Centers for Disease Control and Prevention (CDC) Lyme Disease Home Page (http://www.cdc.gov/ncidod/dvbid/lymeinfo.htm).

involve humans as an accidental host. In East Africa, *B. duttonii* is carried by the domestic soft tick, *Ornithodoros moubata*, and the reservoir for this spirochete appears to be the human population. The relapsing fever spirochetes are the primary borreliae transmitted by the soft tick (Fig. 3). The numerous species of *Borrelia* are responsible for the tick-borne relapsing fevers and are distributed worldwide in both tropical and temperate climates (Table 1). Soft ticks of the genus *Ornithodoros* serve as the primary vectors of these relapsing fever spirochetes, with wild rodents being the major reservoir. Occasionally, lizards, toads, turtles and owls can also serve as competent reservoirs for these spirochetes.

Soft ticks become infected while obtaining a blood meal from a spirochetemic animal. Following ingestion of a blood meal on an infected mammal, *Borrelia* rapidly multiply within the tick. Within hours, a generalized infection ensues such that spirochetes can be found in a variety of tissues, including the salivary glands, the coxal glands on the legs, and the ovaries. Consistent with the presence of spirochetes in the ovaries, the female *Ornithodoros* spp. tick, unlike the body louse, can transmit borreliae transovarially to their progeny. Humans and animals become infected while the tick is feeding, through infectious saliva, excrement, and/or coxal fluid entering via the bite wound or skin. The presence of the *Ornithodoros* spp. vector on humans often goes unnoticed since this vector 1) typically feeds at night, 2) usually completes its feeding in 5 to 20 minutes, and 3) does not cause pain. Soft ticks are particularly durable vectors as they are able to survive for months to years without a blood meal. The spirochetes are similarly able to survive in the ticks for extended periods of time. In addition, the spirochetes can be maintained within the vector during the transition from one developmental stage to another (e.g., larva to nymph to adult).

Presence of *Borrelia* in Hard Ticks

The Ixodidae (hard) ticks are the primary vectors of *B. burgdorferi*, the etiological agent of Lyme disease (Table 1). The association between *B. burgdorferi* and *I. scapularis*, the vector in the northeastern and north-central United States (Fig. 4), has been studied extensively (Burgdorfer et al., 1982; Burgdorfer et al., 1989b; Spielman et al., 1984; Benach et al., 1987; Ribeiro et al., 1987; Piesman, 1989; Dolan et al., 1998; Humair and Gern, 2000). This relationship is similar to that occurring between the European Lyme disease (Table 5) spirochetes and ticks of the *Ixodes ricinus* complex. The larval and nymphal forms of *I. scapularis* commonly become infected with *B. burgdorferi* when obtaining a blood meal from infected rodents, with the white-footed mouse, *Peromyscus leucopus*, being the primary reservoir (Table 2). A variety of birds, in addition to small mammals, may also serve as a reservoir for the Lyme disease spirochete (Anderson and Magnarelli, 1984). One recent study suggests that latent *Borrelia burgdorferi* sensu lato infection in migratory birds (i.e., redwing thrush) may be reactivated by the stress associated with migration. Many of these birds are infested with ixodid ticks and, subsequently, may serve as an efficient mechanism of spreading ticks carrying this pathogen over long distances (Gylfe et al., 2000).

Recent studies using *B. burgdorferi*-infected *I. pacificus* nymphs fed on the western fence lizard (*Sceloporus occidentalis*) suggest that lizard serum contains a thermolabile, borreliacidal factor, probably a protein, that destroys spirochetes in the midgut of feeding *I. pacificus* ticks. The presence of lizards in the enzootic cycle may help to reduce the force of transmission of *B. burgdorferi* by diverting a competent vector tick from an alternative reservoir-competent host (i.e., the dusky-footed wood rat or the California kangaroo rat; Lane and Quistad, 1998).

In contrast to the soft tick- (see Presence in Soft Ticks) and louse-borne (see Presence in Lice) relapsing fever *Borrelia* spp., *B. burgdorferi* remains in the midgut of the hard tick (Fig. 4) until the next blood meal (Ribeiro et al., 1987; Burgdorfer, 1989a), at which time it penetrates the basal membrane of the midgut, invades the hemolymph and various tissues, including the salivary glands, and is transmitted to the host via infectious saliva (Schwan, 1996). Recent studies suggest that the differential expression of lipo-

Table 2. *Borrelia* species associated with Lyme borreliosis, and their ecological and pathogenic characteristics, and their geographic distribution.

Taxon	Vector	Animal host	Human Disease	Distribution
B. burgdorferi sensu lato	*I. scapularis*	Mammals, birds	EM, **arthritis**, carditis, neuroborreliosis	United States
	I. pacificus			
	I. ricinus			Europe
	I. persulatus?			Asia?
B. garinii	*I. ricinus*	Birds, small	EM, arthritis, **neuroborreliosis**	Europe
	I. persulatus	mammals		Asia
B. afzelii	*I. ricinus*	Small mammals	EM, arthritis, neuroborreliosis, **ACA**	Europe
	I. persulatus			Asia
B. japonica	*I. ovatus*	Small mammals	No	Japan
B. miyamotoi	*I. persulatus*	Small mammals	Unclear	Japan
B. valaisiana	*I. ricinus*	Birds	Unclear	Europe
	I. granulatus			Asia
B. lusitaniae	*I. ricinus*	Unknown	No	Central Europe
B. andersonii	*I. dentatus*	Rabbit	No	United States
B. bissettii sp. nov.	*I. pacificus*	Rodents, birds	Unclear	United States
	I. neotomae			
	I. scapularis			
	I. ricinus		EM, lymphocytoma	Slovenia
B. takunii	*I. takunus*	Small mammals	No	Japan
B. turdi[b]	*I. turdus*	Small mammals	No	Japan

[a]The clinical syndrome associated with distinct *B. burgdorferi* sensu lato species are in boldface type.
[b]The name has been corrected from *B. turdae* (Fukunaga et al., 1996) by International Union of Microbiological Societies (1998).

proteins (see Differential Gene Expression) on the surface of the spirochete in response to the blood meal facilitates migration from the midgut to the salivary glands and subsequent transmission to the mammalian host (Schwan et al., 1995; de Silva et al., 1996; Ohnishi et al., 2001). Transovarial transmission by *Ixodes* spp. ticks is thought to occur infrequently. For a review comparing spirochete transmission by hard and soft ticks, see Schwan (1996).

Isolation

The study of *Borrelia* was greatly facilitated by the development of a culture medium by Kelly that would support the growth of the spirochetes (Kelly, 1971). In 1982, Stoenner enriched this formulation by adding yeast extract and tissue culture medium (referred to as "fortified Kellys medium"; Stoenner et al., 1982). Subsequent modifications by Barbour (1984a) resulted in Barbour-Stoenner-Kelly (BSK I) medium, which has allowed the isolation of borreliae from a variety of sources (Barbour, 1986a). For historical reviews of the cultivation of *Borrelia* spp., see Barbour and Hayes (1986b), Barbour (1984a), and Anderson and Magnarelli (1992). The successful cultivation of *Borrelia* spp. is usually dependent on the quality of the animal serum or albumin used in the preparation of the medium (Callister et al., 1990). These reagents are thought to provide necessary long-chain fatty acids, but other, as yet unidentified, trace components may also be important. A commercial source for this medium has now become available (http://www.sigma-aldrich.com, Sigma-Aldrich, BSK [catalog # B 8291]). Semisolid medium is prepared by the addition of 100 ml of sterile 1.89% agarose (dissolved in boiling water) to the BSK I recipe given above to yield BSK II. The agarose solution (cooled to 55°C) is added just prior to dispensing medium. Several methods have been described for plating *Borrelia* as a surface lawn (Kurtti et al., 1987) or by subsurface plating (Samuels et al., 1994a).

Isolation of *Borrelia* from Ticks

The hemolymph and most tissues of *Ornithodoros* spp. ticks can be used as a source of the relapsing fever borreliae and *B. coriaceae*. In contrast, *B. burgdorferi* is usually restricted to the midgut in flat (unfed) *Ixodes* ticks. *Borrelia burgdorferi* has been successfully cultured from the midguts of live, infected *Ixodes* nymphs and adults by crushing the entire contents of the midgut in a sterile Petri dish or on a glass slide, mixing the contents with a small amount (0.5 ml) of BSK II (Table 3) medium, transferring the contents to a sterile screwcap microcentrifuge tube containing an additional 1 ml of BSK II medium and then incubating at 30–35°C for 7–21 days. Cultures should be checked every sev-

Table 3. Liquid BSK II medium for growth of *Borrelia* spp. (Barbour, 1984a).

1. To 900 mls of glass-distilled water, add the following:	
HEPES (Sigma)	6.0 g
Neopeptone (Difco)	5.0 g
Sodium citrate	0.7 g
Glucose	5.0 g
Sodium bicarbonate	2.2 g
TC Yeasolate (Difco)	2.5 g
Sodium pyruvate	0.8 g
N-acetyl glucosamine (Sigma)	0.4 g
Bovine serum albumin, fraction V	50.0 g
2. Adjust pH of medium at room temperature to 7.6 with 1N NaOH.	
3. Sterilize by filtration (0.2 μm).	
4. Add 100 mls of sterile 10× CMRL 1066 without glutamine (Gibco/BRL).	
5. Add 200 mls of sterile 7% gelatin (Difco), which has been dissolved in boiling water.	
6. Add 64 mls of sterile, heat-inactivated rabbit serum (Pel-Freeze Biologicals, Inc).	
7. Dispense to glass or polystyrene tubes or bottles.	
8. Fill to 90% capacity and cap tightly.	
9. Store complete medium at 4°C.	

eral days for spirochetes by microscopy. Owing to their slender shape, they are best visualized by darkfield microscopy (Fig. 1). *Borrelia* also can be isolated from *Ornithodoros* spp. and *Ixodes* spp. tick tissues using BSK II (Table 3) medium.

Isolation of *Borrelia* from Patient Specimens

The *Borrelia* strains causing relapsing fever (Table 1) are typically present in high numbers circulating in the blood of patients during acute (febrile) phases of this disease. The tick-borne relapsing fever spirochete, *B. hermsii*, is readily cultivated from patient samples, primarily blood. Several reports also have described the cultivation of the louse-borne relapsing fever spirochete, *B. recurrentis* (Cutler et al., 1997; Cutler et al., 1999), and the tick-borne relapsing fever spirochete, *B. duttonii* (Cutler et al., 1999), from the blood of spirochetemic patients. Both of these types of relapsing fever spirochetes may be isolated from blood by inoculating one to two drops per 10 ml of liquid or semisolid BSK II (Table 3) media. Cutler et al. (1999) suggest that the critical factor in determining the success of cultivation of *B. duttonii* is the quality of the albumin used in preparation of the BSK II (Table 3) medium; over two-thirds of the batches of this critical medium ingredient tested were rejected because they failed to support growth of the relapsing fever spirochete. Larger tissue and organ samples (bladder, heart, spleen and joint) can be cultured by homogenization in liquid BSK II (Table 3) medium (10% wt/vol) with a Stomacher Lab Blender (Tekmar, Cincinnati, OH). Larger debris is allowed to settle and serial dilutions (10-fold) of the supernatant are made in the semisolid BSK II (Table 3) medium. Cultures are incubated at 30–35°C and examined by darkfield microscopy for spirochetes at weekly intervals.

In contrast to in relapsing fever patients, relatively low densities of spirochetes are present in the blood and tissues of Lyme disease patients. Nevertheless, numerous laboratories have reported the successful isolation of *B. burgdorferi* from skin, blood and other tissues obtained from Lyme disease patients. Spirochetes are most readily cultured from biopsy specimens of the skin lesion of erythema migrans (EM; Fig. 20) and acrodermatitis chronicum atrophicans (ACA; Berger et al., 1985; Berger et al., 1992; Schwartz et al., 1992; van Dam et al., 1993; Nadelman et al., 1996; Picken et al., 1998). *Borrelia burgdorferi*, however, has been isolated and subcultured from the blood and plasma of Lyme disease patients (Benach et al., 1983; Nadelman et al., 1989; Berger et al., 1994; Wormser et al., 1998; Wormser et al., 2000). Significantly improved rates of spirochete cultivation were obtained when plasma (instead of serum or blood) and/or large sample sizes (up to 9 ml of whole blood or 3 ml of serum) were used to inoculate BSK II (Table 3) medium (Wormser et al., 1998; Wormser et al., 2000). Although spirochetes have been isolated and cultured from the cerebrospinal fluid (CSF) of patients exhibiting neurological symptoms (Steere et al., 1983; Nocton and Steere, 1995), attempts to recover spirochetes from this tissue are usually not productive. Although this fastidious organism is clearly difficult to cultivate in vitro, the lack of successful cultivation of spirochetes from CSF may be more a reflection of the limited invasion of the central nervous system by *B. burgdorferi* (Steere et al., 1983; Barthold et al., 1991; Estanislao and Pachner, 1999; Cadavid et al., 2000).

Isolation of *Borrelia* from Animal Tissues

The most common method of cultivating *Borrelia* spp. from field-caught or experimentally infected animals is by inoculation of BSK II (Table 3) medium with several drops of blood (relapsing fever) or by ear punch biopsy (Lyme borreliosis; Sinsky and Piesman, 1989). For ear punch biopsy, a small sample of skin tissue (3 mm) may be taken from an infected animal's ear using a punch tool (available from most biological and veterinary suppliers) or by using fine point scissors to excise a 3-mm long "pie cut" from the animal's ear. The entire ear biopsy is placed in a small volume (1–2 ml) of liquid BSK

II (Table 3) medium. In this case, use of antibiotic additives (rifampicin [5 mg/ml], phosphomycin [2 mg/ml], and amphotericin B [250 mg/ml]), commercially available as a cocktail from Sigma-Aldrich Chemical Corp. (http://www.sigma-aldrich.com) (catalog # A 1956), is recommended to reduce the risk of contamination with animal and/or environmental flora. Although borreliae usually can be detected in culture medium within one to three weeks of incubation, some isolates may not be visible for several months. *Spirochetes* also may be cultured from other animal tissues, including heart, bladder and joint (Anderson et al., 1986; Schwan et al., 1988a; Sinsky and Piesman, 1989; Anderson and Magnarelli, 1992; Pachner et al., 1993).

Uncultivatable *Borrelia* spp.

Despite the successful use of artificial media in cultivating spirochetes from both ticks and mammalian tissues, several examples of uncultivatable *Borrelia* spp. have been reported. Anda et al. (1996) described the isolation of a new relapsing fever *Borrelia* following isolation of this agent from the blood of three febrile patients. Although refractory to in vitro cultivation attempts using numerous BSK II (Table 3) formulations and growth conditions, this organism can be passaged in mice. In addition to being visible in the blood of infected mice, spirochetes were also detected in liver and spleen by Dieterle stain 15 days post-inoculation (Anda et al., 1996). A second notable example of an uncultivatable *Borrelia* spp. is the *Amblyomma americanum* spirochete, *Borrelia lonestari* (*B. barbouri*). This spirochete is thought to be responsible for a tick-associated Lyme disease-like illness in the southern United States (Barbour et al., 1996a). Although immunological and molecular reagents have been developed to detect and partially characterize this spirochete using field-collected tick specimens (Barbour et al., 1996b; Rich et al., 2001), it has yet to be successfully cultivated in vitro using media that support the growth of *B. burgdorferi* (i.e., BSK; Table 3). The inability to cultivate this spirochete (Barbour et al., 1996a) from patient samples and distinguish it from the closely related *B. burgdorferi* may result in the true incidence of *B. lonestari* borreliosis being significantly underestimated. This is a particular concern in areas where the vectors of both spirochetes coexist and coassociate with the human population. In such areas, *B. lonestari*-associated borreliosis could result in physician-diagnosed or -suspected Lyme disease that could be both culture-negative and seronegative. In addition, coinfection with both species of spirochetes may also go undetected.

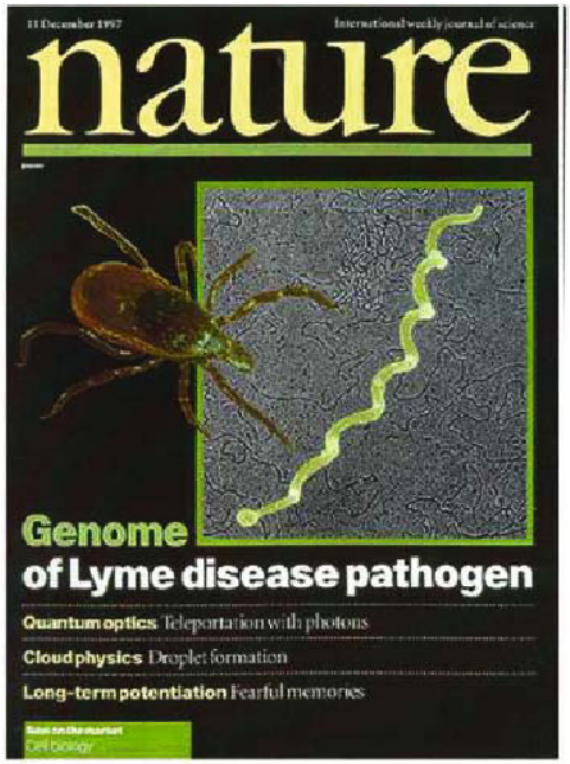
Use of PCR for Detection of *Borrelia* in Tissues

In addition to the complex media requirements and the fastidious nature of *Borrelia*, several additional factors, including the low density of spirochetes in blood and other tissues during infection, the large volume of sample required, and the expertise needed to biopsy skin and other tissues (i.e., cerebrospinal fluid [CSF] and synovial fluid [SF]) from Lyme disease patients, have made clinical diagnosis based on spirochete isolation unreliable. The development of assays based on the sensitive polymerase chain reaction (PCR), however, has made the detection of *Borrelia* within relatively small samples of blood and other tissues feasible. PCR-based assays have been used to detect spirochetes in EM skin lesions (Fig. 20) and lesion exudate (Melchers et al., 1991; Schwartz et al., 1992), ACA lesions (Moter et al., 1994), plasma (Goodman et al., 1995; von Stedingk et al., 1995), blood (Guy and Stanek, 1991), urine (Lebech and Hansen, 1992; Huppertz et al., 1993; Liebling et al., 1993; Priem et al., 1997), CSF (Keller et al., 1992; Lebech and Hansen, 1992; Huppertz et al., 1993; Liebling et al., 1993; Nocton et al., 1994; Nocton et al., 1996; Priem et al., 1997), and SF (Liebling et al., 1993; Bradley et al., 1994; Nocton et al., 1994; Jaulhac et al., 1996; Priem et al., 1997). Although these studies demonstrate that PCR-based methodologies can successfully detect *Borrelia* spp. from a variety of tissues, the clinical utility of these detection methods, however, has not been fully established. For a comprehensive review of PCR-based detection of *Borrelia*, see Schmidt (1997).

Culture Bias

The application of culture-independent techniques (i.e., direct PCR-amplification) has revealed that sequences retrieved from bacteria cultured from tick and patient samples often do not fully reflect the degree of sequence diversity present in the uncultured sample. This phenomenon, called "culture bias," is thought to result from the preferential growth of one genetically distinct subpopulation present within a sample such that it outgrows other members within the population. One of the first examples of borrelial culture bias was discovered during a comparative analysis of cultured and uncultured (i.e., PCR-amplified directly) tick samples collected from an area of the country (Colorado) where Lyme disease is not endemic (Fig. 16). In these studies, Norris et al. (1997) demonstrated that the genetic diversity of alleles at three different loci (*flaB* [see "Flagella"], *p66* [*Borrelia* Molecular Architecture], and *ospA* [see "Reciprocal

Expression of OspA and OspC"]) was consistently lower in cultured spirochetes, suggesting that culturing of *B. burgdorferi* in BSK-H medium may select for specific genotypes. In addition, some alleles were restricted to either cultured organisms or ticks alone (Norris et al., 1997). The potential clinical relevance of culture bias became evident when a similar study was performed using samples from Lyme disease patients (Liveris et al., 1999). In this study, DNA from in vitro-cultivated *B. burgdorferi* or from blood and tissue biopsy samples obtained from Lyme disease patients was characterized by restriction length polymorphism (RFLP) typing of the 16S-23S ribosomal DNA intergenic spacer region. The RFLP diversity seen by direct tissue analysis was significantly greater than that present in the cultured samples. These results are consistent with the conclusions drawn from the Colorado tick study cited above. The combined results of these studies suggest that the diversity of genotypes present within either tick or human populations may be significantly underestimated if based on in vitro cultivation or direct PCR-amplification alone.



Nature Cover

Fig. 5. From Fraser et al. (1997). (www.ncbi.nlm.nih.gov). Reprinted by permission from Nature (http://www.nature.com) © 1997 Macmillan Magazines Ltd.

Preservation

Borreliae are best preserved by the addition of glycerol (final concentration, 10–15%) to blood or plasma from infected animals (relapsing fever borreliae), or to culture medium, and stored at –70°C or in liquid nitrogen. Spirochetes may be recovered from glycerol stocks by the addition of a small amount (0.5 ml) of BSK II (Table 3) medium to the top layer of the frozen culture, pipetted several times, removed, and transferred to a sterile test tube containing an additional 1–3 ml of BSK II (Table 3) medium. The "resurrected" spirochetes should be visible by darkfield microscopy within 1–2 weeks.

Physiology

The publication of the *Borrelia burgdorferi* genome (The *B. burgdorferi* Genome Sequence) has vastly increased our understanding of the physiology of this *Borrelia* species. For a complete outline of the results of this project, visit The Institute for Genome Research (TIGR)). A survey of the *B. burgdorferi* genome revealed a paucity of genes associated with the biosynthesis of amino acids, fatty acids, enzyme cofactors, and nucleotides (Fraser et al., 1997). The absence of genes involved in fatty acid synthesis is in agreement with the biochemical evidence that 1) borreliae are unable to elongate or beta-oxidize fatty acids (Livermore and Johnson, 1978; Belisle et al., 1994) and 2) the fatty acid composition of borrelial cells reflects that of the growth medium (Livermore and Johnson, 1978; Belisle et al., 1994). These findings are thought to explain, at least in part, the fastidious nature of this organism and the requirement for serum-supplemented mammalian tissue culture medium (i.e., CMRL; see Table 3). Genes encoding all of the enzymes of the glycolytic pathway were identified (Fraser et al., 1997). Work by Gebbia et al. (1997) identified the glycolytic enzyme operons in *B. burgdorferi* and *B. hermsii*, and as expected, these loci exhibit very high sequence identity and similar orientations on the linear chromosomes of both organisms. Analysis of the metabolic pathways identified suggests that glucose serves as the major energy source (Fraser et al., 1997). Consistent with the microaerophilic nature of borreliae, genes involved in the conversion of pyruvate (pro-

duced by glycolysis) to lactate were identified (Fraser et al., 1997). This finding also is supported by biochemical studies that identified lactic acid as the predominant metabolic end product of glucose utilization (Kelly, 1976). None of the genes encoding proteins of the tricarboxylic acid cycle or oxidative phosphorylation were identified. These findings, along with the lack of the necessary components of a respiratory transport chain, may explain the slow growth rate (12–30-hour doubling time) of this organism. Analysis of the genome also revealed genes involved in the utilization of glycerol, glucosamine, fructose and maltose, suggesting that the bacterium may also use these substrates as alternative carbon sources (Fraser et al., 1997). Although primarily serving as a component of the cell wall, genes (*N*-acetylglucosamine-6-phosphate deacetylase [BB0151] and glucosamine-6-phosphate isomerase [BB0152]) were identified which would allow *N*-acetylglucosamine (NAG) to be used as an alternative carbon source. The NAG is a necessary additive to BSK II (Table 3) medium and is required for growth of *B. burgdorferi* (Kelly, 1971; Barbour and Hayes, 1986b). Additionally, a model has been proposed for the potential utilization of the tick cuticle component chitin (composed primarily of NAG) as an alternative carbon source for *Borrelia*, which may be contained within the midgut of the tick for extended periods of time (months to a year) between feeding and subsequent transmission to a new mammalian host (Fraser et al., 1997). Support for this hypothesis is provided by the identification of a phosphotransferase system (PTS) cellobiose transporter operon (BBB04, BBB05, and BBB06) that could possibly transport chitobiose (di-*N*-acetyl-D-glucosamine), a molecule with structural similarity to NAG, and a gene product with sequence similarity to chitobiase BBB02, which may convert chitobiose to NAG (Fraser et al., 1997).

The genes encoding essential transcription (sigma factors σ70, σ54, and *rpoS*, translation *nusA*, *nusB*, *nusG*, *rho*, and 31 tRNAs), and DNA replication and repair machinery (DNA pol III, ligase, gyrase, *uvrA-D*, and *recA*) were identified, along with the chromosomal origin of replication (*ori*; Fraser et al., 1997; Picardeau et al., 1999a). In addition, two independent studies have examined the effect of DNA replication and transcription on triplet codon usage for *B. burgdorferi* (McInerney, 1999; Lafay et al., 1999). These studies suggest that evolutionary pressures have resulted in an enrichment for highly expressed genes on the leading strand during the asymmetrical replication of this organism's linear chromosome.

Growth of *Borrelia burgdorferi*: No Iron Requirement

A common theme in microbial pathogenesis is a requirement for iron during growth within a mammalian host. For reviews, see Payne (1993), Litwin and Calderwood (1993), Vasil and Ochsner (1999), and Ratledge (2000). The level of free iron present within human serum and/or tissues is well below that required for growth of many microorganisms. This level is further limited by the production and secretion of the iron-binding protein lactoferrin by the human host during infection. To overcome this host limitation, many bacteria have evolved specialized systems for the acquisition and assimilation of iron in vivo. A recent survey of the *Borrelia* genome suggests that this bacterium has overcome iron limitation within the human host by dispensing with those proteins that utilize iron as a cofactor (i.e., catalase, peroxidase, cytochromes, respiratory proteins, and metalloenzymes; Fraser et al., 1997; Posey and Gherardini, 2000). In support of this hypothesis, Posey and Gherardini (2000) demonstrated that 1) *Borrelia* were able to grow normally in iron-limited medium and 2) the level of iron (either free or bound to proteins) detected in *B. burgdorferi* cell lysates was well below a physiologically relevant concentration. The genes encoding a number of metal-requiring proteins, however, were identified within the *Borrelia* genome, including superoxide dismutase, RNA polymerase and DNA polymerase (Fraser et al., 1997). Although the metal requirements of these putative borrelial metalloenzymes have not been determined, it has been suggested that these proteins may have evolved to utilize alternative metal ions (i.e., manganese and zinc; Posey and Gherardini, 2000).

Borrelia Molecular Architecture

Borrelia share the same basic ultrastructural features as other members of the Family Spirochaetaceae. These features include an outer membrane that encloses a protoplasmic cylinder encased within a cytoplasmic membrane (Fig. 6). Although similar to Gram-negative bacteria in possessing both cytoplasmic and outer membranes, several fundamental differences in the outer membranes of these two types of bacteria have been revealed using combined molecular, ultrastructural and immunological methods. The outer membranes of Gram-negative bacteria characteristically contain a high density of proteins with extensive β-pleated sheet structure (i.e., porins) and no or only rare surface-exposed lipoproteins (Lugtenberg and van Alphen, 1983; Pugsley, 1993). In addition to a

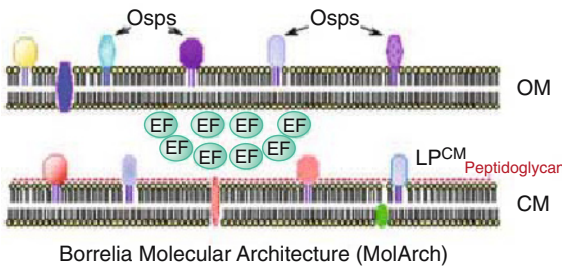


Fig. 6. *Borrelia burgdorferi* molecular architecture. The outer membrane (OM) of *B. burgdorferi* contains a limited number of integral membrane proteins (outer surface proteins or Osps). The majority of proteins within borrelial outer (OM) and inner cytoplasmic (CM) membranes are lipoproteins, anchored via associated lipid moieties. Lipoproteins (LP) anchored to the periplasmic leaflet of the CM are in close association to the peptidoglycan layer. Endoflagella (EF; see "Flagella") are located in the periplasmic space.

significantly lower protein-to-lipid ratio, *B. burgdorferi* outer membranes, in contrast to Gram-negative membranes, contain predominantly lipoproteins (many of which are lipid-anchored to the periplasmic face of the cytoplasmic membrane) and relatively small amounts of proteins with transmembrane (membrane-spanning) domains (Walker et al., 1991; Radolf et al., 1994b; Radolf et al., 1995b; Jones et al., 1995). In concert with the spirochete's ability to alter its outer membrane composition (see "Antigenic Variation") during the course of infection, these two characteristics of the borrelial membrane may help to explain the Lyme disease spirochete's ability to avoid host clearance mechanisms and establish persistent infection. In addition, *Borrelia* spp. have the potential to differentially express (see "Differential Gene Expression") (i.e., downregulate) many of their surface-exposed lipoproteins, thereby further limiting the ability of the host immune system to recognize these immunogens.

One of the most striking characteristics of spirochetal outer membranes, particularly those of *B. burgdorferi* and *Treponema pallidum* (see The Genus *Treponema* in this Volume), is their lability. As a result, the outer membranes of these organisms are often disrupted during routine experimental manipulations (i.e., centrifugation), initially leading some investigators to conclude (using immunofluorescence) that a number of borrelial lipoproteins (i.e., outer surface protein OspA [see "Reciprocal Expression of OspA and OspC"] and OspB) were exclusively surface-exposed (Barbour et al., 1983; Barbour et al., 1984b; Luft et al., 1989). Later studies, using techniques that do not disrupt the spirochetal outer membrane prior to analysis (immunocryoultramicrotomy, Brusca et al., 1991, and immunofluorescence using spirochetes encapsulated within agarose beads, Cox et al., 1996) demonstrated that most of the cell's complement of OspA and OspB is located in the periplasmic space. A similar localization pattern has now been established for a third lipoprotein, OspC (Cox et al., 1996; see "Reciprocal Expression of OspA and OspC"). The presence of some lipoproteins simultaneously in both the inner and outer membranes suggests that the content of borrelial membranes is dynamic (Brusca et al., 1991; Cox et al., 1996). This hypothesis is further supported by a recent study in which exogenously provided recombinant lipoproteins were shown to be incorporated into the outer membranes of viable spirochetes (Bunikis et al., 2001). It is tempting to speculate whether *Borrelia* spp. use the inner membrane as a "holding depot" for lipoproteins that may eventually be transferred to the outer membrane and bacterial surface as the organism encounters different environmental/growth conditions.

A second class of proteins within the outer membranes of *Borrelia* is the membrane-spanning proteins. To date, only a handful of such proteins have been identified. Of these, P66 has attracted the most attention. This 66-kDa protein was identified as a component of borrelial membrane-enriched cell fraction (Barbour et al., 1984b; Bunikis et al., 1995) and also, by an independent group, using a protease screening assay (Probert et al., 1995). Computer analyses predict that, in addition to having an N-terminal signal peptidase I cleavable leader peptide, P66 contains a C-terminal intramembranous domain (amino acid residues 498–597) composed of two transmembrane-spanning hydrophobic α-helices (Bunikis et al., 1998). This prediction is supported by experimental studies, immunoelectron microscopy (Bunikis et al., 1995) and protease (trypsin and proteinase K) sensitivity (Probert et al., 1995), which identified a small (~5 kDa) flexible loop fragment near the C-terminus. This 5-kDa loop domain appears to be the only portion of P66 that is exposed on the spirochetal surface (Bunikis et al., 1996; Bunikis and Barbour, 1999). Recent evidence suggests that P66 is physically associated with OspA and that OspA may shield P66 from proteolytic attack (Bunikis and Barbour, 1999). Consistent with its having surface-exposed epitopes, mice immunized with recombinant P66 were partially protected against *B. burgdorferi*-challenge (Exner et al., 2000). Antibodies against P66 also were readily detectable in immune sera from human Lyme disease patients (Sadziene et al., 1993; Bunikis et al., 1996; Ntchobo et al., 2001), confirming that this protein is expressed during infection.

Two different functions have been proposed for P66. Coburn et al. (1999) reported that recombinant P66 binds to β_3 chain integrins, whereas Skare and coworkers (Skare et al., 1997) reported that the recombinant protein has porin-like activity in planar lipid bilayers. While neither of these functions is mutually exclusive, they are problematic, however, when viewed against the topological analyses indicating that P66 has only limited surface exposure. The P66 integrin-binding domain was mapped to a region that does not appear to be surface-exposed (Coburn et al., 1999). Porins typically contain multiple membrane-spanning domains (Nikaido, 1996). Further work will be needed to resolve these ostensible discrepancies.

Two other membrane-spanning proteins identified in *B. burgdorferi* are *Borrelia* glycosaminoglycan (GAG)-binding protein (Bgp) and an outer membrane-spanning protein, Oms28, with an observed mass of 28 kDa. The Bgp protein was identified from membrane-enriched extracts of *B. burgdorferi* on the basis of its glycosaminoglycan-binding and hemagglutination activities (Parveen and Leong, 2000). Indirect immunofluorescence and Western blotting of purified outer membrane vesicles (both with anti-Bgp antiserum) were used to localize this protein to the spirochetal surface. Purified recombinant Bgp also was shown to inhibit attachment of *B. burgdorferi* to the surface of several different mammalian cell lines (293, Vero and C6), a result consistent with this protein being present on the surface of spirochetes (Parveen and Leong, 2000). The GAG-binding specificity of Bgp, however, has not been determined. The Oms28 protein was identified in *Borrelia burgdorferi* outer membrane preparations based on its porin activity; this activity was demonstrated in a planar lipid bilayer assay using *Escherichia coli* overexpressing recombinant Oms28 (Skare et al., 1996).

Peptidoglycan

The peptidoglycan of *Borrelia* spp. is responsible for imparting structural stability to the protoplasmic cylinder of the spirochete and is the primary component of the bacterial cell wall (Beck et al., 1990; Fig. 6). Additional data demonstrating a structural role of the borrelial peptidoglycan were reported by Charon and coworkers (Motaleb et al., 2000); spirochetes with their outer membranes removed were readily lysed by the addition of the peptidoglycan-degrading enzyme streptozyme (Motaleb et al., 2000). Muramic acid (Ginger, 1963) and ornithine (Klaviter and Johnson, 1979) have been identified as components of the peptidoglycan in relapsing fever *Borrelia*. Although biochemical analyses of the peptidoglycan of other *Borrelia* have not been performed to date, it is likely that both of these components are present in all borrelial cell walls.

Flagella

Spirochetes are unique in that their organelles of motility are located beneath their surface (Fig. 6). For detailed reviews of spirochetal and borrelial flagella ultrastructure, see Holt (1978) and Barbour and Hayes (1986b), respectively. For a comprehensive contemporary review of spirochete flagella and motility, see Li et al. (2000a). Encased between the outer and cytoplasmic (inner) membranes are the periplasmic flagella, also referred to as "endoflagella" (Fig. 6); in this respect, spirochetes are distinct from other bacteria in having their organelles for motility reside inside the cell within the periplasmic space. The number of periplasmic flagella in borreliae varies depending on the species, as well as within the same strain; the relapsing fever spirochetes generally have between 15 and 30 flagella per cell end, whereas Lyme spirochetes have from 7 to 11 per end (Hovind-Hougen, 1976; Barbour and Hayes, 1986b). The periplasmic flagella are composed of two classes of proteins, FlaB, core protein, flagellin, and FlaA (a possible sheath protein, with FlaB being the major constituent). Although the periplasmic flagella of other spirochete species (i.e., *Brachyspira*) have been shown to be composed of at least three core proteins (FlaB1, FlaB2 and FlaB3) and two sheath proteins (FlaA1 and FlaA2; Li et al., 2000b), the periplasmic flagella of *Borrelia burgdorferi* have been shown to be composed of only one each of these two types of proteins (Ge and Charon, 1997a; Fraser et al., 1997; Ge et al., 1998). In addition, the relatively low abundance of FlaA in *B. burgdorferi* suggests that it is unlikely to form a sheath along the entire flagellar length (Li et al., 2000a). A comparison of the periplasmic flagella proteins shows a high degree of conservation (immunological crossreactivity and sequence similarity) within the spirochete phylum (Limberger and Charon, 1986; Cockayne et al., 1987; Norris et al., 1988; Koopman et al., 1992; Trueba et al., 1992; Limberger et al., 1993; Ruby et al., 1997; Ge et al., 1998).

The flagella of *Borrelia* spp. have been shown to be 1) inserted subterminally and bipolarly, 2) wrapped around the protoplasmic cylinder, and 3) overlapping in the middle of the protoplasmic cylinder (Holt, 1978). Electron microscopy studies revealed that the periplasmic flagella form a bundle and are not dispersed around the cell in the periplasmic space (Hovind-Hougen, 1984). Purified periplasmic flagella have been shown to be left-handed with a defined helix pitch and

diameter. These dimensions, however, are dramatically altered when measured in situ (i.e., in association with the protoplasmic cylinder; Goldstein et al., 1996). The helical shape of the flagella, wrapped in a bundle around the peptidoglycan-encased protoplasmic cylinder, imparts to the spirochete a flat-wave morphology (Motaleb et al., 2000; Fig. 7). Charon and coworkers (Motaleb et al., 2000) hypothesize that the outer membrane juxtaposes the peptidoglycan and periplasmic flagella so that these structures can optimally interact with one another to promote cell motility. The dynamics of *Borrelia* spp. motility also have been examined (Goldstein et al., 1994). These studies have demonstrated that, in contrast to their helical shape, translating (i.e., moving) spirochetes swim with a planar waveform similar to that of eukaryotic flagella and propagate backward moving spiral waves. The waves on individual spirochetes were observed to gyrate in a counterclockwise direction (Goldstein et al., 1994; Fig. 7). In addition, periplasmic flagella, inserted at opposite ends of the protoplasmic cylinder, were shown to rotate in opposite directions around one another, causing the cell to bend or exhibit characteristic "flexing" morphology (Goldstein et al., 1994). This rotation, however, was not required for the spirochetes to maintain their helical morphology (Motaleb et al., 2000). The contribution of the periplasmic flagella to both motility and cell morphology has been confirmed using both a spontaneous *Borrelia* mutant (Sadziene et al., 1991) and a *flaB* mutant strain created by allelic exchange (Motaleb et al., 2000). In both studies, the spirochetes not only were nonmotile, but also exhibited a long, straight, rod-shaped morphology. The periplasmic flagella also help the cells maintain their motility in environments of relatively high viscosity (Berg and Turner, 1979; Kimsey and Spielman, 1990; Goldstein et al., 1994). Although borreliae are extracellular parasites, this vigorous form of motility has also been implicated in the spirochete's ability to invade and infect various tissues (including connective tissue and extracellular matrix) and to traverse endothelial cell junctions (Comstock and Thomas, 1989; Comstock and Thomas, 1991; Szczepanski et al., 1990; Sadziene et al., 1991; Klempner et al., 1993).

The 35 genes encoding the proteins required for the synthesis of the complete flagellar organelle are grouped into seven clusters (Fig. 8) on the *B. burgdorferi* chromosome (Li et al., 2000a). Promoter analyses of the *B. burgdorferi* *flaA* and *flaB* genes revealed a striking difference between the regulation of flagellin gene (*flaB*) expression in this spirochete and in other bacteria; transcription of the *flaB* gene in *B. burgdorferi* appears to be initiated by the housekeeping transcription factor, σ 70. This is in contrast to the flagellin genes, *flaB* and *fliC*, in other spirochetes and bacteria, respectively, which are known to be or thought to be controlled by the alternative σ factor, σ 28 (Li et al., 2000b). These data also are consistent with the absence of 1) σ 28 promoter recognition sequences, 2) a σ 28-encoding gene, and 3) a gene encoding the anti-σ factor, FlgM, within the *B. burgdorferi* genome sequence (Fraser et al., 1997; Li et al., 2000a). Interestingly, transcription of the *flaB* gene from *T. pallidum* (the only other sequenced spirochetal genome) does appear to be initiated by σ 28, and a gene encoding this σ factor was identified within this treponemal genome.

Lipoproteins

The study of borrelial lipoproteins has long been a focus of research involving this bacterium. For a recent review, see Haake (2000). Borrelial lipo-

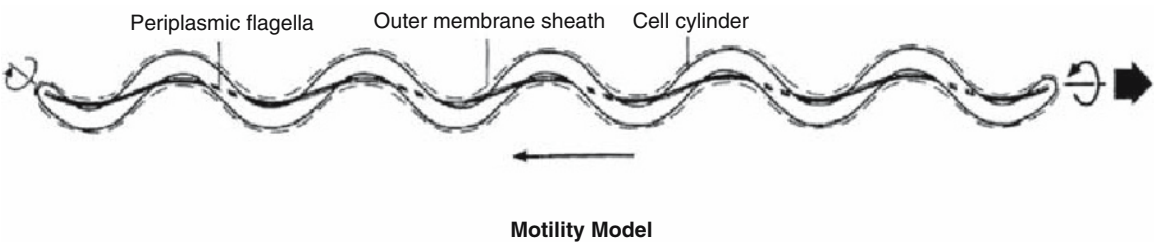


Fig. 7. Proposed model of *Borrelia burgdorferi* motility translating toward the right (wide arrow). The ridge, which is composed of the periplasmic flagella, extends down the length of the cell, and is left-handed with respect to the cell axis. The periplasmic flagella rotate counterclockwise (CCW) as viewed from the back of the cell (thin arrow at anterior end). This rotation causes waves to move from the anterior to the posterior ends of the cell (center arrow). Concomitant with this rotation, the cell rolls clockwise (CW) about the body axis (thin arrow at the posterior end). Image taken from Li et al. (2000b), with permission of Horizon Scientific Press.

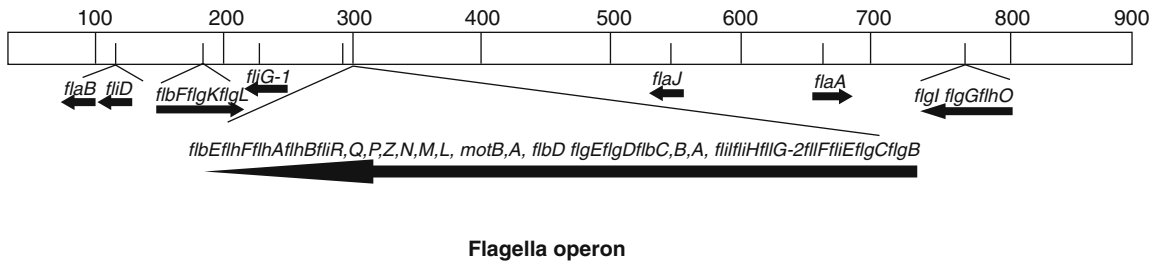


Fig. 8. Organization of the motility genes of *B. burgdorferi* (Fraser et al., 1997; Ge and Charon, 1997a; Ge and Charon, 1997b). Black arrows indicate direction of transcription of genes, and promoters have been identified by primer extension and operons by RT-PCR (Ge and Charon, 1997a; Ge and Charon, 1997b). Gray stippled arrows indicate presumed promoters, operons, and direction of transcription based solely on sequence analysis. Adapted from Li et al. (2000b), with permission of Horizon Scientific Press.

proteins are of interest for several reasons: 1) they are often found at the interactive surface (*Borrelia* Molecular Architectiture) between the spirochete and its growth environment, 2) many have been shown to be differentially expressed (see "Differential Gene Expression") depending on the growth environment, with some being preferentially expressed within either the arthropod or mammalian host, 3) some have assigned physiological roles (see "Differential Gene Expression"), 4) they may serve as potential protective immunogens (see "Lyme Disease Vaccine"), and 5) they are potent proinflammatory agonists (Lipoproteins and the Innate Immune Response). The significance of this class of membrane proteins is highlighted by the large number of putative lipoproteins identified (>150 on the linear chromosome and the plasmids) in the *Borrelia burgdorferi* genome, comprising nearly 10% of the total coding region (Fraser et al., 1997; Casjens et al., 2000). Not surprisingly, the majority of borrelial membrane proteins were found to be typical bacterial lipoproteins, possessing consensus lipoboxes (leader peptides required for lipid modification and transport to the membrane; Fraser et al., 1997). As also would be expected, genes encoding the necessary enzymes for lipid modification (prolipoprotein diacylglycerol transferase *lgt* and prolipoprotein signal peptidase *lsp*) also were identified. A gene encoding the enzyme (Lnt) responsible for the next step in lipoprotein biosynthesis, transfer of a fatty acid from a membrane phospholipid to the N-terminal cysteine of the mature polypeptide, however, was not identified. Since this activity has been shown by biochemical and structural analyses to exist in *Borrelia* (Brandt et al., 1990; Belisle et al., 1994; Beermann et al., 2000), it is presumed that the organism contains a novel form of this enzyme.

Transporters

A number of genes thought to be involved in the transport of solutes and small molecules (Fig. 9) were identified within the *B. burgdorferi* genome; the linear chromosome encodes 46 ORFs and the plasmids of an additional six ORFs related to the transport and binding of proteins. The combined ORFs form 16 distinct membrane transporters of amino acids (i.e., glycine/betaine/L-proline and oligopeptides), carbohydrates (i.e., methylgalactoside, ribose, galactose, fructose, glucose and maltose), small molecules (glutamate, chromate and spermadine/putrescine), anions (i.e., phosphate), and cations (i.e., magnesium and potassium; Fraser et al., 1997). The majority of these transport systems are members of the family of ATP-dependent, multi-subunit ABC-transporters, with many exhibiting broad substrate specificity (Fraser et al., 1997). The ability of *Borrelia* to transport a wide variety of solutes is thought to compensate for this organism's restricted coding potential and its inability to synthesize amino acids de novo. A survey of the genome sequence failed to identify transport systems for nucleotides, nucleosides, NAD/NADH, or fatty acids, although proteins responsible for these functions are likely to be present (Fraser et al., 1997).

The oligopeptide transporters (Opp) are the best studied of the *Borrelia* transporter families. This family is composed of 1) five peptide-binding proteins, OppA1, OppA2, OppA3, OppAIV, and OppAV (all thought to be lipoproteins, but lipid modification has only been confirmed for OppA-1, OppA-2 and OppA-3; Kornacki and Oliver, 1998); 2) two transmembrane proteins (OppB and OppC); and 3) two ATPases (OppD and OppF, Fraser et al., 1997; Bono et al., 1998). OppA1, OppA2 and

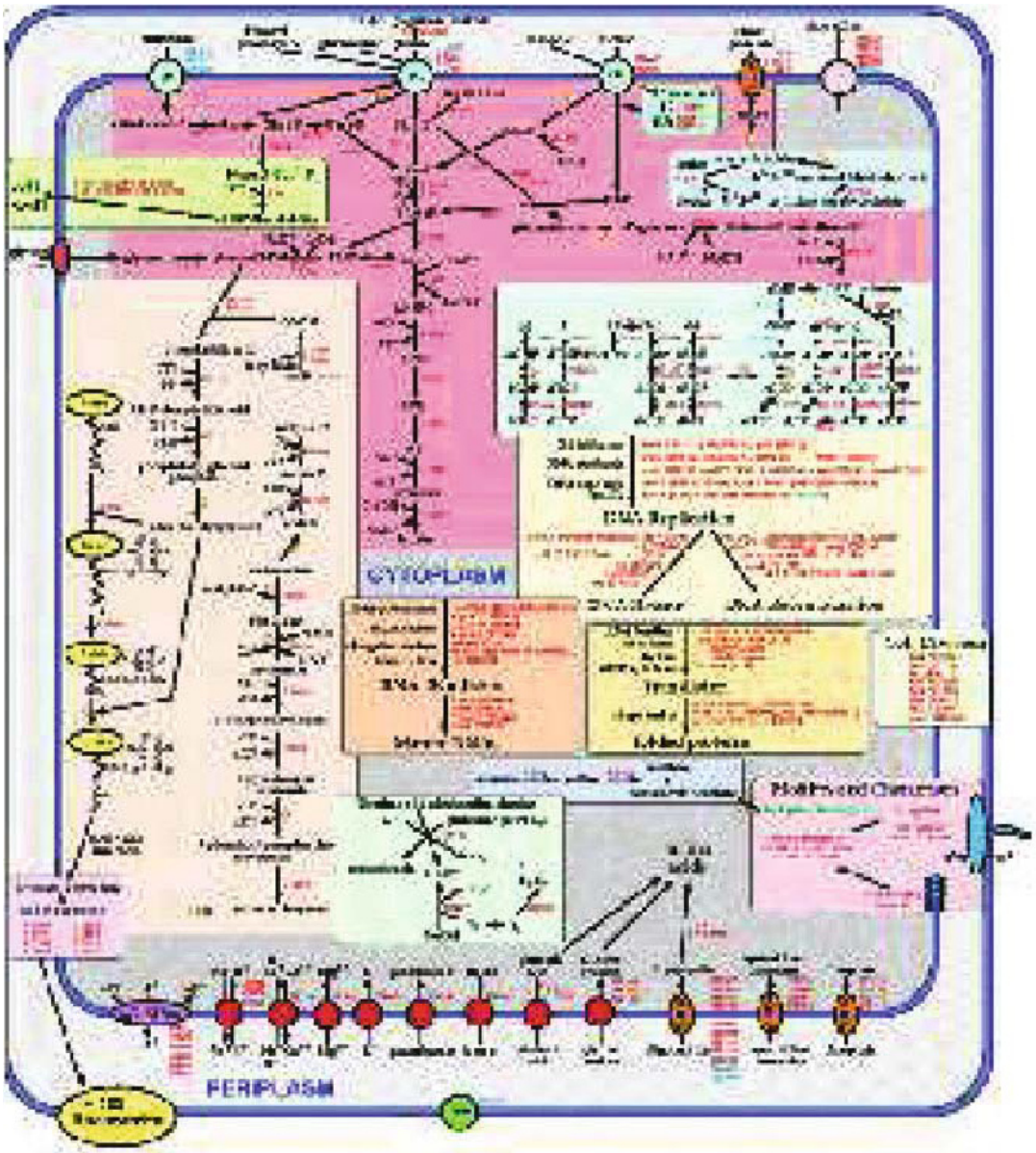


Fig. 9. Solute transport and metabolic pathways in *B. burgdorferi*. A schematic of a *B. burgdorferi* cell, providing an integrated view of the transporters and the main components of the metabolism of this organism, as deduced from the genes identified in the genome. The ORF numbers correspond to those listed in Table 2 of Fraser et al. (1997). Red indicates chromosomal and blue indicates plasmid ORFs. Presumed transporter specificity is indicated. Yellow circles indicate places where particular uncertainties exist as to the substrate specificity, subcellular location, direction of catalysis, or absence of expected activities. Taken from Fraser et al. (1997) and www.nature.com (http://www.nature.com/cgi-taf/DynaPage.taf?file=/nature/journal/v390/n6660/full/390580a0_fs.html) and reprinted by permission from Nature (http://www.nature.com), © 1997 Macmillan Magazines Ltd.

OppA3, along with OppB-F, are encoded in tandem on the chromosome, whereas OppAIV and OppAV are encoded on a circular (cp26) and linear (lp54) plasmid, respectively (Bono et al., 1998). This genetic organization is unique among oligopeptide transporters, which typically are present at a single chromosomal locus. Paralogs of these genes have also been identified in the *B. burgdorferi* sensu lato strains, *B. hermsii* and *B. afzelii* (Kornacki and Oliver, 1998).

The putative physiological roles for this, and other oligopeptide transporters, include sensing pertinent environmental signals and nutrient uptake (by recycling cell wall and external peptides; Bono et al., 1998). The OppA (OppA-1, OppA-2, OppA IV and OppAV) proteins were able to function as substrate binding proteins by complementation of an *opp*$^{-}$ *E. coli* strain (Lin et al., 2001). To explore the role of this oligopeptide permease in the transmission and adaptation of *B. burgdorferi* between the arthropod and mammalian environments, an *oppAIV* mutant was created by insertion inactivation; this mutant did not show impaired growth in BSK II medium (Bono et al., 1998; Table 3). Unfortunately, the current state of borrelial genetics did not allow for this mutation to be constructed in an infectious *Borrelia* strain, and therefore its ability to cycle between the tick and mammalian host could not be determined. A *B. burgdorferi* strain N40 containing a spontaneous truncation in *oppA2*, however, was successfully maintained in the tick-mammal infectivity cycle. This latter observation may be due to functional redundancy among the five OppA peptide-binding proteins, or this system is not essential for maintenance of the enzootic cycle.

Genetics

The organization of the *Borrelia* genomes is unique within the Family Spirochaetaceae and perhaps among the prokaryotes. Most significantly, the chromosome is linear in nature, having single-stranded hairpin loops at each termini, resembling telomeres, a form of DNA thought to be restricted to eukaryotes and viruses (Barbour and Garon, 1987; Baril et al., 1989; Ferdows and Barbour, 1989; Hinnebusch et al., 1990; Bergstrom et al., 1992; Casjens and Huang, 1993; Casjens et al., 1997a; Casjens, 1999; Casjens et al., 2000; Volff and Altenbuchner, 2000). Borrelial DNA has a G+C content of ~29 mol%, which is lower than that (www.tigr.org) reported for *Treponema pallidum* and *Leptospira* (Hyde and Johnson, 1984; Fraser et al., 1997; Fraser et al., 1998). The chromosome copy number of one borrelial species, *B. hermsii*, was determined to be 16 copies per cell (Kitten and Barbour, 1992). These data were also supported using individual agarose-embedded spirochetes (*B. burgdorferi* and *B. hermsii*) labeled in situ and visualized directly by epifluorescence microscopy (Hinnebusch and Bendich, 1997). This number is significantly higher than that of the prototypic bacterial species, *E. coli*, which when actively growing, has an average of 6.5 chromosomal origins per cell (Bremer and Dennis, 1987). In situ visualization of the bacterial nucleoids of *E. coli* and *Borrelia* spp. by fluorescence microscopy also suggests that the *Borrelia* spp. chromosome is not organized into the tightly packed, or condensed, nodes found in *E. coli*, but instead consists of a loose meshwork of DNA strands that lack a central organizing node (Hinnebusch and Bendich, 1997).

The putative origin of replication (*ori*) of the *B. burgdorferi* chromosome was recently mapped using a combination of theoretical (GC skew analysis) and molecular (nascent DNA strand analysis) methodologies (Picardeau et al., 1999b). Analysis of the GC skew pattern identified a region in which there was a switch in polarity from positive to negative at the middle of the chromosome. A cumulative GC skew diagram of complete series of *B. burgdorferi* gene sequences showed a minimum between the *dnaA* and *dnaN* genes, analogous to the position of *oriC* in an identical analysis of *E. coli*. Further analysis of this region, however, failed to identify other sequences (DnaA initiator protein-binding sites and AT-rich regions) characteristic of both Gram-negative and Gram-positive origins (Picardeau et al., 1999a). The identification of a DnaA ortholog in *B. burgdorferi* suggests that this protein may bind to a unique site within the origin (Fraser et al., 1997).

Plasmids of *Borrelia*

In addition to containing linear chromosomes, any one spirochete may contain 21 or more different extrachromsomal elements (plasmids), ranging in size from 5–56 kb, in either linear or supercoiled circular forms (Fraser et al., 1997; Casjens et al., 2000; Fig. 11). Linear plasmids also have covalently closed termini similar to those on the linear chromosome. The unusual linear plasmids were first described in *B. hermsii*, but multiple linear and circular plasmids have also been described for many other *Borrelia* including *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. crocidurae*, *B. burgdorferi*, *B. coriaceae* and *B. anserina* (Hyde and Johnson, 1984; Barbour, 1988; Goodman et al., 1991; Kitten and Barbour, 1992; Xu and Johnson, 1995; Marconi et al., 1996a; Barbour et al., 1996b; Stevenson et al.,

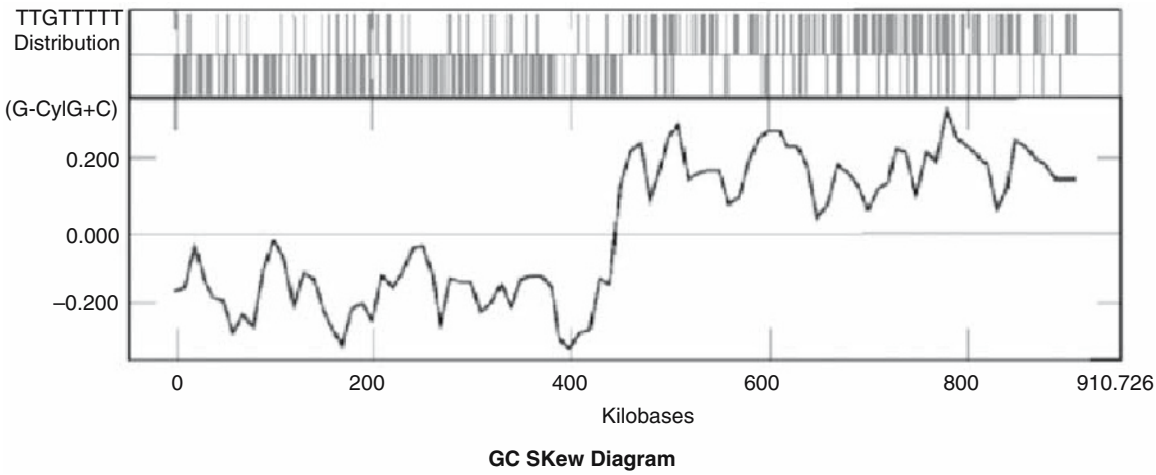


Fig. 10. Distribution of TTGTTTTT and the GC skew in the *B. burgdorferi* chromosome. Top, distribution of the octamer TTGTTTTT. The lines in the top panel represent the location of this octamer in the plus strand of the sequence, and those in the second panel represent the location of this oligomer in the minus strand of the sequence. Bottom, GC skew. Taken from Fraser et al. (1997) and www.nature.com (http://www.nature.com/cgi-taf/DynaPage.taf?file=/nature/journal/v390/n6660/full/390580a0_ fs.html) and reprinted by permission from Nature (http://www.nature.com), © 1997 Macmillan Magazines Ltd.

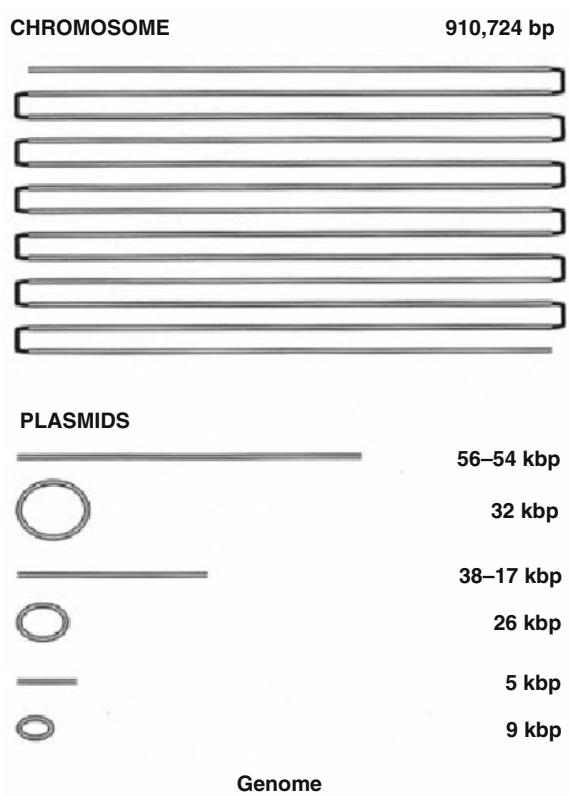


Fig. 11. Schematic representation of the linear chromosome and plasmids (linear and circular) of *Borrelia burgdorferi*.

2000a; Stevenson et al., 2000b; Hendson and Lane, 2000).

The only fully sequenced, and therefore the best characterized, genome is that of *B. burgdorferi* (The *B. burgdorferi* Genome Sequence). In this species, the full complement of linear and circular plasmids comprises approximately one-third of the total genomic content (Fraser et al., 1997; Casjens et al., 2000). Studies examining the copy number of *Borrelia* plasmids indicate that the linear and circular plasmids are closely maintained at approximately one plasmid copy per chromosome in a population of cells (Kitten and Barbour, 1992; Hinnebusch and Barbour, 1992; Casjens and Huang, 1993). This tight control of plasmid copy number suggests that replication of these extrachromosomal elements may be controlled by a mechanism similar to that of chromosomal replication. It has been proposed that the *Borrelia* linear plasmids are actually "minichromosomes" (Casjens, 1999; Barbour, 1993). The mechanism by which the linear and circular plasmids are replicated is currently a matter of much speculation, although recent data would suggest that the linear plasmids initiate replication at their centers and that replication in both types of plasmids proceeds in a bidirectional manner (Picardeau et al., 1999b; Casjens, 1999; Casjens et al., 2000). In proposed models (Fig. 12), the *Borrelia* linear plasmids are thought to use a circular intermediate for replication; circular replication intermediates have been isolated from some *Borrelia* spp. (Ferdows et al., 1996). The formation of covalently closed hairpin loops (telomeres) and the initiation of DNA replication near the center of the chromosome and linear plasmids present, however, a dilemma for the cell. How does the cell resolve two parental strands that are covalently joined at the telomeres, but destined to end up in different

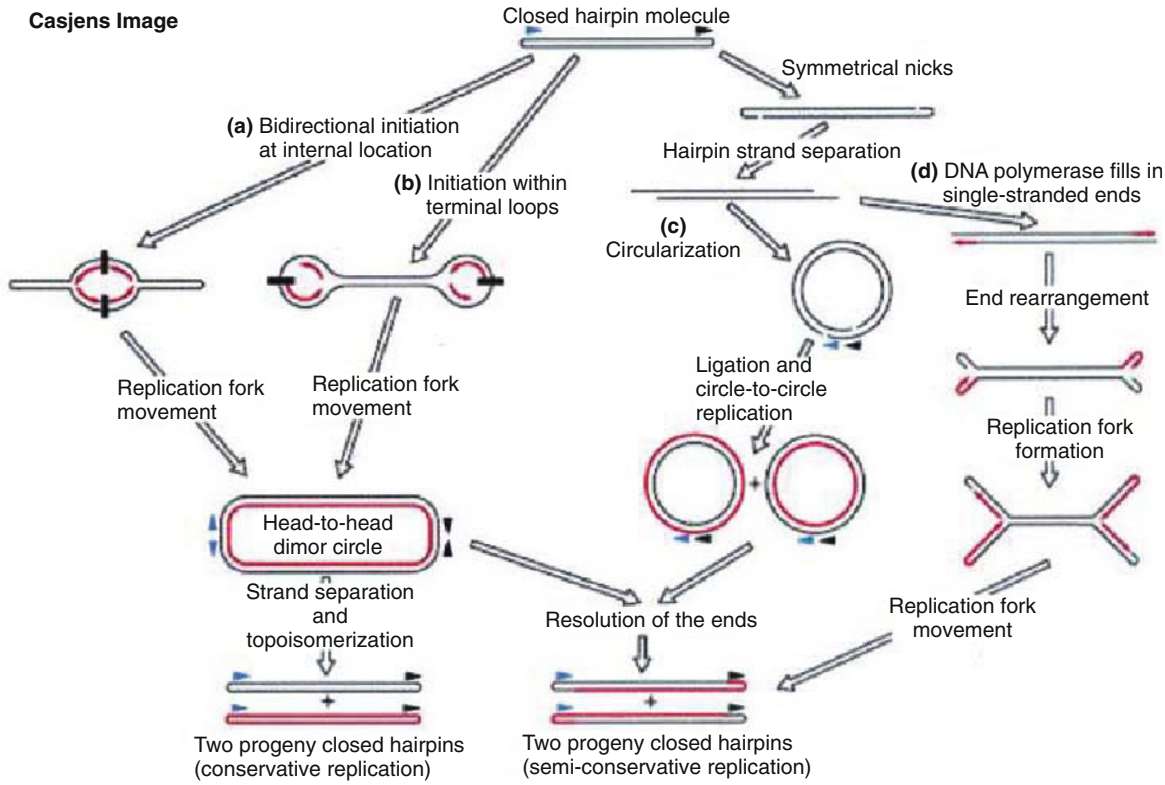


Fig. 12. Four strategies to replicate linear DNAs with covalently closed hairpin telomeres. In scheme (a), bidirection initiation of replication occurs near the center of the chromosome, whereas in scheme (b), initiation occurs within the terminal loops. In both (a) and (b), replication is followed by strand nicking and reassortment during end resolution (semiconservative replication) or strand separation and topoisomerization (conservative replication). In scheme (c), strand nicking occurs both during circularization and after replication. In scheme (d), strand nicking precedes duplication. Red lines indicate newly synthesized strands; black bars indicate replication origins on an unbroken template strand; the black and blue arrowheads indicate different sequences near the two telomeres to indicate the head-to-head and tail-to-tail joints in the possible dimer circle intermediate and the head-to-tail joints in the possible monomer circle intermediate. These strategies need not be mutually exclusive; for example, strategies (a) and (d) could combine to replicate the bulk and ends of the DNA, respectively. Reprinted from *Current Opinion in Microbiology*, 2, Casjens, S., Evolution of the linear DNA replicons of *Borrelia burgdorferi*. pp. 529–534, © 1999, with permission from Excerpta Medica, Inc. (Casjens, 1999).

daughter cells? Several mechanisms of strand resolution have been proposed, each utilizing a strand breakage to allow for segregation of the two parental strands, and also may involve the use of specific proteins, (i.e., transposases, integrases and telomerases). Recent studies by Chaconas et al. (2001) have proposed a model by which the hairpin telomeres are processed during replication. According to this model, the final step in the replication of linear replicons is a site-specific DNA breakage and reunion event to regenerate covalently closed hairpin ends. For recent reviews of *B. burgdorferi* DNA replication, see Casjens (1999) and Garcia-Lara et al. (2000).

The origin of linear DNA hairpin telomeres in *Borrelia* is also a matter of much speculation. Hinnebusch and Barbour (1991) noticed a low level of sequence similarity between the *Borrelia* telomeres and the hairpin ends of the iridopoxvirus African swine fever virus (ASFV). One very interesting hypothesis suggests that if a linear ASFV-like element had integrated into an ancestral *Borrelia* circular DNA element, it could have generated a linear DNA molecule and brought along with it the necessary machinery for end-resolution. Although no convincing DNA similarities were detected between *Borrelia* and poxvirus genes, most of the poxvirus genome could have been lost during evolution, leaving only the end-resolution apparatus required for the replication of the linear DNA molecules. This scenario is made more plausible by the fact that both *Borrelia* and ASFV are transmitted by arthropods; *Ornithodoros moubata* (Table 2) is known to transmit both *B. duttonii* and ASFV. This colocalization within an arthropod vector brings these very distinct

phyla into close enough proximity of each other to allow for genetic exchange (Casjens, 1999).

Plasmids and *Borrelia* Virulence

Shortly after the discovery of *Borrelia* plasmids, it was noted that this bacterium will often lose plasmids as a result of continuous in vitro cultivation (Barbour, 1988). It also was demonstrated that a loss of plasmid content was often associated with changes in protein profile and/or a reduction of infectivity (Schwan et al., 1988b; Persing et al., 1994; Golde and Dolan, 1995; Norris et al., 1995; Xu et al., 1996; Busch et al., 1997; Zhang et al., 1997; Anguita et al., 2000; Purser and Norris, 2000; Labandeira-Rey and Skare, 2001). Two recent studies have more fully characterized the relationship between plasmid content and infectivity in *B. burgdorferi* using clones with their plasmid content fully (Purser and Norris, 2000) or partially (Labandeira-Rey and Skare, 2001) characterized. Both of these studies demonstrated that the loss of one or more linear plasmids can be correlated with a reduction or loss of infectivity. While both of these studies represent a significant step towards furthering our understanding of the role of plasmid-encoded genes in borrelial virulence, the contribution of many of the circular plasmids could not be assessed because 1) they were not lost spontaneously during the isolation of clones (Purser and Norris, 2000), or 2) they were not included in the test panel to determine plasmid content (Labandeira-Rey and Skare, 2001). These data are, however, consistent with the notion that one possible role for the plasmids of *Borrelia* spp., like the virulence-associated plasmids of many bacteria (i.e., *Salmonella* spp, *Shigella flexneri*, *Bacillus anthrasis* and *Agrobacterium tumefaciens*), is to encode proteins that enable this bacterium to survive in vastly different environmental conditions and hosts. Also consistent with this hypothesis is the presence of genes that are expressed preferentially within either of these different host environments. To date, all of the differential gene expressions described have been localized to the linear and circular plasmids, further supporting a significant role for plasmids in the biology of these spirochetes (Wallich et al., 1993; Champion et al., 1994; Akins et al., 1995; Akins et al., 1999; Schwan et al., 1995; Stevenson et al., 1995; Stevenson et al., 1998a; Suk et al., 1995; Porcella et al., 1996; Lahdenne et al., 1997; Cassatt et al., 1998; Fikrig et al., 1999; Yang et al., 1999; Caimano et al., 2000; Miller et al., 2000; Hefty et al., 2001).

The *Borrelia burgdorferi* Genome Sequence

One of the most significant recent developments in borrelial research was the publication of the complete DNA sequence of the *B. burgdorferi* chromosome (Fig. 5) and associated plasmid elements (Fraser et al., 1997; Casjens et al., 2000; Fig. 11). The *B. burgdorferi* strain chosen for this project was the type strain B31 (ATCC 35210), an isolate obtained from an *I. scapularis* tick caught on Shelter Island, New York (Burgdorfer et al., 1982; Johnson et al., 1984). Prior to beginning the sequencing project, the American Type Culture Collection (ATCC) isolate was cloned by limiting dilution to yield an isolate now referred to by many as "B31-MedImmune" (B31-MI; Fraser et al., 1997). While this isolate has been minimally passaged in vitro, B31-MI is missing three circular plasmids (cp32-2, cp32-5 and cp9-2) as compared to the original isolate (Stevenson et al., 1998c; Miller et al., 2000). Briefly, the data from this project revealed that the *B. burgdorferi* linear chromosome is approximately 911 kb and is predicted to encode 853 open reading frames (ORFs). Biological roles were assigned to 59% of these ORFs. Of the remaining ORFs, 12% matched hypothetical coding sequences of unknown function from other organisms, and 29% were unique to *B. burgdorferi* (Fraser et al., 1997). In addition to the large linear chromosome, 12 linear and 9 circular extrachromosomal elements (plasmids) were also sequenced, totaling an additional 611 kb (Fraser et al., 1997; Casjens et al., 2000). In contrast to the ORFs encoded on the chromosome, greater than half of the ORFs contained on the plasmids did not have significant database matches to organisms other than *Borrelia*.

In the pre-genomic era, a number of laboratories had published reports calling attention to the fact that *B. burgdorferi* contains a large number of "repeated" sequences (i.e., related sequences present in multiple copies per genome; Simpson et al., 1990; Zückert et al., 1994; Zückert et al., 1999; Marconi et al., 1996b; Porcella et al., 1996; Zückert and Meyer, 1996; Fraser et al., 1997; Stevenson et al., 1997; Stevenson et al., 2000a; Carlyon and Marconi, 1998a; Carlyon et al., 1998b; Caimano et al., 2000; Carlyon et al., 2000), reviewed in Casjens et al. (2000). The full extent of this sequence redundancy has become apparent with the availability of the complete genomic sequence (Fraser et al., 1997; Casjens et al., 2000). One hundred and fifty-six paralogous families have been identified, with each paralogous family having at least two and up to 42 members. Although biological roles have been assigned to some of these paralogous families (e.g., ABC transporters [PF4, PF37,

PF41, PF105], methyl-accepting chemotaxis proteins [PF13], and plasmid partitioning [PF32, PF49]), the majority represent proteins with no known function. The large number of paralogous families is strong evidence that recombinatorial processes have conferred a remarkable degree of plasticity upon the *B. burgdorferi* genome, presumably in the context of enhancing its adaptation to diverse ecological niches.

The cp32 Family of Supercoiled Circular Plasmids

One of the best characterized examples of genetic redundancy in *Borrelia* is the family of 32- and 18-kb supercoiled circular plasmids (cp32s; Zückert et al., 1994; Zückert and Meyer, 1996; Casjens et al., 1997b; Stevenson et al., 1997; Stevenson et al., 1998c; Stevenson et al., 2000b; Akins et al., 1999; Caimano et al., 2000; Casjens et al., 2000). *Borrelia burgdorferi* sensu stricto isolates may contain up to 9 different cp32 plasmids (Fig. 11), each encoding *ospE*/*ospF*/*elp*/*erp* loci (Akins et al., 1995; Akins et al., 1999; Stevenson et al., 1996; Stevenson et al., 1997; Stevenson et al., 1998c; Casjens et al., 1997a; Caimano et al., 2000; see "OspE/OspF/Elp Lipoproteins"), as reviewed in Stevenson et al. (2000b). Each member of this plasmid family shares a high degree of similarity throughout its sequence, with only limited regions of sequence heterogeneity observed. Interestingly, the three regions, which are variable between members of this plasmid family, contain loci thought to be involved in the stable maintenance and partitioning of these plasmids and two paralogous families of differentially expressed lipoproteins (the Mlp Lipoproteins and OspE/OspF/Elp lipoproteins; Porcella et al., 1996; Stevenson et al., 1996; Stevenson et al., 1998c; Stevenson et al., 2000a; Akins et al., 1999; Yang et al., 1999; Caimano et al., 2000). Evidence has been presented that suggests these latter two variable regions represent sites of extensive recombination during the molecular evolution of this plasmid family (Stevenson et al., 1998c; Akins et al., 1999; Caimano et al., 2000). In addition, the presence of "unique" sequences downstream of some of the OspE/OspF/Elp loci suggests that *B. burgdorferi* may be capable of acquiring and incorporating exogenous DNA (Caimano et al., 2000). The relatively high density of spirochetes in the tick midgut, compared to the density within mammalian tissues, makes the midgut the most likely milieu in which this recombination occurs.

Linear forms of cp32-related plasmids have been described in *B. burgdorferi* (lp56) and *B. parkeri* (Fraser et al., 1997; Casjens et al., 2000; Stevenson et al., 2000b). An analysis of one linear plasmid, lp56, revealed that it contains within it an essentially intact copy of a cp32-like plasmid. The region of lp56 that is cp32-like appears to have resulted from an integration event (by an unknown mechanism) between a progenitor cp32-like plasmid and an unique linear plasmid. Although the inserted cp32-like progenitor shares a higher degree of homology with several cp32s (cp32-4, cp32-6 and cp32-9), it is not identical to any of the seven known cp32 plasmids and therefore potentially represents an eighth member of this family (Casjens et al., 2000). A second example of recombination between the linear and circular plasmids is evident on lp54 (Casjens et al., 2000; Caimano et al., 2000; Fig. 13). This plasmid is notable because it encodes the *ospA/B* (see "Reciprocal Expression of OspA and OspC") operon and the decorin-binding protein operon *dbpBA* (see "Decorin- and Fibronectin-binding Proteins"); both of these operons have been shown to be differentially expressed (*ospA/B* is downregulated, whereas *dbpBA* is upregulated in the mammalian host). The linear lp54 plasmid contains several stretches with high degrees of homology to paralogs which are otherwise exclusively encoded on cp32 plasmids (Fraser et al., 1997; Caimano et al., 2000; Casjens et al., 2000; Fig. 13). The presence of subsequent insertions and replacements within the region of cp32-like sequence suggests that the recombinatorial event(s) responsible for shaping lp54 is likely to have occurred much earlier in the evolution of the *Borrelia* plasmid complement than the event that resulted in the lp56 plasmid (Casjens et al., 2000).

Two different types of recombinatorial events are thought to have occurred within the cp32 family of plasmids (Caimano et al., 2000). The first involved exchanges between these plasmids and the bacterium's other genetic elements. This process appears to have been relatively unrestricted in that it utilized diverse donor sequences and recipient sites; its principal constraint is that it was limited to sequences already present within the borrelial genome. One of its primary functions appears to have been plasmid building (Fig. 13), that is, generating the scaffolding or framework needed to assemble novel genetic elements. The second type of recombinatorial event, in contrast, was confined to relatively small regions of the cp32 plasmids but was less restrictive in that it utilized nonparalogous DNAs, including what may be exogenous genes (i.e., the unique sequences downstream of some of the OspE/OspF/Elp lipoproteins loci). A major consequence of this second type was to individualize plasmids via the variable regions.

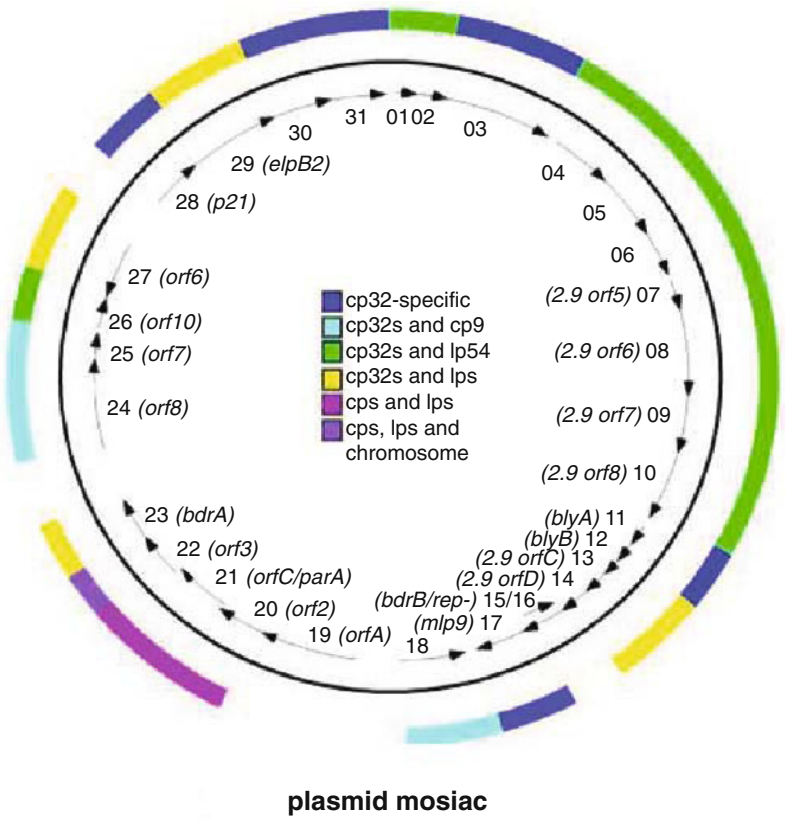


Fig. 13. *Borrelia burgdorferi* strain 297 cp18-2. Representative depiction of the mosiac nature of the cp32/18 plasmid family. Taken from Caimano et al. (2000), with permission of American Society of Microbiology (ASM) Press (http://www.journals. asm.org).

Recently, members of the cp32 family were also described in the relapsing fever spirochete, *B. hermsii* (Stevenson et al., 2000a). Although this plasmid contains many of the sequences that typify the cp32 family of plasmids (i.e., Mlp Lipoproteins, two Bdr paralogs, and partitioning loci (PF32 and PF50 paralogs), it does not contain an OspE/OspF/Elp (Erp) lipoprotein (Stevenson et al., 2000b; OspE/OspF/Elp Lipoproteins). Even more striking, however, is that the position of the Mlp Lipoproteins on the *B. hermsii* plasmid is in the position normally occupied by the *ospE*/*ospF*/*elp*/*erp* (see "OspE/OspF/Elp Lipoproteins") locus. An analysis of the *B. hermsii* genome by Southern hybridization did not reveal any sequences that hybridized with a *B. burgdorferi ospE*/*ospF*/*elp*/*erp* probe (Stevenson et al., 2000a), suggesting that the relapsing fever spirochete lacks homologs of these lipoproteins, or if they are present, their sequences are divergent beyond detection at the level of stringency used. The *B. hermsii* cp32 also encodes a novel protein, Bhm, a putative nucleotide methylase (Stevenson et al., 2000b).

Borrelia Pseudogenes

In addition to the duplications and rearrangements identified in the *B. burgdorferi* genome, a significant proportion of the genome was found to contain a large number of mutationally damaged genes and/or genes thought not to be expressed due to frameshift, inframe stop codons, and fused or truncated genes (pseudogenes; Casjens et al., 2000). In some instances, the majority of a plasmid's copy capacity is comprised of pseudogenes; for example, lp28-1 encodes 36 pseudogenes (78% of its total coding capacity), lp21 encodes 6 pseudogenes (50% of its coding capacity), and lp56 encodes 22 pseudogenes (61% of its total coding capacity; Casjens et al., 2000). In total, 670 potentially functional (non-pseudo)genes and 167 pseudogenes were identified on the 21 plasmids sequenced (Fraser et al., 1997; Casjens et al., 2000). Interestingly, ten of the strain B31 plasmids (lp5, lp17, lp21, lp25, lp28-1, lp28-3, lp28-4, lp36, lp38 and the non-cp32-like portion of lp56) contain 87% of the pseudogenes and have a total non-pseudogene coding capacity of only 41%, with the majority (43%) of these encoding genes ≥300 bp in length (Casjens et al., 2000). It has been hypothesized that the large number of pseudoegenes present on some borrelial plasmids may represent remnants of genes that are no longer useful but have not yet been completely eliminated from the genome (Casjens et al., 2000). Two recent publications may help explain why at least two of the pseudogene-rich plasmids are

consistently maintained in the *B. burgdorferi* genome: 1) the localization of a locus, *vlsE* (*B. burgdorferi* vls Locus), thought to be responsible for antigenic variation in *B. burgdorferi* to lp28-1 (Zhang et al., 1997), and 2) lp28-1 and lp25 were both correlated with infectivity in mice (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001).

Bacteriophage of *Borrelia*

Bacteriophage-like particles have been described for a number of spirochetes, including *Borrelia*, *Treponema* (see The Genus *Treponema* in this Volume), *Leptonema*, *Leptospira* and *Brachyspira* (see review by Eggers et al., 2000). Hayes and coworkers (Hayes et al., 1983) identified the first bacteriophage-like particles in *B. burgdorferi* in 1983. Two similar phage-like particles were described from a human isolate of *B. burgdorferi*, but differed (at the ultrastructural level) from those particles reported by Hayes and co-workers. These data suggest that borreliae may harbor more than one type of bacteriophage (Neubert et al., 1993; Schaller and Neubert, 1994). Recently, Eggers and coworkers have characterized a bacteriophage ΦBB-1 (Fig. 14) from *B. burgdorferi* stain CA-11.2A (Eggers and Samuels, 1999) and demonstrated that phage particles could be produced spontaneously or by the use of a DNA alkylating compound (MMNG, 1-methyl-3-nitro-nitrosoguanidine) as an inducing agent. A molecular characterization of ΦBB-1 revealed that the phage heads were packaged with a discrete piece of linear, double-stranded DNA molecule 32 kb in size as its genome. These linear molecules lack the covalently closed ends characteristic of the linear plasmids typically found in *Borrelia*. Further analyses demonstrated that the prophage form of ΦBB-1 is maintained as one or more members of the cp32 plasmid family and that genes present on these plasmids (BBP41 to BBP26) most likely constitute the genes necessary for a phage late regulon (Eggers et al., 2000). Eggers and coworkers have also been able to use ΦBB-1 as a tool for transduction (the transfer of DNA between bacteria using bacteriophage) by transferring an antibiotic-resistance marker (*kan*^{r}) between cells of different *B. burgdorferi* strains (Eggers et al., 2001).

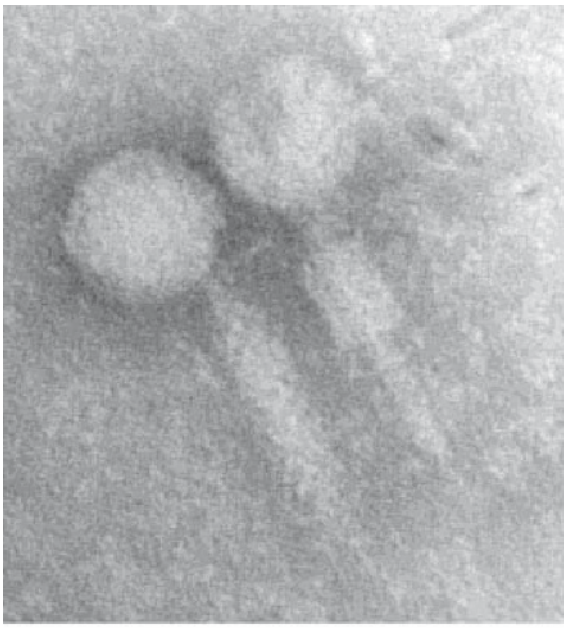


Fig. 14. *Borrelia burgdorferi* phage ΦBB-1. Samples were collected from polyethylene glycol (PEG)-precipitated cell-free supernatants of a *B. burgdorferi* culture, stained with phosphotungstic acid, and viewed by transmission electron microscopy. The contractile tail is either extended (left) or contracted (right). Image taken from Eggers et al. (2001), with permission of ASM Press (http://www.journals.asm.org).

Genetic Transformation of *Borrelia*

The inability to introduce exogenous (borrelial or non-borrelial) DNA into *Borrelia* spp. had long been a hindrance to using molecular biology techniques (i.e., transformation, gene inactivation, and transfection/transduction) that have become routine in most other well-characterized bacterial strains such as *E. coli*, *Bacillus subtilis* and *Streptococcus pneumoniae*. This was due in part to both the difficulties associated with the development of a method for the introduction of exogenous DNA into *Borrelia* spp. and the lack of a suitable cloning (shuttle) vector; these two basic elements are necessary to perform many of the classical molecular genetics experiments. Recent work on *B. burgdorferi*, however, has demonstrated that both of these hurdles may soon be cleared (see review by Tilly et al., 2000). One of the first major advances in molecular genetics was the demonstration of transformation of *B. burgdorferi*. In this pioneering experiment, Samuels and co-workers (Samuels et al., 1994b) were able to introduce a genetic marker (coumermycin A_1 resistance [*gyrB*^{r}]) into *Borrelia* using electroporation. This methodology, combined with a solid medium plating technique, allowed for the isolation of single colony isolates of transformed bacteria (Samuels and Garon, 1993; Samuels et al., 1994a; Samuels et al., 1994b; Samuels et al., 1995). These studies were soon followed by the first allelic exchange studies using targeted insertions of borrelial

genes (Rosa et al., 1996; Tilly et al., 1997). A number of additional borrelial genes involved in peptide transport (*oppAIV*), nucleotide biosynthesis (*guaB*), and environmental sensing and / or virulence (*ospC*, see "Reciprocal Expression of OspA and OspC") have since been mutated by gene inactivation and their phenotypes examined (Tilly et al., 1997; Tilly et al., 1998; Bono et al., 1998). The limitations of using coumermycin soon necessitated the development of tools for the introduction of heterologous (foreign) DNA to monitor gene expression without antibiotic selection (Stevenson et al., 1998b). The most recent breakthrough came with the development of the first extrachromosomal cloning vector system for *B. burgdorferi*. In this system, a broad-host range vector, pGK12 (containing replication functions from *Lactococcus lactis* and erythromycin- and chloramphenicol-resistance markers from *Staphylococcus aureus*), was used to transform *B. burgdorferi* and confer resistance to erythromycin. This plasmid was shown to replicate autonomously in strain B31 and conferred resistance to erythromycin to these transformed bacteria. These studies permitted the development of a reporter plasmid containing enhanced green fluorescent protein (EGFP) under the control of a borrelial promoter (Saratokova et al., 2000). These combined studies represent major steps toward furthering our understanding of borrelial genomics, particularly the regulation of gene expression and function. The majority of this work was performed using noninfectious strains of *B. burgdorferi*. To fully realize the potential of these molecular tools, methods for the transformation of infectious strains of *Borrelia* will need to be developed. Towards that end, recent studies by Stewart et al. (2001) have reported the isolation of a circular plasmid region from a *B. burgdorferi* circular plasmid (cp9) that is capable of autonomous replication and represents the first shuttle vector based on a borrelial origin of replication and plasmid partitioning. Stewart and co-workers also were able to demonstrate that this cp9-based shuttle vector can be electroporated into an infectious strain of *B. burgdorferi* (Stewart et al., 2001). One notable consequence of this transformation, however, was the rapid displacement of the endogenous cp9 plasmid of the recipient strain N40, a phenomenon almost certainly related to a plasmid compatibility issue between the shuttle vector (containing the cp9 replication region) and the endogenous cp9. While this displacement event did not affect the ability of the bacterial transformants to grow in culture medium, it does present a significant limitation of using this and similar shuttle vectors in a genetic background containing the full genetic complement of borrelial plasmids, whose roles in bacterial physiology and/or pathogenesis have not been fully determined.

Epidemiology

Relapsing Fever

Two major forms of relapsing fever have been described, epidemic (louse-borne) and endemic (tick-borne). During large epidemics of louse-borne relapsing fever (LBRF), millions of people have been infected. The last large outbreak of epidemic relapsing fever occurred during and following World War II in the Mediterranean region, North Africa, and the Middle East (Felsenfeld, 1965; Bryceson et al., 1970; Cadavid and Barbour, 1998). Current outbreaks of LBRF are primarily reported from African countries, particularly Ethiopia and Sudan (Cadavid and Barbour, 1998), with some outbreaks occurring in the South American Andes.

Endemic tick-borne relapsing fever (TBRF), transmitted by soft ticks of the genus *Ornithodoros* (Table 1), is more sporadic in occurrence than LTRF. TBRF is endemic in the western United States, southern British Columbia, the plateau regions of Mexico, and Central and South America, the Mediterranean, Central Asia, and throughout most of Africa. In the United States, the environment in which a human host comes into contact with a tick vector is largely determined by the geographical location. In Texas, in the relapsing fever spirochete, *B. turicatae*, TBRF is transmitted by *O. turicatae* and is primarily acquired by residents or tourists cave exploring. In most other western states and southern British Columbia, *B. hermsii* is transmitted by *O. hermsi* and primarily acquired at higher elevations by visitors of rustic cabins that may house rodent nests. A recent outbreak of TBRF (due to *B. hermsii*) in Colorado was associated with a visit by vacationers from Nebraska and Kansas to a rustic cabin in Colorado (Trevejo et al., 1998). In this report, 11 out of 23 family members vacationing at one rustic cabin developed symptoms suggesting TBRF, and although no spirochetes were visible in the case-patients blood smears, *B. hermsii* was cultured from the blood of one case-patient and two chipmunks trapped near the cabin (Trevejo et al., 1998). A survey of previous visitors to that same cabin identified five additional persons from Nebraska, Kansas, Florida and Texas who had developed similar symptoms following their visit. A recent review by Dworkin et al. (1998), however, identified 133 confirmed cases and 49 probable cases in northwestern United States and southwestern Canada. Confirmed cases were defined as both a febrile illness and detection

of spirochetes by microscopic examination of peripheral blood smears. A probable case was defined as a typical clinical history and either an exposure associated with a confirmed case or a positive serology test or enzyme-linked immunosorbent assay (ELISA) confirmed by Western blot. The distribution of these cases was primarily in Washington (33%), Oregon (25.3%), Idaho (22%), and British Columbia (14.3%), with the remaining cases in Montana (0.5%) or not specified (4.9%). The majority of these cases were thought to be acquired by visiting rural cabin dwellings or homes (76.3%), often located on or near a body of water. Potential rodent reservoirs (mice, squirrels, chipmunks and rats) were also present at the exposure sites. The median elevation at which exposure occurred in 89 of the 182 cases was 2,131 feet (range, 100–6,436 feet). The most frequent months of onset were the summer months (July and August), but cases were identified in all months except November. The cases were fairly evenly distributed among the sexes (male, 57.1%; female, 42.9%), but a higher number of cases were seen in children younger than 5 (6%) or under 18 years of age (28.9%). Four women (11.4%) pregnant during their illness were included in the review, one of whom gave birth to an infected infant. Many cases in the southern United States (i.e., Texas) are often associated with cave exploration. In this region, the tick vector (*O. turicata*) of the relapsing fever spirochete, *B. turicatae*, is found in burrows made by cave-dwelling rodents, owls and snakes (Goubau, 1984). TBRF in the southern United States exhibits a different seasonality from TBRF in the northwestern areas, with the majority of cases having a late fall, early winter month of onset (Burgdorfer, 1976b).

Unlike Lyme disease (see "Epidemiology"), TBRF is not a nationally reportable communicable disease and is likely to be underreported. TBRF also may be incorrectly identified as Lyme disease owing to false positivity in Lyme disease serological diagnostic testing (Dworkin et al., 1998). Unless a patient history of wilderness exposure or camping in rodent-infected areas is given and the patient is aware of a tick bite, the illness is usually not suspected during the initial period of fever (Fihn and Larson, 1980).

Lyme Disease

According to the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/ncidod/dvbid/lymeinfo.htm), Lyme disease is the most common vector-borne infection in the United States. According to surveillance data submitted to the CDC between 1992 and 1998, a total of 88,967 cases of Lyme disease were reported by 49 states, the District of Columbia, and Guam (2 cases), with a mean annual average of 5.1 reported cases/100,000 persons/year (Orloski et al., 2000; Fig. 17). Of the total cases reported nationwide, the majority were from only four Northeastern states, New York (32.8%), Connecticut (17.4%), Pennsylvania (14.6%), and New Jersey (12.2%; Orloski et al., 2000). The reported incidence of Lyme disease (Fig. 16), when plotted by county, closely mirrors the regional distribution of the arthropod vector (Fig. 15) *Ixodes scapularis* in the Northeast and Midwest and *Ixodes pacificus* in the pacific northwest United States (Orloski et al., 2000). Cases have also been reported in most European countries where the Lyme spirochete is transmitted by *Ixodes ricinus*. In addition, the disease has been reported in China, Japan, Africa and Australia (Table 2).

In the United States, Lyme disease is a reportable disease, and epidemiological data are collected and cataloged by the CDC (http://www.cdc.gov/ncidod/dvbid/lymeinfo.htm). Lyme disease in Europe are not reportable, and therefore the epidemiological features of borreliosis in this region are often limited. An extensive epidemiologic study of Lyme disease in Europe, however, was performed in southern Sweden in 1996 by Berglund et al. (1995). This study, conducted over a one-year period, used case definitions that generally corresponded to those used by the CDC for surveillance purposes (clinically diagnosed erythema migrans or one or more clinical manifestations of disease plus serological confirmation) to include a total of 1,471 patients with Lyme disease, with *B. afzelii* being the causative agent in all cases. This number reflects an overall annual incidence of 69 cases per 100,000 inhabitants, a number similar to that of endemic areas in the United States (24.2/100,000 and 98/100,000 in New York and Connecticut, respectively). As with cases in the United States, the incidence varied markedly according to geographic region, and the disease was widely prevalent in several areas, with incidences ranging between 26 per 100,000 to as high as 160 per 100,000 (Berglund et al., 1995). While no differences were observed in the number of cases between men and women, incidence in children (5–9 yrs old) and the elderly (60–74 yrs old) was higher (Berglund et al., 1995; Fig. 18).

In the northeastern United States, the onset of Lyme disease is greatest during the months of June, July and August (Orloski et al., 2000). The European study also reported a similar seasonality to Lyme disease, with tick bites being most frequent in July and the highest number of erythema migrans cases occurring in August and September (Berglund et al., 1995). These months coincide with the questing (feeding) period of the *Ixodid* nymph (Fig. 4), the life

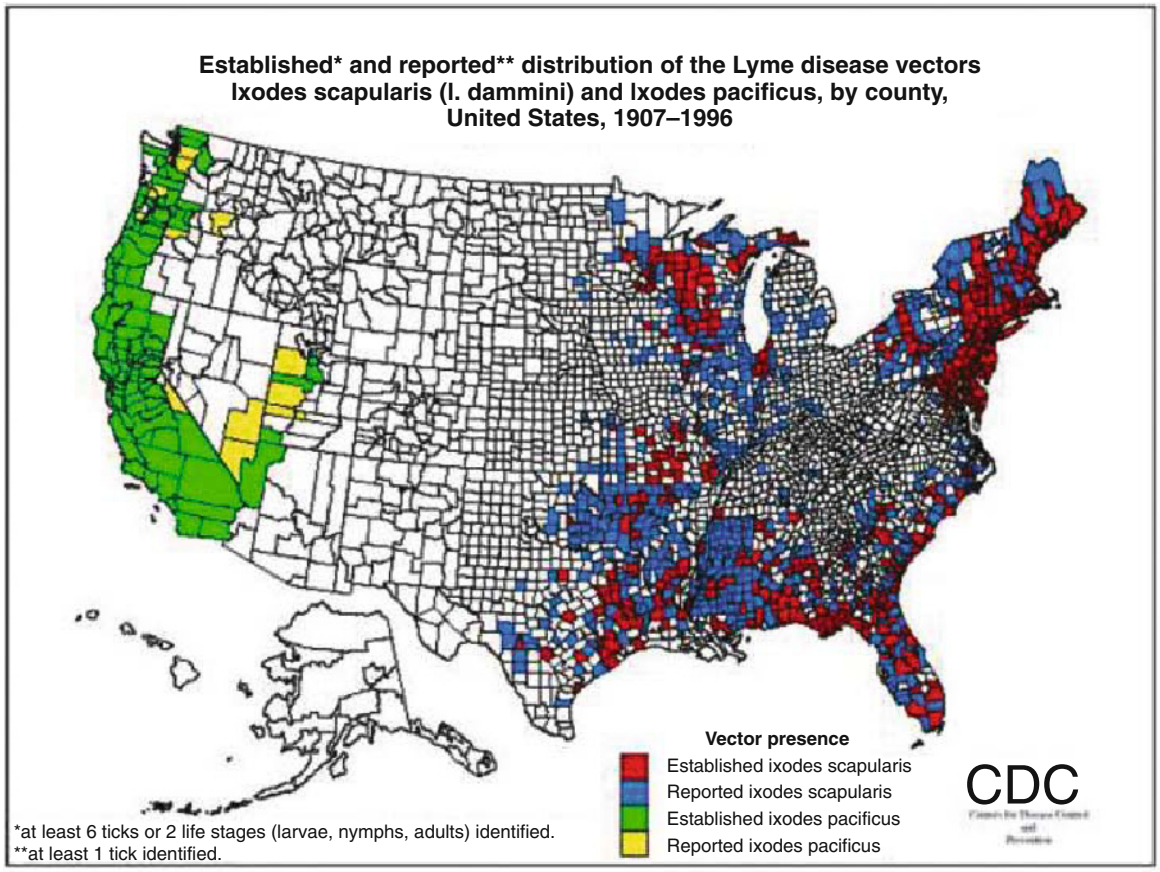


Fig. 15. Geographic distribution of *Ixodes* spp. Image adapted from the CDC (http://www.cdc.gov/ncidod/dvbid/lymeepi.htm).

stage primarily responsible for the transmission of *B. burgdorferi*. In addition, these months are often associated with increased outdoor activities, such as walking and hiking, which bring humans into closer proximity to the arthropod vector habitat.

Recent reports have described a Lyme disease-like illness in the south-central and southeastern United States in patients who have no known exposure to the Lyme disease vector, *Ixodes scapularis* (Barbour et al., 1996a; Fig. 15). In these cases, a skin rash resembling erythema migrans (EM; Fig. 20) has been associated with bites by the hard tick, *Ambylomma americanum*, the Lone Star tick. *Amblyomma americanum* has not been shown to be a competent vector for *B. burgdorferi* (Piesman and Happ, 1997; Dolan et al., 1998) and the infectious agent, *Borrelia lonestari* (*B. barbouri*), thought to be responsible for this Lyme disease-like illness (Barbour et al., 1996b) has yet to be cultivated (Uncultivatable *Borrelia* spp.).

Of mice and moths .... One interesting study by Jones et al. (1998) described a chain reaction of events beginning first with the level of acorns in a wooded area determining the winter survival, reproduction, and the resulting density of mice (both a predator of gypsy moth pupae and a reservoir of the Lyme disease spirochete, *B. burgdorferi*) in the oak forests of the eastern United States. Second, the relationship between mouse and moth densities can either suppress or increase subsequent acorn densities (i.e., high gypsy moth populations can result in deforestation and reduced acorn crops, whereas high mouse populations can result in increased pupae predation and increased acorn crops). Third, the location of high autumnal acorn density determines the next summer's larval tick densities by effecting the use of oak forests by deer (used by

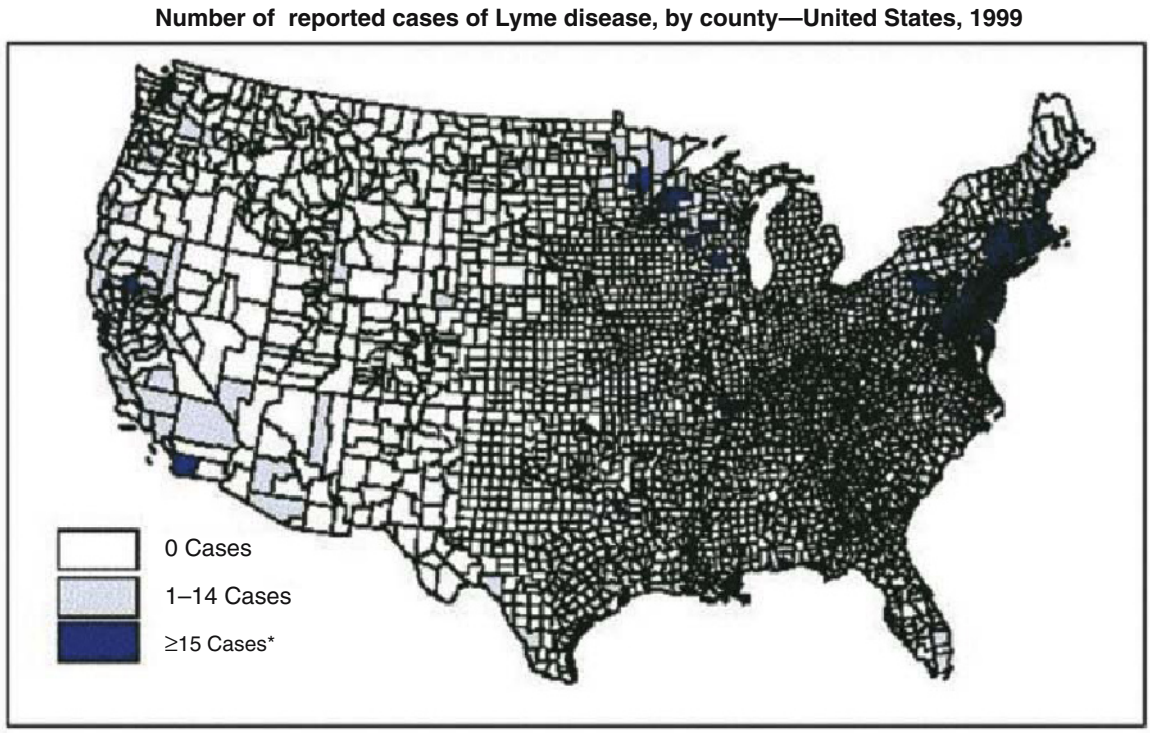


Fig. 16. Geographic distribution of reported cases of Lyme disease. Taken from Orloski et al. (2000).

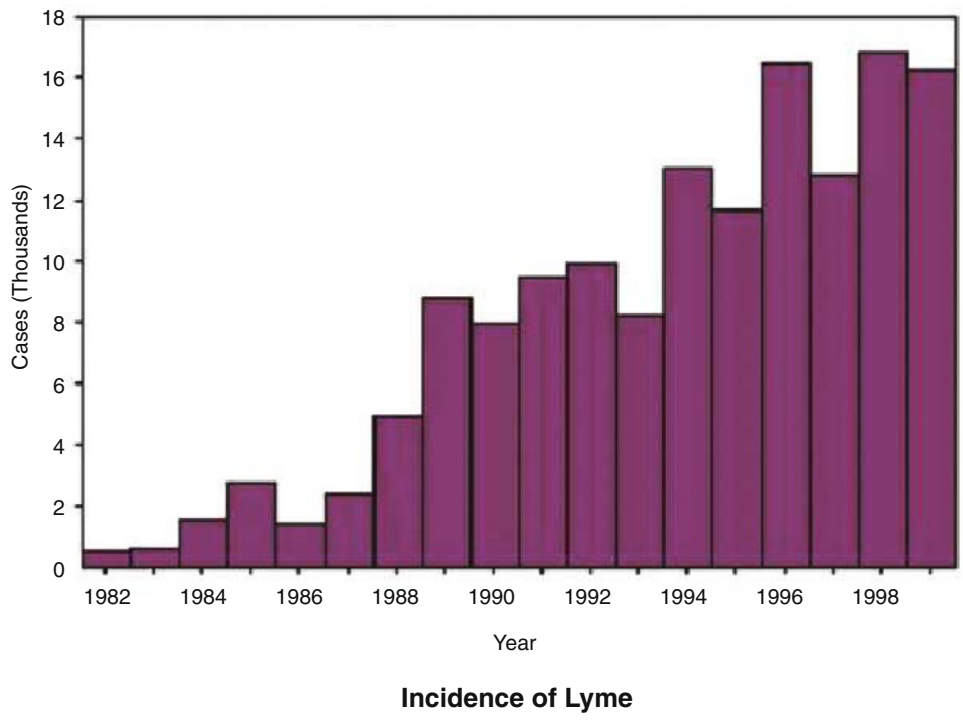


Fig. 17. Incidence of Lyme disease, by year—United States 1982–99 (http://www.cdc.gov/ncidod/dvbid/images/ld_case_barchart.gif). Taken from Orloski et al. (2000), with permission.

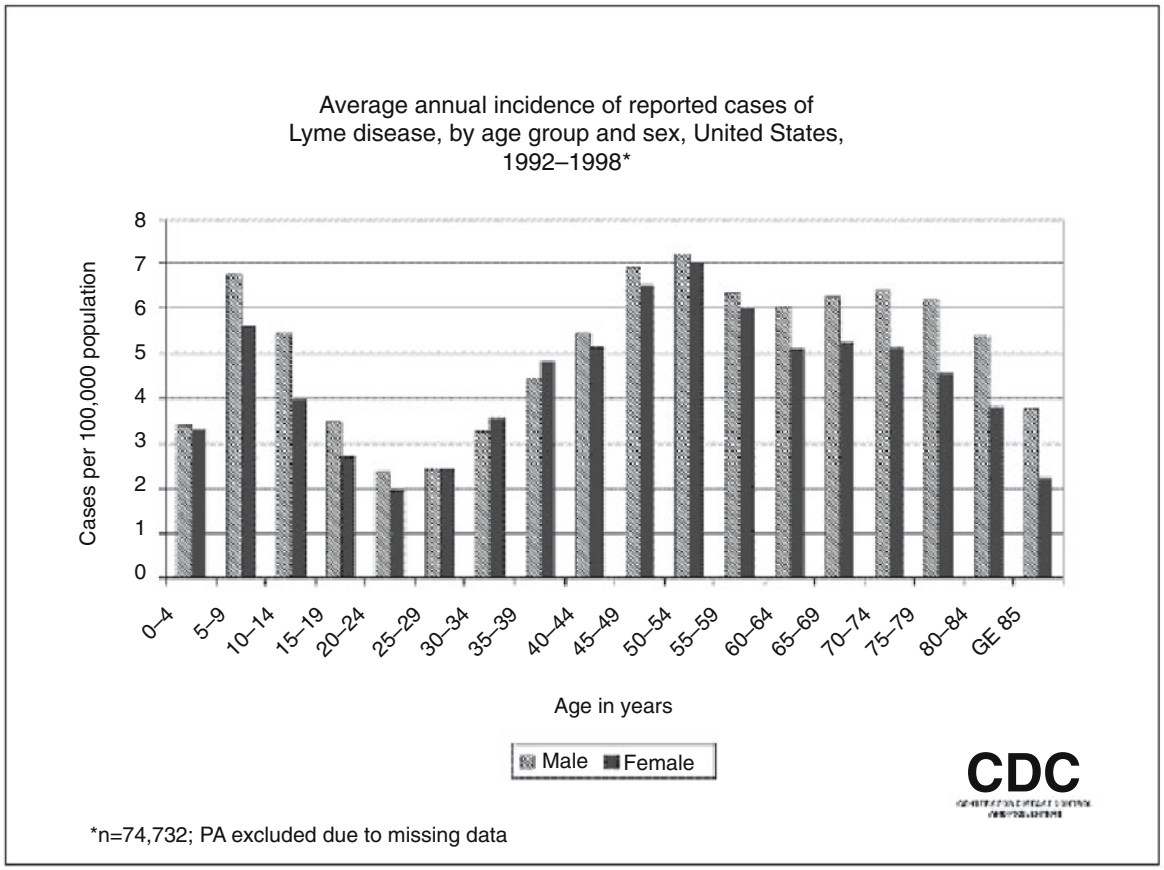


Fig. 18. Average annual incidence of reported cases of Lyme disease (http://www.cdc.gov/ncidod/dvbid/images/ld_case_barchart.gif), by age group and sex in the United States during 1992–1998 (http://www.cdc.gov/ncidod/dvbid/lyme/92-98agesex.htm).

adult ticks for mating); this situation in turn results in high densities of uninfected larval ticks and nymphal ticks questing when and where potentially spirochete-infected mice are most abundant. The authors suggest that it may be feasible to predict the risk of contracting Lyme disease from infected nymphal ticks in oak forests on the basis of autumnal acorn crops (masting), with the risk being greatest two years after an abundant acorn crop (Jones et al., 1998).

Pathogenicity

Relapsing Fevers

Two major forms of relapsing fever have been described, epidemic (louse-borne) and endemic (tick-borne; Table 1). For recent reviews of relapsing fever, see Dworkin et al. (1998) and Cadavid and Barbour (1998). In either of these forms, the relapsing fever borreliae gain access to the mammalian blood and lymph following transmission from an infected arthropod. After an incubation period of 4–18 days and after the spirochetes have multiplied to levels of 10^6–10^8 cells per ml of blood, the illness begins abruptly with shaking chills, fever, headache and fatigue, which persist for 3–7 days. Following an afebrile period of 5–10 days, a second symptomatic episode may occur. Additional relapses of decreasing time and intensity are characteristic of the tick-borne disease but are uncommon in the louse-borne disease. In either case, the relapses are the result of antigenic variation, where the spirochetes are able to undergo a number of cyclic antigenic variations (see "Antigenic Variation"). When antibodies to the prevailing antigenic type appear, organisms "disappear" from the peripheral blood and are replaced by a different antigenic variant within a few days. If the host is untreated, this process may be

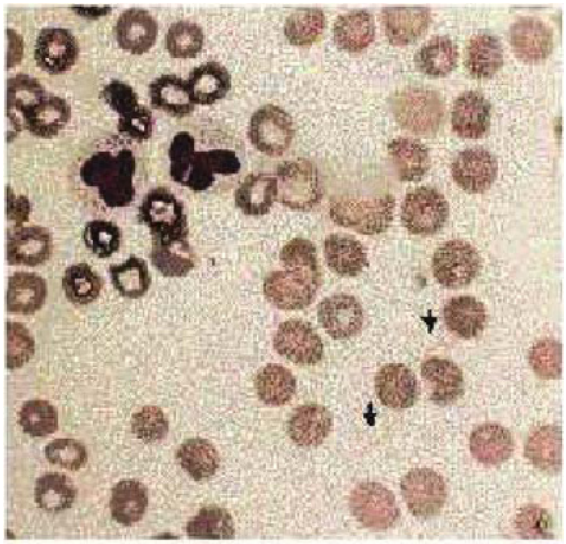


Fig. 19. Relapsing fever. A Wright's-stained peripheral-blood smear contains two borrelia spirochetes isolated from a patient with recurring fever (arrows) (http://www.nejm.org/content/1996/0335/0016/1197.asp). Taken from Newton and Pepper (1996).

Relapsing fever

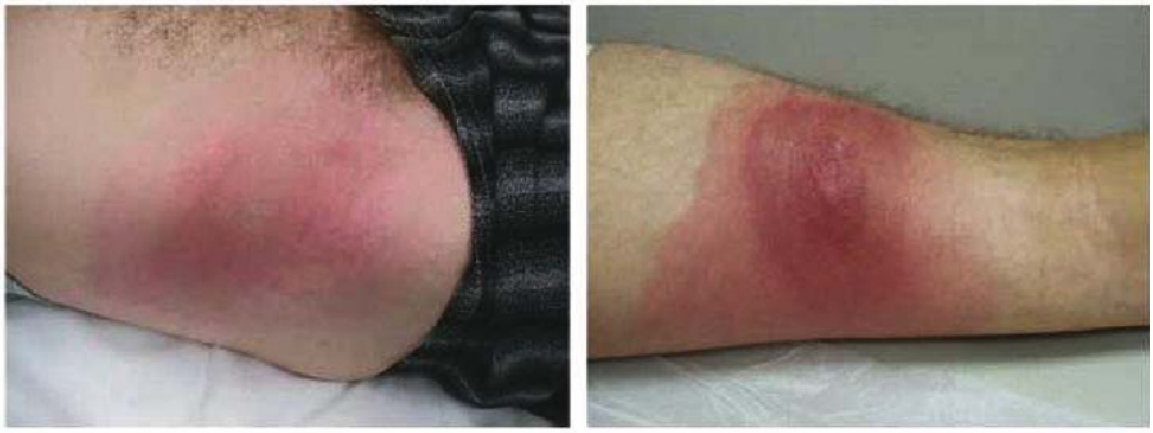
repeated several times, depending upon the infecting strain of *Borrelia*. During the acute phases of the illness, borreliae may be seen in blood smears stained with Giemsa or Wright stain (Fig. 19) and counterstained with crystal violet (Felsenfeld, 1971). The mortality rate in louse-borne relapsing fever ranges as high as 40% in untreated cases, but can be less than 5% following antibiotic treatment. For a recent excellent review of the clinical manifestations and pathology of tick-borne relapsing fever, see Cadavid and Barbour (1998).

Relapsing fever is well recognized as an infection of the blood, but a number of other tissues also may be involved (Felsenfeld, 1965; Barbour and Hayes, 1986b; Pachner, 1986; Garcia-Monco and Benach, 1995). In this respect, the multisystemic nature of the relapsing fevers is similar to infections with other pathogenic spirochetes (i.e., *B. burgdorferi*, *Treponema pallidum* (see The Genus *Treponema* in this Volume), and *Leptospira interrogans* (see The Genus *Leptospira* in this Volume). Patients with TBRF may exhibit symptoms of ocular involvement, including iritis, cyclitis, choroiditis and optic neuritis (Falcone, 1952; Cadavid and Barbour, 1998). In such cases, vision can rapidly deteriorate, leaving most patients with residual visual defects (Quin and Perkins, 1946; Bergeret and Raoult, 1948). Ocular disease has also been associated with relapse episodes (Hamilton, 1943; Falcone, 1952). Ocular complications are most frequently associated with relapsing fevers due to *B. duttonii*, *B. hispanica* and *B. turicatae* (Cadavid and Barbour, 1998).

The frequency of neurological involvement (i.e., lymphocytic meningitis and peripheral facial palsy, crossing of the blood-brain barrier, and persistence in brain tissue) in this disease is similar to that of Lyme disease (Reik et al., 1979; Pachner et al., 1989; Cadavid and Barbour, 1998; Table 4). Spirochetes have been observed by silver stain within the cerebral microvasculature and interstitial spaces and between neurons and glia of autopsied TBRF patients (Buschke and Kroo, 1922; Martínez-Báez and Villasana, 1945). No spirochetes were observed within the brain cells themselves (Lavaditi et al., 1929), a finding which is consistent with the extracellular nature of these pathogens. The relapsing fever spirochetes, however, have differing abilities to invade and persist in brain tissue. *Borrelia duttonii*, *B. crocidurae* and *B. hispanica* were able to infect the brains of all mammalian hosts examined (Cadavid and Barbour, 1998). In contrast, *B. recurrentis* infected the brains of primates (Garnham et al., 1947), but not the brains of mice (Sparrow, 1956). These differences may be due, in part or in whole, to the expression of particular variable membrane proteins (i.e., VspA, see "Antigenic Variation"), some of which have been associated with the increased central nervous system (CNS) involvement and neuroborreliosis (Cadavid et al., 1997; Cadavid and Barbour, 1998).

Although neurological involvement is rare during LBRF, edema and subarachnoid and parenchymal brain hemorrhages were consistently observed during the autopsy of patients who succumbed to infection (Belezky and Umanskaja, 1930; Anderson and Zimmerman, 1955; Judge et al., 1974; Salih et al., 1977; Ahmed et al., 1980). No ocular involvement has been reported in patients with LBRF.

As the geographical distribution of cases of TBRF and Lyme disease (Fig. 15) begin to over-



EM Lesions Images

Fig. 20. Examples of early erythema migrans (EM). Left, an example of a typical EM rash with a central zone of clearing (visible 18 cm in cross diameter) was present on the right iliac crest of a 25-year-old Connecticut man. Image was taken 10 days after initial detection. Image kindly provided by Juan Salazar, M.D., University of Connecticut Health Center. Right, an example of an intensely erythematous rash (15 cm in cross diameter) over the dorsum of the right thigh of a 32-year-old Texas man who most likely contracted Lyme disease while on vacation in Connecticut. The image shown was taken 4 days after initial detection. Image kindly provided by Timothy Sellati, Ph.D., University of Connecticut Health Center.

Table 4. Comparison of tick-borne relapsing fever (TBRF), louse-borne relapsing fever (LBRF), and Lyme disease.

Characteristics	TBRF	LBRF	Lyme disease
Agent	Several species	*B. recurrentis*	*B. burgdorferi* sensu lato[a]
Vector	*Ornithodoros* spp. (soft tick)	*Pediculus humanus* (human body louse)	*Ixodes* spp. (hard tick)
Usual reservoir	Rodents[b]	Humans	Rodents
Epidemiology	Endemic	Epidemic	Endemic
Distribution	Tropical and temperate regions	East Africa[c]	North America, Eurasia
In vitro cultivation	Yes	Yes[d]	Yes
Fever (temperature ≥39°C)	Common	Common	Rare
Fever relapses	Multiple	Few	None
Neurological involvement	Common[e]	Rare	Common
Local skin rash	No	No	Common (EM[f])
Arthritis	No	No	Common
Spirochetes on blood smear	Yes	Yes	No
Serological assay specificity	Fair to poor	Fair to poor	Good to excellent
Antibiotic therapy	Several doses	Single or few doses	Several doses
Jarisch-Herxheimer reaction	Moderate	Moderate to severe	Mild

[a]Included are *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*.
[b]The reservoir for *B. duttonii* in some locations may be humans.
[c]LBRF has a potential worldwide distribution because of association with human body louse.
[d]*In vitro* cultivation of *B. recurrentis* has been reported (Cutler et al., 1999).
[e]Common in >10% of patients with disseminated disease.
[f]Erythema migrans, Fig. 20.

lap, an underappreciation of the frequency of neurological (and ocular) involvement during TBRF could result in this disease being misdiagnosed as Lyme disease, or vice versa, in areas where both diseases are found (Barbour et al., 1996a). These two diseases, however, may be readily distinguishable in some cases by the presence of spirochetes at a density sufficiently high enough as to be visible in the blood during a febrile episode or by a temperature above 38°C (hallmarks of relapsing fever) or by the presence of a localized EM lesion (a hallmark of early Lyme (Borreliosis) Disease; Fig. 20). Molecular and serological techniques can be used to definitively differentiate between these *Borrelia* spp.

Lyme (Borreliosis) Disease

Lyme disease is a multisystem illness that primarily affects the skin, nervous system, heart and joints (Steere, 1989). The hallmark skin lesion of Lyme disease, erythema migrans, was first described in Europe in 1908 (Afzelius, 1921) and in the United States in 1969 (Scrimenti, 1970). The disease was brought to the attention of the medical community in the United States as the result of an outbreak of arthritis in adults and children in Lyme, Connecticut, United States, and the adjacent communities of Old Lyme and East Haddam. The resulting epidemiological and clinical investigations led to the identification of a new disease entity, Lyme disease (Steere et al., 1977). The causative agent of Lyme disease was first described by Willy Burgdorfer and colleagues in 1982, and the species bears his name, *Borrelia burgdorferi* (Burgdorfer et al., 1982; Burgdorfer et al., 1991; Burgdorfer et al., 1993). For an excellent review of Lyme borreliosis, see Steere (1998a).

Lyme disease can be divided into early and late infection (Table 5). Approximately seven to nine days following the initial tick bite, the early (localized) infection is marked by the pathognomonic skin lesion, erythema migrans (in 60–90% of patients; Fig. 20), and is accompanied by fever, regional lymphadenopathy, or minor constitutional symptoms (Steere, 1989).

The EM lesions (Fig. 20) usually fade within three to four weeks, even when untreated, but they may recur at secondary sites coincidental or distal to the initial EM lesion. Weeks to months later (disseminated disease), patients may develop meningoencephalitis, Bell's palsy, myocarditis, migrating musculoskeletal pain and intermittent attacks of arthritis, especially in the large joints such as the knee. As the infection progresses (late or persistent infection), the episodes of arthritis last for months rather than weeks. Also, syndromes of both the central and peripheral nervous system may occur more than a year after the initial infection. Persistent infections in Lyme disease may also develop as acrodermatitis chronicum atrophicans (ACA), the late skin manifestation of the disease, which has primarily been observed in Europe (Asbrink and Hovmark, 1988; Kaufman et al., 1989). The skin lesion typically begins as a bluish-red discoloration of swollen skin on an extremity. This lesion may occur at an earlier site of EM. The inflammatory stage of ACA may persist for years or decades and may lead to atrophy of the skin. Transplacental transmission of *B. burgdorferi* has been reported; however, congenital infection with adverse outcome, such as perinatal death, appears to be unusual (Schlesinger et al., 1985; Weber et al., 1988).

Since Lyme disease is not a notifiable disease in Europe, the knowledge of the epidemiological features of this illness in this region is limited. One extensive study of Lyme borreliosis in southern Sweden, however, does represent an excellent source for reliable epidemiologic (see Epidemiology) and clinical data for this disease (Berglund et al., 1995). The most frequent clinical manifestation (Table 5) was EM (seen in 77% of all cases; Fig. 20), followed by neuroborreliosis (16%) and arthritis (7%). With the exception of a slightly lower rate of carditis (0.5–4%), the pattern of disease found in this study was similar to that reported in the United States.

After the first several weeks of infection, most patients have a positive antibody response to the spirochete. The specific immunoglobulin (Ig)M response is detectable after the third week and peaks after 6–8 weeks (Steere et al., 1983). The *B. burgdorferi*-specific IgG response peaks during the second to third month of infection and may persist for years. Since the direct visualization and culture of *B. burgdorferi* from patient specimens are difficult, serologic determinations are currently the most practical mode of laboratory diagnosis. False negative results occur early in the disease, whereas false positives may occur in patients with a variety of other diseases, including syphilis, Rocky Mountain spotted fever, autoimmune disease, and neurologic disorders. Differential diagnosis is most often made using the CDC surveillance case definition (http://www.cdc.gov/ncidod/dvbid/casedef2.htm) as a guide. If Lyme disease is suspected in an endemic area, patients are often started on antibiotic therapy based solely on the presence of an EM rash (5 cm in diameter; Fig. 20) and/or a history of a recent ixodid tick bite accompanied by one or more of the early Lyme disease symptoms outlined by the CDC surveillance case definition.

Borrelia Genotype and Pathogenesis

Currently, all borreliae capable of causing Lyme disease are members of the *B. burgdorferi* sensu lato complex that is comprised of ten different *Borrelia* genospecies (Wang et al., 1999; Table 2). North American isolates are limited primarily to *B. burgdorferi* sensu stricto, whereas *B. garinii* and *B. afzelii* isolates, in addition to *B. burgdorferi* sensu stricto, are responsible for the majority of cases of Lyme disease in Europe (Table 2). These latter genospecies are more often associated with neuroborreliosis (*B. garinii*; van Dam et al., 1993; Lebech et al., 1994; Wilske et al., 1996; Marconi et al., 1999; Cauwels et al., 2001) and chronic skin manifestations (i.e., ACA; *B. afzelii*; van Dam et al., 1993; Wienecke et al., 1994; Strle et al., 1996; Picken et al., 1998;

Table 5. Major clinical manifestations of Lyme borreliosis in North America and Europe.

Stage[a]	Clinical feature[b]	Incidence in: North America[c]	Incidence in: Europe[d]
I	*Early local infection*		
	EM	Common (60–90%)	Common (~77%)
	Tick bite recalled	25%	64%
	Central clearing of EM	35%	68%
	Systemic symptoms	50–69%	38–51%
II	*Early disseminated infection*		
	Multiple EM	Common (≥18%)	Unusual (6%)
	Neuroborreliosis	Common (10–20%)	Common (16–80%)
	Meningoradiculitis	3–21%	37–61%
	Meningitis	2–17%	4–27%
	Carditis	0.5–10%	0.5–4%
	Borrelial lymphocytoma	Rare	Well documented (3%)
III	*Late LB*		
	Lyme arthritis	Common (51–57%)	Uncommon (~7%)
	ACA	Rare	Well documented (3%)
	Peripheral neuropathy	30–70% late NB	40–63% ACA patients
	CNS involvement	Well documented	<9%
	Encephalomyelitis	Rare (0.1%)	4–6%
	Menigencephalitis	9%	0.5–4%

[a]Stages of the clinical features are those of Steere (Steere, 1989, ID: 185).

[b]NB, neuroborreliosis; CNS, central nervous system.

[c]Data were mainly on an earlier report by the Centers for Disease Control and Prevention on LB surveillance from 1984–1986 (Ciesielski, Markowitz, et al., 1989, ID: 4217) and a population-based study in children in Southern Connecticut (Gerber, Shapiro, et al., 1996, ID: 11924), except for those indicated specifically.

[d]Data were based mainly on a population-based study in Southern Sweden (Berglund, Eitrem, et al., 1995, ID: 9264), except for those indicated specifically.

[e]Data were based on 76 American (Nadelman, Nowakowski, et al., 1996, ID: 12196) and 231 European (Strle, Nelson, et al., 1996, ID: 19085) culture-confirmed patients with EM.

[f]NB patients were used as denominator to calculate the relative prevalence. European data were based on data from 330 NB cases from Germany (Oschmann, Dorndorf, et al., 1998, ID: 19084) and 176 NB cases from Denmark (Hansen & Lebech, 1992, ID: 6973).

[g]A recent population-based study showed that only 7% of children with LB developed Lyme arthritis (Gerber, Shapiro, et al., 1996, ID: 11924). About 10% of patients with Lyme arthritis may develop chronic antibiotic treatment-resistant arthritis (Gross, Forsthuber, et al., 1998, ID: 14570).

Cauwels et al., 2001). Lyme arthritis, first described in the United States, is still more commonly reported in North American cases of Lyme disease (Steere et al., 1983; Table 5), where the causative agent is *B. burgdorferi* sensu stricto isolates, than in European cases, where Lyme disease is attributed to the much more heterogeneous *B. burgdorferi* sensu lato complex. The reverse, however, is true for ACA, which is fairly common in Europe, but rarely seen in North America (Table 5).

For Lyme disease in North America, a correlation between *B. burgdorferi* sensu stricto genotypes and clinical manifestations is not as clear as it is for *B. afzelii* (ACA) and *B. garinii* (neuroborreliosis) in Europe. Two recent studies present evidence suggesting that genetic heterogeneity within *B. burgdorferi* sensu stricto isolates in North America may influence invasiveness and pathogenicity. In the first study, Seinost et al. (1999) examined 140 *B. burgdorferi* strains isolated from primary EM lesions, blood and CSF from patients seen at the Lyme Disease Center at Stony Brook, New York, the Lyme Disease Diagnostic Center at New York Medical College, or at local private physicians. Strains were genotyped according to their *ospC* alleles using a combination of PCR and cold single-strand conformation polymorphism (SSCP) analysis, a method that gave 100% sensitivity and specificity when comparing alleles. Additional samples were obtained by analyzing spirochetes present in locally collected *I. scapularis* ticks. Twenty-two additional alleles were added from sequences present in GenBank. The authors concluded 1) that some *B. burgdorferi* sensu stricto isolates rarely, if ever, cause disease in humans, 2) that some *B. burgdorferi* sensu stricto isolates cause a local infection at the tick bite site but not systemic disease, and 3) that systemic *B. burgdorferi* sensu stricto infections are associated with four *ospC* groups. It is not clear, how-

ever, whether *ospC* is directly influencing pathogenicity or serving as a marker for other, probably unlinked, determinants of invasiveness. Independent work by Baranton et al. (2001), examining genetic diversity of *ospC* loci in European isolates of *B. afzelii* and *B. garinii*, also came to a similar conclusion.

The second study (Wormser et al., 1999) examined the correlation between genetic subtypes and hematogenous dissemination in 104 untreated patients with EM. In this study, the authors used restriction length polymorphism (RFLP) methodology to compare the *B. burgdorferi* sensu stricto 16S-23S rDNA spacer region of spirochetes cultured from EM skin biopsies, whole blood, serum and plasma. Results from this study suggest that the genetic subtype of the infecting strain in the skin is a major determinant of the risk for hematogenous dissemination. The continued characterization of genetic markers associated with different subtypes of *B. burgdorferi* sensu stricto is likely to further our understanding of the relationship between genotype and the clinical presentations of patients with Lyme disease. Additionally, a clearer understanding of the genotypes responsible for disseminated infection in humans may facilitate the selection of novel vaccine candidates by identifying antigenic targets common to these isolates that might be essential to their survival and maintenance in the mammalian host.

Differential Gene Expression in *Borrelia*

To be maintained in its enzootic life cycle, borreliae must adapt to two strikingly different environments, the arthropod vector and the mammalian host. It is, therefore, not surprising that this bacterium undergoes extensive changes in protein composition as it cycles between these two hosts (de Silva and Fikrig, 1997). An extensive amount of work during the past several years has been devoted to elucidating the phenomenon of differential gene expression.

Reciprocal Expression of OspA and OspC

The best studied example of differential gene expression by *B. burgdorferi* involves the synthesis of outer surface protein A (OspA) and C (OspC) during tick feeding. In a pioneering study (http://www.niaid.nih.gov/dir/labs/lmsf.htm), Schwan et al. (1995) observed that *B. burgdorferi*, when present in the midguts of unfed ticks, expressed large amounts of OspA and little to no OspC. When infected ticks were allowed to feed on a suitable mammalian host (i.e., mice), spirochetes within the tick midgut downregulated the expression of OspA and expressed substantial amounts of OspC. Although increased temperature (tick versus mammal) has been shown to be involved in this expression-pattern (see "Environmental Signals as Gene Regulators"), upregulation of OspC independent of temperature was recently demonstrated by the in vitro incubation of spirochetes at 28°C in the presence of tick hemolymph plasma (Johns et al., 2000), suggesting that expression of OspC is enhanced by, but does not require, increased temperature. These data are supported by studies examining the expression of *ospA* and *ospC* mRNA by RT-PCR (Montgomery et al., 1996) and the production of antibodies against OspA and OspC during early murine (Montgomery et al., 1996) and human Lyme borreliosis.

Recent advances in elucidating the potential roles for OspA and OspC in Lyme disease pathogenesis were provided by the publication of the crystal structures (Fig. 21) of these lipoproteins (Li et al., 1997; Eicken et al., 2001; Kumaran et al., 2001). These structures are also of particular interest as OspA is the antigen on which the current Lyme disease vaccine is based, while OspC is considered a highly promising candidate for the next generation of Lyme disease vaccine. The OspA protein displays an unusual, elongated fold composed of 21 consecutive antiparallel β-strands connected by turns or short loops followed by a single short α-helix (Fig. 21). The presence of a cavity and partially buried charged residues with the cleft suggests that the C-terminal domain of OspA might be a binding site for a negatively charged or hydrophobic ligand, small peptide, linear polysaccharide, or exposed protein loop (Li et al., 1997). In contrast to OspA, OspC is comprised of four long helices plus a short fifth helix (Eicken et al., 2001; Kumaran et al., 2001; Fig. 21). An elongated, kidney-shaped OspC dimer (Fig. 21) is thought to form through hydrophobic interactions primarily between the α1 and α1' helices on two OspC monomers. While OspA has no known structural homologs, OspC has similarity to the periplasmic domain of the *Salmonella* aspartate receptor (AR), which is also a dimer. Interestingly, when the crystal structures (Fig. 21) of the OspCs from the HB19 and B31 (both invasive strains) were used to model the structures of noninvasive isolates (Seinost et al., 1999), notable differences were detected in the electrostatic surface potential of the region of OspC furthest away from the surface of the membrane; the surface potential is highly negative in the invasive strains, but not so for the noninvasive strains (Kumaran et al., 2001). The authors speculate that this region's interaction with a positively charged ligand, such as fibronectin (see "Decorin- and Fibronectin-binding Proteins") or a similar molecule, might play a role in the pathogenesis of Lyme disease

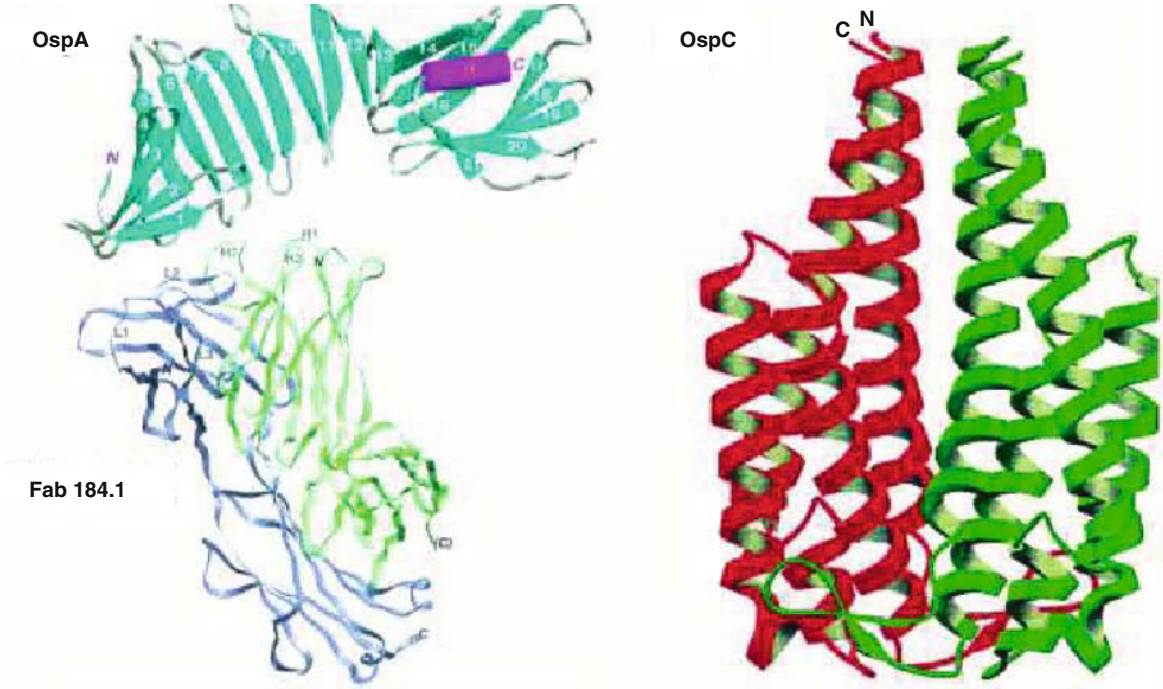


Fig. 21. Crystal structure of OspA and OspC. Left, schematic view of the OspA-Fab 184.1 complex. OspA is displayed with its secondary structural elements, β-strands (cyan, numbered 1–21) and α-helix (magenta, labeled), connected by gray turns or loops. The front face of the central sheet is seen in this view. Fab 184.1 is represented by violet and green ribbons for the light and heavy chains, respectively. The three complementarity determining region (CDR) loops on each chain are displayed as thin ropes and labeled (e.g., L1 for CDR1 of the light chain). Image was taken from Li et al. (1997), © 1997, National Academy of Sciences, with permission. Right, RIBBONS representation of the OspC-HB19 dimer. The two monomers are colored red and green, respectively. The close proximity of the two innermost helices indicates that it is a tight dimer. Image taken from Kumaran et al. (2001) and reproduced by permission of Oxford University Press.

(Kumaran et al., 2001). This finding also has significant implications for the development of an OspC-based vaccine, which is tailored towards recognition of those (invasive) strains that are more likely to cause disseminated disease (see "Lyme Disease Vaccine").

Both OspA and OspC are thought to play critical roles in the transmission of *B. burgdorferi* from the tick to the mammalian host. Two domains within the N40 OspA (amino acids [aa] 85–103 and aa 229–247) were recently identified and proposed to contain functional binding sites responsible for the adherence of spirochetes to the tick midgut (Pal et al., 2000). Consistent with this proposed binding activity, the location of one of these domains (229–247) is predicted by the OspA crystal structure (Fig. 21) data mentioned above to be within the putative ligand binding domain. The role of OspC in the transmission process is unclear. A recent study suggests that this lipoprotein plays a role in the transmission of spirochetes from tick to mammal, but not from mammal to tick (Schwan and Piesman, 2000). A second report demonstrated that immunization with OspC significantly reduced the migration of *Borrelia* from the midgut to the salivary glands of infected *I. scapularis* ticks (Gilmore and Piesman, 2000). These data are consistent with numerous studies demonstrating a role for OspC antibodies in the protection of laboratory animals against tick transmission using both passive and active immunization (Gilmore et al., 1996; Mbow et al., 1999; Zhong et al., 1999).

Recent work by Ohnishi and coworkers (Ohnishi et al., 2001), in which the authors examined the temporal expression patterns of OspA and OspC in both ticks and the skin adjacent to the feeding site in a murine model of Lyme disease, suggests that the relationship between OspA and OspC expression patterns is perhaps even more complex than initially envisioned. Results from these studies demonstrate that the complete downregulation of OspA and the expression of OspC are not required for migration of spirochetes from the midgut to the salivary glands. In fact, OspC was not expressed by a significant percentage of spirochetes in the salivary glands until feeding had progressed for >61 hours. This coincided with the time required to consistently transmit the spirochetes and establish infection. The authors also noted that

early during transmission, mice with spirochetes detectable in the skin directly adjacent to the bite site, expressing mostly OspA compared to OspC, failed to develop infection. In contrast, those mice that had spirochetes expressing higher levels of OspC and reduced OspA consistently became infected. From these data (combined with other studies described above), Ohnishi et al. suggest a model by which spirochetes are retained in the midgut by OspA-mediated adherence until such time as they are adequately "adapted" for migration from the gut lumen to the hemocele and salivary glands by an OspC-facilitated process. Once they have exited the midgut, spirochetes appear to downregulate the expression of OspC, as well as continue to downregulate OspA. In some instances, however, spirochetes ($OspA^+/OspC^-$) are apparently "mislocalized" to the salivary glands and/or prematurely transmitted to the host before being fully "adapted"; these spirochetes fail to establish infection in the mammalian host. This model also suggests that the transmission of *B. burgdorferi* from the arthropod to mammalian host may occur in a steady wave of spirochetes over an extended period (days). These studies also demonstrate that the signal(s) responsible for the temporal expression patterns of OspA and OspC (and most likely other Osps) are clearly complex and likely to include stimuli from both the arthropod and mammalian hosts.

OspE/OspF/Elp Lipoproteins

In addition to the *B. burgdorferi* OspA and OspC lipoproteins (see "Reciprocal Expression of OspA and OspC"), a second group of differentially expressed proteins, the OspE/OspF/Elp lipoproteins, has garnered much interest in the *Borrelia* field. For a recent review of this family of cp32-encoded lipoproteins, see Stevenson et al. (2000a). These lipoproteins were once all considered as one large group of related lipoproteins and referred to by a generic term, "Erp" (OspE/F-related proteins), in the B31 strain (Stevenson et al., 1996; Casjens et al., 2000). A more comprehensive analysis has revealed that the similarities among these proteins are largely restricted to their leader peptides, whereas the mature portions of the polypeptides actually fall into three evolutionary distinct groups: 1) OspE-related orthologs, 2) OspF-related orthologs, and 3) Elps, which contain OspE/F-like leader peptides, but are otherwise unrelated to both OspE and OspF (Akins et al., 1999; Caimano et al., 2000). It is likely that this family of lipoproteins arose from a series of gene fusion events in which a common N-terminus was fused upstream of otherwise unrelated sequences. The resulting fusions formed the progenitors for each of the three groups of OspE, OspF, and Elp lipoproteins, each of which underwent subsequent genetic polymorphisms to form the family of lipoproteins (Fig. 22). In addition to their group designations, members of this larger family of lipoproteins have been referred to by a variety of names in the literature, including p21, pG, BbK2.10, BbK2.11, and "upstream homology box" (UHB) lipoproteins (Lam et al., 1994; Akins et al., 1995; Akins et al., 1999; Suk et al., 1995; Wallich et al., 1995; Marconi et al., 1996a).

The genes encoding all members of this family are encoded on 32- or 18-kb supercoiled circular plasmids (referred to collectively as "cp32s"; see "The cp32 Family of Supercoiled Circular Plasmids") as either mono- or bicistronic operons. The first member of this family shown to be differentially expressed was the p21 lipoprotein, identified within a *Borrelia* expression library as an antigen recognized by sera from infected mice but not by sera from mice immunized with in vitro-cultivated, killed spirochetes (Suk et al., 1995). A number of additional proteins within this family, pG, BbK2.10 and ElpA1, from several different strains have since been characterized as "in vivo-expressed" antigens (i.e., proteins selectively expressed during infection but not by spirochetes grown in artificial medium; Akins et al., 1995; Wallich et al., 1995; Hefty et al., 2001). The remaining OspE/OspF/Elp lipoproteins have all shown to be expressed both in vitro and within the mammalian host (Akins et al., 1995; Akins et al., 1998; Stevenson et al., 1998a; Hefty et al., 2001). Recently, Hefty et al. (2001) performed a comprehensive study of the OspE/OspF/Elp family in *B. burgdorferi* strain 297, which suggested that the differential patterns of antigen expression observed during tick feeding and mammalian infection might represent a temporal hierarchy in which proteins are expressed sequentially as the spirochetes adapt in preparation for the transmission from arthropod to mammal. An examination of the upstream promoter regions has revealed that although this region (upstream homology box, UHB) is highly conserved among all of the *ospE*/*ospF*/*elp*/*erp* genes (Marconi et al., 1996b; Hefty et al., 2001), sequence polymorphisms (insertions/deletions and transitions/transversions) were present (Fig. 23). The majority of these differences were in the putative -10 and -35 hexamers and spacer region between (Hefty et al., 2001); these regions are all known to be critical for RNA polymerase recognition and binding in prokaryotes. A comparative (phylogenetic) analysis of the *ospE*/*ospF*/*elp*/*erp* promoter regions (Fig. 23) demonstrated that these regions do not segregate according to their polypeptide-encoding regions, but instead

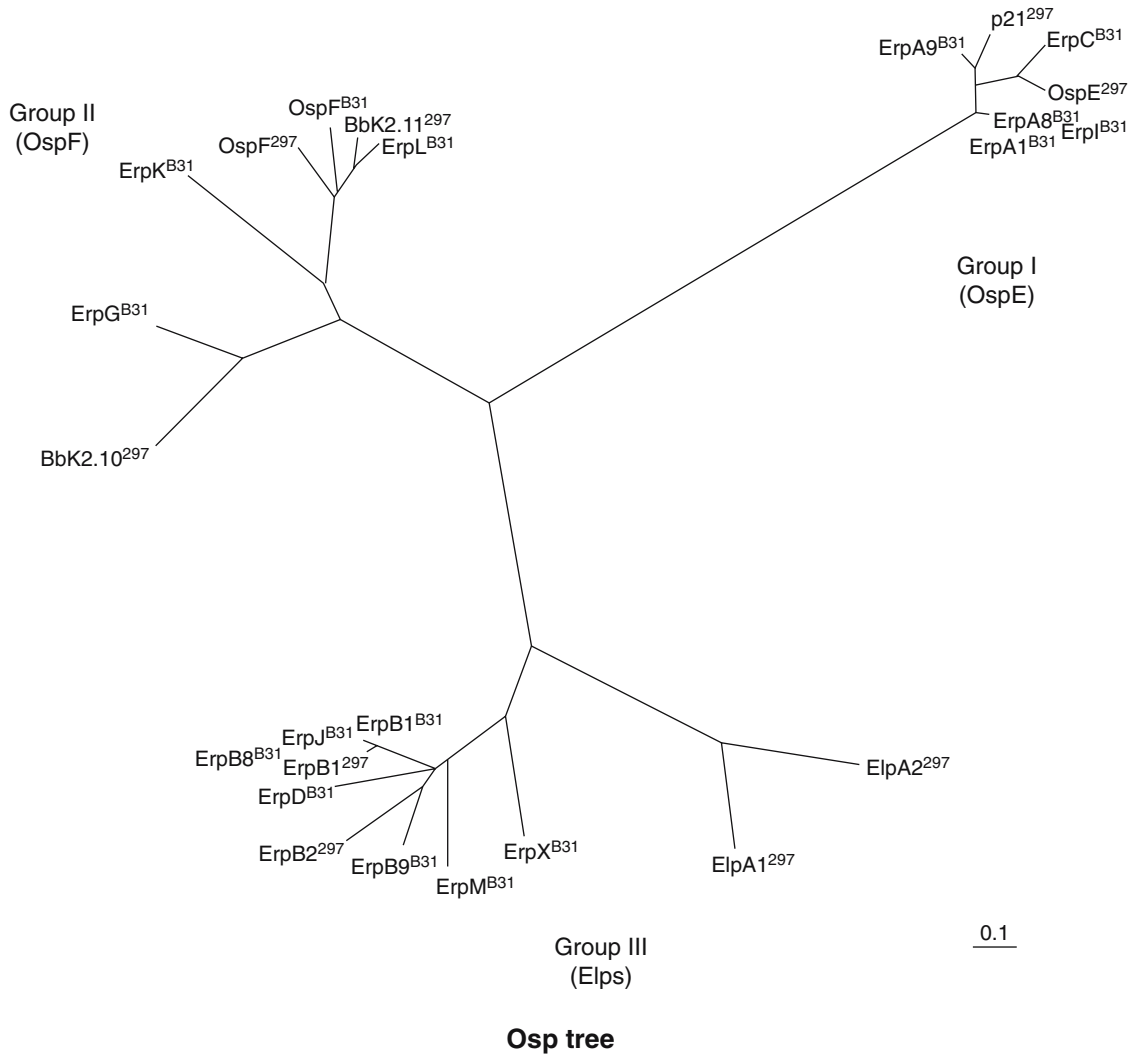
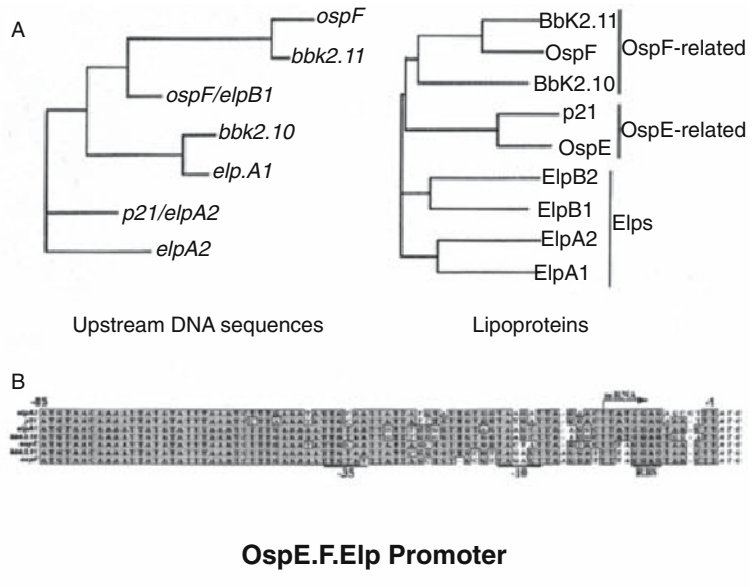


Fig. 22. Unrooted neighbor-joining phylograms of the *B. burgdorferi* strains 297 and B31 OspE/OspF/Elp/Erp lipoproteins. Taken from Caimano et al. (2000), with permission of ASM Press (http://www.journals.asm.org).

appear to segregate according to their temporal expression pattern. These data suggest that minor sequence differences between the promoters may result in altered affinity of RNA polymerase and/or altered expression under different environmental conditions (Hefty et al., 2001).

Mlp Lipoproteins

In addition to the *ospE*/*ospF*/*elp*/*erp* loci (see "OspE/OspF/Elp Lipoproteins"), the cp32/18 family of circular plasmids encodes a second variable lipoprotein family, the *mlp* loci (Porcella et al., 1996; Yang et al., 1999; Porcella et al., 2000; Stevenson et al., 2000b). This family is comprised of two evolutionarily distinct classes that possess highly similar N-termini but divergent C-termini (Caimano et al., 2000; Fig. 24). The recombinatorial process that resulted in the formation of these two lipoprotein classes is likely to be mechanistically similar to that of the process responsible for the formation of the OspE/OspF/Elp family (see "OspE/OspF/Elp Lipoproteins") of lipoproteins (Caimano et al., 2000). Four of the strain 297 Mlps (Mlp7A, Mlp7B, Mlp9 and Mlp10) were shown to be preferentially expressed within a mammalian model (Akins et al., 1998; Yang et al., 1999). Immunoblotting using antiserum from tick-infected C3H/HeJ mouse serum suggests that the strain 297 Mlp lipoproteins are not all expressed early in infection, but may be turned on in a temporal pattern as the spirochetes transition from the arthropod vector to first the skin and then other tissues of the mammalian host (Yang et al., 1999). Preliminary protection studies using a multivalent for-



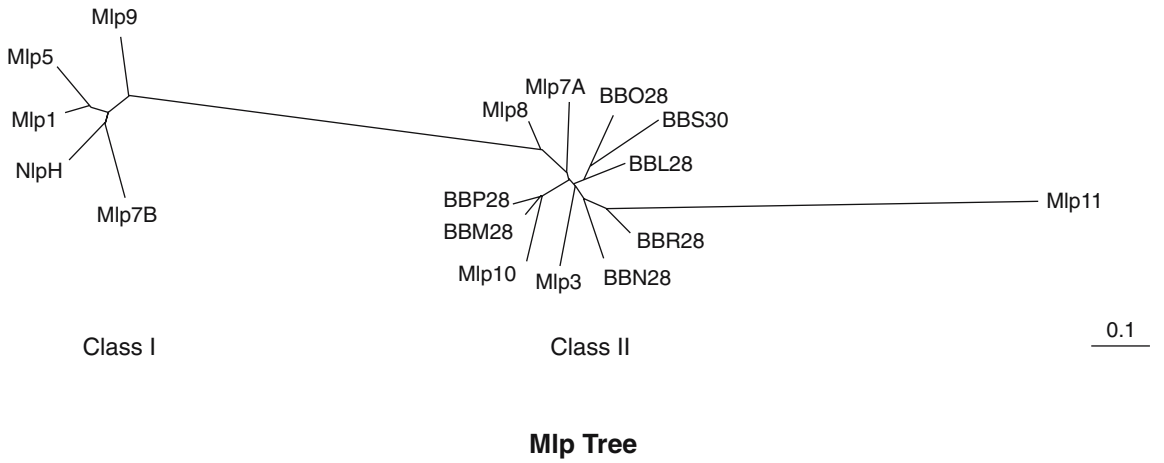


Fig. 23. *Borrelia burgdorferi* strain 297 *ospE*/*ospF*/*elp*/*erp* loci promoter regions. (A) Phenogram analysis of the upstream 85 bp of the putative promoter regions for the *ospE*-related, *ospF*-related, and *elp* loci (left panel). For comparison, a phenogram of the full-length lipoproteins is also shown (right panel). (B) Clustal W alignment analysis of the upstream 85 bp of the putative promoter regions for the *ospE*-related, *ospF*-related, and *elp* loci. Taken from Hefty et al. (2001), with permission of ASM Press (http://www.journals.asm.org).

Fig. 24. Unrooted neighbor-joining phylogram of the *B. burgdorferi* strains 297 and B31 Mlp lipoproteins. Taken from Caimano et al. (2000), © 2001, ASM Press (http://www.journals.asm.org), with permission.

mulation of the Class 2 Mlps from strain 297 as the immunogen gave rise to 80% protection of mice (Yang et al., 1999). Immunoblotting using sera from Lyme disease patients also confirms that members of this family are expressed during human infection (Porcella et al., 2000). While preliminary, these data suggest that this family of lipoproteins may have some vaccinogenic potential that warrants further consideration (Yang et al., 1999).

Decorin- and Fibronectin-binding Proteins

The mechanism(s) by which borreliae are able to disseminate following transmission from the arthropod vector to cause a multisystemic infection in the mammalian host remain largely unknown. The recent identification of two *B. burgdorferi* proteins, DbpA and DbpB, which have the ability to bind decorin (a collagen-associated extracellular matrix proteoglycan found in skin and other tissues), represented a significant step towards characterization of the early stage of Lyme disease (Table 5), as the spirochete first enters the mammalian host through the site of tick feeding (Cassatt et al., 1998; Hagman et al., 1998; Hanson et al., 1998). Based on their ability to interact specifically with extracellular matrix components, these molecules have been placed within a class of cell surface adhesins designated "MSCRAMMs" (microbial surface components recognizing

adhesive matrix molecules; Patti et al., 1994). Interestingly, neither *dbpA* nor *dbpB* was detected in *B. hermsii*, *B. turicatae* or *B. coriaceae* by Southern hybridization (Roberts et al., 1998). Data from proteinase K sensitivity assays (DbpA and DbpB) and immunoelectron microscopy (DbpA) suggest that these proteins are surface-exposed, a location consistent with their putative role in adherence to basement membrane (Hagman et al., 1998; Hanson et al., 1998). Brown et al. (1999) have identified three highly conserved lysine residues (Lys-82, Lys-163 and Lys-170), found in all DbpA polypeptide sequences examined, and all appear to be critical for association with decorin. Several studies have shown that immunization with DbpA, but not DbpB, protects mice against syringe inoculation (Cassatt et al., 1998; Feng et al., 1998; Hagman et al., 1998; Hanson et al., 1998), although DbpA-immunized mice were not protected when inoculated by tick infestation (Hagman et al., 2000). A role for decorin in Lyme disease pathogenesis was recently demonstrated using a decorin-deficient transgenic mouse (Brown et al., 2001). In these studies, decorin-deficient mice were more resistant to infection with *B. burgdorferi* (i.e., fewer spirochetes were found in joints) and showed lower incidence and severity of arthritis than either wildtype or heterozygotes.

A second differentially expressed protein with a potential role in spirochetal attachment to extracellular matrix (Probert and Johnson, 1998) is the fibronectin-binding protein, BBK32; this protein has also been referred to as P35 and P47 (Fikrig et al., 1997; Fikrig et al., 2000; Probert and Johnson, 1998). The BBK32 protein was initially thought to be expressed only within the mammalian host (Fikrig et al., 1997), but this later was shown to be a stain-specific phenomenon, with some *B. burgdorferi* sensu lato isolates (B31, 297, ACA1 and IP3) expressing detectable amounts of BBK32 when cultivated in BSK II (Table 3) medium (Probert and Johnson, 1998). A comprehensive analysis of *bbk32* expression in *B. burgdorferi* strain N40 (which preferentially synthesizes BBK32 during murine and human Lyme disease) was performed by Fikrig et al. (2000) to elucidate what, if any, role this protein plays in tick transmission and/or mammalian infection. The results from these studies demonstrated that 1) BBK32 was not detectable by immunofluorescence on spirochetes within flat ticks, but was present on spirochetes within the engorged ticks, 2) *bbk32* mRNA was first detected two days following syringe inoculation and was maximal at 30 days postinfection, 3) *bbk32* message was detectable in all tissues tested (skin, joints, heart, spleen and bladder) at 14 days postinfection and remained detectable for 60 days, albeit at a significantly reduced level, and 4) BBK32 antibodies provided partial protection from tick infection with *B. burgdorferi* (in a guinea pig model) by killing the spirochetes within the engorged tick, thereby inhibiting the transmission of spirochetes to an immunized animal host (Fikrig et al., 2000).

Environmental Signals as Gene Regulators

A major theme in microbial pathogenesis is the effect of environmental signals on bacterial gene expression. To be maintained within its enzootic cycle, borreliae must possess the ability to adapt to growth within two strikingly different milieus, the arthropod vector and the mammalian host. During this cycle, the spirochete is likely to encounter differences in, among other things, nutrients, temperature and pH. One of the best-studied environmental conditions is temperature. Increased temperature has been associated with increased expression of OspC (see "Reciprocal Expression of OspA and OspC"), OppAV (Bono et al., 1998; see "Transporters"), and the OspE/OspF/Elp (Stevenson et al., 1995; Stevenson et al., 1998a; Hefty et al., 2001; see "OspE/OspF/Elp Lipoproteins") and Mlp (Porcella et al., 2000; Yang et al., 2000; see "Mlp Lipoproteins") lipoprotein families. Several lines of evidence, however, suggest that temperature alone is not solely responsible for the increased expression of these lipoproteins. The incubation of unfed *B. burgdorferi*-infected *I. scapularis* nymphs at 37°C for 24, 72 or 144 hours did not stimulate expression of OspC (Schwan et al., 1995). In addition, the expression of OspC has been shown to wane with continued passage in vitro at 37°C (Schwan and Piesman, 2000). Similarly, a number of observations have been made on the expression of the OspE/OspF/Elp lipoproteins: 1) expression in a mammalian host-adaptation model is significantly greater than the levels observed in spirochetes cultivated in vitro following a temperature shift from 23 to 37°C (Akins et al., 1998), 2) the p21 lipoprotein (see "OspE/OspF/Elp Lipoproteins"), which is not expressed in vitro following temperature shift, is readily detected in a mammalian host-adaptation model (Akins et al., 1998), and 3) animals infected by either tick- or needle-inoculation with *B. burgdorferi* produce antibodies against p21 (see "OspE/OspF/Elp Lipoproteins") and BbK2.10 early during infection, but these sera fail to detect these lipoproteins in spirochetes cultivated in vitro following temperature shift (Wallich et al., 1993; Akins et al., 1995; Akins et al., 1998; Suk et al., 1995; Das et al., 1997). The expression pattern of the Mlp lipoproteins family has been examined in two strains, B31 and 297. Using Northern analyses (Porcella et al., 2000)

and RT-PCR (Yang et al., 1999), little or no *mlp* expression was seen at 23°C, whereas all were shown to be upregulated in response to temperature. A number of environmental factors other than temperature also have been shown to result in differential gene expression in *B. burgdorferi*. Alban et al. (2000) examined the effects of serum starvation in *B. burgdorferi* (grown in RPMI). This study reported that growth of *Borrelia* under this fatty acid- and lipid-limited growth condition resulted in the formation of cysts-like "starvation" forms containing nonmotile, but otherwise intact and viable spirochetes, which rapidly revert to a "vegetative" form when transferred to BSK II (Table 3) medium. In addition, the expression of approximately 20 proteins was induced during growth in RPMI as compared to *B. burgdorferi* grown in BSK II (Table 3) medium. It is intriguing to speculate whether some of these "starvation-induced" proteins are involved in the ability of these bacteria to withstand the extended periods of nutrient limitation they undoubtedly experience in the arthropod host (Alban et al., 2000). Carroll et al. (1999) identified at least 37 changes within borrelial membrane preparations by two-dimensional nonequilibrium pH gradient gel electrophoresis (2D-NEPHGE) in response to changes in pH (pH 6.0 vs 7.0 vs 8.0). In these studies, outer surface protein C (OspC, see "Reciprocal Expression of OspA and OspC") was shown to be decreased at the higher pH, whereas a second unidentified protein was shown to be increased at pH 8.0 (Carroll et al., 1999). Two studies examining spirochetes cultivated in BSK-H (Table 3) medium demonstrated that increased cell density (at constant pH) resulted in increased expression of both *ospC* (see "Reciprocal Expression of OspA and OspC") and *mlp8* (Indest et al., 1997; Ramamoorthy and Philipp, 1998; Mlp Lipoproteins). Although a protein with homology to a quorum-sensing autoinducer synthetase (AI-2) has been identified within the *B. burgdorferi* genome (BB0377; Surette et al., 1999), it has yet to be determined whether a quorum-sensing autoinducer synthetase-like mechanism is responsible for cell-density mediated gene expression. The contribution of growth phase has also been examined. These studies identified a number of protein bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (including P35, BmpD and P7.5), which were upregulated between 2- to 66-fold as the spirochetes in BSK II (Table 3) medium transitioned from mid-log to stationary phase (Ramamoorthy and Philipp, 1998). Using a combination of Northern analyses and immunoblotting, Yang et al. (2000) furthered these studies by examining the interdependence of pH, temperature and cell density on spirochetes cultivated in vitro in BSK-H medium. In these studies, a decrease in pH, in conjunction with an increase in temperature and cell density, acted interdependently, resulting in the reciprocal expression of *ospC* and *ospA* (see "Reciprocal Expression of OspA and OspC"). Examination of other environmentally regulated genes identified patterns of expression that fall roughly into two groups, group I (*ospC*-like) and group II (*ospA*-like). In the proposed model, the group I genes (i.e., *ospC*, *ospF* and *mlp8*) exhibit reduced expression when the concentration of spirochetes and environmental temperature are low, whereas the pH is relatively high. This condition is thought to represent the microenvironment of the unfed tick midgut. The group II genes (i.e., *ospA/B*, *p22* and *lp6.6*) behave in a reciprocal manner and exhibit increased expression under these same conditions. Yang et al. (2000) also propose a role for the alternative σ factor *rpoS* (Fraser et al., 1997; Elias et al., 2000) in the regulation of the group I genes. One group I gene, *dbpA*, did not fit the proposed model; unlike *ospC*, expression of *dbpA* was substantially reduced at pH 6.8 (relative to 7.5; Yang et al., 2000). This result is, however, consistent with the recent finding that DbpA (see "Decorin- and Fibronectin-binding Proteins") is not expressed by *B. burgdorferi* in the midguts of fed *I. scapularis* ticks (Hagman et al., 2000). While these studies represent significant steps toward understanding the complex interplay between multiple environmental factors, each of the above-mentioned studies involves the characterization of spirochetes cultivated in vitro using artificial medium and therefore needs to be seen as suggestive. The development of a tick cell culture model for cultivating *B. burgdorferi* and the demonstration that the reciprocal expression of *ospA* and *ospC* (see "Reciprocal Expression of OspA and OspC") can be modulated by temperature using this model offer an exciting potential for further environmental studies that more closely reflect the arthropod phase of growth (Obonyo et al., 1999).

While alterations in temperature, medium composition and pH are undoubtedly important, mammalian host-derived signals also play a role in inducing dramatic changes in gene and protein expression. The preferential expression of genes in the mammalian host is considered to be indicative of a role in virulence, although this role has not always been readily apparent. The identification and characterization of borrelial genes preferentially expressed in the mammalian host, however, is stymied by the inability to genetically manipulate these organisms and the paucity of organisms found in tissues during infection, particularly in Lyme disease. As a consequence, borrelial gene expression studies have had to rely on

more conventional molecular and immunological assays, examining the expression of only a limited number of genes within a given sample. Ideally, however, one would want to examine the simultaneous expression of multiple genes and the polypeptides they encode in the context of mammalian infection. Towards this end, several recent developments have significantly advanced the field towards overcoming the abovementioned limitations and allowed for the application of more contemporary methodologies. These developments include 1) the development of an electroporation protocol for the introduction of genetic material into *Borrelia burgdorferi* (Genetic Transformation of *Borrelia*), 2) the development of at least two published *Borrelia* shuttle vectors (Genetic Transformation of *Borrelia*), and 3) the development of new animal models for the isolation of spirochetes in a "host-adapted" state. The first two developments are covered elsewhere in the chapter. Animal models for the isolation of spirochetes in a more mammalian host-adapted state were developed by several independent research groups and include the transplantation of skin containing *B. burgdorferi* from infected animals to naive animals developed for either a murine (Barthold et al., 1995) or rabbit (Shang et al., 2000) model of Lyme disease and the cultivation of spirochetes with dialysis membrane chambers (DMC) implanted into the peritoneal cavities of rats (Akins et al., 1998) and subsequently rabbits (Sellati et al., 1999). Future studies utilizing these methodologies will undoubtedly provide insights into both spirochetal and mammalian factors and stimuli involved in the pathogenesis of Lyme disease.

Antigenic Variation in *Borrelia*

VMP LIPOPROTEINS Antigenic variation, a mechanism of immune evasion often employed by bacterial pathogens (Borst, 1991; Donelson, 1995; Deitsch et al., 1997), has been defined as the changes in the structure or expression of antigenic proteins that occur at a frequency greater than the usual mutation rate (Seifert and So, 1990). The best example of antigenic variation in the borreliae is that of the variable major proteins of the relapsing fever spirochetes. One of the hallmarks of relapsing fever is the periodic cycling of acute and afebrile episodes. The acute febrile episodes are characterized by the presence of a large number of spirochetes in the bloodstream of the infected mammal or patient (spirochetemia) and are followed by a change in the serotype and major lipoproteins associated with the bacterial membrane. Multiphasic antigenic variation was first described in the relapsing fever spirochete *B. hermsii* (Barbour et al., 1982; Stoenner et al., 1982) and was associated with a family of lipoproteins, collectively named the "variable major lipoproteins" (VMPs). The VMPs were later shown to be composed of two multigene families, the *vsp* (variable small protein) family, which encodes lipoproteins 20–23 kDa in size, and the *vlp* (variable large protein) family, which encodes lipoproteins 36–40 kDa in size (Barstad et al., 1985; Restrepo et al., 1992; Cadavid et al., 1997; Hinnebusch et al., 1998). The *vlp* family can further be divided into four subfamilies (α, β, γ and δ; Hinnebusch et al., 1998). These proteins share several features of the variant surface glycoproteins (VSGs) responsible for antigenic variation in African trypanosomes, including the post-translational addition of a lipid moiety that mediates attachment to the cell membrane and similar secondary structures of the mature polypeptides (Burman et al., 1990). In addition, these two organisms use a similar recombinatorial mechanism for the genetic exchange of DNA during antigenic variation (Kitten and Barbour, 1990; Donelson, 1995). The mechanism for the conversion of VMP types (and related VMP-like proteins) has been described for several *Borrelia* strains, but here *B. hermsii* will be used as the prototype. Briefly, 28–32-kb linear plasmids of *B. hermsii* have greater than 30 silent copies of serotype-specific variable major protein genes (*vsp* and *vlp*; Meier et al., 1985; Kitten and Barbour, 1990; Barbour, 1993). These silent loci were originally named by variable major protein designations (*vmp1*, *vmp2*, *vmp3*, etc.), but have since been renamed by either *vsp* or *vlp* family designations (Cadavid et al., 1997). The expression locus containing a promoter sequence and the active copy of either a *vsp* or *vlp* is located on the same or a different 28–32-kb linear plasmid (Fig. 11) adjacent to the covalently closed telomeric end (Kitten and Barbour, 1990; Plasterk et al., 1985). Only one *vmp (vsp/vlp)* gene present at one telomere end of the single linear expression plasmid is expressed in each organism (Kitten and Barbour, 1990; Barbour et al., 1991a). Antigenic variation (switching) occurs at a frequency of 10^{-3} to 10^{-4} per generation (Stoenner et al., 1982) and results from the recombination of one of the silent (archival) genes into the telomeric expression locus through inter- and intraplasmic recombination (Plasterk et al., 1985; Barbour et al., 1991b; Restrepo et al., 1994b). This recombination, which is thought to utilize homologous sequences present within the 5′ and 3′ ends of all silent and expressed *vmp* loci, results in the partial or complete replacement of the former VMP serotype with a new serotype. In some instances, incomplete recombination has resulted in the formation of chimeric expression loci containing partial or complete copies of

more than one *vmp* gene (Kitten et al., 1993; Restrepo et al., 1994b). Additional sequence heterogeneity is provided by the introduction of point mutations post-switch (Restrepo and Barbour, 1994a).

Two Vsp serotypes, VspA and VspB, also have been described in relapsing fever agent *B. turicatae* (Cadavid et al., 1997; Pennington et al., 1999a). The Vsp system in *B. turicatae* differs most significantly from the VMPs of *B. hermsii* in that the archival silent loci and the expressed locus are both located near the center of 37–50-kb linear plasmids and not near the telomeres (Pennington et al., 1999b). The silent and expressed loci are, however, encoded on different linear plasmids (Pennington et al., 1999a). The antigenic switching mechanism in *B. turicatae* occurs when a silent *vsp* locus and downstream 3′ sequence (totaling approximately 13–14 kb) are duplicated at a unique expression site, replacing the existing expressed copy and its corresponding downstream sequence (Pennington et al., 1999a). As with the VMPs of *B. hermsii*, only one Vsp serotype is expressed at a time in *B. turicatae*. Vsp/Vlp-related sequences have also been described in *B. parkeri*, *B. crocidurae* and *B. coriaceae* (Hinnebusch et al., 1998).

Antigenic switching of the VMPs has been shown to occur both during in vitro cultivation and during infection of the mammalian host (Stoenner et al., 1982; Schwan and Hinnebusch, 1998). During infection of a mammalian host, each acute relapse episode is predominated by an almost entirely new serotype (Stoenner et al., 1982), presumably allowing evasion from the mammalian adaptive immune response. Until recently, it was not known whether a similar switch occurred as the spirochete passaged from the arthropod-vector to the mammalian host during tick feeding, but a recent study by Schwan and Hinnebusch (1998), using *O. hermsi* infected with either of two distinct *B. hermsii* VMP serotypes, demonstrated that the *vmp* gene present at the telomeric expression site did not change during passage through ticks.

While there are now extensive data on the mechanisms of *vmp*/*vls* serotype switching in borreliae, much less is known about the contribution of these lipoproteins to pathogenesis. Recent studies with *B. turicatae* using a mouse model of Lyme disease demonstrated that organisms expressing the VspA lipoprotein have more extensive CNS involvement, whereas organisms expressing the VspB lipoprotein exhibit more severe arthritic manifestations (Cadavid et al., 1994). In addition, Pennington and coworkers (Pennington et al., 1997) demonstrated that mice infected with the VspB-expressing serotype showed increased numbers of spirochetes in their joints and blood than did mice infected with serotype A. Magoun and co-workers (Magoun et al., 2000) provided further evidence for a possible role of the VMPs in virulence and/or differences in tissue tropism by demonstrating that VspB, but not VspA, mediated attachment of *B. turicatae* to cultured mammalian cells through an interaction with cell-associated glycosaminoglycans (heparin and dermatan sulfate).

Vsp-OspC Lipoprotein Family A third type of variable major protein serotype was also described in *B. hermsii* by Stoenner et al. (1982) and designated "serotype C." This serotype was of interest due to its tendency to predominate in serially passaged populations of *B. hermsii* (Barbour et al., 1982; Stoenner et al., 1982). Once it was determined that antibodies specific for serotype C were directed against a variable small lipoprotein (Vsp), the designation was later changed to "Vsp33" in keeping with the established nomenclature of VMP serotypes (Carter et al., 1994). The *vsp33* gene differs from the other *vsp* and *vlp* genes in that 1) it is expressed from a site distinct from that of the telomeric site typically used by the *vsp/vlp* genes (Barbour et al., 2000), and 2) the *vsp33* promoter is more closely related to the promoter for the *ospC* gene of *B. burgdorferi* than the promoters for the *vsp/vlp* genes of *B. hermsii* (Barbour et al., 1991a; Pennington et al., 1999b).

Phylogenetic analyses of Vsp33 from *B. hermsii* with the other small Vsps of the relapsing fever *Borrelia* spp., a Vmp from *B. miyamotoi* (Fukunaga et al., 1995), and the OspCs (see "Reciprocal Expression of OspA and OspC") from *B. burgdorferi* sensu lato isolates suggest that these proteins form a family of related proteins, termed "the Vsp-OspC family" (Carter et al., 1994; Cadavid et al., 1997). These data are supported by recent structural studies (circular dichroism) comparing the *B. turicatae* VspA with three related proteins, VspB of *B. turicatae*, Vsp26 of *B. hermsii* and OspC (see "Reciprocal Expression of OspA and OspC") of *B. burgdorferi* strain B31. Results from these studies suggest that 1) despite as little as 40% identity in primary sequence, these proteins share a common highly α-helical secondary structure predicted to form a four-helix bundle (Fig. 21), 2) non-conservative amino acid changes, insertions and deletions could potentially affect secondary structure cluster in regions outside the predicted helices, and 3) a common secondary structure results in the formation of similarly sized protease-resistant cores (Zückert et al., 2001). In addition, each of the proteins examined had the ability to dimerize in solution (Zückert et al., 2001). Further structure-function analyses of the Vsp-OspC family of lipoproteins will likely increase our understanding of the role of these

proteins in antigenic variation, facilitating transmission from the arthropod vector, and mammalian infection, particularly the establishment of tissue-specific niches.

Although they share some sequence similarity in their promoter regions and plasmid origins (Barbour et al., 2000), *vsp33* from *B. hermsii* and *ospC* (see "Reciprocal Expression of OspA and OspC") from *B. burgdorferi* sensu lato differ in their patterns of expression. The expression of Vmp33 has been shown to be induced by cultivation either in artificial medium or within tick midguts at 23°C (Schwan and Hinnebusch, 1998). The OspC (see "Environmental Signals as Gene Regulators"), on the other hand, is poorly expressed at this lower temperature (either in vitro or within the unfed tick midgut) and instead is induced by the higher temperatures (33–37°C) experienced by spirochetes during infection of the mammalian host (Schwan et al., 1995). Based on these and other data, Schwan and Hinnebusch (1998) have proposed roles for the Vmp33-OspC lipoproteins in tick-transmission and/or early colonization of the mammalian host. According to the proposed model, Vmp33 is produced by spirochetes in the midguts of soft tick's (*O. hermsi*) following a short period required for the cooling of the bloodmeal contents. Vmp33 is then constitutively expressed by spirochetes as they persistently infect the tick's salivary glands, awaiting an opportunity to be transmitted to the next mammalian host. In addition, expression of *vmp33* has a "silencing" effect on the expression of the *vsp/vlp* present at the telomeric expression site, and vice versa; the mechanism for this inactivation is not known (Barbour et al., 2000). The OspC of *B. burgdorferi*, on the other hand, is not expressed in the midgut of unfed ticks. The Lyme disease spirochete remains within the midgut until the infected tick takes a next bloodmeal, during which the expression of OspC (see "Reciprocal Expression of OspA and OspC") is induced and the spirochetes migrate to the salivary glands to "prepare" for transmission to the mammalian host (Ribeiro et al., 1987; Zung et al., 1989; Lane et al., 1991; Coleman et al., 1997; Ohnishi et al., 2001). In contrast to the constitutive expression of Vmp33 and the rapid transmission (15–90 minutes) of the tick-borne relapsing fever spirochetes, expression of OspC (see "Reciprocal Expression of OspA and OspC") by the Lyme disease spirochete and subsequent transmission to the mammalian host can take a minimum of 2–3 days (Schwan et al., 1995; Schwan and Piesman, 2000; Ohnishi et al., 2001). This period, however, is well within the 3–5 days required for a hard tick to feed to repletion. The expression patterns of Vmp33 and OspC following transmission to the mammalian host are also different. Although readily visualized on spirochetes within the salivary glands of infected soft ticks by immunofluroescence, expression of the Vmp33 lipoprotein is undetectable once the density of spirochetes is high enough for visualization of the organism in the blood (Schwan and Hinnebusch, 1998). The loss of Vsp33 expression is accompanied by the appearance of one of the other VMP lipoproteins (Vsp or Vlp; Schwan and Hinnebusch, 1998). On the other hand, OspC is constitutively expressed by (most) spirochetes as they enter the salivary glands of the tick and as they are transmitted to the mammalian host, and expression continues during infection. These data could suggest that although Vmp33 and OspC may both facilitate the transmission of the relapsing fever and Lyme disease spirochetes, respectively, from their arthropod vectors to the mammalian host, the continued expression of Vmp33 (for the sake of not expressing a new VMP serotype, Barbour et al., 2000) is disadvantageous for the relapsing fever spirochete. In contrast, the continued expression of OspC may not offer any disadvantage or, alternatively, OspC (see "Reciprocal Expression of OspA and OspC") may be required for some *B. burgdorferi*-specific function.

Borrelia burgdorferi Vls System It has been long recognized that the Lyme disease spirochete, *B. burgdorferi*, can alter its antigenic composition during growth through differential gene expression (see "Differential Gene Expression"). The identification of a VMP-like system (*vls*), however, suggests that this spirochete may also employ antigenic variation as a mechanism for immune evasion and/or host-adaptation. The genetic organization of the *vls* (Fig. 25) system in *B. burgdorferi* is similar to that of the VMP system from relapsing fever spirochetes in the utilization of 15 variable silent loci (*vls* cassette) and a single "expression" locus (*vlsE*). In contrast to *B. hermsii*, both the silent and expression loci are located adjacent to each other on a telomeric end of a single 28-kb linear plasmid (lp28-1) in *B. burgdorferi* strain B31. Each *vls* cassette silent locus is flanked by a conserved 17-bp direct repeat that may be involved either in the alignment and recombination or in binding of a proposed site-specific recombinase(s) within the *vlsE* site. The VlsE from *B. burgdorferi* stain B31 contains two highly conserved domains, one at the amino terminus (96 amino acids) and one at the carboxyl terminus (51 amino acids; Zhang et al., 1997; Fig. 25). The amino acid sequence between these two invariable domains is composed of a central variable domain that contains six variable regions (VRs) and six invariable regions (IRs). These two types of regions are interspersed with each other, and each consti-

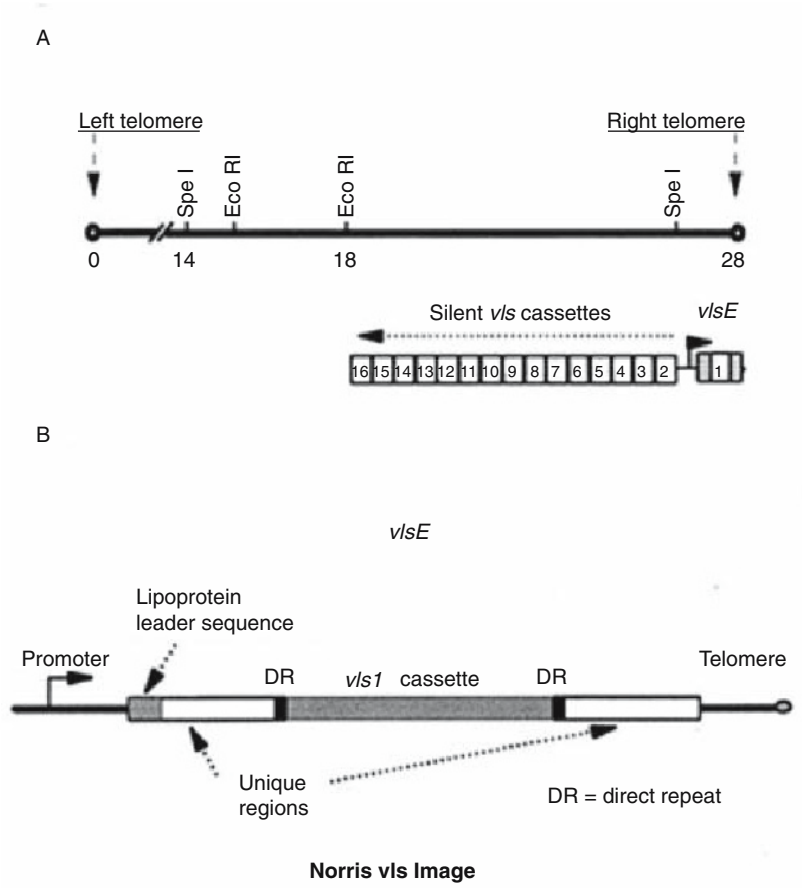


Fig. 25. *Borrelia burgdorferi* clone B31-5A3 *vls* locus. (A) Diagrammatic illustration of the overall arrangement of the *vls* locus in *B. burgdorferi* strain B31 plasmid lp28–1. (B) Structure of *B. burgdorferi* strain B31 *vlsE*. Taken from Zhang et al. (1997), © 1997, Cell Press, with permission.

tutes approximately one-half of the variable domain's length (Zhang et al., 1997; Liang et al., 1999). Additional variability within the *vls* system is achieved via combinatorial antigenic variation and promiscuous recombination resulting from replacement of portions of the expression sites by segments from several silent loci within the other *vls* cassette genes (Zhang et al., 1997; Liang et al., 1999). These recombinatorial events are dramatically induced during mammalian infection (Zhang and Norris, 1998). Also in contrast to the VMP system in the relapsing fever spirochetes in which each phase (relapse) of infection is predominated by one serotype (Barbour, 1993), multiple VlsE serotypes may be expressed within a given population of *B. burgdorferi* during infection of a single mammalian host (Zhang et al., 1997). Zhang and Norris (1998) examined the kinetics of *vlsE* sequence variation in C3H/HeN and severe combined immunodeficiency (SCID) mice. Results from these studies demonstrated that *vlsE* variation occurred in both mouse strains in as few as four days postinfection. The rate of accumulation of amino acid changes was significantly higher in the C3H/HeN mice, suggesting that immune selection may play a role in the persistence of *vlsE* variants. The finding that the rate of accumulation of amino acid changes in the *vlsE* loci within *B. burgdorferi*-infected SCID mice was higher than that in spirochetes cultured in vitro cannot be explained by adaptive immune selection and suggests that *vls* recombination is induced by a mammalian host factor(s). Subsequent immune selection, however, may explain the higher rate of changes in the surviving variant clones (over the parental clone) in the C3H/HeN mice compared to SCID mice (Zhang and Norris, 1998). Recent studies by Ohnishi et al. (2001) suggest that tick feeding may increase recombination at the *vlsE* locus.

To date, the *vls* (Fig. 25) system of only one *B. burgdorferi* strain (B31) has been characterized, but the *vls* cassette region (Kawabata et al., 1998) and *vlsE* expression (M. J. Caimano and J. D. Radolf, unpublished observation) loci have been identified for *B. burgdorferi* sensu stricto strain 297. Southern blot analysis of 22 blood

and erythema migrans biopsy (low-passage) isolates from human Lyme disease patients in Westchester County, New York, determined that all 22 isolates contained a *vlsE*-like sequence (Iyer et al., 2000). Although the size of the plasmid bands varied (from 21 to 38 kb) within the group of isolates, the *vlsE*-hybridizing bands were restricted to one plasmid species within an individual isolate. The consistent presence of *vls* sequences in Lyme disease isolates provides evidence supporting the view that the *vls* locus or associated sequences may be required for infection of mammalian hosts (Iyer et al., 2000). The *vls* system in *B. burgdorferi* is also implicated in virulence; isolates of *B. burgdorferi* strain B31 lacking the linear plasmid (lp28-1) encoding the *vls* locus exhibited reduced infectivity in a mouse model of Lyme disease (Purser and Norris, 2000). Additional infection data suggest that isolates lacking other linear plasmids may be similarly attenuated in virulence (Labandeira-Rey and Skare, 2001).

Studies have shown that five of the six invariable regions (IRs; Fig. 25) present within all VlsE polypeptides were not exposed on the surface of spirochetes cultivated in vitro (Liang et al., 1999; Liang et al., 2000). In addition, a recent study by Liang et al. (2001) examined the surface localization of the conserved C-terminal invariable domain of VlsE and found that it also was not exposed on the spirochetal surface. Consistent with these localization data, antibodies against the conserved C-terminal domain, although immunodominant during mammalian infection, failed to protect against challenge with *B. burgdorferi* (Liang et al., 2001). These data, along with the accumulation of substantial genetic heterogeneity within the variable regions during mammalian infection, suggest that although this molecule may play a role in infectivity, VlsE may not be useful as a vaccinogen (Liang et al., 2001).

Lipoproteins and the Innate Immune Response

Although many aspects of Lyme disease pathogenesis remain ill defined, it is generally accepted that clinical manifestations result primarily, perhaps entirely, from the host's local immune response to spirochetes in infected tissues (Duray and Steere, 1988; Szczepanski and Benach, 1991). For a more comprehensive review of the immunology of Lyme disease, see Sigal (1997). There is now a substantial body of evidence that borrelial lipoproteins are the major proinflammatory agonists in Lyme disease and the relapsing fevers and that lipid modification is a prerequisite for this biological property (Radolf et al., 1991; Radolf et al., 1995a; Ma and Weis, 1993; Boggemeyer et al., 1994; Norgard et al., 1996; Sellati et al., 1996; Morrison et al., 1997; Vidal et al., 1998). Although both borrelial lipoproteins and LPS initiate cell signaling by binding to CD14 (Sellati et al., 1998; Wooten et al., 1998) and both activate cells via nuclear factor-kappa B (NF-β; Norgard et al., 1996; Wooten et al., 1996), key differences in the signaling pathways utilized by these two agonists have come to light. Activation by lipoproteins is serum-independent and does not require LPS-binding protein (Sellati et al., 1998; Wooten et al., 1998). More importantly, the lipoprotein-CD14 complex signals through toll-like receptor (TLR)2 (Aliprantis et al., 1999; Brightbill et al., 1999; Hirschfeld et al., 1999; Lien et al., 1999), whereas LPS signals through TLR4 (Poltorak et al., 1998; Takeuchi et al., 1999). The importance of innate immunity in the establishment of Lyme disease is highlighted by the observation that *B. burgdorferi*-infected severe combined immunodeficiency (SCID) mice exhibit persistent spirochetemia and chronic progressive inflammation of the joints, heart and liver (Schaible et al., 1990; Barthold et al., 1992).

Role of Plasminogen/Plasmin in *Borrelia* Pathogenesis

Plasmin, a broad spectrum serine protease, is responsible for fibrin degradation during thrombolysis, but also has been proposed to be involved in other physiological and pathological processes such as skin, corneal, and arterial wound repair, tumor progression, and ischemic and excitotoxic brain damage. The discovery that pathogenic microorganisms (Gram-positive and -negative) can convert plasminogen into enzymatically active plasmin to increase their invasiveness and enhance their ability to cross tissue barriers has received much attention (reviewed in Broder et al., 1991). The observation that *Borrelia* species (*B. burgdorferi* and *B. hermsii*) can incorporate plasminogen onto their surfaces where it can be activated to plasmin by the host's plasminogen activation system (PAS) has lent itself to a study of the PAS and spirochetal virulence (Fuchs et al., 1994; Fuchs et al., 1996; Coleman et al., 1995; Coleman et al., 1997; Hu et al., 1995; Hu et al., 1997; Klempner et al., 1995; Perides et al., 1996; Gebbia et al., 1999; Coleman and Benach, 2000). Once incorporated onto the surface of the bacterium, the enzymatically active plasmin has the potential to degrade components of the extracellular matrix, such as fibronectin and laminin. The incorporated plasmin proteolytic activity on the surface of the pathogenic bacteria, such as *Borrelia* spp., must disseminate from their site of entry in the skin to the blood and other tissues (Gebbia et al., 1999). Evidence supporting this role for plasmin has

been reported by Benach and coworkers. Using an in vitro assay, a *B. burgdorferi* human isolate, once complexed with plasmin and supplied an exogenous plasminogen activator (uPA), had the ability to degrade purified extracellular matrix (ECM) components and an interstitial ECM. In two separate animal models of borrelial infection, relapsing fever and Lyme disease, the in vivo role for plasminogen/plasmin has been examined by using plasminogen-deficient knockout mice ($plg^{-/-}$). In the Lyme disease model, plasminogen was shown to be required for efficient spirochete dissemination in ticks and establishment of spirochetemia in mice (Coleman et al., 1997). Plasminogen was also shown to be an important host factor in the relapsing fever model. Plasminogen-deficient mice ($plg^{-/-}$) showed significantly decreased spirochetal burdens in both heart and brain tissues (as measured by PCR amplification). Furthermore, the decreased spirochetal load in the brains of $plg^{-/-}$ mice was associated with a significant decrease in the degree of inflammation of the leptomeninges in these mice, as compared to the control ($plg^{+/+}$ and $plg^{+/-}$) mice (Gebbia et al., 1999). Together these data strongly support a role for the PAS in *Borrelia* spp. infection.

Chemotherapy

Relapsing Fevers

For a review of antibiotic therapies for the relapsing fevers, see Cadavid and Barbour (1998). Relapsing fever has been successfully treated with tetracycline, chloramphenicol, penicillin and erythromycin (Sanford, 1976; Perine and Tekle, 1983; Butler, 1985; Horton and Blaser, 1985), although chloramphenicol treatment was not highly successful in the treatment of relapsing fever in animal models of infection (Cadavid and Barbour, 1998). For louse-borne relapsing fever, tetracycline, in a single oral dose (0.5 g), is the preferred therapy, except in pregnant women and children younger than 8 years (Perine and Tekle, 1983). Erythromycin in a single oral dose (0.5 g) is an equally effective alternative therapy (Perine and Tekle, 1983). For tick-borne relapsing fever, tetracycline (0.5 g), given in four divided doses at 6-h intervals for 5–10 days, is considered the drug regime of choice, owing to the higher rate of treatment failure and relapses in these patients (Sanford, 1976; Foster, 1977; Horton and Blaser, 1985). Although effective in cases of LBRF, erythromycin did not prevent relapse or neurological complications in patients with TBRF (Horton and Blaser, 1985; Colebunders et al., 1993). Parenteral antibiotics (i.e., penicillin G, cefotaxime or ceftriaxone) should be given for 14 or more days in patients with meningitis or encephalitis (Cadavid and Barbour, 1998).

Lyme Borreliosis

Early Lyme (Borreliosis) disease usually responds well to antimicrobial therapy. However, cases with persistent joint or central nervous system complications tend to be more resistant to treatment. Adults with the early manifestations generally respond to doxycycline (100 mg orally, 2 × daily, 10B30 days) or amoxicillin (500 mg orally, 4 × daily, 10B30 days). Children younger than 8 years of age are generally treated with amoxicillin (250 mg orally, 3 × daily, 10B30 days). Neurologic manifestations (Table 5) and other abnormalities are treated with ceftriaxone (Rocephin; 2 g, intravenously, 1 × daily, 14 days). Treatment failures have occurred with all of these regimens, and retreatment may be necessary. Since patients who have already developed a mature anti-borrelia IgG response often remain seropositive after apparently successful antibiotic therapy, the presence or absence of circulating antibodies to *Borrelia* following treatment is not a reliable indicator of cure (Dattwyler et al., 1989). Descriptions of clinical presentations and the medical progress of Lyme disease have been reviewed by Steere (1989) and Edlow (1999).

Jarisch-Herxheimer Reaction

Shortly (10–30 minutes) following the initiation of antibiotic therapy in a large number of relapsing fever patients, a Jarisch-Herxheimer reaction may occur, manifested by rigors, sudden fever, headache, and persistent hypertension, followed over the next few hours by profuse sweating and a slow decline in temperature and fall in blood pressure. The discovery of this distressing and potentially life-threatening (case fatality rate of approximately 5%) phenomenon is usually credited to Jarisch and Herxheimer, who first described the components of this syndrome in patients with secondary syphilis (see The Genus *Treponema* in this Volume) who received mercury treatment. This reaction has been described in a variety of bacterial infections, including five spirochetal infections (louse-borne and tick-borne relapsing fevers, Lyme disease, leptospirosis (see The Genus *Leptospira* in this Volume), and syphilis (see The Genus *Treponema* in this Volume) and closely resembles a classic endotoxin reaction (or systemic inflammatory response syndrome), which typically occurs when endotoxin (LPS) from cell walls of Gram-negative bacteria or other viral, protozoal, fungal

pyrogen, or bacterial toxin is released. More recently, bacterial lipoproteins (see "Lipoproteins and the Innate Immune Response") have been added to the list of molecules capable of inducing an acute inflammatory response (Radolf et al., 1991; Radolf et al., 1995a; Ma and Weis, 1993; Tai et al., 1994; Weis et al., 1994; Norgard et al., 1995; Norgard et al., 1996; Sellati et al., 1996; Sellati et al., 1998; Wooten et al., 1996; Morrison et al., 1997; Theus et al., 1998; Vincent et al., 1998). One general characteristic of all Jarisch-Herxheimer reactions is a substantial increase in circulating levels of tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8) just before symptoms develop. In a recent study by Fekade et al. (1996), treatment with Fab fragments of anti-TNF-α antibodies significantly reduced the incidence and severity of the Jarisch-Herxheimer reaction in patients with louse-borne (endemic) relapsing fever caused by *B. recurrentis*. This report was one of the first to establish that passive immunization against TNF-α can block the development of a shock-like illness in humans and has received support within the medical community as an attractive and viable therapy for the prevention of the Jarisch-Herxheimer reaction in spirochetal and potentially other bacterial infections (Beutler and Munford, 1996).

Lyme Disease Vaccine

In two large clinical trials, more than 10,000 adult volunteers received three doses (intramuscularly) of one of two Lyme disease vaccines directed against the *B. burgdorferi* outer surface lipoprotein, OspA (see "Reciprocal Expression of OspA and OspC"). Individuals were vaccinated at time 0, 1 and 12 months. After three doses, the OspA-based vaccine was shown to be 76–92% effective (Sigal et al., 1998; Steere et al., 1998b). The Food and Drug Administration (FDA) gave final approval, in 1998, to a Lyme disease vaccine, LymeRix, based on a recombinant form of OspA and manufactured by SmithKline Beecham Pharmaceuticals. There are several caveats to the use of the LymeRix vaccine (http://www.cdc.gov/epo/mmwr/preview/mmwrhtml/rr4807a1.htm). Although long studied using *Borrelia* cultivated in BSK II (Table 3) medium, the target of the vaccine, OspA (see "Reciprocal Expression of OspA and OspC"), was recently shown to be expressed only on the surface of spirochetes in the flat (unfed) tick and not on the surface of spirochetes in the tick salivary glands or on spirochetes transmitted to the human host. Once a tick begins to take a bloodmeal on a vaccinated individual, any spirochetes within the midgut of that tick are susceptible to the host's bactericidal anti-OspA antibodies. The end result is a lowering of the density of organisms within the tick salivary gland to below a critical threshold required for initiating events linked to transmission (de Silva et al., 1999). Those spirochetes that are not killed in the midgut prior to the downregulation and disappearance of OspA from the bacterial cell surface, however, are no longer susceptible to killing by antibodies directed against OspA (see "Reciprocal Expression of OspA and OspC"), thereby reducing the vaccine's efficacy. In addition, individuals who receive the vaccine will become ELISA positive (but Western-blot negative), making future diagnosis of those patients who subsequently become infected more difficult. Uncertainties about who should receive the vaccine (http://www.cdc.gov/epo/mmwr/preview/mmwrhtml/rr4807a1.htm), concerns about the limited duration of protective immunity (Onrust and Goa, 2000; Taege, 2000), and the unfortunate misperception that it has a high incidence of serious side effects, including autoimmune reactions (Sigal, 2000; OspA and Autoimmunity), have produced a less than enthusiastic reception among practitioners and the lay public. To date, these concerns have failed to materialize as there have been no significant side effects among the vaccinated public.

One attractive alternative to a vaccine based solely on OspA is the development of a multivalent vaccine based on a number of borrelial antigens that are expressed preferentially in either the arthropod (i.e., OspA Reciprocal Expression of OspA and OspC) or the mammalian host (i.e., Osp A/Osp C Reciprocal Expression of OspA and OspC, and OspE/OspF/Elp, Mlp, and DbpA. The increased expression of OspC in the mammalian host, the identification of OspC subtypes of *B. burgdorferi*, which are primarily responsible for invasive disease in humans (see *Borrelia* Genotype and Pathogenesis in the second edition), and the ability of OspC antibodies to prevent, as well as resolve, infection with *B. burgdorferi* (Zhong et al., 1999) make this molecule particularly attractive.

The vaccine, however, does not offer any protection against other tick-borne diseases, *Babesia microti* and the agent of human granulocytic ehrlichiosis (HGE), common to and transmitted by the same ixodid vectors. An area of active interest is the development of anti-arthropod vaccines. Such vaccines could be designed to interfere with one or more of the steps involved in pathogen transmission, including 1) preventing tick attachment and feeding, and 2) preventing pathogen attachment to and migration within the arthropod vector tissues. Such vaccines could have the potential to simultaneously protect

against multiple pathogens transmitted by the same vector.

For recent vaccine reviews, see Thanassi and Schoen (2000) and Poland and Jacobson (2001).

OspA and Autoimmunity

A study by Steere and coworkers (Gross et al., 1998), looking at patients with treatment-resistant and -responsive Lyme arthritis, suggested that infection with *B. burgdorferi* may lead to the development of an immune response to the OspA (see "Reciprocal Expression of OspA and OspC") lipoprotein that cross-reacts with a human antigen, human leukocyte function-associated antigen-1 (hLFA-1). In a small number of patients who possess a particular Major Histocompatability Class (MHC) II allele, HLA-DRB1*0401, the response against hLFA-1 may lead to the subsequent development of an autoimmune-mediated arthritis (Gross et al., 1998). The identification of OspA-reactive type-1 T helper (T_H1) cells that cross-reacted with a related-region within hLFA-1 in the synovial fluid of individuals with treatment-resistant Lyme arthritis supports this hypothesis (Gross et al., 1998). The association between an anti-OspA immune response and hLFA-1 cross-reactivity and the development of treatment-resistant Lyme arthritis is not fully understood. Some patients who develop hLFA-1 autoreactive T-cells do not possess the HLA-DRB1*0401 allele; conversely, some patients who possess that HLA-DRB1*0401 allele do not respond to either OspA or hLFA-1 (Gross et al., 1998). The implication(s) of these data for vaccine (see "Lyme Disease Vaccine") recipients remains to be seen.

Veterinary Diseases

Borrelia anserina is the etiological agent of avian borreliosis (Table 1), a highly fatal disease of geese, ducks, turkeys and chickens. Mammals are resistant to this infection. The disease is widespread, occurring in many countries including those in Europe, Asia, Australia, and South, Central, and North America. The vectors of *B. anserina* are species of *Argas* ticks. The disease begins with a high fever; then the birds become cyanotic and develop diarrhea. Spirochetes are present in the blood early during the disease, and relapses do not occur.

Borrelia coriaceae is the putative etiological agent of epizootic bovine abortion (Table 1), a disease of major economic importance in California (Lane et al., 1985; Johnson et al., 1987). This species of *Borrelia* (Johnson et al., 1987) is carried by the human-biting soft tick *Ornithodoros coriaceus* (Lane et al., 1985). Until recently, only one isolate of *B. coriaceae* Co53 had been characterized genetically and phenotypically (Johnson et al., 1987; LeFebvre and Perng, 1989). The publication of two additional strains of *B. coriaceae*, isolated from adult *O. coriaceus* ticks collected from northwestern California, has significantly increased our understanding of this little-known borrelial genospecies (Hendson and Lane, 2000). Hybridization studies of these two new isolates, along with the initial Co53 isolate, demonstrated low homology between these strains and the relapsing fever spirochete *B. parkeri* among the linear plasmids of these isolates. Pulsed field gel electrophoresis demonstrated that *B. coriaceae* exhibits genetic diversity within the linear plasmid profiles of these three isolates, a characteristic common among the borreliae (Hendson and Lane, 2000). Unlike most *Borrelia* spp., *B. coriaceae* can be passed transstadially and occasionally transovarially (Lane et al., 1985; Lane and Manweiler, 1988a). The Columbian black-tailed deer (*Odocoileus hemionus columbianus*) has been implicated as a probable natural host (Lane and Manweiler, 1988b).

Borrelia theileri is responsible for a mild disease of cattle, horses and sheep (Table 1). The illness is characterized by one to two episodes of fever, weight loss, weakness and anemia. The vectors of this spirochete are species of the hard ticks *Rhipicephalus* and *Boophilus*. *Borrelia theileri* also has been found to be transmitted transovarially in *Boophilus microplus* eggs (Smith et al., 1985). The disease has been reported in South Africa and Australia.

Literature Cited

Afzelius, A. 1921. Erythema chronicum migrans. Acta Dermatologica Venereologica 2:120–125.

Ahmed, M., S. Abdel-Wahab, and M. Abdel Malik. 1980. Louse-borne relapsing fever in the Sudan: A historical review and a clinico-pathological study. Trop. Geogr. Med. 32:106–111.

Akins, D. R., S. F. Porcella, T. G. Popova, D. Shevchenko, S. I. Baker, M. Li, M. V. Norgard, and J. D. Radolf. 1995. Evidence for in vivo but not in vitro expression of a Borrelia burgdorferi outer surface protein F (OspF) homolog. Molec. Microbiol. 18:507–520.

Akins, D. R., K. W. Bourell, M. J. Caimano, M. V. Norgard, and J. D. Radolf. 1998. A new animal model for studying Lyme disease spirochetes in a mammalian host-adapted state. J. Clin. Invest. 101:2240–2250.

Akins, D. R., M. J. Caimano, X. Yang, F. Cerna, M. V. Norgard, and J. D.Radolf. 1999. Molecular and evolutionary analysis of Borrelia burgdorferi 297 circular plasmid-encoded lipoproteins with OspE- and OspF-like leader peptides. Infect. Immun. 67:1526–1532.

Alban, P. S., P. W. Johnson, and D. R. Nelson. 2000. Serum-starvation-induced changes in protein synthesis and morphology of Borrelia burgdorferi. Microbiology 146:119–127.

Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky. 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 285:736–739.

Anda, P., W. Sanchez-Yebra, M. del Mar Vitutia, E. P. Pastrana, I. Rodriguez, N. S. Miller, P. B. Backenson, and J. L. Benach. 1996. A new Borrelia species isolated from patients with relapsing fever in Spain. Lancet 348:162–165.

Anderson, T., and L. Zimmerman. 1955. A clinicopathologic study of eleven fatal cases with special attention to association with salmonella infections. Am. J. Pathol. 31:1083–1109.

Anderson, J. F., and L. A. Magnarelli. 1984. Avian and mammalian hosts for spirochete-infected ticks and insects in a Lyme disease focus in Connecticut. Yale J. Biol. Med. 57:627–641.

Anderson, J. F., R. C. Johnson, L. A. Magnarelli, and F. W. Hyde. 1986. Culturing Borrelia burgdorferi from spleen and kidney tissues of wild-caught white-footed mice, Peromyscus leucopus. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 263:34–39.

Anderson, J. F., and L. A. Magnarelli. 1992. Epizootiology of Lyme disease and methods of cultivating Borrelia burgdorferi [Review]. Ann. NY Acad. Sci. 653:52–63.

Anguita, J., S. Samanta, B. Revilla, K. Suk, S. Das, S. W. Barthold, and E. Fikrig. 2000. Borrelia burgdorferi gene expression in vivo and spirochete pathogenicity. Infect. Immun. 68:1222–1230.

Asbrink, E., and A. Hovmark. 1988. Early and late cutaneous manifestations in Ixodes-borne borreliosis (Erythema migrans borreliosis. Lyme borreliosis). Ann. NY Acad. Sci. 539:4–15.

Baranton, G., G. Seinost, G. Theodore, D. Postic, and D. Dykhuizen. 2001. Distinct levels of genetic diversity of Borrelia burgdorferi are associated with different aspects of pathogenicity. Res. Microbiol. 152:149–156.

Barbour, A. G., S. L. Tessier, and H. G. Stoenner. 1982. Variable major proteins of Borrelia hermsii. J. Exp. Med. 156:1312–1324.

Barbour, A. G., S. L. Tessier, and W. J. Todd. 1983. Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect. Immun. 41:795–804.

Barbour, A. G. 1984a. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. 57:521–525.

Barbour, A. G., S. L. Tessier, and S. F. Hayes. 1984b. Variation in a major surface protein of Lyme disease spirochetes. Infect. Immun. 45:94–100.

Barbour, A. G. 1986a. Cultivation of Borrelia: A historical overview. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 263:11–14.

Barbour, A. G., and S. F. Hayes. 1986b. Biology of Borrelia species. Microbiol. Rev. 50:381–400.

Barbour, A. G., and C. F. Garon. 1987. Linear plasmids of the bacterium Borrelia burgdorferi have covalently closed ends. Science 237:409–411.

Barbour, A. G. 1988. Plasmid analysis of Borrelia burgdorferi, the Lyme disease agent. J. Clin. Microbiol. 26:475–478.

Barbour, A. G. 1989. The molecular biology of Borrelia. Rev. Infect. Dis. 11, Suppl. 6:S1470–S1474.

Barbour, A. G., N. Burman, C. J. Carter, T. Kitten, and S. Bergstrom. 1991a. Variable antigen genes of the relapsing fever agent Borrelia hermsii are activated by promoter addition. Molec. Microbiol. 5:489–493.

Barbour, A. G., C. J. Carter, N. Burman, C. S. Freitag, C. F. Garon, and S. Bergstrom. 1991b. Tandem insertion sequence-like elements define the expression site for variable antigen genes of Borrelia hermsii. Infect. Immun. 59:390–397.

Barbour, A. G. 1993. Linear DNA of Borrelia species and antigenic variation. Trends Microbiol. 1:236–239.

Barbour, A. G., C. J. Carter, V. Bundoc, and J. Hinnebusch. 1996a. The nucleotide sequence of a linear plasmid of Borrelia burgdorferi reveals similarities to those of circular plasmids of other prokaryotes. J. Bacteriol. 178:6635–6639.

Barbour, A. G., G. O. Maupin, G. J. Teltow, C. J. Carter, and J. Piesman. 1996b. Identification of an uncultivable Borrelia species in the hard tick Amblymomma americanum: Possible agent of a Lyme-like illness. J. Infect. Dis. 173:403–409.

Barbour, A. G., C. J. Carter, and C. D. Sohaskey. 2000. Surface protein variation by expression site switching in the relapsing fever agent Borrelia hermsii. Infect. Immun. 68:7114–7121.

Baril, C., C. Richaud, G. Baranton, and I. Saint Girons. 1989. Linear chromosome of Borrelia burgdorferi. Res. Microbiol. 140:507–516.

Barstad, P. A., J. E. Coligan, M. G. Raum, and A. G. Barbour. 1985. Variable major proteins of Borrelia hermsii. Epitope mapping and partial sequence analysis of CNBr peptides. J. Exp. Med. 161:1302–1314.

Barthold, S. W., D. H. Persing, A. L. Armstrong, and R. A. Peeples. 1991. Kinetics of Borrelia burgdorferi dissemination and evolution of disease after intradermal inoculation of mice. Am. J. Pathol. 139:263–273.

Barthold, S. W., C. L. Sidman, and A. L. Smith. 1992. Lyme borreliosis in genetically resistant and susceptible mice with severe combined immunodeficiency. Am. J. Trop. Med. Hyg. 46:605–613.

Barthold, S. W., E. Fikrig, L. K. Bockenstedt, and D. H. Persing. 1995. Circumvention of outer surface protein A immunity by host-adapted Borrelia burgdorferi. Infect. Immun. 63:2255–2261.

Beck, G., J. L. Benach, and G. S. Habicht. 1990. Isolation, preliminary chemical characterization, and biological activity of Borrelia burgdorferi peptidoglycan. Biochem. Biophys. Res. Commun. 167:89–95.

Beermann, C., G. Lochnit, R. Geyer, P. Groscurth, and L. Filgueira. 2000. The lipid component of lipoproteins from Borrelia burgdorferi: structural analysis, antigenicity, and presentation via human dendritic cells. Biochem. Biophys. Res. Commun. 267:897–905.

Belezky, W., and R. Umanskaja. 1930. Die Recurrensspirochatose des zentralen Nervensystems des Menschen. Zeitschr. Gesamte Neurol. Psychiatr. 129:21–41.

Belisle, J. T., M. E. Brandt, J. D. Radolf, and M. V. Norgard. 1994. Fatty acids of Treponema pallidum and Borrelia burgdorferi lipoproteins. J. Bacteriol. 176:2151–2157.

Benach, J. L., E. M. Bosler, J. P. Hanrahan, J. L. Coleman, G. S. Habicht, T. F. Bast, D. J. Cameron, J. L. Ziegler, A. G. Barbour, W. Burgdorfer, R. Edelman, and R. A. Kaslow. 1983. Spirochetes isolated from the blood of two patients with Lyme disease. N. Engl. J. Med. 308:740–742.

Benach, J. L., J. L. Coleman, R. A. Skinner, and E. M. Bosler. 1987. Adult Ixodes dammini on rabbits: a hypothesis for

the development and transmission of Borrelia burgdorferi. J. Infect. Dis. 155:1300–1306.

Berg, H. C., and L. Turner. 1979. Movement of microorganisms in viscous environments. Nature 278:349–351.

Berger, B. W., M. H. Kaplan, I. R. Rothenberg, and A. G. Barbour. 1985. Isolation and characterization of the Lyme disease spirochete from the skin of patients with erythema chronicum migrans. J. Am. Acad. Dermatol. 13:444–449.

Berger, B. W., R. C. Johnson, C. Kodner, and L. Coleman. 1992. Cultivation of Borrelia burgdorferi from erythema migrans lesions and perilesional skin. J. Clin. Microbiol. 30:359–361.

Berger, B. W., R. C. Johnson, C. Kodner, and L. Coleman. 1994. Cultivation of Borrelia burgdorferi from the blood of two patients with erythema migrans lesions lacking extracutaneous signs and symptoms of Lyme disease. J. Am. Acad. Dermatol. 30:48–51.

Bergeret, C., and A. Raoult. 1948. Notes sur les formes nerveuses de la fièvre récurrente: Fièvre récurrente à tiques en Afrique Occidentale Française. Bulletin Médicale de l'Afrique Occidentale Française 5:271–279.

Berglund, J., R. Eitrem, K. Ornstein, A. Lindberg, A. Ringner, H. Elmrud, M. Carlsson, A. Runehagen, C. Svanborg, and R. Norrby. 1995. An epidemiologic study of Lyme disease in southern Sweden. N. Engl. J. Med. 333:1319–1324.

Bergstrom, S., C. F. Garon, A. G. Barbour, and J. MacDougall. 1992. Extrachromosomal elements of spirochetes. Res. Microbiol. 143:623–628.

Beutler, B., and R. S. Munford. 1996. Tumor necrosis factor and the Jarisch-Herxheimer reaction. N. Engl. J. Med. 335:347–348.

Boggemeyer, E., T. Stehle, U. E. Schaible, M. Hahne, D. Vestweber, and M. M. Simon. 1994. Borrelia burgdorferi upregulates the adhesion molecules E-selectin, P-selectin, ICAM-1, and VCAM-1 on mouse endothelioma in vitro. Cell Adhes. Commun. 2:145–157.

Bono, J. L., K. Tilly, B. Stevenson, D. Hogan, and P. A. Rosa. 1998. Oligopeptide permease in Borrelia burgdorferi: putative peptide-binding components encoded by both chromosomal and plasmid loci. Microbiology 144:1033–1044.

Borst, P. 1991. Molecular genetics of antigenic variation. Immunol. Today 12:A29–A33.

Bradley, J. F., R. C. Johnson, and J. L. Goodman. 1994. The persistence of spirochetal nucleic acids in active Lyme arthritis. Ann. Int. Med. 120:487–489.

Brandt, M. E., B. S. Riley, J. D. Radolf, and M. V. Norgard. 1990. Immunogenic integral membrane proteins of Borrelia burgdorferi are lipoproteins. Infect. Immun. 58:983–991.

Bremer, H., and P. P. Dennis. 1987. Modulation of chemical composition and other parameters of the cell by growth rate. *In:* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (Eds.). Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology. American Society for Microbiology. Washington DC, 1527–1542.

Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, P. J. Brennan, B. R. Bloom, P. J. Godowski, and R. L. Modlin. 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 285:732–736.

Broder, C. C., R. Lottenberg, G. O. von Mering, K. H. Johnston, and M. D. P. Boyle. 1991. Isolation of a prokaryotic plasmin receptor: Relationship to a plasminogen activator produced by the same micro-organism. J. Biol. Chem. 266:4922–4928.

Brown, E. L., B. P. Guo, P. O'Neal, and M. Höök. 1999. Adherence of Borrelia burgdorferi: Identification of critical lysine residues in DbpA required for decorin binding. J. Biol. Chem. 274:26272–26278.

Brown, E. L., R. M. Wooten, B. J. B. Johnson, R. V. Iozzo, A. Smith, M. C. Dolan, B. P. Guo, J. J. Weis, and M. Höök. 2001. Resistance to Lyme disease in decorin-deficient mice. J. Clin. Invest. 107:845–852.

Brusca, J. S., A. W. McDowall, M. V. Norgard, and J. D. Radolf. 1991. Localization of outer surface proteins A and B in both the outer membrane and intracellular compartments of Borrelia burgdorferi. J. Bacteriol. 173:8004–8008.

Brusca, J. S., and J. D. Radolf. 1994. Isolation of integral membrane proteins by phase partitioning with Triton X-114. Methods Enzymol. 228:182–193.

Bryceson, A. D. E., E. H. O. Parry, P. L. Perine, D. A. Warrell, D. Vukotich, and C. S. Leithead. 1970. Louse-borne relapsing fever. A clinical and laboratory study of 62 cases in Ethiopia and a reconsideration of the literature. J. Med. 39:129–170.

Bunikis, J., L. Noppa, and S. Bergstrom. 1995. Molecular analysis of a 66-kDa protein associated with the outer membrane of Lyme disease Borrelia. FEMS Microbiol. Lett. 131:139–145.

Bunikis, J., L. Noppa, Y. Ostberg, A. G. Barbour, and S. Bergstrom. 1996. Surface exposure and species specificity of an immunoreactive domain of a 66-kilodalton outer membrane protein (P66) of the Borrelia species that cause Lyme disease. Infect. Immun. 64:5111–5116.

Bunikis, J., C. J. Luke, E. Bunikiene, S. Bergstrom, and A. G. Barbour. 1998. A surface-exposed region of a novel outer membrane protein (P66) of Borrelia spp. is variable in size and sequence. J. Bacteriol. 180:1618–1623.

Bunikis, J., and A. G. Barbour. 1999. Access of antibody and trypsin to an integral outer membrane protein (p66) of Borrelia burgdorferi is hindered by Osp lipoproteins. Infect. Immun. 67:2874–2883.

Bunikis, J., H. Mirian, E. Bunikiene, and A. G. Barbour. 2001. Non-heritable change of a spirochaete's phenotype by decoration of the cell surface with exogenous lipoproteins. Molec. Microbiol. 40:387–396.

Burgdorfer, W. 1976a. The diagnosis of the relapsing fevers. *In:* R. C. Johnson (Ed.). The Biology of Parasitic Spirochetes. Academic Press. New York, NY. 225–234.

Burgdorfer, W. 1976b. The epidemiology of the relapsing fevers. *In:* R. C. Johnson (Ed.). The Biology of Parasitic Spirochetes. Academic Press. New York, NY. 191–200.

Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis. 1982. Lyme disease—a tick-borne spirochetosis? Science 216:1317–1319.

Burgdorfer, W. 1989a. Vector/host relationships of the Lyme disease sprirochete, Borrelia burgdorferi. Rheum. Dis. Clin. N. Amer. 15:775–787.

Burgdorfer, W., S. F. Hayes, and D. Corwin. 1989b. Pathophysiology of the Lyme disease spirochete, Borrelia burgdorferi, in Ixodid ticks. Rev. Infect. Dis. 11, Suppl. 6:S1442–S1450.

Burgdorfer, W. 1991. Lyme borreliosis: Ten years after discovery of the etiologic agent, Borrelia burgdorferi. Infection 19:257–262.

Burgdorfer, W. 1993. How the discovery of Borrelia burgdorferi came about. Clin. Dermatol. 11:335–338.

Burman, N., S. Bergstrom, B. I. Restrepo, and A. G. Barbour. 1990. The variable antigens Vmp7 and Vmp21 of the relapsing fever bacterium Borrelia hermsii are structurally analogous to the VSG proteins of the African trypanosome. Molec. Microbiol. 4:1715–1726.

Busch, U., G. Will, C. Hizo-Teufel, B. Wilske, and V. Preac-Mursic. 1997. Long-term in vitro cultivation of Borrelia burgdorferi sensu lato strains: influence on plasmid patterns, genome stability and expression of proteins. Res. Microbiol. 148:109–118.

Buschke, A., and H. Kroo. 1922. Experimental recurrentis infection. Klin. Wochenschr. 1:2470.

Butler, T. 1985. Relapsing fever: New lessons about antibiotic action. Ann. Int. Med. 102:397.

Cadavid, D., D. D. Thomas, R. Crawley, and A. G. Barbour. 1994. Variability of a bacterial surface protein and disease expression in a possible mouse model of systemic Lyme borreliosis. J. Exp. Med. 179:631–642.

Cadavid, D., P. M. Pennington, T. A. Kerentseva, S. Bergstrom, and A. G. Barbour. 1997. Immunologic and genetic analyses of VmpA of a neurotropic strain of Borrelia turicatae. Infect. Immun. 65:3352–3360.

Cadavid, D., and A. G. Barbour. 1998. Neuroborreliosis during relapsing fever: Review of the clinical manifestations, pathology, and treatment of infections in humans and experimental animals. Clin. Infect. Dis. 26:151–164.

Cadavid, D., T. O'Neill, H. Schaeffer, and A. R. Pachner. 2000. Localization of Borrelia burgdorferi in the nervous system and other organs in a nonhuman primate model of Lyme disease. Lab. Invest. 80:1043–1054.

Caimano, M. J., X. Yang, T. G. Popova, M. L. Clawson, D. R. Akins, M. V. Norgard, and J. D. Radolf. 2000. Molecular and evolutionary characteriztaion of the cp32/18 family of supercoiled plasmids in Borrelia burgdorferi 297. Infect. Immun. 68:1574–1586.

Callister, S. M., K. L. Case, W. A. Agger, R. F. Schell, R. C. Johnson, and J. L. Ellingson. 1990. Effects of bovine serum albumin on the ability of Barbour- Stoenner-Kelly medium to detect Borrelia burgdorferi. J. Clin. Microbiol. 28:363–365.

Carlyon, J. A., and R. T. Marconi. 1998a. Cloning and molecular characterization of a multicopy, linear plasmid-carried, repeat motif-containing gene from Borrelia turicatae, a causative agent of relapsing fever. J. Bacteriol. 180:4974–4981.

Carlyon, J. A., C. LaVoie, S.-Y. Sung, and R. T. Marconi. 1998b. Analysis of the organization of multicopy linear- and circular-plasmid-carried open reading frames in Borrelia burgdorferi sensu lato isolates. Infect. Immun. 66:1149–1158.

Carlyon, J. A., D. M. Roberts, and R. T. Marconi. 2000. Evolutionary and molecular analyses of the Borrelia bdr super gene family: delineation of distinct sub-families and demonstration of the genus wide conservation of putative functional domains, structural properties and repeat motifs. Microb. Pathog. 28:89–105.

Carroll, J. A., C. F. Garon, and T. G. Schwan. 1999. Effects of environmental pH on membrane proteins in Borrelia burgdorferi. Infect. Immun. 67:3181–3187.

Carter, C. J., S. Bergstrom, S. J. Norris, and A. G. Barbour. 1994. A family of surface-exposed proteins of 20 kilodaltons in the genus Borrelia. Infect. Immun. 62:2792–2799.

Casjens, S., and W. M. Huang. 1993. Linear chromosomal physical and genetic map of Borrelia burgdorferi, the Lyme disease agent. Molec. Microbiol. 8:967–980.

Casjens, S., M. Murphy, M. Delange, L. Sampson, R. van Vugt, and W. M. Huang. 1997a. Telomeres of the linear chromosome of Lyme disease spirochaetes: nucleotide sequence and possible exchange with linear plasmid telomeres. Molec. Microbiol. 26:581–596.

Casjens, S., R. van Vugt, K. Tilly, P. A. Rosa, and B. Stevenson. 1997b. Homology throughout the multiple 32-kilobase circular plasmids present in Lyme disease spirochetes. J. Bacteriol. 179:217–227.

Casjens, S. 1999. Evolution of the linear DNA replicons of Borrelia burgdorferi. Curr. Opin. Microbiol. 2:529–534.

Casjens, S., N. Palmer, R. van Vugt, W. M. Huang, B. Stevenson, P. Rosa, R. Lathigra, G. Sutton, J. Peterson, R. J. Dodson, D. Haft, E. Hickey, M. Gwinn, O. White, and C. M. Fraser. 2000. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete Borrelia burgdorferi. Molec. Microbiol. 35:490–516.

Cassatt, D. R., N. K. Patel, N. D. Ulbrandt, and M. S. Hanson. 1998. DbpA, but not OspA, is expressed by Borrelia burgdorferi during spirochetemia and is a target for protective antibodies. Infect. Immun. 66:5379–5387.

Cauwels, A., K. Frei, S. Sansano, C. Fearns, R. Ulevitch, W. Zimmerli, and R. Landmann. 2001. The origin and function of soluble CD14 in experimental bacterial meningitis. J. Immunol. 162:4762–4772.

Chaconas, G., P. E. Stewart, K. Tilly, J. L. Bono, and P. Rosa. 2001. Telomere resolution in the Lyme disease spirochete. EMBO J.

Champion, C. I., D. R. Blanco, J. T. Skare, D. A. Haake, M. Giladi, D. Foley, J. N. Miller, and M. A. Lovett. 1994. A 9.0 kilobase-pair circular plasmid of Borrelia burgdorferi encodes an exported protein: evidence for expression only during infection. Infect. Immun. 62:2653–2661.

Ciesielski, C. A., L. E. Markowitz, R. Horsley, A. W. Hightower, H. Russell, and C. V. Broome. 1989. Lyme disease surveillance in the United States, 1983–1986. Rev. Infect. Dis. 2, Suppl. 6:S1435–S1440.

Coburn, J., W. Chege, L. Magoun, S. C. Bodary, and J. M. Leong. 1999. Characterization of a candidate Borrelia burgdorferi chain integrin ligand identified using a phage display library. Molec. Microbiol. 34:926–940.

Cockayne, A., M. J. Bailey, and C. W. Penn. 1987. Analysis of sheath and core structures of the axial filament of Treponema pallidum. J. Gen. Microbiol. 133:1397–1407.

Colebunders, R., P. De Serrano, A. Van Gompel, H. Wynants, K. Blot, E. Van den Enden, and J. van den Ende. 1993. Imported relapsing fever in European tourists. Scand. J. Infect. Dis. 25:533–536.

Coleman, J. L., T. J. Sellati, J. E. Testa, R. R. Kew, M. B. Furie, and J. L. Benach. 1995. Borrelia burgdorferi binds plasminogen, resulting in enhanced penetration of endothelial monolayers. Infect. Immun. 63:2478–2484.

Coleman, J. L., J. A. Gebbia, J. Piesman, J. L. Degen, T. H. Bugge, and J. L. Benach. 1997. Plasminogen is required for efficient dissemination of B. burgdorferi in ticks and for enhancement of spirochetemia in mice. Cell 89:1111–1119.

Coleman, J. L., and J. L. Benach. 2000. The generation of enzymatically active plasmin on the surface of spirochetes. Methods 21:133–141.

Comstock, L. E., and D. D. Thomas. 1989. Penetration of endothelial cell monolayers by Borrelia burgdorferi. Infect. Immun. 57:1626–1628.

Comstock, L. E., and D. D. Thomas. 1991. Characterization of Borrelia burgdorferi invasion of cultured endothelial cells. Microb. Pathog. 10:137–148.

Cox, D. L., D. R. Akins, M. V. Norgard, and J. D. Radolf. 1994. Localization of Treponema pallidum and Borrelia burgdorferi proteins using a gel microdroplet immunofluorescence assay. Abstr. Ann. Meet. Am. Soc. Microbiol. J-4:273.

Cox, D. L., D. R. Akins, K. W. Bourell, P. Lahdenne, M. V. Norgard, and J. D. Radolf. 1996. Limited surface exposure of Borrelia burgdorferi outer surface lipoproteins. Proc. Natl. Acad. Sci. USA 93:7973–7978.

Cutler, S. J., J. Moss, M. Fukunaga, D. J. M. Wright, D. Fekade, and D. Warrell. 1997. Borrelia recurrentis characterization and comparison with relapsing-fever, Lyme-associated, and other Borrelia spp. Int. J. Syst. Bacteriol. 47:958–968.

Cutler, S. J., C. O. K. Akintunde, J. Moss, M. Fukunaga, K. Kurtenbach, A. Talbert, H. Zhang, D. J. M. Wright, and D. A. Warrell. 1999. Successful in vitro cultivation of Borrelia duttonii and its comparison with Borrelia recurrentis. Int. J. Syst. Bacteriol. 49:1793–1799.

Das, S., S. W. Barthold, S. S. Giles, R. R. Montgomery, S. R. Telford 3rd, and E. Fikrig. 1997. Temporal pattern of Borrelia burgdorferi p21 expression in ticks and mammalian host. J. Clin. Invest. 99:987–995.

Dattwyler, R. J., D. J. Volkman, and B. J. Luft. 1989. Immunologic aspects of Lyme borreliosis. Rev. Infect. Dis. 11, Suppl. 6:S1494–S1498.

Davis, G. E. 1956. The identification of spirochetes from human cases of relapsing fever by xenodiagnosis with comments on local specificity of tick vectors. Exp. Parasitol. 5:271–275.

Deitsch, K. W., E. R. Moxon, and T. E. Wellems. 1997. Shared themes of antigenic variation and virulence in bacterial, protozoal, and fungal infections. Microbiol. Molec. Biol. Rev. 61:281–293.

de Silva, A. M., S. R. Telford, L. R. Brunet, S. W. Barthold, and E. Fikrig. 1996. Borrelia burgdorferi OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. J. Exp. Med. 183:271–275.

de Silva, A. M., and E. Fikrig. 1997. Arthropod- and host-specific gene expression by Borrelia burgdorferi. J. Clin. Invest. 99:377–379.

de Silva, A., N. S. Zeidner, Y. Zhang, M. C. Dolan, J. Piesman, and E. Fikrig. 1999. Influence of outer surface protein A antibody on Borrelia burgdorferi within feeding ticks. Infect. Immun. 67:30–35.

Dolan, M. C., J. Piesman, M. L. Mbow, G. O. Maupin, O. Peter, M. Brossard, and W. T. Golde. 1998. Vector competence of Ixodes scapularis and Ixodes ricinus (Acari: Ixodidae) for three genospecies of Borrelia burgdorferi. J. Med. Entomol. 35:465–470.

Donelson, J. E. 1995. Mechanisms of antigenic variation in Borrelia hermsii and African trypanosomes. J. Biol. Chem. 270:7783–7786.

Duray, P. H., and A. C. Steere. 1988. Clinical pathological correlations of Lyme disease by stage. Ann. NY Acad. Med. 53:65–79.

Dworkin, M. S., D. E. Anderson Jr., T. G. Schwan, P. C. Shoemaker, S. N. Banerjee, B. O. Kassen, and W. Burgdorfer. 1998. Tick-borne relapsing fever in the Northwestern United States and Southwestern Canada. Clin. Infect. Dis. 26:122–131.

Edlow, J. A. 1999. Lyme disease and related tick-borne illnesses. Ann. Emerg. Med. 33:680–693.

Eggers, C. H., and D. S. Samuels. 1999. Molecular evidence for a new bacteriophage of Borrelia burgdorferi. J. Bacteriol. 181:7308–7313.

Eggers, C. H., S. Casjens, S. F. Hayes, C. F. Garon, C. J. Damman, D. B. Oliver, and D. S. Samuels. 2000. Bacteriophages of spirochetes. J. Molec. Microbiol. Biotechnol. 2:365–373.

Eggers, C. H., B. J. Kimmel, J. L. Bono, A. F. Elias, P. Rosa, and D. S. Samuels. 2001. Transduction by BB-1, a bacteriophage of Borrelia burgdorferi. J. Bacteriol.

Eicken, C., V. Sharma, T. Klabunde, R. T. Owens, D. S. Pikas, M. Höök, and J. C. Sacchettini. 2001. Crystal structure of Lyme disease antigen outer surface protein C from Borrelia burgdorferi. J. Biol. Chem. 276:10010–10015.

Elias, A. F., J. L. Bono, J. A. Carroll, P. Stewart, K. Tilly, and P. Rosa. 2000. Altered stationary-phase response in a Borrelia burgdorferi rpoS mutant. J. Bacteriol. 182:2809–2918.

Estanislao, L. B., and A. R. Pachner. 1999. Spirochetal infection of the nervous system. Neurol. Clin. 17:783–800.

Exner, M. M., X. Wu, D. R. Blanco, J. N. Miller, and M. A. Lovett. 2000. Protection elicited by native outer membrane protein Osm66 (p66) against host-adapted Borrelia burgdorferi: Conformational nature of bactericidal epitopes. Infect. Immun. 68:2647–2654.

Falcone, G. 1952. Le complicazioni oculari mella febbre ricorrente de zecca in Somalia. Bolletino Societa Italiana di Medicina e Igiene Tropicale in Eritrea Special Edition:319–333.

Fekade, D., K. Knox, K. Hussein, A. Melka, D. G. Lalloo, R. E. Coxon, and D. A. Warrell. 1996. Prevention of Jarisch-Herxheimer reactions by treatment with antibodies against tumor necrosis factor? N. Engl. J. Med. 335:311–315.

Felsenfeld, O. 1965. Borrelia, human relapsing fever, and parasite-vector-host relationship. Bacteriol. Rev. 29:46–74.

Felsenfeld, O. 1971. Borrelia. Warren H. Green Publishing. St. Louis, MO.

Feng, S., E. Hodzic, B. Stevenson, and S. W. Barthold. 1998. Humoral immunity to Borrelia burgdorferi N40 decorin binding proteins during infection of laboratory mice. Infect. Immun. 66:2827–2835.

Ferdows, M. S., and A. G. Barbour. 1989. Megabase-sized linear DNA in the bacterium Borrelia burgdorferi, the Lyme disease agent. Proc. Natl. Acad. Sci. USA 86:5969–5973.

Ferdows, M. S., P. Serwer, G. A. Griess, S. J. Norris, and A. G. Barbour. 1996. Conversion of a linear to a circular plasmid in the relapsing fever agent Borrelia hermsii. J. Bacteriol. 178:793–800.

Fihn, S., and E. B. Larson. 1980. Tick-borne relapsing fever in the Pacific Northwest: an underdiagnosed illness? West. J. Med. 133:203–209.

Fikrig, E., S.W. Barthold, W., W. Sun, W. Feng, S. R. Telford, and R. A. Flavell. 1997. Borrelia burgdorferi p35 and p37 proteins, expressed in vivo, elicit protective immunity. Immunity 6:531–539.

Fikrig, E., M. Chen, S. W. Barthold, J. Anguita, W. Feng, S. R. Telford 3rd, and R. A. Flavell. 1999. Borrelia burgdorferi erpT expression in the arthropod vector and murine host. Molec. Microbiol. 31:281–280.

Fikrig, E., W. Feng, S. W. Barthold, S. R. Telford 3rd, and R. A. Flavell. 2000. Arthropod- and host-specific Borrelia burgdorferi bbk32 expression and the inhibition of spirochete transmission. J. Immunol. 164:5344–5351.

Foster, M. T. 1977. Relapsing fever. *In:* H. F. Conn (Ed.). Current Therapy. W. B. Saunders. Philadelphia, PA. 64–66.

Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, J. Weidman, T. Utterback, L. Watthey, L. McDonald, P. Artiach, C. Bowman, S. Garland, C. Fujii, M. D. Cotton, K. Horst, K. Roberts, B. Hatch, H. O. Smith, and J. C. Venter. 1997. Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi. Nature 390:580–586.

Fraser, C. M., S. J. Norris, G. M. Weinstock, O. White, G. C. Sutton, R. Dodson, M. Gwinn, E. K. Hickey, R. Clayton, K. A. Ketchum, E. Sodergren, J. M. Hardham, M. P. McLeod, S. Salzberg, J. Peterson, H. Khalak, D. Richardson, J. K. Howell, M. Chidambaram, T. Utterback, L. McDonald, P. Artiach, C. Bowman, M. D. Cotton, C. Fujii, S. Garland, B. Hatch, K. Horst, K. Roberts, M. Sandusky, J. Weidman, H. O. Smith, and J. C. Venter. 1998. Complete genome sequence of Treponema pallidum, the syphilis spirochete. Science 281:375–388.

Fuchs, H., R. Wallich, M. M. Simon, and M. D. Kramer. 1994. The outer surface protein A of the spirochete Borrelia burgdorferi is a plasmin(ogen) receptor. Proc. Natl. Acad. Sci. USA 91:12594–12598.

Fuchs, H., M. M. Simon, R. Wallich, M. Bechtel, and M. D. Kramer. 1996. Borrelia burgdorferi induces secretion of pro-urokinase-type plasminogen activator by human monocytes. Infect. Immun. 64:4307–4312.

Fukunaga, M., Y. Takahashi, Y. Tsuruta, O. Matsushita, D. Ralph, M. McClelland, and M. Nakao. 1995. Genetic and phenotypic analysis of Borrelia miyamotoi sp. nov., isolated from the ixodid tick Ixodes persulcatus, the vector for Lyme disease in Japan. Int. J. Syst. Bacteriol. 45:804–810.

Fukunaga, M., A. Hamase, K. Okada, and M. Nakao. 1996. Borrelia tanukii sp. nov. and Borrelia turdae sp. nov. found from ixodid ticks in Japan: rapid species identification by 16S rRNA gene-targeted PCR analysis. Microbiol. Immunol. 40:877–881.

Garcia-Lara, J., M. Picardeau, B. J. Hinnebusch, W. M. Huang, and S. Casjens. 2000. The role of genomics in approaching the study of Borrelia DNA replication. J. Molec. Microbiol. Biotechnol. 2:447–454.

Garcia-Monco, J. C., and J. L. Benach. 1995. Lyme neuroborreliosis. Ann. Neurol. 37:691–702.

Garnham, P., C. Davis, R. Heisch, and G. Timms. 1947. An epidemic of louse-borne relapsing fever in Kenya. Trans. R. Soc. Trop. Med. Hyg. 41:141–170.

Ge, Y. G., and N. W. Charon. 1997a. An unexpected flaA homolog is present and expressed in Borrelia burgdorferi. J. Bacteriol. 179:552–556.

Ge, Y. G., and N. W. Charon. 1997b. Identification of a large motility operon in Borrelia burgdorferi by semi-random PCR chromosome walking. Gene 189:195–201.

Ge, Y. G., C. Li, L. Corum, C. Slaughter, and N. W. Charon. 1998. Structure and expression of the FlaA periplasmic flagellar protein of Borrelia burgdorferi. J. Bacteriol. 180:2418–2425.

Gebbia, J. A., P. B. Backenson, J. L. Coleman, P. Anda, and J. L. Benach. 1997. Glycolytic enzyme operon of Borrelia burgdorferi: Characterization and evolutionary implications. Gene 188:221–228.

Gebbia, J. A., J. C. Monco-Garcia, J. L. Degen, T. H. Bugge, and J. L. Benach. 1999. The plasminogen activation system enhances brain and heart invasion in murine relapsing fever borreliosis. J. Clin. Invest. 103:81–87.

Gerber, M. A., E. D. Shapiro, G. S. Burke, V. J. Parcells, G. L. Bell, and the Lyme Disease Pediatric Study Group. 1996. Lyme disease in children in southeastern Connecticut. N. Engl. J. Med. 335:1270–1274.

Gilmore Jr., R. D., K. J. Kappel, M. C. Dolan, T. R. Burkot, and B. J. B. Johnson. 1996. Outer surface protein C (OspC), but not P39, is a protective immunogen against tick-transmitted Borrelia burgdorferi challenge: Evidence for a conformational protective epitope in OspC. Infect. Immun. 64:2234–2239.

Gilmore Jr., R. D., and J. Piesman. 2000. Inhibition of Borrelia burgdorferi migration from the midgut to the salivary glands following feeding by ticks on OspC-immunized mice. Infect. Immun. 68:411–414.

Ginger, C. D. 1963. Isolation and characterization of muramic acid from two spirochetes: Borrelia duttonii and Leptospira biflexa. Nature 199:159–159.

Golde, W. T., and M. C. Dolan. 1995. Variation in antigenicity and infectivity of derivatives of Borrelia burgdorferi, strain B31, maintained in the natural, zoonotic cycle compared with maintenance in culture. Infect. Immun. 63:4795–4801.

Goldstein, S. F., N. W. Charon, and J. A. Kreiling. 1994. Borrelia burgdorferi swims with a planar waveform similar to that of eukaryotic flagella. Proc. Natl. Acad. Sci. USA 91:3433–3437.

Goldstein, S. F., K. F. Buttle, and N. W. Charon. 1996. Structural analysis of the Leptospiraceae and Borrelia burgdorferi by high-voltage electron microscopy. J. Bacteriol. 178:6539–6545.

Goodman, J. L., P. Jurkovich, J. M. Kramber, and R. C. Johnson. 1991. Molecular detection of persistent Borrelia burgdorferi in the urine of patients with active Lyme disease. Infect. Immun. 59:269–278.

Goodman, J. L., J. F. Bradley, A. E. Ross, P. Goellner, A. Lagus, B. Vitale, B. W. Berger, S. Luger, and R. C. Johnson. 1995. Bloodstream invasion in early Lyme disease: results from a prospective, controlled, blinded study using the polymerase chain reaction. Am. J. Med. 99:6–12.

Goubau, P. F. 1984. Relapsing fevers: A review. Ann. Soc. Belge. Med. Trop. 64:335–364.

Gross, D. M., T. Forsthuber, M. Tary-Lehmann, C. Etling, K. Ito, Z. A. Nagy, J. A. Field, A. C. Steere, and B. T. Huber. 1998. Identification of LFA-1 as a candidate autoantigen in treatment-resistant Lyme arthritis. Science 281:703–706.

Guy, E. C., and G. Stanek. 1991. Detection of Borrelia burgdorferi in patients with Lyme disease by the polymerase chain reaction. J. Clin. Pathol. 44:610–611.

Gylfe, A., S. Bergstrom, J. Lundstrom, and B. Olsen. 2000. Reactivation of Borrelia infection in birds. Nature 403:724–725.

Haake, D. A. 2000. Spirochaetal lipoproteins and pathogenesis. Microbiology 146:1491–1504.

Hagman, K. E., P. Lahdenne, T. G. Popova, S. F. Porcella, D. R. Akins, J. D. Radolf, and M. V. Norgard. 1998. Decorin-binding protein of Borrelia burgdorferi is encoded within a two-gene operon and is protective in the murine model of Lyme borreliosis. Infect. Immun. 66:2674–2683.

Hagman, K. E., X. Yang, S. K. Wikel, G. B. Schoeler, M. J. Caimano, J. D. Radolf, and M. V. Norgard. 2000. Decorin-binding protein (Dbp) of Borrelia burgdorferi is not protective when immunized mice are challenged via tick infestation and correlates with the lack of DbpA expression by B. burgdorferi in ticks. Infect. Immun. 68:4759–4764.

Hamilton, J. 1943. Ocular complications in relapsing fever. Br. J. Opthamol. 27:68–80.

Hansen, K., and A. M. Lebech. 1992. The clinical and epidemiological profile of Lyme neuroborreliosis in Denmark 1985–1990: A prospective study of 187 patients with Borrelia burgdorferi specific intrathecal antibody production. Brain 115:399–423.

Hanson, M. S., D. R. Cassatt, B. P. Guo, N. K. Patel, M. P. McCarthy, D. W. Dorward, and M. Höök. 1998. Active and passive immunization against Borrelia burgdorferi decorin binding protein A protects against infection. Infect. Immun. 66:2143–2153.

Hayes, S. F., W. Burgdorfer, and A. G. Barbour. 1983. Bacteriophage in the Ixodes dammini spirochete, etiological agent of Lyme disease. J. Bacteriol. 154:1436–1439.

Hayes, S. F., and W. Burgdorfer. 1993. Ultrastructure of Borrelia burgdorferi. *In:*K. Weber and W. Burgdorfer (Eds.). Aspects of Lyme Borreliosis. Springer-Verlag. New York, NY. 29–43.

Hefty, P. S., S. E. Joliff, M. J. Caimano, S. K. Wikel, J. D. Radolf, and D. R. Akins. 2001. Regulation of the OspE-related, OspF-related and Elp lipoproteins of Borrelia burgdorferi strain 297 by mammalian host-specific signals. Infect. Immun.

Hendson, M., and R. S. Lane. 2000. Genetic characteristics of Borrelia coriaceae isolates from the soft tick Ornithodoros coriaceus (Acari:Argasidae). J. Clin. Microbiol. 38:2678–2682.

Hinnebusch, J., S. Bergstrom, and A. G. Barbour. 1990. Cloning and sequence analysis of linear plasmid telomeres of the bacterium Borrelia burgdorferi. Molec. Microbiol. 4:811–820.

Hinnebusch, J., and A. G. Barbour. 1991. Linear plasmids of Borrelia burgdorferi have a telomeric structure and sequence similar to those of a eukaryotic virus. J. Bacteriol. 173:7233–7239.

Hinnebusch, J., and A. G. Barbour. 1992. Linear and circular plasmid copy numbers in Borrelia burgdorferi. J. Bacteriol. 174:5251–5257.

Hinnebusch, B. J., and A. J. Bendich. 1997. The bacterial nucleoid visualized by fluorescence microscopy of cells lysed within agarose: comparison of Escherichia coli and spirochetes of the genus Borrelia. J. Bacteriol. 179:2228–2237.

Hinnebusch, B. J., A. G. Barbour, B. I. Restrepo, and T. G. Schwan. 1998. Population structure of the relapsing fever spirochete Borrelia hermsii as indicated by polymorphism of two multigene families that encode immunogenic outer surface lipoproteins. Infect. Immun. 66:432–440.

Hirschfeld, M., C. J. Kirschning, R. Schwandner, H. Wesche, J. H. Weis, R. M. Wooten, and J. J. Weis. 1999. Inflammatory signaling by Borrelia burgdorferi lipoporteins is mediated by toll-like receptor 2. J. Immunol. 163:2382–2386.

Holt, S. C. 1978. Anatomy and chemistry of spirochetes. Microbiol. Rev. 42:114–160.

Horton, J. M., and M. J. Blaser. 1985. The spectrum of relapsing fever in the Rocky Mountains. Arch. Int. Med. 145:871–875.

Hovind-Hougen, K. 1976. Treponema and Borrelia morphology. *In:*R. C. Johnson (Ed.). The Biology of Parasitic Spirochetes. Academic Press. New York, NY. 7–18.

Hovind-Hougen, K. 1984. Ultrastructure of spirochetes isolated from Ixodes ricinus and Ixodes dammini. Yale J. Biol. Med. 57:543–548.

Hu, L. T., G. Perides, R., Noring, and M. S. Klempner. 1995. Binding of human plasminogen to Borrelia burgdorferi. Infect. Immun. 63:3491–3496.

Hu, L. T., S. D. Pratt, G. Perides, L. Katz, R. A. Rogers, and M. S. Klempner. 1997. Isolation, cloning, and expression of a 70-kilodalton plasminogen binding protein of Borrelia burgdorferi. Infect. Immun. 65:4989–4995.

Humair, P.-F., and L. Gern. 2000. The wild hidden face of Lyme borreliosis in Europe. Microb. Infect. 2:915–922.

Huppertz, H.-L., H. Schmidt, and H. Karch. 1993. Detection of Borrelia burgdorferi by nested polymerase chain reaction in cerebrospinal fluid and urine of children with neuroborreliosis. Eur. J. Pediatr. 152:414–417.

Hyde, F. W., and R. C. Johnson. 1984. Genetic relationship of Lyme disease spirochetes to Borrelia, Treponema, and Leptospira spp. J. Clin. Microbiol. 20:151–154.

Indest, K. J., R. Ramamoorthy, M. Sole, R. D. Gilmore, B. J. B. Johnson, and M. T. Philipp. 1997. Cell-density-dependent expression of Borrelia burgdorferi lipoproteins in vitro. Infect. Immun. 65:1165–1171.

International Union of Microbiological Societies. 1998. Validation of the publication of new names and new combinations previously effectively published outside the IJSB. Int. J. Syst. Bacteriol. 47:1274.

Iyer, R., J. M. Hardham, G. P. Wormser, I. Schwartz, and S. J. Norris. 2000. Conservation and heterogeneity of vlsE among human and tick isolates of Borrelia burgdorferi. Infect. Immun. 68:1714–1718.

Jaulhac, B., I. Chary-Valckenaere, J. Sibilia, R. M. Javier, Y. Piemont, H. Monteil, and J. Pourel. 1996. Detection of Borrelia burgdorferi by DNA amplification in synovial tissue samples from patients with Lyme arthritis. Arthritis Rheum. 39:736–745.

Johns, R. H., D. E. Sonenshine, and W. L. Hynes. 2000. Enhancement of OspC expression by Borrelia burgdorferi in the presence of tick hemolymph. FEMS Microbiol. Lett. 193:137–141.

Johnson, R. C., F. W. Hyde, G. Schmid, and D. Brenner. 1984. Borrelia burgdorferi sp. nov. etiological agent of Lyme disease. Int. J. Syst. Bacteriol. 34:496–497.

Johnson, R. C., W. Burgdorfer, R. S. Lane, A. G. Barbour, S. F. Hayes, and F. W. Hyde. 1987. Borrelia coriaceae sp. nov.: Putative agent of epizootic bovine abortion. Int. J. Syst. Bacteriol. 37:72–74.

Jones, J. D., K. W. Bourell, M. V. Norgard, and J. D. Radolf. 1995. Membrane topology of Borrelia burgdorferi and

Treponema pallidum lipoproteins. Infect. Immun. 63:2424–2434.

Jones, C. G., R. S. Ostfedl, M. P. Richard, E. M. Schauber, and J. O. Wolff. 1998. Chain reactions linking acorns to gypsy moth outbreaks and Lyme disease. Science 279:1023–1026.

Judge, D., I. Samuel, P. Perine, and D. Vukotic. 1974. Louse-borne relapsing fever in man. Arch.Pathol. 97:136–140.

Kaufman, L. D., B. L. Gruber, M. E. Phillips, and J. L. Benach. 1989. Late cutaneous Lyme disease: Acrodermatitis chronica atrophicans. Am. J. Med. 86:828–830.

Kawabata, H., F. Myouga, Y. Inagaki, N. Murai, and H. Watanabe. 1998. Genetic and immunological analyses of Vls (VMP-like) sequences of Borrelia burgdorferi. Microb. Pathog. 24:155–166.

Keller, T. L., J. J. Halperin, and M. Whitman. 1992. PCR detection of Borrelia burgdorferi DNA in cerebrospinal fluid of Lyme neuroborreliosis patients. Neurol. 42:32–42.

Kelly, R. 1971. Cultivation of Borrelia hermsi. Science 173:443–444.

Kelly, R. T. 1976. Cultivation and physiology of relapsing fever borreliae. *In:* R. C. Johnson (Ed.). The Biology of Parasitic Spirochetes. Academic Press. New York, NY. 87–94.

Kimsey, R. B., and A. Spielman. 1990. Motility of Lyme disease spirochetes in fluids as viscous as the extracellular matrix. J. Infect. Dis. 162:1205–1208.

Kitten, T., and A. G. Barbour. 1990. Juxtaposition of expressed variable antigen genes with a conserved telomere in the bacterium Borrelia hermsii. Proc. Natl. Acad. Sci. USA 87:6077–6081.

Kitten, T., and A. G. Barbour. 1992. The relapsing fever agent Borrelia hermsii has multiple copies of its chromosome and linear plasmids. Genetics 132:311–324.

Kitten, T., A. V. Barrera, and A. G. Barbour. 1993. Intragenic recombination and a chimeric outer membrane protein in the relapsing fever agent Borrelia hermsii. J. Bacteriol. 175:2516–2522.

Klaviter, E. C., and R. C. Johnson. 1979. Isolation of a spirochete from the soft tick, Ornithodoros coriaceus: A possible agent of epizootic bovine abortion. Acta Trop. 36:123–131.

Klempner, M. S., R. Noring, and R. A. Rogers. 1993. Invasion of human skin fibroblasts by the Lyme disease spirochete, Borrelia burgdorferi. J. Infect. Dis. 167:1074–1081.

Klempner, M. S., R. Noring, M. P. Epstein, B. McCloud, R. Hu, S. A. Limentani, and R. A. Rogers. 1995. Binding of human plasminogen and urokinase-type plasminogen activator to the Lyme disease spirochete, Borrelia burgdorferi. J. Infect. Dis. 171:1258–1265.

Koopman, M. B., E. Baats, C. J. van Vorstenbosch, B. A. van der Zeijst, and J. G. Kusters. 1992. The periplasmic flagella of Serpulina (Treponema) hyodysenteriae are composed of two sheath proteins and three core proteins. J. Gen. Microbiol. 138:2697–2706.

Kornacki, J. A., and D. B. Oliver. 1998. Lyme disease-causing Borrelia species encode multiple lipoproteins homologous to peptide-binding proteins of ABC-type transporters. Infect. Immun. 66:4115–4122.

Kumaran, D., S. Eswaramoorthy, B. J. Luft, S. Koide, J. J. Dunn, C. L. Lawson, and S. Swaminathan. 2001. Crystal structure of outer surface protein C (OspC) from the Lyme disease spirochete, Borrelia burgdorferi. EMBO J. 20:971–978.

Kurtti, T. J., U. G. Munderloh, R. C. Johnson, and G. G. Ahlstrand. 1987. Colony formation and morphology in Borrelia burgdorferi. J. Clin. Microbiol. 25:2054–2058.

Labandeira-Rey, M., and J. T. Skare. 2001. Decreased infectivity in Borrelia burgdorferi strain B31 is associated with loss of linear plasmid 25 or 28-1. Infect. Immun. 69:446–455.

Lafay, B., A. T. Lloyd, M. J. McLean, K. M. Devine, P. M. Sharp, and K. H. Wolfe. 1999. Proteome composition and codon usage in spirochetes: species-specific and DNA strand-specific mutational biases. Nucleic Acids Res. 27:1642–1649.

Lahdenne, P., S. F. Porcella, K. E. Hagman, D. R. Akins, T. G. Popova, D. L. Cox, J. D. Radolf, and M. V. Norgard. 1997. Molecular characterization of a 6.6-kilodalton Borrelia burgdorferi outer membrane-associated lipoprotein (lp6.6) which appears to be downregulated during mammalian infection. Infect. Immun. 65:412–421.

Lam, T. T., T. P. K. Nguyen, R. R. Montgomery, F. S. Kantor, E. Fikrig, and R. A. Flavell. 1994. Outer surface proteins E and F of Borrelia burgdorferi, the agent of Lyme disease. Infect. Immun. 62:290–298.

Lane, R. S., W. Burgdorfer, S. F. Hayes, and A. G. Barbour. 1985. Isolation of a spirochete from the soft tick, Ornithodoros coriaceus: a possible agent of epizootic bovine abortion. Science 230:85–87.

Lane, R. S., and S. A. Manweiler. 1988a. Borrelia coriaceae in its tick vector Borrelia coriaceae, Ornithodoros coriaceus (Acari: Argasidae), with emphasis on transstadial and transovarial infection. J. Med. Entomol. 25:172–177.

Lane, R. S., and W. Burgdorfer. 1988b. Spirochetes in mammals and ticks (Acari: Ixodidae) from a focus of Lyme borreliosis in California. J. Wildl. Dis. 24:1–9.

Lane, R. S., J. Piesman, and W. Burgdorfer. 1991. Lyme borreliosis: relation of its causative agent to its vectors and hosts in North America and Europe. Ann. Rev. Entomol. 36:587–609.

Lane, R. S., and G. B. Quistad. 1998. Borreliacidal factors in the blood of the western fence lizard (Sceleporus occidentalis). J. Parasitol. 84:29–34.

Lavaditi, C., T. Anderson, F. Selbie, and R. Schoen. 1929. Présence du spirille de la fièvre récurrente (Sp. duttoni) dans le cerveau des animaux immuns. Bulletin del'Académie de Médicine 102:705–710.

Lebech, A.-M., and K. Hansen. 1992. Detection of Borrelia burgdorferi DNA in urine samples and cerebrospinal fluid samples from patients with early and late Lyme neuroborreliosis by polymerase chain reaction. J. Clin. Microbiol. 30:1646–1653.

Lebech, A. M., K. Hansen, B. Wilske, and M. Theisen. 1994. Taxonomic classification of 29 Borrelia burgdorferi strains isolated from patients with Lyme borreliosis: A comparison of five different phenotypic and genotypic typing schemes. Med. Microbiol. Immunol. 183:325–341.

LeFebvre, R. B., and G.-C. Perng. 1989. Genetic and antigenic characterization of Borrelia coriaceae, putative agent of epizootic bovine abortion. J. Clin. Microbiol. 27:389–393.

Li, H., J. J. Dunn, B. J. Luft, and C. L. Lawson. 1997. Crystal structure of Lyme disease antigen outer surface protein A complexed with an Fab. Proc. Natl. Acad. Sci. USA 94:3584–3589.

Li, C., L. Corum, D. Morgan, E. L. Rosey, T. B. Stanton, and N. W. Charon. 2000a. The spirochete FlaA periplasmic flagellar sheath protein impacts flagellar helicity. J. Bacteriol. 182:6698–6706.

Li, C., M. A. Motaleb, M. Sal, S. F. Goldstein, and N. W. Charon. 2000b. Spirochete periplasmic flagella and motility. J. Molec. Microbiol. Biotechnol. 2:345–354.

Liang, F. T., A. L. Alvarez, Y. Gu, J. M. Nowling, R. Ramamoorthy, and M. T. Philipp. 1999. An immunodominant conserved region within the variable domain of VlsE, the variable surface antigen of Borrelia burgdorferi. J. Immunol. 163:5566–5573.

Liang, F. T., J. M. Nowling, and M. T. Philipp. 2000. Cryptic and exposed invariable regions of VlsE, the variable surface antigen of Borrelia burgdorferi. J. Bacteriol. 182:3597–3601.

Liang, F. T., M. B. Jacobs, and M. T. Philipp. 2001. C-terminal invariable domain of VlsE may not serve as target for protective immune response against Borrelia burgdorferi. Infect. Immun. 69:1337–1343.

Liebling, M. R., M. Nishio, A. Rodriguez, L. H. Sigal, T. Jin, and J. S. Louie. 1993. The polymerase chain reaction for the detection of Borrelia burgdorferi in human body fluids. Arthritis Rheum. 36:665–675.

Lien, E., T. J. Sellati, A. Yoshimura, T. H. Flo, G. Rawadi, R. W. Finberg, J. Carroll, T. Espevik, R. R. Ingalls, J. D. Radolf, and D. T. Golenbock. 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J. Biol. Chem. 274:33419–33425.

Limberger, R. J., and N. W. Charon. 1986. Antiserum to the 33,000-dalton periplasmic-flagellum protein of "Treponema phagedenis" reacts with other treponemes and Spirochaeta aurantia. J. Bacteriol. 168:1030–1032.

Limberger, R. J., L. L. Slivienski, D. B. Yelton, and N. W. Charon. 1993. Molecular genetic analysis of a class B periplasmic-flagellum gene of Treponema phagedenis. J. Bacteriol. 174:6404–6410.

Lin, B., S. A Short, M. Eskildsen, M. S. Klempner, and L. T. Hu. 2001. Functional testing of putative oligopeptide permease (Opp) proteins of Borrelia burgdorferi: A complementation model in opp- Escherichia coli. Biochim. Biophys. Acta 1499:222–231.

Litwin, C. M., and S. B. Calderwood. 1993. Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. 6:137–149.

Liveris, D., S. Varde, R. Iyer, S. Koenig, S. Bittker, D. Cooper, D. McKenna, J. Nowakowski, R. B. Nadelman, G. P. Wormser, and I. Schwartz. 1999. Genetic diversity of Borrelia burgdorferi in Lyme disease patients as determined by culture versus direct PCR with clinical specimens. J. Clin. Microbiol. 37:565–569.

Livermore, B. P., and R. C. Johnson. 1978. Lipid metabolism of Borrelia hermsii. Infect. Immun. 20:215–220.

Luft, B. J., W. Jiang, P. Munoz, R. J. Dattwyler, and P. D. Gorevic. 1989. Biochemical and immunological characterization of the surface proteins of Borrelia burgdorferi. Infect. Immun. 57:3637–3645.

Lugtenberg, B., and L. van Alphen. 1983. Molecular architecture and functioning of the outer membrane of Escherichia coli and other Gram-negative bacteria. Biochim. Biophys. Acta 737:51–115.

Ma, Y., and J. J. Weis. 1993. Borrelia burgdorferi outer surface lipoproteins OspA and OspB possess B-cell, mitogenic, and cytokine-stimulatory properties. Infect. Immun. 61:3843–3853.

Magoun, L., W. R. Zückert, D. Robbins, N. Parveen, K. R. Alugupalli, T. G. Schwan, A. G. Barbour, and J. M. Leong. 2000. Variable small protein (Vsp)-dependent and Vsp-independent pathways for glycosaminoglycan recognition for relapsing fever spirochetes. Molec. Microbiol. 36:886–897.

Marconi, R. T., S. Casjens, U. G. Munderloh, and S. D. Samuels. 1996a. Analysis of linear plasmid dimers in Borrelia burgdorferi sensu lato isolates: Implications concerning the potential mechanism of linear plasmid replication. J. Bacteriol. 178:3357–3361.

Marconi, R. T., S. Y. Sung, C. A. Norton Hughes, and J. A. Carlyon. 1996b. Molecular and evolutionary analyses of a variable series of genes in Borrelia burgdorferi that are related to ospE and ospF, constitute a gene family, and share a common upstream homology box. J. Bacteriol. 178:5615–5626.

Marconi, R. T., S. Hohenberger, S. Jauris-Heipke, U. Schulte-Spechtel, C. P. Lavoie, D. Robler, and B. Wilske. 1999. Genetic analysis of Borrelia garinii OspA serotype 4 strains associated with neuroborreliosis: Evidence for extensive genetic homogeneity. J. Clin. Microbiol. 37:3965–3970.

Martínez-Báez, M., and A. Villasana. 1945. Sobre la histopatología de la fiebre recurrente experimental. Rev. Inst. Salubr. Enferm. Trop. 6:185–194.

Mbow, M. L., R. D. Gilmore Jr., and R. G. Titus. 1999. An OspC-secific monoclonal antibody passively protects mice from tick-transmitted infection by Borrelia burgdorferi. Infect. Immun. 67:5470–5472.

McInerney, J. O. 1999. Replicational and transcriptional selection on codon usage in Borrelia burgdorferi. Proc. Natl. Acad. Sci. USA 95:10698–10703.

Meier, J. T., M. I. Simon, and A. G. Barbour. 1985. Antigenic variation is associated with DNA rearrangements in a relapsing fever Borrelia. Cell 41:403–409.

Melchers, W., J. Meis, P. A. Rosa, E. Claas, L. Nohlmans, R. Koopman, A. Horrevorts, and J. Galama. 1991. Amplification of Borrelia burgdorferi DNA in skin biopsies from patients with Lyme disease. J. Clin. Microbiol. 29:2401–2406.

Miller, J. C., J. L. Bono, K. Babb, N. El-Hage, S. Casjens, and B. Stevenson. 2000. A second allele of eppA in Borrelia burgdorferi strain B31 is located on the previously undetected circular plasmid cp9-2. J. Bacteriol. 182:6254–6258.

Montgomery, R. R., S. E. Malawista, K. J. M. Feen, and L. K. Bockenstedt. 1996. Direct demonstration of antigenic substitution of Borrelia burgdorferi ex vivo: Exploration of the paradox of the early immune response to outer surface proteins A and C in Lyme disease. J. Exp. Med. 183:261–269.

Morrison, T. B., J. H. Weis, and J. J Weis. 1997. Borrelia burgdorferi outer surface protein A (OspA) activates and primes human neutrophils. J. Immunol. 158:4838–4845.

Motaleb, M. A., L. Corum, J. L. Bono, A. F. Elias, P. Rosa, D. S. Samuels, and N. W. Charon. 2000. Borrelia burgdorferi periplasmic flagella have both skeletal and motility functions. Proc. Natl. Acad. Sci. USA 97:10899–10904.

Moter, S. E., H. Hofmann, R. Wallich, M. M. Simon, and M. D. Kramer. 1994. Detection of Borrelia burgdorferi sensu latu in lesional skin of patients with erythema migrans and acrodermatitis chronica atrophicans by ospA-specific PCR. J. Clin. Microbiol. 32:2980–2988.

Nadelman, R. B., C. S. Pavia, L. A. Magnarelli, and G. P. Wormser. 1989. Isolation of Borrelia burgdorferi from the blood of seven patients with Lyme disease. Am. J. Med. 88:21–26.

Nadelman, R. B., J. Nowakowski, G. Forseter, N. S. Goldberg, S. Bittker, D. Cooper, M. Aguero-Rosenfeld, and G. P. Wormser. 1996. The clinical spectrum of early Lyme borreliosis in patients with culture-confirmed erythema migrans. Am. J. Med. 100:502–508.

Neubert, U., M. Schaller, E. Januschke, W. Stolz, and H. Schmieger. 1993. Bacteriophages induced by ciprofloxacin in a Borrelia burgdorferi skin isolate. Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis. 279:307–315.

Newton, J. A., and P. V. Pepper. 1996. Relapsing fever. N. Engl. J. Med. 335:1197.

Nikaido, H. 1996. Outer membrane. *In:*F. C. Neidhart, R. Curtiss 3rd, J. Ingraham, E. Lin, K. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, and H. Umbarger (Eds.). Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology. American Society for Microbiology. Washington DC, 29–47.

Nocton, J. J., F. Dressler, B. J. Rutledge, P. N. Rys, D. H. Persing, and A. C. Steere. 1994. Detection of Borrelia burgdorferi DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. N. Engl. J. Med. 330:229–234.

Nocton, J. J., and A. C. Steere. 1995. Lyme disease. Adv. Int. Med. 40:69–117.

Nocton, J. J., B. J. Bloom, B.J. Rutledge, D. H. Persing, E. L. Logigian, C. H. Schmid, and A. C. Steere. 1996. Detection of Borrelia burgdorferi DNA by polymerase chain reaction in cerebrospinal fluid in Lyme neuroborreliosis. J. Infect. Dis. 174:623–627.

Norgard, M. V., B. S. Riley, J. A. Richardson, and J. D. Radolf. 1995. Dermal inflammation elicted by synthetic analogs of Treponema pallidum and Borrelia burgdorferi lipoproteins. Infect. Immun. 63:1507–1515.

Norgard, M. V., L. L. Arndt, D. R. Akins, L. L. Curetty, D. A. Harrich, and J. D. Radolf. 1996. Activation of human monocytic cells by Treponema pallidum and Borrelia burgdorferi lipoproteins and synthetic lipopeptides proceeds via a pathway distinct from that of lipopolysaccharide but involves the transcriptional activator NF-κB. Infect. Immun. 64:3845–3852.

Norris, S. J., N. W. Charon, R. G. Cook, M. D. Fuentes, and R. J. Limberger. 1988. Antigenic relatedness and N-terminal sequence homology define two classes of periplasmic flagellar proteins of Treponema pallidum subsp. pallidum and Treponema phagedenis. J. Bacteriol. 170:4072–4082.

Norris, S. J., J. K. Howell, S. A. Garza, M. S. Ferdows, and A. G. Barbour. 1995. High- and low-infectivity phenotypes of clonal populations of in vitro-cultured Borrelia burgdorferi. Infect. Immun. 63:2206–2212.

Norris, D. E., B. J. Johnson, J. Piesman, G. O. Maupin, J. L. Clark, and W. C. Black 4th. 1997. Culturing selects for specific genotypes of Borrelia burgdorferi in an enzootic cycle in Colorado. J. Clin. Microbiol. 35:2359–2364.

Ntchobo, H., H. Rothermel, W. Chege, A. C. Steere, and J. Coburn. 2001. Recognition of multiple antibody epitopes throughout Borrelia burgdorferi p66, a candidate adhesin, in patients with early or late manifestations of Lyme disease. Infect. Immun. 69:1953–1956.

Obonyo, M., U. G. Munderloh, V. Fingerle, B. Wilske, and T. Kurtti. 1999. Borrelia burgdorferi in tick cell culture modulates expression of outer surface proteins A and C in response to temperature. J. Clin. Microbiol. 37:2137–2141.

Ohnishi, J., J. Piesman, and A. M. Desilva. 2001. Antigenic and genetic heterogeneity of Borrelia burgdorferi populations transmitted by ticks. Proc. Natl. Acad. Sci. USA 98:670–675.

Onrust, S. V., and K. L. Goa. 2000. Adjuvanted Lyme disease vaccine: a review of its use in the management of Lyme disease. Drugs 59:281–299.

Orloski, K. A., E. B. Hayes, G. L. Campbell, and D. T. Dennis. 2000. Surveillance for Lyme Disease—United States, 1992–1998. Morbid. Mortal. Weekly Rep. 49(SS03):1–11.

Oschmann, P., W. Dorndorf, C. Hornig, C. Schafer, H. J. Wellensiek, and K. W. Pflughaupt. 1998. Stages and syndromes of neuroborreliosis. J. Neurol. 245:262–272.

Pachner, A. R. 1986. Spirochetal diseases of the CNS. Neurol. Clin. 4:207–222.

Pachner, A. R., P. Duray, and A. C. Steere. 1989. Central nervous system manifestations of Lyme disease. Arch. Neurol. 46:790–795.

Pachner, A. R., N. Ricalton, and E. Delaney. 1993. Comparison of polymerase chain reaction with culture and serology for diagnosis of murine experimental Lyme borreliosis. J. Clin. Microbiol. 31:208–214.

Pal, U., A M. de Silva, R. R. Montgomery, D. Fish, J. Anguita, J. F. Anderson, and E. Fikrig. 2000. Attachment of Borrelia burgdorferi within Ixodes scapularis mediated by outer surface protein A. J. Clin. Invest. 106:561–569.

Parveen, N., and J. M. Leong. 2000. Identification of a candidate glycosaminoglycan-binding adhesin of the Lyme disease spirochete Borrelia burgdorferi. Molec. Microbiol. 35:1220–1234.

Paster, B. J., F. E. Dewhirst, W. G. Weisburg, L. A. Tordoff, G. J. Fraser, R. B. Hespell, T. B. Stanton, L. Zablen, L. Mandelco, and C. R. Woese. 1991. Phylogenetic analysis of the spirochetes. J. Bacteriol. 173:6101–6109.

Patti, J. M., B. L. Allen, M. J. McGavin, and M. Hook. 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. Ann. Rev. Microbiol. 48:585–617.

Payne, S. M. 1993. Iron acquisition in microbial pathogenesis. Trends Microbiol. 1:66–69.

Pennington, P. M., C. D. Allred, C. S. West, R. Alvarez, and A. G. Barbour. 1997. Arthritis severity and spirochete burden are determined by serotype in the Borrelia turicatae-mouse model of Lyme disease. Infect. Immun. 65:285–292.

Pennington, P. M., D. Cadavid, and A. G. Barbour. 1999a. Characterization of VspB of Borrelia turicatae, a major outer membrane protein expressed in blood and tissues of mice. Infect. Immun. 67:4637–4645.

Pennington, P. M., D. Cadavid, J. Bunikis, S. J. Norris, and A. G. Barbour. 1999b. Extensive interplasmidic duplications change the virulence phenotype of the relapsing fever agent Borrelia turicatae. Molec. Microbiol. 34:1120–1134.

Perides, G., R. Noring, and M. S. Klempner. 1996. Inhibition of Borrelia burgdorferi-bound fibrinolytic enzymes by alpHA(2)-antiplasmin, PAI-1 AND PAI-2. Biochem. Biophys. Res. Commun. 219:690–695.

Perine, P. L. and B. Tekle. 1983. Antibiotic treatment of louseborne fever in Ethiopia: A report of 377 cases. Am. J. Trop. Med. Hyg. 32:1096–1100.

Persing, D. H., D. Mathiesen, D. Podzorski, and S. W. Barthold. 1994. Genetic stability of Borrelia burgdorferi recovered from chronically infected immunocompetent mice. Infect. Immun. 62:3521–3527.

Picardeau, M., J. R. Lobry, and B. J. Hinnebusch. 1999a. Analyzing DNA strand compositional asymmetry to

identify candidate replication origins in Borrelia burgdorferi linear and circular plasmids.

Picardeau, M., J. R. Lobry, and B. J. Hinnebusch. 1999b. Physical mapping of an origin of bidirectional replication of the centre of the Borrelia burgdorferi linear chromosome. Molec. Microbiol. 32:437–445.

Picken, R. N., F. Strle, M. M. Picken, E. Ruzic-Sablijic, V. Maraspin, S. Lotric-Furlan, and J. Cimperman. 1998. Identification of three species of Borrelia burgdorferi sensu lato (B. burgdorferi sensu strico, B. garinii, and B. afzelii) among isolates from acrodermatitis chronica atrophicans lesions. J. Invest. Dermatol. 110:211–214.

Piesman, J. 1989. Transmission of Lyme disease spirochetes (Borrelia burgdorferi) [Review]. Exp. Appl. Acarol. 7:71–80.

Piesman, J., and C. M. Happ. 1997. Ability of the Lyme disease spirochete Borrelia burgdorferi to infect rodents and three species of human-biting ticks (blacklegged tick, American dog tick, Lone star tick) (Acari, Ixodidae). J. Med. Entomol. 34:451–456.

Plasterk, R. H., M. I. Simon, and A. G. Barbour. 1985. Transposition of structural genes to an expression sequence on a linear plasmid causes antigenic variation in the bacterium Borrelia hermsii. Nature 318:257–263.

Poland, G. A., and R. M. Jacobson. 2001. The prevention of Lyme disease with vaccine. Vaccine 19:2303–2308.

Poltorak, A., X. He, I. Smirnova, M.-Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57Bl/10ScCr mice: Mutations in Tlr4 gene. Science 282:2085–2088.

Porcella, S. F., T. G. Popova, D. R. Akins, M. Li, J. D. Radolf, and M. V. Norgard. 1996. Borrelia burgdorferi supercoiled plasmids encode multiple tandem open reading frames and a lipoprotein gene family. J. Bacteriol. 178:3293–3307.

Porcella, S. F., C. A. Fitzpatrick, and J. L. Bono. 2000. Expression and immunological analysis of the plasmid-borne mlp genes of Borrelia burgdorferi B31. Infect. Immun. 68:4992–5001.

Posey, J. E., and F. C. Gherardini. 2000. Lack of a role for iron in the Lyme disease pathogen. Science 288:1651–1653.

Priem, S., M. G. Rittig, T. Kamradt, G. R. Burmester, and A. Krause. 1997. An optimized PCR leads to rapid and highly sensitive detection of Borrelia burgdorferi in patients with Lyme borreliosis. J. Clin. Microbiol. 35:685–690.

Probert, W. S., K. M. Allsup, and R. B. LeFebvre. 1995. Identification and characterization of a surface-exposed 66-kilodalton protein from Borrelia burgdorferi. Infect. Immun. 63:1933–1939.

Probert, W. S., and B. J. B. Johnson. 1998. Identification of a 47 kDa fibronectin-binding protein expressed by Borrelia burgdorferi isolate B31. Molec. Microbiol. 30:1003–1015.

Pugsley, A. P. 1993. The complete general secretory pathway in Gram-negative bacteria. Microbiol. Rev. 57:50–108.

Purser, J. E., and S. J. Norris. 2000. Correlation between plasmid content and infectivity in Borrelia burgdorferi. Proc. Natl. Acad. Sci. USA 97:13865–13870.

Quin, C., and E. Perkins. 1946. Tick-borne relapsing fever in East Africa. Trop. Med. Hyg. 49:30–32.

Radolf, J. D., M. V. Norgard, M. E. Brandt, R. D. Isaacs, P. A. Thompson, and B. Beutler. 1991. Lipoproteins of Borrelia burgdorferi and Treponema pallidum activate cachectin/tumor necrosis factor synthesis: Analysis using a CAT reporter construct. J. Immunol. 147:1968–1974.

Radolf, J. D. 1994a. Role of outer membrane architecture in immune evasion by Treponema pallidum and Borrelia burgdorferi. Trends Microbiol. 2:307–311.

Radolf, J. D., K. W. Bourell, D. R. Akins, J. S. Brusca, and M. V. Norgard. 1994b. Analysis of Borrelia burgdorferi membrane architecture by freeze-fracture electron microscopy. J. Bacteriol. 176:21–31.

Radolf, J. D., L. L. Arndt, D. R. Akins, L. L. Curetty, M. E. Levi, Y. Shen, L. S. Davis, and M. V. Norgard. 1995a. Treponema pallidum and Borrelia burgdorferi lipoproteins and synthetic lipopeptides activate monocytes/macrophages. J. Immunol. 154:2866–2877.

Radolf, J. D., M. S. Goldberg, K. W. Bourell, S. I. Baker, J. D. Jones, and M. V. Norgard. 1995b. Characterization of outer membranes isolated from Borrelia burgdorferi, the Lyme disease spirochete. Infect. Immun. 63:2154–2163.

Ramamoorthy, R., and M. T. Philipp. 1998. Differential expression of Borrelia burgdorferi proteins during growth in vitro. Infect. Immun. 66:5119–5124.

Ratledge, C. 2000. Iron metabolism in pathogenic bacteria. Ann. Rev. Microbiol. 54:881–941.

Reik, L., A. C. Steere, N. H. Bartenhagen, R. E. Shope, and S. E. Malawista. 1979. Neurologic abnormalities of Lyme disease. Medicine 58:281–294.

Restrepo, B. I., T. Kitten, C. J. Carter, D. Infante, and A. G. Barbour. 1992. Subtelomeric expression regions of Borrelia hermsii linear plasmids are highly polymorphic. Molec. Microbiol. 6:3299–3311.

Restrepo, B. I., and A. G. Barbour. 1994a. Antigen diversity in the bacterium B. hermsii through "somatic" mutations in rearranged vmp genes. Cell 78:867–876.

Restrepo, B. I., C. J. Carter, and A. G. Barbour. 1994b. Activation of a vmp pseudogene in Borrelia hermsii: An alternate mechanism of antigenic variation during relapsing fever. Molec. Microbiol. 13:287–299.

Ribeiro, J. M. C., T. N. Mather, J. Piesman, and A. Spielman. 1987. Dissemination and salivary delivery of Lyme disease spirochetes in vector ticks (Acari: Ixodae). J. Med. Entomol. 24:201–205.

Rich, S. M., P. M. Armstrong, R. D. Smith, and S. R. Telford 3rd. 2001. Lone star tick-infecting Borreliae are most closely related to the agent of bovine borrelisos. J. Clin. Microbiol. 39:494–497.

Roberts, W. C., B. A. Mullikin, R. Lathigra, and M. S. Hanson. 1998. Molecular analysis of sequence heterogeneity among genes encoding decorin binding proteins A and B of Borrelia burgdorferi sensu lato. Infect. Immun. 66:5275–5285.

Rosa, P. A., D. S. Samuels, D. Hogan, B. Stevenson, S. Casjens, and K. Tilly. 1996. Directed insertion of a selectable marker into a circular plasmid of Borrelia burgdorferi. J. Bacteriol. 178:5946–5953.

Ruby, J. D., H. Li, H. Kuramitsu, S. J. Norris, S. F. Goldstein, K. F. Buttle, and N. W. Charon. 1997. Relationship of Treponema denticola periplasmic flagella to irregular cell morphology. J. Bacteriol. 179:1628–1635.

Sadziene, A., D. D. Thomas, V. G. Bundoc, S. C. Holt, and A. G. Barbour. 1991. A flagella-less mutant of Borrelia burgdorferi: Structural, molecular, and in vitro functional characterization. J. Clin. Invest. 88:82–92.

Sadziene, A., P. A. Thompson, and A. G. Barbour. 1993. In vitro inhibition of Borrelia burgdorferi by antibodies. J. Infect. Dis. 167:165–172.

Salih, S., D. Mustafa, S. Abdel-Wahab, M. Ahmed, and A. Omer. 1977. Louse-borne relapsing fever. I: A clinical and laboratory study of 363 cases in the Sudan. Trans. R. Soc. Trop. Med. Hyg. 71:43–48.

Samuels, D. S., and C. F. Garon. 1993. Coumermycin A1 inhibits growth and induces relaxation of supercoiled plasmids in Borrelia burgdorferi, the Lyme disease agent. Antimicrob. Agents Chemother. 37:46–50.

Samuels, S., K. E. Mach, and C. F. Garon. 1994a. Genetic transformation of the Lyme disease agent Borrelia burgdorferi with coumarin-resistant gyrB. J. Bacteriol. 176:6045–6049.

Samuels, D. S., R. T. Marconi, W. M. Huang, and C. F. Garon. 1994b. gyrB mutations in coumermycin A1-resistant Borrelia burgdorferi. J. Bacteriol. 176:3072–3075.

Samuels, D. S. 1995. Electrotransformation of the spirochete Borrelia burgdorferi: Electrotransformation protocols for microorgansims. Methods Molec. Biol. 47: 253–259.

Sanford, J. P. 1976. Relapsing fever treatment and control. *In:* R. C. Johnson (Ed.). The Biology of Parasitic Spirochetes. Academic Press. New York, NY. 389–394.

Saratokova, M., E. Dobrikova, and F. C. Cabello. 2000. Development of an extrachromosomal cloning vector system for use in Borrelia burgdorferi. Proc. Natl. Acad. Sci. USA 97:4850–4855.

Schaible, U. E., S. Gay, C. Museteanu, M. D. Kramer, G. Zimmer, K. Eichmann, U. Museteanu, and M. M. Simon. 1990. Lyme borreliosis in the severe combined immunodeficiency (scid) mouse manifests predominantly in the joints, heart, and liver. Am. J. Pathol. 137:811–820.

Schaller, M., and U. Neubert. 1994. Ultrastructure of Borrelia burgdorferi after exposure to benzylpenicillin. Infection 22:401–406.

Schlesinger, P. A., P. H. Duray, B. A. Burke, A. C. Steere, and M. T. Stillman. 1985. Maternal-fetal transmission of the Lyme disease spirochete, Borrelia burgdorferi. Ann. Int. Med. 103:67–69.

Schmidt, B. L. 1997. PCR in laboratory diagnosis of human Borrelia burgdorferi infections. Clin. Microbiol. Rev. 10:185–201.

Schwan, T. G., W. Burgdorfer, and C. F. Garon. 1988a. Changes in infectivity and plasmid profile of the Lyme disease spirochete, Borrelia burgdorferi, as a result of in vitro cultivation. Infect. Immun. 56:1831–1836.

Schwan, T. G., W. Burgdorfer, M. E. Schrumpf, and R. H. Karstens. 1988b. The urinary bladder, a consistent source of Borrelia burgdorferi in experimentally infected white-footed mice (Peromyscus leucopus). J. Clin. Microbiol. 26:893–895.

Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa. 1995. Induction of an outer surface protein on Borrelia burgdorferi during tick feeding. Proc. Natl. Acad. Sci. USA 92:2909–2913.

Schwan, T. G. 1996. Ticks and Borrelia: model systems for investigating pathogen-arthropod interactions. Infect. Agents Dis. 5:167–181.

Schwan, T. G., and B. J. Hinnebusch. 1998. Bloodstream-versus tick-associated variants of a relapsing fever bacterium. Science 280:1938–1940.

Schwan, T. G., and J. Piesman. 2000. Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, Borrelia burgdorferi, during the chain of infection in ticks and mice. J. Clin. Microbiol. 38:382–388.

Schwartz, I., G. P. Wormser, J. J. Schwartz, D. Cooper, P. Weissensee, A. Gazumyan, E. Zimmermann, N. S. Goldberg, S. Bittker, G. L. Campbell et al. 1992. Diagnosis of early Lyme disease by polymerase chain reaction amplification and culture of skin biopsies from Erythema migrans lesions. J. Clin. Microbiol. 30:3082–3088.

Scrimenti, R. J. 1970. Erythem chronicum migrans. Arch. Dermatol. 102:104–105.

Seifert, H. S., and M. So. 1990. Genetic mechanisms of bacterial antigenic variation. Microbiol. Rev. 52:327–336.

Seinost, G., D. E. Dykhuizen, R. J. Dattwyler, W. T. Golde, J. J. Dunn, I.-N. Wang, G. P. Wormser, M. E. Schriefer, and B. J. Luft. 1999. Four clones of Borrelia burgdorferi sensu stricto cause invasive infection in humans. Infect. Immun. 67:3518–3524.

Sellati, T. J., L. D. Abrescia, J. D. Radolf, and M. B. Furie. 1996. Outer surface lipoproteins of Borrelia burgdorferi activate vascular endothelium in vitro. Infect. Immun. 64:3180–3187.

Sellati, T. J., D. A. Bouis, R. L. Kitchens, R. P. Darveau, J. Pugin, R. J. Ulevitch, S. M. Goyert, M. V. Norgard, and J. D. Radolf. 1998. Treponema pallidum and Borrelia burgdorferi lipoproteins and synthetic lipopeptides activate monocytic cells via a CD14-dependent pathway distinct from that utilized by lipopolysaccharide. J. Immunol. 160:5455–5464.

Sellati, T. J., D.A. Bouis, M. J. Caimano, A. J. Feulner, C. Ayers, E. Lien, and J. D. Radolf. 1999. Activation of human monocytic cells by Borrelia burgdorferi and Treponema pallidum is facilitated by CD14 and correlates with surface exposure of spirochetal lipoproteins. J. Immunol. 163:2049–2056.

Shang, E. S., C. I. Champion, X. Y. Wu, J. T. Skare, D. R. Blanco, J. N. Miller, and M. A. Lovett. 2000. Comparison of protection in rabbits against host-adapted and cultivated Borrelia burgdorferi following infection-derived immunity or immunization with outer membrane vesicles or outer surface protein A. Infect. Immun. 68:4189–4199.

Sigal, L. H. 1997. Lyme disease: A review of aspects of its immunology and immunopathogenesis. Ann. Rev. Immunol. 15:63–92.

Sigal, L. H., J. M. Zahradnik, P. Lavin, S. J. Patella, G. Bryant, R. Haselby, E. Hilton, M. Kunkel, D. Adler-Klein, T. Doherty, J. Evans, S. E. Malawista, and the Recombinant Outer-Surface Protein A Lyme Disease Vaccine Study Consortium. 1998. A vaccine consisting of recombinant Borrelia burgdorferi outer-surface protein A to prevent Lyme disease. N. Engl. J. Med. 339:216–222.

Sigal, L. 2000. Should speculations about immune-mediated adverse effects of Lyme disease vaccine deter us from giving it? Cleveland Clin. J. Med. 67:873–874.

Simpson, W. J., C. F. Garon, and T. G. Schwan. 1990. Borrelia burgdorferi contains repeated DNA sequences that are species specific and plasmid associated. Infect. Immun. 58:847–853.

Sinsky, R. J., and J. Piesman. 1989. Ear punch biopsy method for detection and isolation of Borrelia burgdorferi from rodents. J. Clin. Microbiol. 27:1723–1727.

Skare, J. T., C. I. Champion, T. A. Mirzabekov, E. S. Shang, D. R. Blanco, H. Erdjument-Bromage, P. Tempest, B. L. Kagan, J. N. Miller, and M. A. Lovett. 1996. Porin activity of the native and recombinant outer membrane

protein Oms28 of Borrelia burgdorferi. J. Bacteriol. 178:4909–4918.

Skare, J. T., T. A. Mirzabekov, E. S. Shang, D. R. Blanco, J. Bunikis, S. Bergstrom, P. Tempst, B. L. Kagan, J. N. Miller, and M. A. Lovett. 1997. The Oms66 (p66) protein is a Borrelia burgdorferi porin. Infect. Immun. 65:3654–3661.

Smith, R. D., G. S. Miranpuri, J. H. Adams, and E. H. Ahrens. 1985. Borrelia theileri : Boophilus microplus isolation from ticks (Boophilus microplus) and tick-borne transmission between splenectomized calves. Am. J. Vet. Res. 46:1396–1398.

Sparrow, H. 1956. Entretien de Borrelia recurrentis (souches Ethiopiennes) par passages sur souriceaux nouveau-nés. Arch. Inst. Pasteur Tunis. 33:163–180.

Spielman, A., J. F. Levine, and M. L. Wilson. 1984. Vectorial capacity of North American Ixodes ticks. Yale J. Biol. Med. 57:507–513.

Steere, A. C., S. E. Malawista, J. A. Hardin, S. Ruddy, W. Askenase, and W. A. Andiman. 1977. Erythema chronicum migrans and Lyme arthritis: The enlarging clinical spectrum. Ann. Int. Med. 86:685–698.

Steere, A. C., R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson, and S. E. Malawista. 1983. The spirochetal etiology of Lyme disease. N. Engl. J. Med. 308:733–740.

Steere, A. C. 1989. Lyme disease. N. Engl. J. Med. 321:586–597.

Steere, A. C. 1998a. Borrelia burgdorferi (Lyme disease, Lyme borreliosis). *In:* G. L. Mandell, J. E. Bennett, and R. Dolin (Eds.). Principles and Practice of Infectious Diseases. Churchill Livingstone. Philadelphia, PA. 2504–2518.

Steere, A. C., V. K. Sikand, F. Meurice, D. L. Parenti, E. Fikrig, R. T. Schoen, J. Nowakowski, C. Schmid, S. Kaukamp, C. Buscarino, D. S. Krause, and the Lyme Disease Vaccine Study Group. 1998b. Vaccination against Lyme disease with recombinant Borrelia burgdorferi outer surface lipoprotein A with adjuvant. N. Engl. J. Med. 339:209–215.

Stevenson, B., T. G. Schwan, and P. A. Rosa. 1995. Temperature-related differential expression of antigens in the Lyme disease spirochete, Borrelia burgdorferi. Infect. Immun. 63:4535–4539.

Stevenson, B., K. Tilly, and P. A. Rosa. 1996. A family of genes located on four separate 32-kilobase circular plasmids in Borrelia burgdorferi. J. Bacteriol. 178:3508–3516.

Stevenson, B., S. Casjens, R. van Vugt, S. F. Porcella, K. Tilly, J. L. Bono, and P. A. Rosa. 1997. Characterization of cp18, a naturally truncated member of the cp32 family of Borrelia burgdorferi plasmids. J. Bacteriol. 179:4285–4291.

Stevenson, B., J. L. Bono, T. G. Schwan, and P. A. Rosa. 1998a. Borrelia burgdorferi Erp proteins are immunogenic in mammals infected by tick bite, and their synthesis is inducible in cultured bacteria. Infect. Immun. 66:2648–2654.

Stevenson, B., J. L. Bono, A. Elias, K. Tilly, and P. Rosa. 1998b. Transformation of the Lyme disease spirochete Borrelia burgdorferi with heterologous DNA. J. Bacteriol. 180:4850–4855.

Stevenson, B., S. Casjens, and P. Rosa. 1998c. Evidence of past recombination events among the genes encoding Erp antigens of Borrelia burgdorferi. Microbiology 144:1869–1879.

Stevenson, B., S. F. Porcella, K. L. Oie, C. A. Fitzpatrick, S. J. Raffel, M. E. Schrumpf, and T. G. Schwan. 2000a. The relapsing fever spirochete Borrelia hermsii contains multiple, antigen-encoding circular plasmids that are homologous to the cp32 plasmids of Lyme disease spirochetes. Infect. Immun. 68:3900–3908.

Stevenson, B., W. R. Zückert, and D. R. Akins. 2000b. Repetition, conservation, and variation: the multiple cp32 plasmids of Borrelia species. J. Molec. Microbiol. Biotechnol. 2:411–422.

Stewart, P. E., R. Thalken, J. L. Bono, and P. Rosa. 2001. Isolation of a circular plasmid region sufficient for autonomous replication and transformation of infectious Borrelia. Molec. Microbiol. 39:714–721.

Stoenner, H. G., T. Dodd, and C. Larson. 1982. Antigenic variation of Borrelia hermsii. J. Exp. Med 156:1297–1311.

Strle, F., J. A. Nelson, E. Ruzic-Sablijic, J. Cimperman, V. Maraspin, S. Lotric-Furlan, Y. Cheng, M. M. Picken, G. M. Trenholme, and R. N. Picken. 1996. European Lyme borreliosis: 231 culture-confirmed cases involving patients with erythema migrans. Clin. Infect. Dis. 23:61–65.

Suk, K., S. Das, W. Sun, B. Jwang, S. W. Barthold, R. A. Flavell, and E. Fikrig. 1995. Borrelia burgdorferi genes selectively expressed in the infected host. Proc. Natl. Acad. Sci. USA 92:4269–4273.

Surette, M. G., M. B. Miller, and B. L. Bassler. 1999. Quorum sensing in Escherichia coli, Salmonella typhimurium, and Vibrio harveyi: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. USA 96:1639–1644.

Szczepanski, A., M. B. Furie, J. L. Benach, B. P. Lane, and H. B. Fleit. 1990. Interaction between Borrelia burgdorferi and endothelium in vitro. J. Clin. Invest. 85:1637–1647.

Szczepanski, A., and J. L. Benach. 1991. Lyme borreliosis: host responses to Borrelia burgdorferi. Microbiol. Rev. 55:21–34.

Taege, A. J. 2000. Who should receive the Lyme disease vaccine? Cleveland Clin. J. Med. 67:239–240.

Tai, K. F., Y. Ma, and J. J. Weis. 1994. Normal human B lymphocytes and mononuclear cells respond to the mitogenic and cytokine-stimulatory activities of Borrelia burgdorferi and its lipoprotein OspA. Infect. Immun. 62:520–528.

Takayama, K., R. J. Rothenberg, and A. G. Barbour. 1987. Absence of lipopolysaccharide in the Lyme disease spirochete, Borrelia burgdorferi. Infect. Immun. 55:2311–2313.

Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. Immunity 11:443–451.

Thanassi, W. T., and R. T. Schoen. 2000. The Lyme disease vaccine: Conception, development, and implementation. Ann. Int. Med. 132:661–668.

Theus, S. A., D. A. Harrich, R. Gaynor, J. D. Radolf, and M. V. Norgard. 1998. Treponema pallidum, treponemal lipoproteins, and synthetic lipoprotein analogs (lipopeptides) induce human immunodeficiency virus-1 gene expression in monocyte/macrophages via nuclear translocation of NF-kB. J. Infect. Dis. 177:941–950.

Thomas, V., J. Anguita, S. Samanta, P. Rosa, P. Stewart, S. W. Barthold, and E. Fikrig. 2001. Dissociation of infectivity and pathogenicity in Borrelia burgdorferi. Infect. Immun. 69:3507–3509.

Tilly, K., S. Casjens, B. Stevenson, J. L. Bono, D. S. Samuels, D. Hogan, and P. A. Rosa. 1997. The Borrelia burgdorferi circular plasmid cp26: Conservation of plasmid structure and targeted inactivation of the ospC gene. Molec. Microbiol. 25:361–373.

Tilly, K., L. Lubke, and P. Rosa. 1998. Characterization of circular plasmid dimers in Borrelia burgdorferi. J. Bacteriol. 180:5676–5681.

Tilly, K., A. F. Elias, J. L. Bono, P. Stewart, and P. Rosa. 2000. DNA exchange and insertional inactivation in spirochetes. J. Molec. Microbiol. Biotechnol. 2:433–442.

Trevejo, R. T., M. E. Schriefer, K. L. Gage, T. J. Saranek, K. A. Orloski, W. J. Pape, J. A. Montenieri, and G. L. Campbell. 1998. An interstate outbreak of tick-borne relapsing fever among vacationers at a Rocky Mountain cabin. Am. J. Trop. Med. Hyg. 58:743–747.

Trueba, G. A., C. A. Bolin, and R. L. Zuerner. 1992. Characterization of the periplasmic flagellum proteins of Leptospira interrogans. J. Bacteriol. 174:4761–4768.

van Dam, A. P., H. Kuiper, K. Vos, A. Widjojokusumo, B. M. de Jongh, L. Spanjaard, A. C. P. Ramselaar, M. D. Kramer, and J. Dankert. 1993. Different genospecies of Borrelia burgdorferi are associated with distinct clinical manifestations of Lyme borreliosis. Clin. Infect. Dis. 17:708–717.

Vasil, M. L., and U. A. Ochsner. 1999. The response of Pseudomonas aeruginosa to iron: genetics, biochemistry, and virulence. Molec. Microbiol. 34:399–413.

Vidal, V., I. G. Scragg, S. J. Cutler, K. A. Rockett, D. Fekade, D. A. Warrell, D. J. M. Wright, and D. Kwiatkowski. 1998. Variable major lipoprotein is a principal TNF-inducing factor of louse-borne relapsing fever. Nature Med. 4:1416–1420.

Vincent, M. S., K. Roessner, T. Sellati, C. D. Huston, L. H. Sigal, S. M. Behar, J. D. Radolf, and R. C. Budd. 1998. Lyme arthritis synovial gamma-delta T cells respond to Borrelial burgdorferi lipoproteins and lipidated hexapeptides. J. Immunol. 161:5762–5771.

Volff, J. N., and J. Altenbuchner. 2000. A new beginning with new ends: linearization of circular chromosomes during bacterial evolution. FEMS Microbiol. Lett. 186:143–150.

von Stedingk, L. V., I. Olsson, H. S. Hanson, E. Asbrink, and A. Hovmark. 1995. Polymerase chain reaction for detection of Borrelia burgdorferi DNA in skin lesions of early and late Lyme borreliosis. Eur. J. Clin. Microbiol. Infect. Dis. 14:1–5.

Walker, E. M., L. A. Borenstein, D. R. Blanco, J. N. Miller, and M. A. Lovett. 1991. Analysis of outer membrane ultrastructures of pathogenic Treponema and Borrelia species by freeze-fracture electron microscopy. J. Bacteriol. 173:5585–5588.

Wallich, R., M. M. Simon, H. Hofmann, S. E. Moter, U. E. Schaible, and M. D. Kramer. 1993. Molecular and immunological characterization of a novel polymorphic lipoprotein of Borrelia burgdorferi. Infect. Immun. 61:4158–4166.

Wallich, R., C. Brenner, M. D. Kramer, and M. M. Simon. 1995. Molecular cloning and immunological characterization of a novel linear-plasmid-encoded gene, pG, of Borrelia burgdorferi expressed only in vivo. Infect. Immun. 63:3327–3335.

Wang, G., A. P. van Dam, I. Schwartz, and J. Dankert. 1999. Molecular typing of Borrelia burgdorferi sensu lato: taxonomic, epidemiological, and clinical implications. Clin. Microbiol. Rev. 12:633–653.

Weber, K., H. J. Bratzke, U. Neubert, B. Wilske, and P. H. Duray. 1988. Borrelia burgdorferi in a newborn despite oral penicillin for Lyme borreliosis during pregnancy. Pediatr. Infect. Dis. J. 7:286–289.

Weis, J. J., Y. Ma, and L. F. Erdile. 1994. Biological activities of native and recombinant Borrelia burgdorferi outer surface protein A: Dependence on lipid modification. Infect. Immun. 62:4632–4636.

Wienecke, R., N. Zochling, U. Neubert, E. M. Schlupen, M. Meurer, and M. Volkenandt. 1994. Molecular subtyping of Borrelia burgdorferi in erythema migrans and acrodermatitis chronica atrophicans. J. Invest. Dermatol. 103:19–22.

Wilske, B., U. Busch, H. Eiffert, V. Fingerle, H. W. Pfister, and D. Rossler. 1996. Diversity of OspA and OspC among cerebrospinal fluid isolates of Borrelia burgdorferi sensu lato from patients with neuroborreliosis in Germany. Med. Microbiol. Immunol. 184:195–201.

Wooten, R. M., V. R. Modur, T. M. McIntyre, and J. J. Weis. 1996. Borrelia burgdorferi outer membrane protein A induces nuclear translocation of nuclear factor NF-kB and inflammatory activation in human endothelial cells. J. Immunol. 157:4584–4590.

Wooten, R. M., T. B. Morrison, J. H. Weis, S. D. Wright, R. Thieringer, and J. J. Weis. 1998. The role of CD14 in signaling mediated by outer membrane lipoproteins of Borrelia burgdorferi. J. Immunol. 160:5485–5492.

Wormser, G. P., J. Nowakowski, R. B. Nadelman, S. Bittker, D. Cooper, and C. S. Pavia. 1998. Improving the yield of blood cultures for patients with early Lyme disease. J. Clin. Microbiol. 36:296–298.

Wormser, G. P., D. Liveris, J. Nowakowski, R. B. Nadelman, L. F. Cavaliere, D. McKenna, D. Holmgren, and I. Schwartz. 1999. Association of specific subtypes of Borrelia burgdorferi with hematogenous dissemination in early Lyme disease. J. Infect. Dis. 180:720–725.

Wormser, G. P., S. Bittker, D. Cooper, J. Nowakowski, R. B. Nadelman, and C. S. Pavia. 2000. Comparison of the yields of blood cultures using serum or plasma from patients with early Lyme disease. J. Clin. Microbiol. 38:1648–1650.

Xu, Y., and R. C. Johnson. 1995. Analysis and comparison of plasmid profiles of Borrelia burgdorferi sensu lato strains. J. Clin. Microbiol. 33:2679–2685.

Xu, Y., C. Kodner, L. Coleman, and R. C. Johnson. 1996. Correlation of plasmids with infectivity of Borrelia burgdorferi sensu stricto type strain B31. Infect. Immun. 64:3870–3876.

Yang, X., T. G. Popova, K. E. Hagman, S. K. Wikel, G. G. Schoeler, M. J. Caimano, J. D. Radolf, and M. V. Norgard. 1999. Identification, characterization, and expression of three new members of the Borrelia burgdorferi Mlp (2.9) lipoprotein gene family. Infect. Immun. 67:6008–6018.

Yang, X., M. S. Goldberg, T. G. Popova, G. B. Schoeler, S. K. Wikel, K. E. Hagman, and M. V. Norgard. 2000. Interdependence of environmental factors influencing reciprocal patterns of gene expression in virulent Borrelia burgdorferi. Molec. Microbiol. 347:1470–1479.

Zhang, J. R., J. M. Hardham, A. G. Barbour, and S. J. Norris. 1997. Antigenic variation in Lyme disease borreliae by promiscuous recombination of Vmp-like sequence cassettes. Cell 89:275–285.

Zhang, J. R., and S. J. Norris. 1998. Kinetics and in vivo induction of genetic variation of vlsE in Borrelia burgdorferi. Infect. Immun. 66:3689–3697.

Zhong, W., L. Gern, T. Stehle, C. Museteanu, M. Kramer, R. Wallich, and M. M. Simon. 1999. Resolution of experimental and tick-borne Borrelia burgdorferi infection in mice by passive but not active immunization using recombinant OspC. Eur. J Immunol. 29:946–957.

Zückert, W. R., E. Filipuzzi-Jenny, M. Stalhammar-Carlemalm, J. Melster-Turner, and J. Meyer. 1994. Repeated DNA sequences on circular and linear plasmids of Borrelia burgdorferi sensu lato. *In:* J. S. Axford and D. H. E. Rees (Eds.). Lyme Borreliosis. Plenum Press. New York, NY. 253–260.

Zückert, W. R., and J. Meyer. 1996. Circular and linear plasmids of Lyme disease spirochetes have extensive homology: characterization of a repeated DNA element. J. Bacteriol. 178:2287–2298.

Zückert, W. R., J. Meyer, and A. G. Barbour. 1999. Comparative analysis and immunological characterization of the Borrelia Bdr protein family. Infect. Immun. 67:3257–3266.

Zückert, W. R., and A. G. Barbour. 2000. Stability of Borrelia burgdorferi bdr loci in vitro and in vivo. Infect. Immun. 68:1727–1730.

Zückert, W. R., T. A. Kerentseva, C. L. Lawson, and A. G. Barbour. 2001. Structural conservation of neurotropism-associated VspA within the Borrelia Vsp-OspC lipoprotein family. J. Biol. Chem. 276:457–463.

Zung, J. L., S. Lewengrug, M. A. Mrdzibnska, and A. Spielman. 1989. Fine structural evidence for the penetration of the Lyme disease spirochete Borrelia burgdorferi through the gut and salivary tissues of Ixodes dammini. Can. J. Zool. 67:1737–1748.

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CHAPTER 4.4

The Genus *Leptospira*

BEN ADLER AND SOLLY FAINE

Introduction

Members of the genus *Leptospira* are helicoidal bacteria that may exist as free-living saprophytes or in association with a diverse range of animal hosts. They either are the cause of acute leptospirosis or are associated with particular host tissues (especially the renal proximal tubules) on a long-term basis (possibly for the life of the animal). Two additional species, *L. parva* and *L. illini*, which were previously included in the genus, have been reclassified as the separate genera *Turneria* and *Leptonema*, respectively (Hovind-Hougen, 1979; Hookey et al., 1993; Ellis, 1995), although the former has not been officially published. These three genera comprise the family Leptospiraceae within the order Spirochaetales, which also contains the families Spirochaetaceae and Serpulinaceae.

Human leptospirosis has clearly existed since the earliest times, but it is impossible to ascribe with any certainty various records of febrile jaundice to leptospirosis or to other causes. Many of the early descriptions are reviewed in Faine et al. (1999). It was in 1860 that Adolph Weil, professor of medicine at the University of Heidelberg, published his description of an "... infectious disease accompanied by splenomegaly, jaundice and nephritis" (Weil, 1886), the most severe form of which now bears his name as Weils disease. Leptospires were first isolated in pure culture by Japanese workers in 1914 (Inada et al., 1916) only a short time before a similar isolation in Europe (Uhlenhuth and Fromme, 1915), although it is clear that the first visual observations of *Leptospira* were made by Stimson a few years earlier in silver stained sections of human kidney from a patient thought to be suffering from yellow fever (Stimson, 1907; Stimson, 1909).

Taxonomy

The taxonomy of *Leptospira* has been both complicated and controversial; at various times over the past 60 years, the genus has contained only two species or almost 200 species. It is only with the relatively recent application of accepted molecular methods that the taxonomic status of members of the genus has been unequivocally determined.

The genus is defined as *Leptospira* Noguchi (1917), type species *Leptospira interrogans* (Stimson, 1907), type strain serovar Icterohaemorrhagiae, Ictero No. I (ATCC 43782).

Following the first isolation of *Leptospira*, the next three decades saw the isolation of scores of strains that were clearly different with respect to epidemiology, disease pattern and a very limited range of phenotypic characteristics. Because of the limited phenotypic differences available, antigenic differences in agglutinating (lipopolysaccharide; LPS) antigens were used as the basis for identification and classification. The serovar became the basic taxon, with each serovar accorded species status, e.g., *L. pomona*, *L. icterohaemorrhagiae*, *L. hardjo*, etc. Antigenically related serovars were placed together in serogroups, which per se have no taxonomic status. From a taxonomic viewpoint, this scheme was clearly erroneous. Accordingly, in 1982 the Subcommittee on the Taxonomy of *Leptospira* adopted a scheme by which all pathogenic leptospires were designated the single species *L. interrogans* (also known as the "*L. interrogans* complex") followed by the serovar name. All saprophytic serovars were placed in the species *L. biflexa* (the *L. biflexa* complex).

The application of DNA hybridization allowed the speciation of members of *Leptospira* based on the accepted definition of >70% hybridization under defined high stringency conditions, with less than 5% divergence. Following the original designation of several species of *Leptospira* (Yasuda et al., 1987), other workers have defined or isolated new species; currently 12 species of *Leptospira* are recognized (Brenner et al., 1999; Table 1), with at least four additional species that have not yet been named. Some confusion arises owing to the retention of the species names *L. interrogans* and *L. biflexa* for one of the genetically defined pathogenic and saprophytic species, respectively. This may be avoided by the use of

Table 1. Species classification of the genus *Leptospira*.

Species	Pathogenesis	G+C content (%)
L. alexanderi	Pathogenic	38.0
L. borgpetersenii		39.8
L. interrogans		34.9
L. kirschneri		ND[b]
L. noguchii		36.5
L. santarosai		40.5
L. weilii		40.5
Unnamed species 1[a]		39.8
Unnamed species 3[a]		43.4
Unnamed species 4[a]		38.9
Unnamed species 5[a]		37.9
L. fainei	Indeterminate	ND[b]
L. inadai		42.6
L. biflexa	Saprophytic	33.5
L. meyeri		35.2
L. wolbachii		37.2

[a]Brenner, 1999.
[b]Not determined.

the terms "sensu stricto" and "sensu lato" to refer to the species or the overall complex, respectively. See Faine et al. (1999) for a complete listing of the more than 230 serovars currently recognized.

A significant outcome of the genetic classification scheme was the finding that many antigenically related serovars, or even subtypes of the same serovar, may belong to different species. For example, the antigenically indistinguishable subtypes of serovar Harjdo (Hardjobovis and Hardjoprajitno) are placed in *L. borgpetersenii* and *L. interrogans*, respectively. Although the two subtypes are by definition identical serovars, they are often referred to as serovars Hardjobovis and Hardjoprajitno. Interestingly, genetic analysis of the LPS biosynthetic loci of the two subtypes has indicated a mosaic arrangement with evidence for lateral acquisition of blocks of functionally identical genes (De la Peña-Moctezuma et al., 1999; De la Peña-Moctezuma et al., 2001).

Cell Structure

By light microscopy, leptospires are best viewed with dark field illumination in which they appear as thin, helical, motile organisms, ranging from 10 to more than 20 μm in length. They are usually hooked at one or both ends (Fig. 1) and exhibit rapid, darting, to-and-fro motility in liquid media, as well as both flexing motion and constant rotational movement about their long axes. In viscous media and on cell surface mucus, leptospires exhibit a snake-like crawling motility. Straight, unhooked variants occur, which are less translationally motile and form dense colonies in solid media, in contrast to the spreading growth of the hooked forms. The straight forms are sometimes avirulent, but the molecular basis for this is unknown.

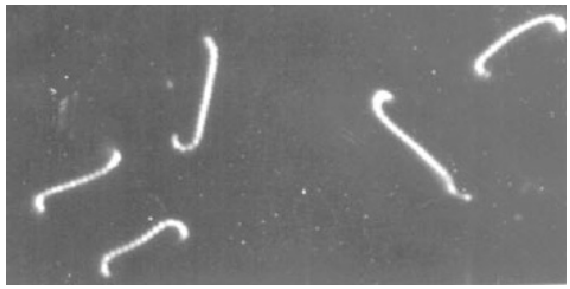

Fig. 1. Darkfield photomicrograph (magnification ca. × 1300) of *Leptospira* sp., showing helical morphology and hooked ends. From Faine et al. (1999).

Fig. 2. Shadowed electron micrograph of *Leptospira* sp. (magnification ca. × 30,000). The characteristic helical shape, periplasmic flagella (red arrow) and outer membrane (green arrow) can be seen. Micrograph by A Chang.

Leptospires notionally stain as Gram negative, but they are very thin and thus stained preparations are difficult to visualize microscopically. They may be seen more readily after staining by silver deposition methods or by immunostaining with fluorescent or immunohistochemical conjugates.

Electron microscopy is necessary to observe the detailed structural components of the leptospiral cell (Fig. 2), which resemble closely the architecture of a typical Gram-negative bacterial cell. There is an outer membrane, external to an inner membrane and peptidoglycan, wound in a helical shape. The direction of coiling is right-handed. Leptospires range in length from about 10–20 μm, with a coil amplitude of approximately 0.1–0.15 μm and a wavelength of about 0.5 μm.

Leptospiral Endoflagella

Each leptospire has two flagella, one arising at each end from an insertion through the inner membrane/peptidoglycan complex and lying in the periplasmic space between the peptidoglycan and the outer membrane. The two polar, peri-

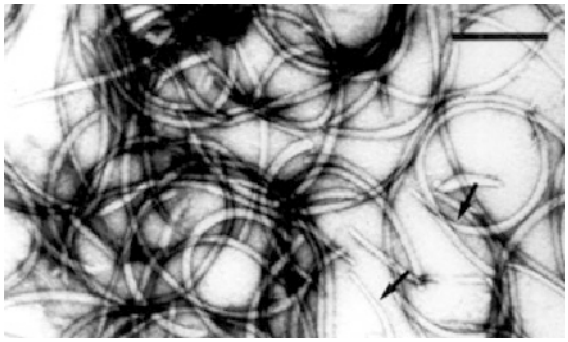

Fig. 3. Negatively stained electron micrograph of purified leptospiral flagella. Arrows indicate flagellar core without sheath. Bar = ca. 200 nm. Micrograph by B. Adler.

plasmic, single flagella are characteristic and distinguishing features of *Leptospira*. When a cell divides, it develops a new flagellum at the newly formed end, immediately after division. Flagella have been isolated from leptospires for morphological, chemical or immunochemical studies. The flagella, freed from the leptospiral body, were concentrated by differential centrifugation or by density gradient centrifugation (Chang and Faine, 1970; Kelson et al., 1988). In negatively stained electron micrographs, purified flagella appear to be approximately 15–18 nm wide, with an inner 12-nm core surrounded by a sheath (Fig. 3).

The flagella are similar in general structure to the flagella of other Gram-negative bacteria, comprising a hooked proximal end and disk rotor insertion structures, intercalated with the layers of the cell wall (Birch-Andersen et al., 1973; Holt, 1978). A hooked region penetrates the wall, outside of which the flagellum is seen to comprise a central core, made up of the flagella subunit protein, FlaB. Because of the periplasmic nature of leptospiral flagella, their role in motility and in the retention of the helical cell morphology has been controversial (Li et al., 2000). The recent inactivation by allelic exchange of the *flaB* gene in *L. biflexa* (Picardeau et al., 2001) demonstrated unequivocally the essential role of the flagella in motility, but not in helical cell morphology. The *flaB* mutants were shown to be nonmotile and devoid of flagella and hooked ends. However, they retained the characteristic helical shape of the wildtype parent.

The Leptospiral Surface

The outer membrane can be seen in shadowed, scanned or sectioned preparations and contains proteins, lipids, lipoproteins and LPS. Crude preparations of outer membrane (so-called "outer envelope") have been made by plasmolysing leptospires or otherwise disrupting them (Auran et al., 1972; Zeigler and VanEseltine, 1975). These preparations have been used as vaccines (Auran et al., 1972), the active component of which is almost certainly LPS. Freeze-fracture studies of *L. kirschneri* serovar Grippotyphosa revealed a low outer-membrane protein content compared with typical Gram-negative bacteria. Virulent strains had 443, and avirulent strains 990, molecules/μm^2, a highly statistically significant difference (Haake et al., 1991) and at least 10- to 100-fold less than the numbers seen in *Escherichia coli*.

The exposed leptospiral surface carries a net negative charge (Brown and Broom, 1929). In laboratory cultures, leptospires are always coated with adherent protein from the culture medium, the last traces of which cannot be removed, even with repeated centrifugal washing.

The leptospiral peptidoglycan layer is likewise similar to that of typical Gram-negative bacteria, lying external to, and associated with, the inner membrane. A role has been suggested for peptidoglycan in stimulating phagocytosis and production of the cytokine TNF-α by human monocytes (Cinco et al., 1993; Cinco et al., 1996). Penicillin-binding proteins have been shown to be associated with the inner membrane/peptidoglycan complex (Brenot et al., 2001a).

Leptospiral Lipids

Leptospires are characterized by a relatively high total lipid content and by unusual fatty acids (Johnson, 1976; Cacciapuoti et al., 1991); however, studies of lipids are complicated by the essential presence in the culture medium of long-chain fatty acids, required for growth, which also influence leptospiral lipid content and composition. Total lipids comprise about 14–26% of dry weight, of which 50–70% are phospholipids, mainly phosphatidyl ethanolamine (80–90%), although smaller amounts of phosphatidyl glycerol (5–10%), diphosphatidyl glycerol (1–5%), and lysophosphatidyl ethanolamine (0–3%) are also present.

Among the long chain fatty acids in lipid fractions, varying amounts of C 10:0, *cis*-12:9^9, 2OH 11:0, 3OH 12:0, 14:2, *cis*-14:1^9, *cis*-14:1^{11}; 14:0, a-15:0, 16:0, 16:2, *cis*-16:1^9, *cis*-16:1^{11}, 16:0, 3OH 15:0, *cis*-18:1^9, and *cis*-18:1^{11} have been found (Johnson et al., 1970; Cacciapuoti et al., 1991). Saprophytes contained more 14:0 than parasites contained, and in Canicola grown on 18:0 (octadecanoic acid), 87% of the C16 acid was the Δ 11 isomer. The *cis*-16:1^{11} isomer is unusual, characteristic and almost unique to *Leptospira* and related bacteria. Characteristic fatty acid methyl ester profiles obtained by gas-liquid chromatog-

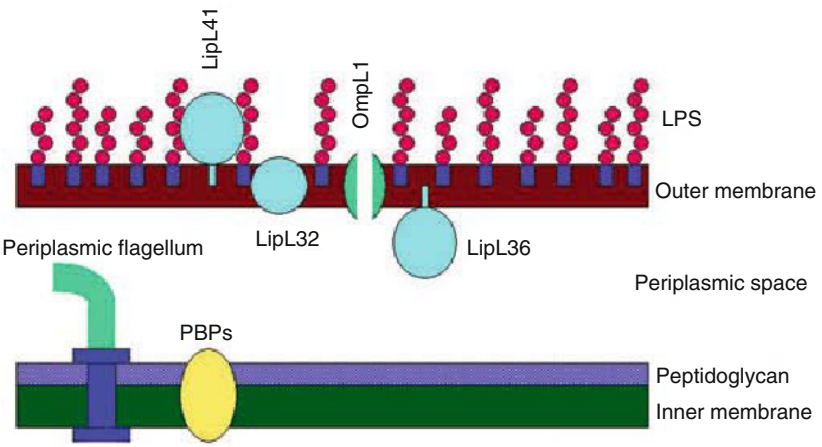

Fig. 4. Schematic representation of the membrane architecture of *Leptospira*. The precise membrane topology of the major OMP, LipL32, has not been defined. OMP, outer membrane protein; LPS, lipopolysaccharide; LipL32, L36 and L41, lipoproteins; and PBP, penicillin-binding proteins.

raphy are useful in chemotaxonomy (Cacciapuoti et al., 1991).

The structure and composition of some leptospiral membrane components are represented schematically in Fig. 4.

Physiology and Metabolism

Cell Division

As leptospiral cells grow, they elongate to maximum length, at which time a constriction appears in the protoplasmic cylinder, extending to close off the fresh ends of each leptospire. Under conditions of nutritional stress, very long (50 µm or more) leptospires may develop that fail to separate so that long chains of elongated leptospires may be observed.

Growth Rates and Conditions

Saprophytic leptospires can be distinguished by their ability to grow at 11–13°C, whereas pathogens will not (Johnson and Harris, 1967), although the optimum temperature in laboratory media for both is 28–30°C. The optimum pH range for growth is 7.2–7.6; alkaline conditions are tolerated better than acidic conditions. Aerobic conditions are essential and growth rates are improved by shaking, sparging or increased surface areas. The typical doubling time for pathogenic leptospires adapted to laboratory cultivation under optimum conditions for growth is about 6–8 h, whereas doubling times of 3–4 h have been reported for *L. biflexa*. Cultures of pathogens therefore usually need to be incubated for 3–10 days, sometimes longer. Growth of primary isolates is much poorer, with doubling times in excess of 24 h.

Starting inocula from stationary phase cultures of as much as 1% (sometimes up to 10%) of the volume of fresh medium are used, so that maximum growth is reached in 4–7 days. Final densities of about 5×10^8–10^9 cells/ml can be achieved in Tween-albumin media for well-adapted laboratory strains of most leptospires.

Technical details of methods of observation, preparation of culture media, manipulation and subculture may be found in Faine et al. (1999). In liquid media, the first appearance of growth is a barely perceptible, but quite characteristic, birefringence, whereas fully grown cultures are obviously turbid to the naked eye at cell concentrations of approximately 5×10^7–10^8 leptospires/ml. However, some strains, especially fresh isolates, may grow to only 1/100 of this cell concentration. Growth must therefore be checked by removing a loopful of culture aseptically for dark-field microscopy.

Most strains of leptospires grow as subsurface colonies on solid media, but colonies may take days or weeks to appear; some serovars or strains may be impossible to grow as colonies on solid media. Plates should be incubated for 3–4 weeks, avoiding dehydration and contamination. Addition of pyruvate or rabbit serum (5–10%) enhances the growth of some strains.

Ordinary bacteriological membrane filters with average pore diameters of 0.2 µm or greater will allow leptospires to pass. Culture media or constituents made with tap water that has not been heat sterilized, or which have been held in vessels containing traces of tap water, have resulted in contamination of leptospiral or tissue cultures by *L. biflexa* and *L. inadai* strains able to pass through bacteriological filters.

Leptospires in cultures may remain viable for a very long time (at least 10 years in Korthof's medium has been recorded in the authors' laboratory) in glass tubes in the dark at room temperatures up to 35°C. Addition of hemoglobin to media in an amount sufficient to produce a pink color and use of 0.1–0.3% sloppy agar

media have been recommended to enhance survival.

Energy Metabolism

Leptospires are chemoorganotrophs that use O_2 as the final electron receptor. Cytochromes *a*, *c* and c_1, catalase and oxidase are present. Differences in the relative distribution of catalase and oxidase between serovars were reported, and no *L. biflexa* strains were catalase-positive (Corin et al., 1978). The only major carbon and energy sources are long-chain (more than C_{14}) fatty acids, from which energy is derived by β-oxidation (Henneberry and Cox, 1970). Sugars are not fermented and cannot be utilized as carbon sources, but carbohydrates may be synthesized by means of the tricarboxylic acid cycle and a nonoxidative pentose phosphate pathway or phosphogluconate and glyoxalate pathways, for which the key enzymes glucosephosphate isomerase, isocitrate lyase and malate synthase were detected in *L. biflexa* grown in synthetic medium (Yanagihara et al., 1984a; Yanagihara et al., 1984b).

Lipid Metabolism

Leptospires can shorten or desaturate essential long-chain fatty acids and incorporate them, but cannot synthesize them de novo (Stern et al., 1969; Johnson et al., 1970). Not surprisingly, the fatty acid composition of the leptospires reflects that of the growth medium.

Short-chain fatty acids can be utilized if longer chain fatty acids are also present (Johnson et al., 1969). Short-chain fatty acids, for example acetate, are metabolized, but their carbon is not selectively used in forming long-chain lipids (Stern et al., 1969). The essential fatty acids required for nutrition and energy metabolism are also toxic, so that they must be presented to the leptospires in the presence of a detoxifier that will adsorb them and release them slowly by equilibrium in low concentrations. Common detoxicants are serum albumin of bovine origin (in oleate-albumin media or in the albumin component of serum in media containing whole serum) and the usually concomitant use of sorbitol-complexed fatty acids (Tweens). In nature, leptospires probably obtain their essential long-chain fatty acids by attaching themselves to, and possibly by integrating their outer membrane with, surfaces on which the essential fatty acids are adsorbed (Kefford and Marshall, 1984).

All *L. biflexa* strains produce lipases (trioleinases), as do *L. inadai* and some *L. interrogans* strains (Kasarov and Addamiano, 1969). The other *interrogans* leptospires and *L. borgpetersenii*, *L. noguchii*, *L. santarosai* and *L. weilii* were reported not to produce lipases (Johnson and Harris, 1968; Yasuda et al., 1987), but more recent molecular analyses have contradicted these early reports (see Virulence Factors).

Amino Acid Metabolism

Alanine, arginine, aspartate, glutamate, glycine, leucine, phenylalanine and valine were incorporated into protein by serovar Pomona. Arginine, leucine, phenylalanine and valine were incorporated directly, almost exclusively in protein, but labeled carbon from glutamate and aspartate was also found in other cellular protein amino acids, whereas labeled carbon from alanine, aspartate, glutamate and glycine was in the lipid-soluble and trichloracetic acid precipitated fractions (Johnson and Rogers, 1964b). Different groups of leptospires may synthesize isoleucine by the classical threonine pathway, by an unusual pyruvate pathway, or by both, with the pyruvate pathway dominant (Charon et al., 1974; Westfall et al., 1983). Genes of the methionine biosynthetic pathway in *L. meyeri* serovar Semaranga have been cloned by complementation of *E. coli* mutants in the *metA* and *metB* genes (Bourhy et al., 1997; Belfaiza et al., 1998). However, despite functional similarity of the *E. coli* and *L. meyeri* enzymes, it is clear that the leptospiral pathway involves direct sulfhydrylation of *O*-acetyl homoserine and thus resembles the pathway in yeast rather than the more common bacterial pathway found in *E. coli*.

Purines and Pyrimidines

There are clear differences in the metabolism of purines and pyrimidines by different groups of leptospires. All species of *L. biflexa* can synthesize their purine and pyrimidine requirements and grow in the presence of 2,6-diaminopurine (10 mg/ml) or 8-azaguanine (225 mg/ml) added to the growth medium. In contrast, among the pathogenic species, *L. borgpetersenii*, *L. santarosai* and some of the *L. interrogans* grow in the presence of 2,6-diaminopurine. Except for *L. inadai*, whose pathogenic status is uncertain, 8-azaguanine inhibits all the pathogenic species.

Carbon Sources

Long-chain fatty acids and CO_2 are essential. Chain lengths of 12–18 carbon atoms are satisfactory, but only *L. biflexa* will grow on saturated fatty acids alone; indeed, they will also grow on long- or short-chain, saturated or unsaturated fatty acids. Unsaturated fatty acids alone will support the growth of pathogenic species, which require unsaturated fatty acids to be present in order to utilize saturated fatty acids. They also require longer chain length fatty acids than *L.*

biflexa does for the utilization of C15-16 fatty acids (Johnson et al., 1969).

The fatty acids may be provided in any nontoxic form or in the presence of a detoxicant. The most elementary detoxicants are surfaces on which the fatty acids are adsorbed, such as glass, charcoal, silica gel, or starch. Serum contains albumin, which is capable of absorbing large amounts of fatty acids, among other compounds, and releasing them into the medium at low, nontoxic concentrations. Alternatively, in place of serum, the serum albumin can be extracted and used in defined serum-free media. Additionally, the fatty acids can be presented in a nontoxic form by combination in a complex such as a polyoxyethylene sorbitan ester (Tween), designated by a number according to its chemical composition. Tween 80 is polyoxyethylene sorbitan monooleate, Tween 60 contains monostearate, and Tween 40 contains palmitate. Tweens are industrial chemicals that also contain unesterified fatty acids, polyethylene glycols and other byproducts, which may inhibit growth or saturate the capacity of the absorbent. Impurities in Tweens may be removed before their use in culture media by absorption with activated charcoal, followed by filtration to remove charcoal particles (Bey and Johnson, 1978b), polyvinylpyrolidone treatment (Schönberg, 1983), partition in lipid solvents or zeolite-silicic acid column chromatography (Kojima et al., 1984).

Glycerol (200 mg/liter) enhances the growth of some leptospires. Pyruvate assists the growth of some strains from small inocula, notably Hardjo and Ballum. Pyruvate and CO_2 enhance colonial growth in solid media (Johnson et al., 1973). No other sources of carbon are recognized.

Other Growth Requirements

Ammonium ions, whether provided as ammonium salts or by the deamination of amino acids, are the only recognized nitrogen source. Cyanocobalamin (vitamin B_{12}), thiamin (vitamin B_1) and for some strains biotin are required. Phosphate is essential, as are calcium, magnesium and iron, optimally as Fe^{+++} or heme compounds such as hemoglobin or hematin. In practice, other compounds containing copper, manganese, and sulfate are added, particularly for isolation and maintenance of pathogens (Faine et al., 1999).

Leptospiral Antigens

Lipopolysaccharide

Leptospiral LPS contributes to the pathology associated with disease and comprises the major surface component of leptospires (Fig. 4). Studies with monoclonal antibodies have shown that LPS is the target for agglutinating and opsonizing antibodies (Farrelly et al., 1987). Thus, LPS plays a key role in immunity to infection. Indeed, anti-LPS monoclonal antibodies provide passive protection against infection, and purified LPS can stimulate active immunity (Jost et al., 1986; Midwinter et al., 1990). As an agglutinating antigen, LPS is also important for serological classification of leptospires.

Early reports of antigenic carbohydrate preparations extracted from leptospires used phenol, alkali, acetone or other solvents. Terms such as TM (for type-specific main) antigen, ESS (for erythrocyte-sensitizing substance), LLS (for LPS-like substance), or PAg (for protective antigen) have been used for these extracts, but all of them clearly contained LPS or LPS components as major constituents. The structure of LPS remains unknown. However, chemical analysis of leptospiral LPS showed the presence of common hexoses, amino hexoses and pentoses, and some sugars found more rarely as LPS components, such as xylose and arabinose (Vinh et al., 1986; Vinh et al., 1989). Methylated and *O*-acetylated sugars have also been reported (Yanagihara et al., 1983). The detection of 2-keto-3-deoxyoctonate (KDO) in the LPS of *L. interrogans* serovar Copenhageni and *L. borgpetersenii* serovar Hardjo is consistent with a composition similar to that of typical Gram-negative LPS.

Despite the structural, biochemical and immunological similarity of leptospiral LPS to Gram-negative bacterial LPS, it is at least 10-fold less toxic for animals or cells. Nevertheless, leptospiral LPS can activate macrophages and act as a B-cell mitogen (Isogai et al., 1990a; Isogai et al., 1990b). It has been proposed that the reduced toxic activity is a result of the absence of β-hydroxy-myristic acid. The activation of macrophages occurs via the CD_{14} and toll-like receptors TLR2, rather than via TLR4 as seen for the LPS of Gram-negative bacteria (Werts et al., 2001). Notably, TLR2 is commonly used as a receptor for Gram-positive bacteria and for other non-LPS components.

Genetic analysis has identified the *rfb* loci involved in the biosynthesis of the LPS O-antigen in several leptospiral serovars. Rhamnose is a component sugar of all serovars analyzed to date. The four genes encoding enzymes required for the biosynthesis of dTDP-rhamnose from glucose were first cloned from serovar Copenhageni and shown to be functional by complementation of a defined *S. flexneri* mutant (Mitchison et al., 1997). Genes encoding rhamnosyl transferases were found downstream of the rhamnose biosynthetic cluster. Subsequent work delineated the *rfb* locus in *L. borgpetersenii* serovar Hardjo

(Kalambaheti et al., 1999). This locus contains at least 31 open reading frames (ORFs) that encode enzymes involved in the biosynthesis of activated sugars, glycosyl transferases, and sugar processing and transport proteins. Three sugar biosynthetic clusters are involved in the synthesis of rhamnose (*orfs24–27*), galactose, mannose and fucosamine (*orfs6–11*) and a dideoxy-aminohexose (*orfs18–20*). By complementation of defined mutations in different Gram-negative species, orf13 was shown to encode an enzyme with galactosyl transferase activity, whereas *orf8* and *orf10* were shown to encode the galactose epimerase, GalE, and an *N*-acetyl glucosamine epimerase, respectively (Bulach et al., 2000). A putative flippase and O-antigen polymerase were also identified, as well as other glycosyl transferases of unknown specificity. Leptospiral LPS thus appears to be assembled via the classical Wzy (Rfc)-dependent pathway. Interestingly, the *L. borgpetersenii* serovar Hardjo locus is bounded by insertion (IS*1533*) elements, raising the possibility of horizontal acquisition. In addition, an IS*5*-like element is found between *orf14* and *orf15*. The *rfb* loci of serologically indistinguishable representatives of serovar Hardjo from *L. interrogans* and *L. borgpetersenii* are similar, but the former lacks IS elements (De la Peña-Moctezuma et al., 1999). However, detailed comparison of the sequences revealed that the *L. interrogans* locus consists of four segments. Two of these (*orf1–14* and *orf21–22*) resemble the *L. borgpetersenii* serovar Hardjo locus, whereas the *orf15–20* and *orf23–31* clusters show greater sequence identity to loci of other *L. interrogans* serovars (De la Peña-Moctezuma et al., 1999). A comparison of the *rfb* loci of 14 other *L. interrogans* serovars revealed conservation in the region from *orf11* to *orf31*. Genes upstream of *orf11* vary among serovars or show more genetic drift (De la Peña-Moctezuma et al., 2001). This region thus appears to encode biosynthetic enzymes and glycosyl transferases responsible for serovar specificity and some genes of unknown function.

Protein and Lipoprotein Antigens

A large number of protein antigens have been described in terms of recognition by convalescent sera or following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole cell lysates, but in most cases the proteins have not been identified or allocated to a particular cell component. Among the proteins described are a 62-kDa nonagglutinating subsurface genus-specific antigenic protein in serovar Kremastos (Sakamoto et al., 1985) and a variety of others in serovar Copenhageni (Niikura et al., 1987). A 35-kDa *L. interrogans* species-specific nonagglutinating, nonopsonic protein antigen (Jost et al., 1988) was related to outer envelope (OE) material in electron-microscopic immunogold staining, but probably originated from periplasmic material or the inner surface of the OE.

The use of immunoprecipitation studies aimed at identifying surface exposed proteins led to the identification of OmpL1, a transmembrane protein with porin activity that exists in a typical trimeric form in the leptospiral outer membrane (Haake et al., 1993; Shang et al., 1995). Subsequent studies identified two outer-membrane lipoproteins, LipL36 and LipL41, produced in *L. kirschneri*. LipL36, containing palmitate, is exported with LipL41 and LPS to the outer membrane. Production of LipL36 occurs more in the early log phase of growth in laboratory cultures than in the later stages and does not appear to occur when bacteria grow in vivo, that is, during infection, at least in the hamster kidney (Haake et al., 1998); however, these results do not preclude a role for LipL36 at other stages of the infectious process. In contrast, the major OMP, LipL32, as well as OmpL1, LipL41 and LPS, is expressed during growth in the hamster kidney (Haake et al., 2000b; Zuerner et al., 2000). Although LipL32 is known to be located in the outer membrane, its precise membrane topology is not yet known (Fig. 4). LipL41 and OmpL1 are highly conserved outer membrane proteins among pathogenic leptospires (Shang et al., 1996) and were the first, and to date only, protein antigens capable of eliciting a protective immune response (Haake et al., 1999). The two proteins in combination, but neither of them alone, could protect hamsters against lethal infection. Interestingly, the usual L-X-Y-C consensus cleavage sequence for transport and processing of Gram-negative lipoproteins varies substantially amongst spirochete lipoproteins, and the spirochete lipobox sequence of L(A/S)-L(V/F/I)-I(V/G)-A(S/G)-C has been proposed (Haake, 2000a; Zuerner et al., 2000).

Flagellar Antigens

In polyacrylamide gels, a doublet of 33–34 kDa, presumed to represent flagellar core proteins, reacts similarly in immunoblots with anti-leptospiral or anti-flagellar antisera (Kelson et al., 1988). Later work confirmed these observations and recognized 7 different proteins; a 34-kDa protein was associated with the core (measured as 11.3 nm) and a 36-kDa protein associated with a 21.5-nm diameter sheath filament (Trueba et al., 1992). The *flaB* gene has been cloned from *L. borgpetersenii* serovar Hardjo and expressed in *E. coli*. The 32-kDa

FlaB protein showed significant sequence similarity with FlaB homologues from *Treponema pallidum*, as well as from other Gram-negative and -positive bacteria (Mitchison et al., 1991). Sequences of *flaB* in Hardjobovis, Hardjoprajitno and Grippotyphosa were highly conserved and recombinant FlaB crossreacted antigenically between Grippotyphosa and Hardjobovis (Woodward and Redstone, 1994). Notably, the *flaB* gene was the first leptospiral gene to be inactivated by recombination-mediated allelic exchange (Picardeau et al., 2001).

Heat Shock Proteins

The operons encoding the major heat shock proteins, GroEL/GroES and DnaK, of *Leptospira* have been identified, cloned and sequenced (Ballard et al., 1993; Ballard et al., 1998). GroEL is a major immunogen recognized during infection in humans and animals, but was shown to play no role in immunity to infection. Interestingly, the regulation of the heat shock response in *Leptospira* shows features of both Gram-positive and Gram-negative regulatory mechanisms. In particular, the genetic organization and regulation of the *dnaK* locus more closely resemble those of Gram-positive bacteria.

Genetics

The Leptospiral Genome

The chromosome of *Leptospira* is characterized by a G+C content of 35–41 mol%, depending on species, with a genome size estimated to be ca. 5000 kb, measured in pulsed-field gel electrophoresis (PFGE) after digestion with *Not*I, *Nhe*I or *Apa*I (Baril and Girons, 1990). Using mainly the restriction enzymes *Not*I and *Sfi*I, a physical and genetic map of *L. interrogans* serovar Pomona was constructed (Zuerner, 1991), indicating a genome size of ca. 4750 kb, similar to that of Icterohaemorrhagiae. The genome consists of two circular replicons, of approximately 4750 kb and 350 kb (Boursaux-Eude et al., 1998). The large and the small replicons were regarded as chromosomes because the essential *asd* gene (for β-semialdehyde dehydrogenase) was located on the smaller unit. The small replicon is present in pathogenic serovars examined and shows very little variation in size. A comparison of genetic maps for strains of *L. interrogans* serovars Icterohaemorrhagiae and Pomona indicated considerable heterogeneity and variability within the species *L. interrogans*, which involves rearrangements of large segments of chromosome (Zuerner et al., 1993; Boursaux-Eude et al., 1998).

Ribosomal RNA Genes

Both *L. biflexa* (*sensu lato*) and *L. interrogans* (*sensu lato*) strains were shown to contain two ribosomal 23S rRNA genes, which not surprisingly cross-hybridized extensively with one another (Fukunaga and Mifuchi, 1989a). Two genes for 23S rRNA and for 16S rRNA, but only one for 5S rRNA, were found in *L. interrogans* serovar Canicola, each located far from other RNA genes. There were two 5S rRNA genes in *L. biflexa* (Fukunaga and Mifuchi, 1989b), one of which was sequenced (Fukunaga et al., 1992). Pathogenic strains of leptospires contained only one highly conserved 5S rRNA gene (Fukunaga et al., 1992). The 23S rRNA was further processed to 14S and 17S rRNA, in a pattern seldom found in prokaryotes, in *L. borgpetersenii* serovars Hardjo (type Hardjoprajitno) and Balcanica (Hsu et al., 1990). The primary, and especially the secondary, structures of the 5S rRNA gene in pathogenic strains of leptospires were found to be well conserved, similar to one another, and essentially similar to eubacterial 5S rRNA. It is uniquely organized, existing as a single copy within the genome, encoding a 117-nucleotide-long RNA molecule. The gene is flanked at both the 5′ and 3′ ends by A+T rich sequences, with a promoter sequence in the 5′ flanking region (Fukunaga et al., 1990; Fukunaga et al., 1991). The two genes for 5S rRNA in *L. biflexa* strains were located several kb apart on the chromosome, with divergent flanking sequences (Fukunaga et al., 1992). Thus, in contrast to the situation in many other bacteria where the 16S (*rrs*), 23S (*rrl*) and 5S (*rrf*) rRNA genes are clustered and co-transcribed, those in *Leptospira* are widely scattered on the large chromosome (Baril et al., 1992; Saint Girons et al., 1992).

IS Elements

Although no compound transposons have been found in the genus *Leptospira*, Zuerner (1994) identified and characterized IS1*533* from *L. borgpetersenii*. Multiple copies of IS1*533* were present on the genome. Two proteins encoded by IS1*533*, expressed and functional in *E. coli*, were related to a family of IS-encoded transposases and recombinases. Subsequently, a PCR assay based on IS1*533* sequences was adapted for identification of *L. interrogans* (*sensu lato*) strains. A similar IS element was reported in serovar Tarassovi (GenBank X77623). An IS*3*-like element, designated "IS1*500*," was present only in pathogenic leptospires (Boursaux-Eude et al., 1995). The IS1*500* insertions were present only on the large chromosome and appeared to coincide with genomic regions that had undergone rearrangement. This element was also used as

the basis for fingerprinting and epidemiological analysis of leptospiral isolates (Zuerner and Bolin, 1997).

Interestingly, the serovar Hardjo (type Hardjobovis, but not Hardjoprajitno) LPS biosynthetic locus is bounded by two IS*1533* elements (De la Peña-Moctezuma et al., 1999; Kalambaheti et al., 1999). A second IS element was also found in an intergenic region of the Hardjobovis locus; it appeared to be intact and is related to the IS*5* family, but no functional studies were performed (Kalambaheti et al., 1999). Similarly, an apparently intact IS*1500* element was found to disrupt the *orf35* gene in Ictero No. I, but not RGA, strains of serovar Icterohaemorrhagiae; this gene encodes a putative membrane protein within the LPS biosynthetic locus (De la Peña-Moctezuma et al., 2001). It has been speculated that the presence of these IS elements may regulate gene expression, leading to differences in LPS structure, but there is no evidence yet for such a role.

Heat Shock Genes

The early branching of spirochetes (before Gram-positive and -negative bacteria; Woese, 1987) is also reflected in the regulation of their heat shock response, which shows characteristics of both Gram-positive and -negative regulatory mechanisms.

The *groE* and *dnaK* operons of *Leptospira* have been identified, cloned and sequenced (Stamm et al., 1991; Ballard et al., 1993; Ballard et al., 1998). The *groE* operon encoding the GroEL and GroES proteins exhibits features found in heat shock operons regulated by either $Sigma^{32}$ or CIRCE (controlling inverted repeat of chaperone expression; Zeilstra-Ryalls et al., 1991; Zuber and Schumann, 1994). These are generally found in Gram-negative and Gram-positive bacteria, respectively. The CIRCE element was also found upstream of the dnaK locus, which contained the genes *hrcA*, *grpE*, *dnaK* and *dnaJ* (Ballard et al., 1998). The genetic organization and regulation of this locus are thus more like those in Gram-positive than in Gram-negative bacteria (Zeilstra-Ryalls et al., 1991; Zuber and Schumann, 1994).

Genetic Exchange Systems

Genetic analysis of *Leptospira* has been impeded by the lack of methods for the introduction of DNA into leptospiral cells. Standard methods such as transformation and gene inactivation have therefore not been available, and in this regard, work on *Leptospira* has lagged somewhat compared with advances in genetic exchange systems for other spirochetes. Early reports from the 1960s of bacteriophage-like particles in leptospires were not confirmed, but Saint Girons and her colleagues (Saint Girons et al., 1990) isolated and characterized three bacteriophages, whose replication was limited to the saprophyte *L. biflexa*. One of these phages, LE1, was shown to replicate as a plasmid in *L. biflexa* and was used as the basis for the first *L. biflexa-E. coli* plasmid shuttle vector, pGKLep4 (Saint Girons et al., 2000). An interesting feature is the necessity for a selectable marker of Gram-positive origin, the kanamycin-resistance gene from *Enterococcus faecalis*. No shuttle vectors or other plasmids have yet been reported for the pathogenic leptospires. A restriction-modification system has been demonstrated, at least in *L. biflexa* (Brenot et al., 2001b).

There is only one published report of successful gene inactivation by recombination-mediated allelic replacement in *Leptospira*. The kanamycin-resistance gene from pGKLep4 was used to inactivate the *flaB* gene encoding the flagellar subunit protein in *L. biflexa* (Picardeau et al., 2001). Mutants were nonmotile and lacked flagella and hooked ends, but retained their helical shape. The commonly used *sacB* gene was found to be an unsuitable counterselectable marker in *L. biflexa*, but the *rpsL* gene, which encodes the S12 ribosomal protein, was used to increase dramatically the number of double crossover transformants obtained. There are no published reports of allelic replacement in pathogenic leptospires.

Identification Methods

Serological Methods

The conventional method for identifying the serovar of a leptospiral isolate (the cross-agglutinin absorption test) by definition relied on a comparison of the agglutinating antigens of the unknown isolate with those of known reference strains. Two strains were considered to belong to different serovars if more than 10% residual homologous agglutinating titer remained after exhaustive absorption of hyperimmune rabbit sera with the heterologous strain (Dikken and Kmety, 1978). Despite both the inherent variability and rather arbitrary nature of this test, the concept of serovar has stood the test of time, probably because in most cases discrimination at this level was meaningful in terms of epidemiology, host association and severity of disease. Nevertheless, this method of identification is expensive and time consuming and is now performed infrequently, and then only by specialized reference laboratories; it is not recom-

mended. However, most of the more modern molecular identification and typing tests are judged by their ability to discriminate strains at about the level of the serovar.

The use of panels of agglutinating monoclonal antibodies has allowed strains to be identified by comparison of their reactivity pattern with those of reference strains, thereby generating a fingerprint for each serovar (Kolk et al., 1984; Terpstra et al., 1985; Terpstra et al., 1987; Korver et al., 1988). Any laboratory that can perform the microscopic agglutination test can therefore serotype isolates with relative ease.

Molecular Methods

The reference method for delineation of bacterial species remains the degree of homology at DNA level, as determined by DNA hybridization (Wayne et al., 1987). However, the procedure is cumbersome and unsuitable as a tool for identification. Restriction fragment length polymorphism (RFLP) based on polymerase chain reaction (PCR) amplification of either 16S rRNA or 23S rRNA genes has been shown to group strains in general agreement with their correct species designation (Ralph et al., 1993; Perolat et al., 1994). Probes derived from random primed PCR fingerprints were also able to discriminate at species level (Letocart et al., 1997).

At subspecies level, the first widely used method was restriction endonuclease analysis (REA) of whole genomic DNA (Marshall et al., 1981). Despite the difficulty (due to the large number of fragments generated) in reading complex restriction profiles, the technique has provided useful epidemiological information. For example, REA first identified the Hardjobovis and Hardjoprajitno subtypes now known to belong to different species, as well as allowed discrimination of three subtypes of Hardjobovis (Thiermann et al., 1986). Similar subtypes have been identified within other serovars. All leptospiral genomes appear to have multiple copies of a range of insertion sequences; some of these have been used as targets for identification and molecular typing schemes (Zuerner et al., 1995; Zuerner and Bolin, 1997).

Interestingly, and perhaps unexpectedly, the analysis by PFGE of restriction fragments generated by digestion with rare cutting enzymes such as *Not*I or *Sgr*AI yielded unique patterns for most serovars (Herrmann et al., 1992; Herrmann and Saint-Girons, 1993; Herrmann, 1994). In reality, PFGE has become accepted as the most appropriate and accurate molecular typing method. A disadvantage is the need for specialized equipment and expertise. For this reason, a range of methods based on random amplification of chromosomal DNA has been reported (Faine et al., 1999; Levett, 2001); in general, they show good correlation with PFGE and can be performed readily by any laboratory with PCR capacity.

Isolation, Cultivation and Preservation

Growth in Liquid Media

Leptospires are grown routinely in liquid media, sometimes supplemented with 0.1% agar for primary isolation or for maintaining stock cultures. Recipes and details of culture media can be found in Faine et al. (1999). Incubation periods may be as long as 3–4 weeks for some pathogens. Obviously, much more care about sterility and avoidance of airborne contamination is required than when working with conventional bacteria grown overnight on solid medium in culture plates. Media are sterilized by filtration because of the presence of heat-labile essential constituents, notably albumin. All containers, including holding vessels used prior to filtration, should be dry and all solutions should be heat sterilized or made with water that is heat sterilized because leptospires exist in tap water and can pass through the 0.2 μm filters used for filter-sterilizing media. An alternative precaution is to heat media at 56°C for 30 min after final dispensing into working containers.

Cultures are usually inoculated using relatively large inocula (1–10% of the volume of the new medium) using a pipette; the use of bacteriological loops for subculturing leptospires is not recommended. Shaking in air enhances the rate and amount of growth. Cotton plugs should be avoided in vessels used in the preparation of media or for cultivation because cotton contains toxic lipids, which melt or vaporize during autoclaving or incubation and contaminate the media. Screw-cap containers are best for cultures that require more than a day or two of incubation to avoid dehydration.

Media Containing Serum

Numerous media containing dilute animal serum, usually rabbit serum, have been described, all buffered to about pH 7.2–7.6 with phosphate and containing peptone and other common medium constituents (Faine et al., 1999). Best results are obtained when the serum is slightly hemolyzed; hemolysis releases essential iron and cyanocobalamin from the erythrocytes. Probably the most commonly used medium containing serum is Korthof's medium (Korthof, 1932), in which a dilute phosphate-

buffered saline containing a small amount of peptone is supplemented with about 8–10% pooled, heated, slightly hemolyzed rabbit serum and finally sterilized by filtration. Most serum media precipitate phosphates during preparation and sometimes afterwards, chelating metals in the process. Precipitates are a disadvantage when dark-field microscopy has to be used to check growth or end-points in a microscopic agglutination test for leptospiral antibodies.

Serum-free Media

Studies of the minimal nutritional requirements for growth of pathogenic leptospires led to the development of a serum-free, oleic-acid albumin medium and derivatives containing Tweens as the sources of fatty acids, with serum albumin as a detoxifier, such as the widely used, commercially available Ellinghausen-McCullough-Johnson-Harris medium (EMJH). Bovine serum albumin (BSA) for use in culture media is expensive. Each batch, even from the same supplier, must be checked for its ability to support the growth of a range of serovars and strains. BSA quality can be improved by extracting it with fat solvents (methanol, chloroform, and ether) before drying and weighing.

Protein-free Media

Protein-free media (Shenberg, 1967; Bey and Johnson, 1978b; Christopher et al., 1982) are those in which conditions are balanced and ingredients are selected or purified to avoid toxicity. Their main application is in use for vaccines (Marshall et al., 1979) where BSA is unacceptable because of the risks of hypersensitivity or autoimmune reactions in vaccinated animals or people. Success in their use depends on scrupulous care and attention to detail in water purification. Use fresh double-glass distilled water in preference to deionized water and take exceptional care in cleaning glassware to avoid all traces of lipids. Tweens must be cleaned freshly before use because they autoxidize to form traces of toxic fatty acids and peroxides on storage; likewise, the media have a limited shelf life. Strains usually need to be adapted to grow in protein-free media and subcultured every few days. Otherwise they die quite suddenly, probably as a result of endogenous lipases produced by the developing leptospires acting on the Tweens in the medium, releasing toxic, lytic lipids that cannot be absorbed in the absence of a detoxicant. Low-protein media (Bey and Johnson, 1978b; Christopher et al., 1982) containing 0.1% or 0.01% BSA are sometimes used as a compromise to cultivate strains that cannot be adapted to protein-free media or for maintenance of strains in protein-free medium.

Growth on Solid Media

Any liquid medium can be solidified by the addition of agar. The liquid medium may be made up double-strength, sterilized by filtration, and warmed to 45°C. Double the required concentration of agar in water is autoclaved, cooled to 45°C, and combined in equal volumes with the medium, mixed thoroughly and dispensed. Media solidified with 0.8–1% agar will support the growth of subsurface colonies, even after surface inoculation. Surface colonies can be seen on media with concentrations of 1.2–2% agar. Colonies can be visualized before they can be seen by eye if the redox dye and indicator (2-6-dichlorophenol-indophenol) is added (1 : 100,000) to the medium. A zone of colorless or pink decoloration of the blue dye precedes the appearance of leptospiral growth by 1–2 days at the position where the colony will appear.

Selective and Differential Media

Difficulties in the use of solid media and the risk of contamination on prolonged incubation or when attempting to isolate leptospires from natural sources, such as soil, mud, animal tissues or carcasses or body fluids, have led to the development of several selective media that contain various inhibitors of other bacteria. These include 5-fluorouracil (50–1000 μg/ml; Johnson and Rogers, 1964a); a combination of nalidixic acid (50 μg/ml), vancomycin (10 μg/ml), and polymyxin-B-sulfate (5 units/ml; Schöberg et al., 1980); or a combination of actidione (100 μg/ml), bacitracin (40 μg/ml), 5-fluorouracil (250 μg/ml), neomycin sulfate (2 μg/ml), polymyxin B (0.2 μg/ml) and rifampicin (10 μg/ml; Adler et al., 1986). Prompt subculturing, after not more than 2 days, into media free of inhibitors is recommended. Saprophytic leptospires can be cultured selectively in medium containing 8-azaguanine (225 μg/ml); the same medium will differentiate saprophytes, which will grow in it, from pathogens, which will not.

Purification of Contaminated Cultures

Leptospires pass through filters of average pore diameter 0.2 μm, which hold back most other bacteria. The contaminated culture is filtered, and the filtrate inoculated into fresh medium. An older method of decontamination is to inoculate a mouse intraperitoneally with approximately 0.5 ml of the culture and then bleed the mouse by heart puncture under anesthesia after about 15 min. Leptospires invade the bloodstream

faster than other bacteria, so they can be selectively recovered from the blood. Hamsters or guinea pigs are also suitable.

Preservation

A variety of methods have been reported over the years for long-term preservation of *Leptospira* strains. The authors' experience is that storage in liquid nitrogen is the most suitable method for preserving viability, antigenicity and virulence for many years. A simple protocol involves the addition of 5–10% dimethyl sulfoxide (DMSO) to late log phase cultures in EMJH medium, followed by slow cooling to –70°C; this is readily achieved by wrapping vials in a thick layer of cotton wool in a –70°C freezer. The vials are then transferred to liquid nitrogen the next day.

Clinical and Epidemiological Aspects of Infection and Disease

It is beyond the scope of this brief section to provide the details of the varied clinical aspects of infection with *Leptospira* in humans and animals. The reader is referred to the following books, chapters or reviews for detailed information on specific findings and clinical presentations in leptospirosis: Bolin (1996), Faine (1998), Faine et al. (1999), and Levett (2001).

General Features

Leptospirosis is in most instances an acute, febrile illness occurring in humans or animals all over the world. In humans, the disease is always contracted from an animal source; human-to-human transmission is for practical purposes nonexistent and the disease is regarded globally as a zoonosis. Pathogenic leptospires live in the proximal renal tubules of the kidneys of carriers, although other tissues and organs may also serve as a source of infection. From the kidneys, leptospires are excreted in urine and may then contaminate soil, surface water, streams and rivers. Infections of animals or humans occur from direct contact with urine or indirectly from contaminated water. The carriers may be wild or domestic animals, especially rodents and small marsupials, cattle, pigs and dogs. Almost every mammal (including aquatic mammals) and marsupial has been shown to be a carrier of leptospires. Humans almost never become chronic carriers, but suffer acute infections, sometimes with longer-term sequelae.

Leptospirosis in humans varies in severity according to the infecting serovar of *Leptospira* and the age, health and nutrition of the patient. It ranges from a mild influenza-like illness to a severe infection with renal and hepatic failure, pulmonary distress, myocarditis, hemorrhages and death (Weil's disease). Congenital infection occurs in humans and in animals, where it causes loss of productivity in livestock through abortion, still-birth or loss of milk production or failure to thrive. There is a worldwide strong occupational association with animal production (dairy farming, pig production, and abattoirs) and a universal risk from rodent-carrier-mediated infection, especially prevalent in tropical countries, where many serovars may be present in a locality. It is important to recognize and identify the infecting leptospires for clinical and epidemiological reasons. There are characteristic associations of particular serovars with certain species of animals as carriers, but the association is not absolute and the molecular basis for any such host "specificity" is unknown. The main associations recognized throughout the world are serovars Hardjo with cattle, Pomona with pigs, cattle, and rodents, and Icterohaemorrhagiae, Copenhageni, Bataviae, *Autumnalis*, *Australis*, and *Zanoni* with rats and a wide range of small rodents, which may become lifelong carriers.

The renal carrier state is thus a key component that is central to the persistence and epidemiology of leptospirosis. Leptospires colonize the surfaces of renal proximal tubular epithelial cells. They may persist there for periods of weeks to years, often for the lifetime of the animal in some leptospiral serovar-host associations, especially rodents. The molecular basis for this bacterial-cell association is unknown and specific adhesion factors have not been described. The ability of leptospires to bind fibronectin via specific fibronectin-binding proteins may be of significance in their ability to colonize epithelial surfaces (Merien et al., 2000). Excretion in urine may be intermittent or continuous and the urinary concentration of bacteria may be as high as 10^8/ml. Antileptospiral immunoglobulin is present in the tubular and bladder urine but does not kill the leptospires, probably because of the absence of complement. Leptospires do not survive well in acid urine, but remain viable in alkaline urine. Consequently herbivores and animals whose diet produces an alkaline urine are relatively more important as shedders than are producers of acid urine.

Leptospirosis in Humans

Leptospirosis commences suddenly with headache, fever (typically to 102°F, 39°C), malaise, myalgia, conjunctival suffusion (red eyes) and sometimes a transient rash on the palate and

skin. Thereafter the illness may be mild and self-limiting or severe and possibly fatal. The mild type may be serious and incapacitating, but seldom leads to kidney or liver failure, hemorrhages or death.

Mild Leptospirosis

This description is applicable worldwide to illness characteristically seen in infection with serovars Hardjo, Grippotyphosa or Pomona, but also with others. On the other hand, infection with some of these usually milder serovars can occasionally lead to severe leptospirosis, which may be life threatening. Human infection with Pomona may lead to renal failure, while severe cholecystitis has been reported for Hardjo infections. The initial symptoms may be followed by a transient remission, in 3–5 days, which may then proceed to an exacerbation and include aseptic meningitis, renal failure and abdominal or chest pains, reflecting the generalized pathology. Clinical differential diagnosis is required between leptospirosis and severe influenza, viral meningitis, acute abdominal conditions or glomerulonephritis. Recovery is usually complete but weakness, tiredness, depression, and even psychosis may prevent a return to normal living for weeks or months. Reports of chronic or recurrent leptospirosis have usually not been adequately proven or investigated, but there is sufficient anecdotal evidence for at least some of these claims to be taken seriously. A clear association was described between uveitis following leptospiral infection and the presence of leptospires (detected by PCR) in the anterior chamber of the eye (Chu et al., 1998).

Severe Leptospirosis

Characteristically this is seen in severe infections with serovars Icterohaemorrhagiae, Copenhageni, Lai and others. The illness worsens, usually rapidly after onset, so that renal failure appears within 7–10 days, sometimes accompanied or followed by skin and mucosal hemorrhages, jaundice, hemoptysis, myocarditis, or liver failure, leading to death if untreated. Case fatality rates approaching 20% have been reported (Everard et al., 1995). A more recently recognized respiratory manifestation involves severe pulmonary edema and hemorrhages, which have been the main cause of death in some epidemics (Shim et al., 1980; Zaki and Shieh, 1996). As with mild leptospirosis, chronic, long-term sequelae have been reported, but frequently not investigated fully. The host and microbial factors, which may lead to long-term persistence, are unknown.

Leptospirosis of either type in pregnancy carries the risks of intrauterine infection and fetal death.

Acute Leptospirosis in Animals

Acute leptospirosis is similar in all animals. In its most flagrant form, it is manifested by listlessness, loss of appetite, irritability, fever, ruffled fur, red eyes, and sometimes diarrhea, occurring 3–7 days after infection. There may be signs of hemorrhages and jaundice. Movement is accompanied by a characteristic arching of the back. Recovery may occur from here, or death may supervene. Recovery may be accompanied, or followed, by weight loss, runting in young animals, chronic renal failure and its signs, and delayed death. In milk-producing cattle, there may be disturbance of milk flow and quality. Congenital infection of fetuses in utero follows a similar course, leading to abortion if the fetus dies. The abortion products may be hemorrhagic or jaundiced or both; they may be heavily loaded with leptospires and a danger to animal handlers. Stillborn fetuses may likewise be hemorrhagic, jaundiced and infectious. Less severe infections, ranging down to the clinically unapparent, occur frequently.

Chronic Leptospirosis in Animals

The chronic persistence of leptospirosis in animals is the central feature that maintains the cycle leading to fresh infection of new animal hosts. In this sense, humans are an accidental host and are irrelevant in the maintenance of leptospires in the environment. Animals that have recovered from acute leptospirosis may develop a carrier condition in which leptospires grow and may remain in the renal tubules for periods of days to years. From here they are passed out in the urine. Leptospires may also persist in other organs, notably the genital tract, the brain and the eye. In horses especially, recurrent, autoimmune uveitis is well documented and involves crossreactivity of anti-leptospiral antibodies with host ocular tissue (Lucchesi and Parma, 1999).

Pathogenesis

Leptospires enter through small cuts or abrasions, via mucous membranes such as the conjunctiva or possibly through wet skin. They spread immediately and circulate in the bloodstream. This bacteremic phase lasts for about 1–7 days. After the numbers of leptospires in the blood and tissues reach a critical level, lesions (due to the action of undefined leptospiral toxin(s) or toxic cellular components) and con-

sequent symptoms appear. The primary lesion is damage to the endothelium of small blood vessels leading to localized ischemia in organs, resulting in renal tubular necrosis, hepatocellular damage, meningitis, myositis and placentitis. Hemorrhages occur in severe cases as do jaundice and frequently, platelet deficiency. There is usually a mild granulocytosis and splenomegaly. Once circulating antibodies appear, leptospires are removed from the circulation and tissues by phagocytosis, following opsonization. Tissue damage, even though it is severe, may be reversible and followed by complete repair (e.g., of the kidney and liver) although long lasting damage (e.g., myocarditis) may be a complication and may lead to scarring, well recognized in the kidneys of pigs and dogs, where it may be observed macroscopically as "white spots."

Epidemiology

Leptospirosis occurs worldwide wherever there is a risk of direct or indirect contact with the urine or kidneys of infected animals or their infected conception products. The organisms die when dry or in acid conditions (pH <7.0), so that transmission is confined to wet environments or circumstances. The most frequent sources of infection are urine, kidneys, and surface of stream waters contaminated with infected urine. Infection is not transmitted by ingestion of food or by inhalation of airborne particles.

Sources of nonoccupational leptospirosis in temperate climates are mainly rodents, dogs, leisure activities and travel (e.g., hobby farming, rafting, camping, boating and swimming), affecting city and rural dwellers. City dwellers may also be occupationally exposed (e.g., sewer workers, drainers, slaughter men, cattle-yarders, butchers, meat inspectors, and veterinarians). Rural dwellers are always at risk, especially in tropical countries where they are in close contact with potentially infected field or domestic rodents and with domestic animals in a warm, wet climate, usually without considerations of protective clothing or footwear. Their habitation is often subjected to flooding. In temperate climates, carriers (pigs, cattle and dogs) are the most important sources of leptospirosis in humans.

Treatment

Antibiotic treatment is effective within the first 7–10 days of infection. It should be given immediately on diagnosis or suspicion. Rapid effective antibiotic treatment can reduce the progression of symptoms and the likelihood of sequelae, thereby preventing the more severe manifestations of infection.

Antibiotics will eliminate leptospires from accessible tissues but will not reverse already established pathological changes, which must run their course and be treated symptomatically or by appropriate supportive therapy. The antibiotic of choice is benzyl penicillin by injection in doses of 5 mega units per day for 5 days. A variation is to give half the dose in procaine penicillin for the first 2 days of the regime. Patients genuinely allergic to penicillin may be given erythromycin, 250 mg four times daily for 5 days. Doxycycline (100 mg twice daily orally for 10 days) is also recommended for patients who can tolerate oral therapy. Tetracyclines were used previously but are contraindicated in patients with renal insufficiency (which occurs in almost all), in children or in pregnant women. They have no place nowadays where alternatives are available. Doxycycline has also been used as a prophylactic for short-term exposure, but it is not recommended for routine continuous use or for long-term occupational exposure.

Virulence Factors

The mechanisms by which leptospires cause host tissue damage and disease are not well defined. In particular, the molecular basis for virulence remains unknown, owing mainly to the absence of genetic tools for the manipulation of *Leptospira*. There have been reports dating back many years of pathogenic mechanisms, but in almost all cases the specific leptospiral components responsible for the activity investigated have not been identified.

For example, virulent leptospires were shown to adhere to cultured renal tubular epithelial cells (Ballard et al., 1986), while the adhesion to cultured fibroblasts was enhanced by antibody (Vinh et al., 1984); in neither case were the leptospiral adhesins identified. Virulent leptospires are resistant to complement and to killing by neutrophils in nonimmune hosts (Cinco and Banfi, 1983a; Wang et al., 1984), but are rapidly killed by either mechanism in the presence of specific antibody (Anderson and Johnson, 1968; Vinh et al., 1982; Farrelly et al., 1987). There is no unequivocal evidence for a classical secreted toxin in *Leptospira*; indeed, in the kidneys of experimentally infected guinea pigs, endothelial damage was closely associated with the presence of remnants of leptospiral cells (de Brito et al., 1992). The ability of leptospires to invade Vero cells and to induce apoptosis in macrophages was correlated with virulence (Merien et al., 1997). Significantly, both properties were lost very rapidly upon in vitro subculture. A cytotoxic, glycolipoprotein fraction was shown to inhibit host ATPase (Younes-Ibrahim et al., 1995), but the

activity was probably due to the presence of long-chain fatty acids (Burth et al., 1997). Likewise, the molecular basis for chemotaxis towards hemoglobin (Yuri et al., 1993) has not been defined. A 36-kDa fibronectin-binding protein was identified in a virulent strain of Icterohaemorrhagiae but was absent in an avirulent variant and in a saprophytic strain (Merien et al., 2000); the variant was not genetically defined.

Hemolysins sphgenes have been reported in a number of leptospiral serovars. Not surprisingly, therefore, a range of genes encoding sphingomyelinases, which are either cell-associated and/or extracellular, has been identified in different *Leptospira* species. Segers et al. (1990) cloned a gene, *sphA*, encoding a cell-associated sphingomyelinase C enzyme in *L. borgpetersenii* serovar Hardjobovis. Similar sequences were identified in all pathogenic strains tested, but were not present in nonpathogenic serovars (Segers et al., 1992). The enzyme activity appeared to be extracellular in Canicola and Pomona. On the basis of hybridization and cloning experiments, at least seven other *sphA*-like genes were detected among the pathogenic species of *Leptospira*. A gene designated "*sphB*" was cloned from *L. borgpetersenii*, *L. weilii* and *L. santarosai*, whereas hybridization studies suggested the presence of a third, related gene, *sphC*. Within the species *L. interrogans*. *L. noguchi* and *L. kirschneri*, an additional five *sph* genes were identified. A further sphingomyelinase, SphH, identified in serovar Lai, was postulated to be a pore-forming hemolysin (Lee et al., 2000). Preliminary studies indicated that SphA and SphB do not elicit a protective immune response. The significance in pathogenesis and/or immunity of the other *sph* genes remains undetermined.

The biological activity of leptospiral LPS and its potential role in pathogenesis is described under Leptospiral Antigens.

Immunity and Vaccines

Immunity to Infection

Immunity in leptospirosis is predominantly, and probably exclusively, humorally mediated. There is no convincing published evidence for a role for cellular immunity in protection from infection. Immunity can be readily transferred by convalescent human or animal serum, by experimentally produced antiserum, or by appropriate monoclonal antibodies (Mabs) such as those against polysaccharide epitopes of leptospiral LPS, which protected guinea-pigs, hamsters, dogs and monkeys against lethal infection (Jost et al., 1986; Schoone et al., 1989). MAbs against LPS determinants are agglutinating and opsonic (Adler and Faine, 1983; Farrelly et al., 1987), and in the presence of specific antibodies, leptospires are rapidly phagocytosed by macrophages and neutrophils, both in vitro and in vivo (Faine, 1964; Cinco et al., 1981; Vinh et al., 1982; Cinco and Banfi, 1983b; Farrelly et al., 1987). In addition, lysis of leptospires occurs in the presence of antibodies and complement within minutes of exposure in the laboratory (Anderson and Johnson, 1968). LPS is highly antigenic whether in its native form in whole or disintegrated leptospires or in chemically purified form, and immunization with purified LPS or LPS components elicits protective immunity (Jost et al., 1989; Midwinter et al., 1990; Midwinter et al., 1994; Masuzawa et al., 1996). Significantly, LPS is a major antigen recognized by human and animal convalescent sera (Chapman et al., 1988; Chapman et al., 1991; Guerreiro et al., 2001), and immunity following naturally acquired infection is restricted to serovars with serologically related LPS. Early work on immunity amongst antigenically unrelated serovars (Kemenes, 1964; Plesko and Lataste-Dorolle, 1970; Plesko and Hlavata, 1971) has not been confirmed; the responsible antigens were not identified.

There is only one published report of unequivocal protective immunity elicited by non-LPS antigens (Haake et al., 1999). Immunization of hamsters with *E. coli* membrane fractions containing a combination of OmpL1 and LipL41 induced significant protection against homologous challenge with *L. kirschneri* serovar Grippotyphosa. No protection was observed with either antigen administered alone. Interestingly, nonmembrane-associated forms of the antigens were not protective, even when used in combination, leading the authors to suggest that the manner in which these proteins associate with the membrane is important in the induction of a protective immune response.

Leptospiral Vaccines

Prevention against leptospirosis without vaccination is difficult. Measures for occupational hygiene such as protective clothing and avoidance of splash from urine or water are often useful but hard to implement because they impede work or are unacceptable to both workers and employers. For example, it is not practicable to advise dwellers in tropical villages to avoid hazardous activities like contact with pigs and other livestock or dogs and walking or working in wet conditions, including in soil or water, such as rice paddies, contaminated by the animals' urine.

Vaccines for humans and animals have been used since the 1920s; almost all of them were prepared from whole leptospiral cells killed by a variety of methods, including heat, formalin,

phenol, irradiation, etc. (Faine et al., 1999). The use of undefined, live avirulent, attenuated, or saprophytic leptospires (Stalheim, 1968; Rottini et al., 1972) has not gained acceptance. Many of these early preparations were too reactogenic for widespread human use. Attempts to reduce reactogenicity have included the use of protein-free media for growth of leptospires (Shenberg and Torten, 1973; Marshall et al., 1979; Christopher et al., 1982; Bey and Johnson, 1983) and the use of subcellular fractions (Bey et al., 1974; Bey and Johnson, 1978a; Bey and Johnson, 1982), the active component of which was almost certainly LPS.

Nevertheless, human vaccines containing whole killed leptospires have been used successfully in China following floods resulting in exposure of large populations to risk of leptospirosis (Chen, 1985) and in Japan and Vietnam. Generally, vaccines contain two or more locally prevalent serovars. For example, a trivalent killed vaccine was recently developed in Cuba following the unavailability of an imported vaccine (Martinez Sanchez et al., 2000). In all cases, repeated annual revaccination is recommended to retain immunity. As with natural infection, immunity is restricted to antigenically related serovars.

Vaccination of livestock can reduce urinary shedding and risk to human handlers especially, and perhaps only, when associated with a high level of education, awareness, and hygiene in the community and with support from the agencies responsible for administration of human and veterinary public health. Prenatal vaccination of sows protects young piglets from acute leptospirosis, but not from being carriers as adults because the acquisition of infection after maternal immunity wanes (Millar et al., 1987), thus failing to reduce the risks to handlers, slaughter men and other meat-workers. Traditionally, the ability of vaccines to elicit the production of agglutinating antibodies has been considered a reasonable measure of their efficacy. However, this association has been refuted at least for Hardjobovis infection in cattle, in which high levels of circulating antibody did not protect cattle against ocular challenge, thought to mimic more closely the natural challenge route (Bolin et al., 1989). Vaccines that are efficacious in this immunization-challenge model may stimulate either cellular immunity or antibodies against unknown nonagglutinating antigens.

Diagnosis

Clinical diagnosis of leptospirosis in humans requires the confidence of current experience supported by laboratory confirmation. The symptoms, while characteristic, are not pathognomonic. The epidemiological environment, clinical history, symptoms and findings on physical examination may allow a clinical diagnosis, but usually laboratory diagnosis is required. The main clinically useful features are the sudden onset of severe headache and fever, almost invariable proteinuria and initially mild or transient nitrogen retention, palatal and skin rash, conjunctival suffusion, and extreme muscle tenderness, especially in the calf and back muscles. Presenting symptoms may mimic or indeed result from an abdominal emergency (e.g., acute cholecystitis), hemorrhages (hemoptysis), renal failure or liver failure (jaundice).

In addition to general laboratory tests for meningitis, liver function, renal function and hematological parameters (Faine et al., 1999; Levett, 2001), specific microbiological tests are necessary. Diagnosis of leptospirosis is essentially similar in humans and animals. The same laboratory tests are available and useful. Checklists for the diagnosis of leptospirosis in humans and animals may be found in Faine et al. (1999).

Culture of Leptospires

As with other bacterial infections, culture and identification of the causative organism provides unequivocal proof of infection. Much mythology surrounds the perceived difficulty of culture of leptospires from clinical material. However, the success rate with blood culture can be very high if appropriate procedures are followed. Leptospiremia commences before the onset of symptoms and usually declines by the end of the first week. Blood cultures must therefore be taken as early as possible and before the initiation of antibiotic therapy. Best results are achieved when medium is inoculated at the bedside with freshly drawn blood. A recommended protocol involves the inoculation of 3 bottles of 5–10 ml EMJH medium with 1, 5 and 20 drops of blood; too much blood can be inhibitory. Provided that proper aseptic procedures are followed, the use of selective media for blood cultures is not recommended. Although leptospires may survive for a few days in conventional blood culture media (Palmer et al., 1984), reliance on this is not recommended. Cultures should be incubated at 28–30°C and examined by dark field microscopy at least weekly for the presence of leptospires; positive cultures should be subcultured immediately. Cultures may need to be kept and observed for several weeks before being discarded as negative. The use of semisolid media is said to increase the isolation rate, but this has not been the authors' experience.

If cerebrospinal fluid (CSF) is taken for other investigations, it should be cultured similarly to

blood. Urine culture in humans is rarely productive because the survival of leptospires in voided urine is poor. Urine must therefore be cultured immediately into selective medium.

The use of animal inoculation for primary isolation of leptospires is in most cases not practical and is not recommended.

Clearly, the slow growth rate of leptospires means that in many cases, a positive culture provides a confirmatory diagnosis. However, in addition to individual diagnosis, it is critical that isolations of locally prevalent serovars are made for epidemiological purposes and also for possible use as antigens for serodiagnosis.

Direct Observation

Direct observation of leptospires in blood and CSF is of very limited diagnostic value; it is not sufficiently sensitive and extremely time consuming. Theoretically 10^4 bacteria/ml would yield one organism per microscope field, but in reality the sensitivity is at least 10- to 100-fold lower. An additional problem is the presence in blood of fibrin strands, which appear to the inexperienced eye as leptospires, so-called "pseudospirochetes." However, unequivocal observation of leptospires by experienced personnel will provide a rapid presumptive diagnosis and facilitate patient management.

In situ staining by immunofluorescence, immunohistochemistry or silver deposition is routinely used for the detection of leptospires in veterinary histopathology, where it serves an important role (Ellis, 1986; Chappel et al., 1992). However, these methods have been largely supplanted by PCR-based detection methods.

Antigen Detection

There have been several reports in the literature of the use of various immunoassays for the detection of leptospiral antigens, including enzyme immunoassay (EIA), radioimmunoassay (RIA), and chemiluminescence. However, they have suffered from sensitivity problems and have not gained widespread acceptance.

Molecular Diagnostic Procedures

The availability of PCR technology has made possible the rapid and sensitive detection of leptospiral DNA in a variety of clinical samples from humans and animals, including serum, urine, CSF, aqueous humor and various organ tissues (see Levett, 2001, for a more extensive review). The most frequent target sequences for amplification have been 16S and 23S rRNA genes (Hookey, 1992; Merien et al., 1992), IS elements (Zuerner et al., 1995; Zuerner and Bolin, 1997) or sequences of unknown function derived from random genomic libraries (Gravekamp et al., 1993). None of the published PCR primers can identify target sequences to serovar level, and a rational PCR-based detection and identification method must await the unequivocal identification of leptospiral genes encoding serovar specificity, most probably within the *rfb* LPS biosynthetic locus. In an analogous situation, a rational PCR-based system was reported recently for *Pasteurella multocida*, based on defined genes encoding specificity of capsular polysaccharide antigens (Townsend et al., 2001).

Serological Diagnosis—Microscopic Agglutination Test

The great majority of cases of leptospirosis in humans and animals are diagnosed serologically. The "gold standard" for serodiagnosis remains the microscopic agglutination test (MAT) in which agglutination of live or killed leptospires by patient serum is assessed by dark field microscopy. Detailed methods and protocols for the performance of the MAT may be found elsewhere (Faine, 1982; Faine et al., 1999). As with blood culture, an undeserved negative mythology surrounds the MAT. It is in fact one of the most sensitive and specific serological tests available, and when performed correctly by trained experienced personnel, it is reliable and reproducible. In the authors' opinion, the oftenquoted drawbacks of occupational danger to laboratory staff and the need to maintain live cultures of *Leptospira* are overstated. However, it does remain a test that is in general performed by specialized laboratories because both the test and the culture of leptospires require particular expertise. As with other serological tests, a fourfold rise in titer in paired sera is the accepted criterion for definitive diagnosis. A single high titer (400 or higher) in a nonendemic area, together with appropriate clinical and epidemiological history, is highly suggestive of current or very recent infection, whereas similar findings in endemic areas may require further confirmation. Titers of greater than 100,000 may be observed with infections by some serovars, especially those of the Icterohaemorrhagiae serogroup (Alston and Broom, 1958). Conversely, very low maximal titers (less than 100) have been reported with Hardjo infections in both humans (Christmas et al., 1974) and animals and with Bratislava infections in animals (Ellis, 1991). Typical titers against Hardjo are in the order of 400 to 1600. Antibiotic therapy may reduce,

delay, or, rarely, abrogate completely the antibody response.

A battery of serovars representing those prevalent in each geographical area must be included in the panel used for MAT, and it is recommended that fresh, authenticated strains be obtained yearly from a certified reference laboratory (listed in Faine et al., 1999). The use of formalin-killed cultures results in lower titers (Palmer et al., 1987). Lyophilized leptospiral suspensions also have been produced and are reported to give similar results.

It must be emphasized that the precise infecting serovar cannot be determined from the patient's serological response, which is at best serogroup specific. However, the MAT response, together with an up-to-date knowledge of locally prevalent serovars, can usually give a reasonable indication of the likely identity of the infecting serovar. This emphasizes the continuing importance of culture as a diagnostic and epidemiological procedure.

Serological Diagnosis—Other Tests

A variety of alternative serological tests have been used over the years, but none of them matches the MAT in terms of specificity and sensitivity. Complement fixation, passive hemagglutination, latex agglutination and slide agglutination tests have all been described (Faine, 1982; Faine et al., 1999; Levett, 2001), but none of them warrants recommendation for routine use.

Enzyme-based antibody assays (ELISA or EIA) were first described over 20 years ago (Adler et al., 1980; Terpstra et al., 1980), and a number of commercial EIA kits are now available for both humans and animals, including a rapid dipstick IgM EIA (Gussenhoven et al., 1997). Most EIAs use disrupted whole leptospires as antigen and the EIA therefore detects antibodies against both agglutinating LPS antigens and subsurface nonagglutinating antigens, such as the heat shock proteins GroEL and DnaK. EIAs are accordingly more broadly reactive than the MAT, although claims of genus-wide reactivity have not been unequivocally demonstrated. A common misconception arises about the significance of a positive IgM EIA test in humans, which is often erroneously taken to indicate a current infection. Because LPS is a dominant antigen in leptospires, it elicits a typical T-independent antibody response in which IgM predominates, even many months or years after infection. The presence of human anti-leptospiral IgM does not therefore necessarily indicate a current or even a recent infection. Certainly, a positive IgM EIA result provides evidence of previous infection, but in all cases the diagnosis must be confirmed by MAT. Because of the occurrence of both false positive and false negative reactions, reliance on IgM EIA as a sole serological test for leptospirosis is not recommended.

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Further Information

See the home page of the International Leptospirosis Society for additional information and links on various aspects of *Leptospira* and leptospirosis: (http://www.med.monash.edu.au/microbiology/staff/adler/ilspage.htm).

Literature Cited

Adler, B., A. M. Murphy, S. Locarnini, and S. Faine. 1980. Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. J. Clin. Microbiol. 11:452–457.

Adler, B., and S. Faine. 1983. A pomona serogroup-specific, agglutinating antigen in Leptospira, identified by monoclonal antibodies. Pathol. 15:247–250.

Adler, B., S. Faine, W. L. Christopher, and R. J. Chappel. 1986. Development of an improved selective medium for isolation of leptospires from clinical material. Vet. Microbiol. 12:377–381.

Alston, J. M., and J. C. Broom. 1958. Leptospirosis in Man and Animals. E. & S. Livingstone. Edinburgh London.

Anderson, D. L., and R. C. Johnson. 1968. Electron microscopy of immune disruption of leptospires: action of complement and lysozyme. J. Bacteriol. 95:2293–2309.

Auran, N. E., R. C. Johnson, and D. M. Ritzi. 1972. Isolation of the outer sheath of Leptospira and its immunogenic properties in hamsters. Infect. Immun. 5:968–975.

Ballard, S. A., M. Williams, B. Adler, and S. Faine. 1986. Interactions of virulent and avirulent leptospires with primary cultures of renal epithelial cells. J. Med. Microbiol. 21:59–67.

Ballard, S. A., R. P. A. M. Segers, N. Bleumink-Pluym, J. Fyfe, S. Faine, and B. Adler. 1993. Molecular analysis of the hsp (groE) operon of Leptospira interrogans serovar copenhageni. Molec. Microbiol. 8:739–751.

Ballard, S., M. Go, R. Segers, and B. Adler. 1998. Molecular analysis of the dnaK locus of Leptospira interrogans serovar Copenhageni. Gene 216:21–29.

Baril, C., and I. S. Girons. 1990. Sizing of the Leptospira genome by pulsed-field agarose gel electrophoresis. FEMS Microbiol. Lett. 71:95–100.

Baril, C., J. L. Herrmann, C. Richaud, D. Margarita, and I. Saint Girons. 1992. Scattering of the rRNA genes on the physical map of the circular chromosome of Leptospira

interrogans serovar icterohaemorrhagiae. J. Bacteriol. 174:7566–7571.

Belfaiza, J., A. Martel, D. Margarita, and I. Saint Girons. 1998. Direct sulfhydrylation for methionine biosynthesis in Leptospira meyeri. J. Bacteriol. 180:250–255.

Bey, R. F., N. E. Auran, and R. C. Johnson. 1974. Immunogenicity of whole cell and outer envelope leptospiral vaccines in hamsters. Infect. Immun. 10:1051–1056.

Bey, R. F., and R. C. Johnson. 1978a. Humoral immune responses of cattle vaccinated with leptospiral pentavalent outer envelope and whole culture vaccines. Amer. J. Vet. Res. 39:1109–1114.

Bey, R. F., and R. C. Johnson. 1978b. Protein-free and low-protein media for cultivation of Leptospira. Infect. Immun. 19:562–569.

Bey, R. F., and R. C. Johnson. 1982. Leptospiral vaccine in dogs: Immunogenicity of whole cell and outer envelope vaccines prepared in protein-free medium. Am. J. Vet. Res. 43:831–834.

Bey, R. F., and R. C. Johnson. 1983. Leptospiral vaccines: immunogenicity of protein-free medium cultivated whole cell bacterins in swine. Am. J. Vet. Res. 44:2299–2301.

Birch-Andersen, A., K. Hovind Hougen, and C. Borg-Petersen. 1973. Electron microscopy of Leptospira. 1: Leptospira strain pomona. Acta Pathol. Microbiol. Scand. 81:665–676.

Bolin, C. A., R. L. Zuerner, and G. Trueba. 1989. Effect of vaccination with a pentavalent leptospiral vaccine containing Leptospira interrogans serovar hardjo type hardjo-bovis on type hardjo-bovis infection of cattle. Am. J. Vet. Res. 50:2004–2008.

Bolin, C. A. 1996. Diagnosis of leptospirosis: a reemerging disease of companion animals. Semin. Vet. Med. Surg. (Small Animal) 11:166–171.

Bourhy, P., A. Martel, D. Margarita, I. Saint Girons, and J. Belfaiza. 1997. Homoserine O-acetyltransferase, involved in the Leptospira meyeri methionine biosynthetic pathway, is not feedback inhibited. J. Bacteriol. 179:4396–4398.

Boursaux-Eude, C., I. Saint Girons, and R. Zuerner. 1995. IS1500, an IS3-like element from Leptospira interrogans. Microbiol. 141:2165–2173.

Boursaux-Eude, C., I. Saint Girons, and R. Zuerner. 1998. Leptospira genomics. Electrophoresis 19:589–592.

Brenner, D. J., A. F. Kaufmann, K. R. Sulzer, A. G. Steigerwalt, F. C. Rogers, and R. S. Weyant. 1999. Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for Leptospira alexanderi sp. nov. and four new Leptospira genomospecies. Int. J. Syst. Bacteriol. 49:839–858.

Brenot, A., D. Trott, I. Saint Girons, and R. Zuerner. 2001a. Penicillin-binding proteins in Leptospira interrogans. Antimicrob. Agents Chemother. 45:870–877.

Brenot, A., C. Werts, C. Ottone, N. Sertour, N. W. Charon, D. Postic, G. Baranton, and I. Saint Girons. 2001b. First evidence for a restriction-modification system in Leptospira sp. FEMS Microbiol. Lett. 201:139–143.

Brown, H. C., and J. C. Broom. 1929. Observations upon electric charge in certain bacteriological problems. Brit. J. Exp. Pathol. 10:219–225.

Bulach, D. M., T. Kalambaheti, A. de la Pena-Moctezuma, and B. Adler. 2000. Functional analysis of genes in the rfb locus of Leptospira borgpetersenii serovar Hardjo subtype Hardjobovis. Infect. Immun. 68:3793–3798.

Burth, P., M. Younes-Ibrahim, F. H. Goncalez, E. R. Costa, and M. V. Faria. 1997. Purification and characterization of a Na+, K+ ATPase inhibitor found in an endotoxin of Leptospira interrogans. Infect. Immun. 65:1557–1560.

Cacciapuoti, B., L. Ciceroni, and D. Attard Barbini. 1991. Fatty acid profiles, a chemotaxonomic key for the classification of strains of the family Leptospiraceae. Int. J. Syst. Bacteriol. 41:295–300.

Chang, A., and S. Faine. 1970. Electron-microscopic evidence for reactions of axial filaments of Leptospira with IgM and IgG antibodies. Bull. WHO 43:571–577.

Chapman, A. J., B. Adler, and S. Faine. 1988. Antigens recognized by the human immune response to infection with Leptospira interrogans serovar hardjo. J. Med. Microbiol. 25:269–278.

Chapman, A. C., C. O. R. Everard, S. Faine, and B. Adler. 1991. Antigens recognized by the human immune response to severe leptospirosis in Barbados. Epidemiol. Infect. 107:143–155.

Chappel, R. J., R. W. Prime, B. D. Millar, L. J. Mead, R. T. Jones, and B. Adler. 1992. Comparison of diagnostic procedures for porcine leptospirosis. Vet. Microbiol. 30:151–163.

Charon, N. W., R. C. Johnson, and D. Peterson. 1974. Amino acid biosynthesis in the spirochete Leptospira: evidence for a novel pathway of isoleucine biosynthesis. J. Bacteriol. 117:203–211.

Chen, T. Z. 1985. Development and situation of and techniques for production of leptospirosis vaccine in China. Jap. J. Bacteriol. 40:755–762.

Christmas, B. W., D. G. Till, and J. M. Bragger. 1974. Dairy farm fever in New Zealand: isolation of L pomona and L hardjo from a local outbreak. New Zealand Med. J. 79:904–906.

Christopher, W. L., B. Adler, and S. Faine. 1982. Immunogenicity of leptospiral vaccines grown in protein-free medium. J. Med. Microbiol. 15:493–501.

Chu, K. M., R. Rathinam, P. Namperumalsamy, and D. Dean. 1998. Identification of Leptospira species in the pathogenesis of uveitis and determination of clinical ocular characteristics in south India. J. Infect. Dis. 177:1314–1321.

Cinco, M., E. Banfi, and M. R. Soranzo. 1981. Studies on the interaction between macrophages and leptospires. J. Gen. Microbiol. 124:409–413.

Cinco, M., and E. Banfi. 1983a. Activation of complement by leptospires and its bactericidal activity. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 254:261–265.

Cinco, M., and H. Banfi. 1983b. Interactions between human polymorphonuclear leukocytes and one strain of pathogenic Leptospira (Leptospira interrogans sp.) and one of saprophytic Leptospira (L. biflexa sp.). FEMS Microbiol. Lett. 19:51–54.

Cinco, M., S. Perticarari, G. Presani, A. Dobrina, and F. Liut. 1993. Biological activity of a peptidoglycan extracted from Leptospira interrogans: in vitro studies. J. Gen. Microbiol. 139:2959–2964.

Cinco, M., E. Vecile, R. Murgia, P. Dobrina, and A. Dobrina. 1996. Leptospira interrogans and Leptospira peptidoglycans induce the release of tumor necrosis factor alpha from human monocytes. FEMS Microbiol. Lett. 138:211–214.

Corin, R. E., E. Boggs, and C. D. Cox. 1978. Enzymatic degradation of H_2O_2 by Leptospira. Infect. Immun. 22:672–675.

de Brito, T., M. J. Prado, V. A. Negreiros, A. L. Nicastri, E. E. Sakata, P. H. Yasuda, R. T. Santos, and V. A. F. Alves. 1992. Detection of leptospiral antigen (L. interrogans serovar copenhageni serogroup Icterohaemorrhagiae) by immunoelectron microscopy in the liver and kidney of experimentally infected guinea-pigs. Int. J. Exp. Pathol. 73:633–642.

De la Peñ-Moctezuma, A., D. M. Bulach, T. Kalambaheti, and B. Adler. 1999. Comparative analysis of the LPS biosynthetic loci of the genetic subtypes of serovar Hardjo: Leptospira interrogans Subtype Hardjoprajitno and Leptospira borgpetersenii Subtype Hardjobovis. FEMS Microbiol. Lett. 177:319–326.

De la Peñ-Moctezuma, A., D. M. Bulach, T. Kalambaheti, and B. Adler. 2001. Genetic differences among the LPS biosynthetic loci of serovars of Leptospira interrogans and Leptospira borgpetersenii. FEMS Immunol. Med. Microbiol. 31:73–81.

Dikken, H., and E. Kmety. 1978. Serological typing methods of leptospires. *In:* T. Bergan and R. Norris (Eds.) Methods in Microbiology. Academic Press. New York, NY. 260–295.

Ellis, W. A. 1986. The diagnosis of leptospirosis in farm animals. *In:* W. A. Ellis and T. W. A. Little (Eds.) The present state of leptospirosis and control. Martinus Nijhoff. Dordrecht, The Netherlands. 13–31.

Ellis, W. A. 1991. Leptospira interrogans serovar bratislava infection in domestic animals. *In:* Y. Kobayashi (Ed.) Leptospirosis: Proceedings of the Leptospirosis Research Conference 1990. Hokusen-Sha. Tokyo, Japan. 20–33.

Ellis, W. A. 1995. International Committee on Systematic Bacteriology Subcomittee on the Taxonomy of Leptospira: Minutes of the Meetings, 1 and 2 July 1994, Prague, Czech Republic. Int. J. Syst. Bacteriol. 45:872–874.

Everard, C. O., C. N. Edwards, J. D. Everard, and D. G. Carrington. 1995. A twelve-year study of leptospirosis on Barbados. Eur. J. Epidemiol. 11:311–320.

Faine, S. 1964. Reticuloendothelial phagocytosis of virulent leptospires. Am. J. Vet. Res. 25:830–835.

Faine, S. 1982. Guidelines for the the control of leptospirosis. World Health Organization. Geneva, Switzerland.

Faine, S. 1998. Leptospirosis. *In:* W. J. Hausler and M. Sussman (Eds.) Topley and Wilson's Microbiology and Microbial Infections. Arnold. London, 849–869.

Faine, S., B. Adler, C. Bolin, and P. Perolat. 1999. Leptospira and Leptospirosis. MediSci. Melbourne, Australia.

Farrelly, H. E., B. Adler, and S. Faine. 1987. Opsonic monoclonal antibodies against lipopolysaccharide antigens of Leptospira interrogans serovar hardjo. J. Med. Microbiol. 23:1–7.

Fukunaga, M., and I. Mifuchi. 1989a. The number of large ribosomal RNA genes in Leptospira interrogans and Leptospira biflexa. Microbiol. Immunol. 33:459–466.

Fukunaga, M., and I. Mifuchi. 1989b. Unique organization of Leptospira interrogans rRNA genes. J. Bacteriol. 171:5763–5767.

Fukunaga, M., I. Horie, and I. Mifuchi. 1990. Isolation and characterization of the 5S rRNA gene of Leptospira interrogans. J. Bacteriol. 172:3264–3268.

Fukunaga, M., I. Horie, N. Okuzako, and I. Mifuchi. 1991. Isolation and characterisation of the 5S rRNA genes in Leptospira and Leptonema. *In:* Y. Kobayashi (Ed.) Leptospirosis: Proceedings of the Leptospirosis Research Conference 1990. Hokusen-sha. Tokyo, Japan. 290–302.

Fukunaga, M., I. Mifuchi, Y. Yanagihara, and N. Okuzako. 1992. Comparison of flanking regions of the 5S ribosomal ribonucleic acid genes in Leptospira biflexa and Leptospira interrogans. Chem. Pharm. Bull. (Tokyo) 40:544–546.

Gravekamp, C., H. Van de Kemp, M. Franzen, D. Carrington, G. J. Schoone, G. J. Van Eys, C. O. Everard, R. A. Hartskeerl, and W. J. Terpstra. 1993. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. J. Gen. Microbiol. 139:1691–1700.

Guerreiro, H., J. Croda, B. Flannery, M. Mazel, J. Matsunaga, M. G. Reis, P. N. Levett, A. I. Ko, and D. A. Haake. 2001. Leptospiral proteins pecognized during the humoral immune response to leptospirosis in humans. Infect. Immun. 69:4958–4968.

Gussenhoven, G. C., M. A. van der Hoorn, M. G. Goris, W. J. Terpstra, R. A. Hartskeerl, B. W. Mol, C. W. van Ingen, and H. L. Smits. 1997. LEPTO dipstick, a dipstick assay for detection of Leptospira-specific immunoglobulin M antibodies in human sera. J. Clin. Microbiol. 35:92–97.

Haake, D. A., E. M. Walker, D. R. Blanco, C. A. Bolin, J. N. Miller, and M. A. Lovett. 1991. Changes in the surface of Leptospira interrogans serovar grippotyphosa during in vitro cultivation. Infect. Immun. 59:1131–1140.

Haake, D. A., C. I. Champion, C. Martinich, E. S. Shang, D. R. Blanco, J. N. Miller, and M. A. Lovett. 1993. Molecular cloning and sequence analysis of the gene encoding OmpL1, a transmembrane outer membrane protein of pathogenic Leptospira spp. J. Bacteriol. 175:4225–4234.

Haake, D. A., C. Martinich, T. A. Summers, E. S. Shang, J. D. Pruetz, A. M. McCoy, M. K. Mazel, and C. A. Bolin. 1998. Characterization of leptospiral outer membrane lipoprotein LipL36: Downregulation associated with late-log-phase growth and mammalian infection. Infect. Immun. 66:1579–1587.

Haake, D. A., M. K. Mazel, A. M. McCoy, F. Milward, G. Chao, J. Matsunaga, and E. A. Wagar. 1999. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect. Immun. 67:6572–6582.

Haake, D. A. 2000a. Spirochaetal lipoproteins and pathogenesis. Microbiol. 146:1491–1504.

Haake, D. A., G. Chao, R. L. Zuerner, J. K. Barnett, D. Barnett, M. Mazel, J. Matsunaga, P. N. Levett, and C. A. Bolin. 2000b. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect. Immun. 68:2276–2285.

Henneberry, R. C., and C. D. Cox. 1970. β-oxidation of fatty acids by Leptospira. Can. J. Microbiol. 16:41–45.

Herrmann, J. L., E. Bellenger, P. Perolat, G. Baranton, and I. Saint Girons. 1992. Pulsed-field gel electrophoresis of NotI digests of leptospiral DNA: A new rapid method of serovar identification. J. Clin. Microbiol. 30:1696–1702.

Herrmann, J. L., and I. Saint-Girons. 1993. Value of pulsed-field gel electrophoresis for genetic and epidemiological studies of Leptospira and Borrelia. Meth. Molec. Cell. Biol. 4:63–67.

Herrmann, J. L. 1994. Genomic techniques for identification of Leptospira strains. Pathol. Biol. (Paris) 41:943–950.

Holt, S. 1978. Anatomy and chemistry of spirochetes. Microbiol. Rev. 42:114–160.

Hookey, J. V. 1992. Detection of Leptospiraceae by amplification of 16S ribosomal DNA. FEMS Microbiol. Lett. 15:267–274.

Hookey, J. V., J. Bryden, and L. Gatehouse. 1993. The use of 16S rDNA sequence analysis to investigate the phylogeny of Leptospiraceae and related spirochaetes. J. Gen. Microbiol. 139:2585–2590.

Hovind-Hougen, K. 1979. Leptospiraceae, a new Family to include Leptospira Noguchi 1917 and Leptonema, gen. nov. Int. J. Syst. Bacteriol. 29:245–251.

Hsu, D., M.-J. Pan, Y. C. Zee, and R. B. LeFebvre. 1990. Unique ribosome structure of Leptospira interrogans is composed of four rRNA components. J. Bacteriol. 172:3478–3480.

Inada, R., Y. Ido, R. Hoki, R. Kaneko, and H. Ito. 1916. The etiology, mode of infection, and specific therapy of Weil's disease (Spirochaetosis icterohaemorrhagica). J. Exp. Med. 23:377–402.

Isogai, E., H. Isogai, N. Fujii, and K. Oguma. 1990a. Biological effects of leptospiral lipopolysaccharide on mouse B, T and NK cells. Japan. J. Vet. Sci. 52:923–930.

Isogai, E., H. Isogai, N. Fujii, and K. Oguma. 1990b. Macrophage activation by leptospiral lipopolysaccharide. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 273:200–208.

Johnson, R. C., and P. Rogers. 1964a. 5-fluorouracil as a selective agent for the growth of leptospirae. J. Bacteriol. 88:422–426.

Johnson, R. C., and P. Rogers. 1964b. Metabolism of leptospirae. I: Utilization of amino acids and purine and pyrimidine bases. Arch. Biochem. Biophys. 107:459–470.

Johnson, R. C., and V. G. Harris. 1967. Differentiation of pathogenic and saprophytic leptospires. I: Growth at low temperatures. J. Bacteriol. 94:27–31.

Johnson, R. C., and V. G. Harris. 1968. Purine analogue sensitivity and lipase activity of leptospires. Appl. Microbiol. 16:1584–1590.

Johnson, R. C., V. G. Harris, and J. K. Walby. 1969. Characterization of leptospires according to fatty acid requirements. J. Gen. Microbiol. 55:399–407.

Johnson, R. C., B. P. Livermore, J. K. Walby, and H. M. Jenkin. 1970. Lipids of parasitic and saprophytic leptospires. Infect. Immun. 2:286–291.

Johnson, R. C., J. K. Walby, R. A. Henry, and N. E. Auran. 1973. Cultivation of parasitic leptospires: Effect of pyruvate. Appl. Microbiol. 26:118–119.

Johnson, R. C. 1976. Comparative spirochete phyisiology and cellular composition. The Biology of Parasitic Spirochetes. Academic Press. New York, NY. 39–48.

Jost, B. H., B. Adler, T. Vinh, and S. Faine. 1986. A monoclonal antibody reacting with a determinant on leptospiral lipolysaccharide protects guinea pigs against leptospirosis. J. Med. Microbiol. 22:269–275.

Jost, B. H., B. Adler, and S. Faine. 1988. Reaction of monoclonal antibodies with species specific determinants in Leptospira interrogans outer envelope. J. Med. Microbiol. 27:51–57.

Jost, B. H., B. Adler, and S. Faine. 1989. Experimental immunisation of hamsters with lipopolysaccharide antigens of Leptospira interrogans. J. Med. Microbiol. 29:115–120.

Kalambaheti, T., D. M. Bulach, K. Rajakumar, and B. Adler. 1999. Genetic organization of the lipopolysaccharide O-antigen biosynthetic locus of Leptospira borgpetersenii serovar Hardjobovis. Microb. Pathog. 27:105–117.

Kasarov, L. B., and L. Addamiano. 1969. Metabolism of the lipoproteins of serum by leptospires: degradation of the triglycerides. J. Med. Microbiol. 2:165–168.

Kefford, B., and K. C. Marshall. 1984. Adhesion of Leptospira at a solid-liquid interface: A model. Arch. Microbiol. 138:84–88.

Kelson, J. S., B. Adler, A. J. Chapman, and S. Faine. 1988. Identification of leptospiral flagellar antigens by gel electrophoresis and immunoblotting. J. Med. Microbiol. 26:47–53.

Kemenes, F. 1964. Cross-immunity studies on virulent strains of leptospires belonging to different serotypes. Zeitschr. Immunitätsf. Exp. Therap. 127:209–229.

Kojima, T., Y. Yanagihara, and I. Mifuchi. 1984. Detoxification of tween for the cultivation of Leptospira. Microbiol. Immunol. 28:949–954.

Kolk, A. H. J., J. Van Leeuwen, H. Korver, and W. J. Terpstra. 1984. Serotyping of leptospires by monoclonal antibodies. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 257: 515.

Korthof, G. 1932. Experimentelles Schlammfieber beim Menschen. Zentralbl. Bakteriol. Parasitenkd. [A] 125: 429–434.

Korver, H., A. H. J. Kolk, J. Vingerhold, J. van Leeuwen, and W. J. Terpstra. 1988. Classification of the icterohaemorrhagiae serogroup by monoclonal antibodies. Israel J. Vet. Med. 44:15–18.

Lee, S. H., K. A. Kim, Y. G. Park, I. W. Seong, M. J. Kim, and Y. J. Lee. 2000. Identification and partial characterization of a novel hemolysin from Leptospira interrogans serovar lai. Gene 254:19–28.

Letocart, M., G. Baranton, and P. Perolat. 1997. Rapid identification of pathogenic Leptospira species (Leptospira interrogans, L. borgpetersenii, and L. kirschneri) with species-specific DNA probes produced by arbitrarily primed PCR. J. Clin. Microbiol. 35:248–253.

Levett, P. N. 2001. Leptospirosis. Clin. Microbiol. Rev. 14:296–326.

Li, C., A. Motaleb, M. Sal, S. F. Goldstein, and N. W. Charon. 2000. Spirochete periplasmic flagella and motility. J. Molec. Microbiol. Biotechnol. 2:345–354.

Lucchesi, P. M., and A. E. Parma. 1999. A DNA fragment of Leptospira interrogans encodes a protein which shares epitopes with equine cornea. Vet. Immunol. Immunopathol. 71:173–179.

Marshall, R. B., E. S. Broughton, and J. S. Hellstrom. 1979. Protection of cattle against natural challenge with Leptospira interrogans serovar hardjo using a hardjo-pomona vaccine. New Zealand Vet. J. 27:114–116.

Marshall, R. B., B. Wilton, and A. J. Robinson. 1981. Identification of Leptospira serovars by restriction-endonuclease analysis. J. Med. Microbiol. 14:163–166.

Martinez Sanchez, R., A. Perez Sierra, M. Baro Suarez, A. M. Alvarez, J. Menendez Hernandez, M. Diaz Gonzalez, R. Cruz de la Paz, G. de los Reyes, B. Montoya Batista, G. Sierra Gonzalez, M. Armesto del Rio, A. Saltaren Cobas, and O. Sabournin Ramos. 2000. Evaluation of the effectiveness of a new vaccine against human leptospirosis in groups at risk. Revista Panama di Saluda Publica 8:385–392.

Masuzawa, T., R. Nakamura, Y. Beppu, and Y. Yanagihara. 1996. Immunochemical characteristics and localization on cells of protective antigen (PAg) prepared from Leptospira interrogans serovar lai. Microbiol. Immunol. 40:237–241.

Merien, F., P. Amouriaux, P. Perolat, G. Baranton, and I. Saint Girons. 1992. Polymerase chain reaction for detection of Leptospira spp. in clinical samples. J. Clin. Microbiol. 30:2219–2224.

Merien, F., G. Baranton, and P. Perolat. 1997. Invasion of Vero cells and induction of apoptosis in macrophages by pathogenic Leptospira interrogans are correlated with virulence. Infect. Immun. 65:729–738.

Merien, F., J. Truccolo, G. Baranton, and P. Perolat. 2000. Identification of a 36-kDa fibronectin-binding protein expressed by a virulent variant of Leptospira interrogans serovar icterohaemorrhagiae. FEMS Microbiol. Lett. 185:17–22.

Midwinter, A., S. Faine, and B. Adler. 1990. Vaccination of mice with lipopolysaccharide (LPS) and LPS-derived immuno-conjugates from Leptospira interrogans. J. Med. Microbiol. 33:199–204.

Midwinter, A., T. Vinh, S. Faine, and B. Adler. 1994. Characterization of an antigenic oligosaccharide from Leptospira interrogans serovar pomona and its role in immunity. Infect. Immun. 62:5477–5482.

Millar, B. D., R. J. Chappel, B. Adler, S. J. Driesen, and R. T. Jones. 1987. Effect of maternal vaccination on the susceptibility of growing pigs to leptospiral infection. Vet. Microbiol. 15:79–87.

Mitchison, M., J. I. Rood, S. Faine, and B. Adler. 1991. Molecular analysis of a Leptospira borpetersenii gene encoding an endoflagellar subunit protein. J. Gen. Microbiol. 137:1529–1536.

Mitchison, M., D. M. Bulach, T. Vinh, K. Rajakumar, S. Faine, and B. Adler. 1997. Identification and characterization of the dTDP-rhamnose biosynthesis and transfer genes of the lipopolysaccharide-related rfb locus in Leptospira interrogans serovar Copenhageni. J. Bacteriol. 179: 1262–1267.

Niikura, M., E. Ono, and R. Yanagawa. 1987. Molecular comparison of antigens on proteins of virulent and avirulent clones of Leptospira interrogans serovar copenhageni strain Shibaura. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 266:453–462.

Palmer, M., S. A. Waitkins, and W. Zochowski. 1984. Survival of leptospires in commercial blood culture systems. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 257:480–487.

Palmer, M. F., S. A. Waitkins, and S. W. Wanyangu. 1987. A comparison of live and formalised leptospiral microscopic agglutination test. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 265:151–159.

Perolat, P., F. Merien, W. A. Ellis, and G. Baranton. 1994. Characterization of Leptospira isolates from serovar hardjo by ribotyping, arbitrarily primed PCR, and mapped restriction site polymorphisms. J. Clin. Microbiol. 32:1949–1957.

Picardeau, M., A. Brenot, and I. Saint Girons. 2001. First evidence for gene replacement in Leptospira spp. Inactivation of L. biflexa flaB results in non-motile mutants deficient in endoflagella. Molec. Microbiol. 40:189–199.

Plesko, I., and C. Lataste-Dorolle. 1970. Intertype immunity relations of Leptospira strains belonging to the "Australis" serogroup. Biologia (Bratislava) 25:403–411.

Plesko, I., and Z. Hlavata. 1971. Cross immunity studies with lipase negative strains of leptospires. Biologia (Bratislava) 26:689–693.

Ralph, D., M. McClelland, J. Welsh, G. Baranton, and P. Perolat. 1993. Leptospira species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. J. Bacteriol. 175:973–981.

Rottini, G. D., M. Cinco, F. Panizon, and E. Agosti. 1972. Comportamento delle Ig seriche nell'uomo dopo vaccinazione con Leptospira saprofitica (ceppo Patoc 1). Giorn. Malatt. Infett. Parassit. 24:879–885.

Saint Girons, I., D. Margarita, P. Amouriaux, and G. Baranton. 1990. First isolation of bacteriophage for a spirochaete: potential genetic tools for Leptospira. Res. Microbiol. 141:1131–1138.

Saint Girons, I., S. J. Norris, U. Gobel, J. Meyer, E. M. Walker, and R. Zuerner. 1992. Genome structure of spirochetes. Res. Microbiol. 143:615–621.

Saint Girons, I., P. Bourhy, C. Ottone, M. Picardeau, D. Yelton, R. W. Hendrix, P. Glaser, and N. Charon. 2000. The LE1 bacteriophage replicates as a plasmid within Leptospira biflexa: construction of an L. biflexa-Escherichia coli shuttle vector. J. Bacteriol. 182:5700–5705.

Sakamoto, N., E. Ono, H. Kida, and R. Yanagawa. 1985. Production of monoclonal antibodies against leptospiral genus specific antigen and localization of the antigen by immunoelectron microscopy. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 259:557–563.

Schöberg, A., U. Käpe, and D. Rohloff. 1980. Der Einsatz von Hemmstoffen beim kulturellen Nachweis der Leptospiren in Schweinesperma. Berlin München Tierärztl. Wochenschr. 93:166–171.

Schöberg, A. 1983. Growth of 10 Leptospira interrogans serovars using polyvinylpyrrolidone(PVP)-treated tween in protein-free medium. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 254:540–544.

Schoone, G. J., C. O. R. Everard, H. Korver, D. G. Carrington, V. A. Inniss, J. Baulu, and W. J. Terpstra. 1989. An immunoprotective monoclonal antibody directed against Leptospira interrogans serovar copenhageni. J. Gen. Microbiol. 135:73–78.

Segers, R. P. A. M., d. D. A. van, N. A. de, P. Corcione, d. Z. B. A. M. van, and W. Gaastra. 1990. Molecular analysis of a sphingomyelinase C gene from Leptospira interrogans serovar hardjo. Infect. Immun. 58:2177–2185.

Segers, R. P., J. A. van Gestel, G. J. van Eys, B. A. van der Zeijst, and W. Gaastra. 1992. Presence of putative sphingomyelinase genes among members of the family Leptospiraceae. Infect. Immun. 60:1707–1710.

Shang, E. S., M. M. Exner, T. A. Summers, C. Martinich, C. I. Champion, R. E. Hancock, and D. A. Haake. 1995. The rare outer membrane protein, OmpL1, of pathogenic Leptospira species is a heat-modifiable porin. Infect. Immun. 63:3174–3181.

Shang, E. S., T. A. Summers, and D. A. Haake. 1996. Molecular cloning and sequence analysis of the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic Leptospira species. Infect. Immun. 64:2322–2330.

Shenberg, E. 1967. Growth of pathogenic Leptospira in a chemically defined media. J. Bacteriol. 93:1598–1606.

Shenberg, E., and M. Torten. 1973. A new leptospiral vaccine for use in man. I: Development of a vaccine from "Leptospira" grown on a chemically defined medium. J. Infect. Dis. 128:642–646.

Shim, Y. H., B. S. Shim, K. H. Choe, D. S. Kim, K. C. Shin, K. L. Lee, Y. W. Lee, Y. J. Kim, C. J. Chin, J. T. Lee, and I. S. Chai. 1980. Epidemic pulmonary hemorrhagic fever. I. Epidemiological and clinical observations. J. Korean Med. Assn. 23:131–144.

Stalheim, O. H. V. 1968. Vaccination against leptospirosis: Immunogenicity of viable, avirulent Leptospira pomona in hamsters, swine and cattle. Am. J. Vet. Res. 29:473–478.

Stamm, L. V., F. C. Gherardini, E. A. Parrish, and C. R. Moomaw. 1991. Heat shock response of spirochetes. Infect. Immun. 59:1572–1575.

Stern, N., E. Shenberg, and A. Tietz. 1969. Studies on the metabolism of fatty acids in Leptospira: the biosynthesis of DELTA 9 and DELTA11-monounsaturated acids. Eur. J. Biochem. 8:101–108.

Stimson, A. M. 1907. Note on an organism found in yellow-fever tissue. Publ. Hlth. Rep. 22:541.

Stimson, A. M. 1909. Notes on Stimson's spirochaete found in the kidney of a yellow fever case. Trans. Roy. Soc. Trop. Med. Hyg. 3:56.

Terpstra, W. J., G. S. Ligthart, and G. J. Schoone. 1980. Serodiagnosis of human leptospirosis by enzyme-linked immunosorbent-assay (ELISA). Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 247:400–405.

Terpstra, W. J., H. Korver, J. van Leeuwen, P. L. Klatser, and A. H. J. Kolk. 1985. The classification of sejroe group serovars of Leptospira interrogans with monoclonal antibodies. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 259:498–506.

Terpstra, W. J., H. Korver, G. J. Schoone, J. van Leeuwen, C. E. Schönemann, S. de Jonge-Aglibut, and A. H. J. Kolk. 1987. Comparative classification of Leptospira serovars of the Pomona group by monoclonal antibodies and restriction-endonuclease analysis. Zentralbl. Bakteriol. Mikrobiol. Hyg. [A] 266:412–421.

Thiermann, A. B., A. L. Handsaker, J. W. Foley, F. H. White, and B. F. Kingscote. 1986. Reclassification of North American leptospiral isolates belonging to serogroups Mini and Sejroe by restriction endonuclease analysis. Am. J. Vet. Res. 47:61–66.

Townsend, K. M., J. D. Boyce, J. Y. Chung, A. J. Frost, and B. Adler. 2001. Genetic organization of Pasteurella multocida cap loci and development of a multiplex capsular PCR typing system. J. Clin. Microbiol. 39:924–929.

Trueba, G. A., C. A. Bolin, and R. L. Zuerner. 1992. Characterization of the periplasmic flagellum proteins of Leptospira interrogans. J. Bacteriol. 174:4761–4768.

Uhlenhuth, P., and W. Fromme. 1915. Experimentelle Untersuchungen über die sogenannte Weilsche Krankheit (ansteckende Gelbsucht). Med. Klin. (München) 44:1202, 1264, and 1296.

Vinh, T., B. Adler, and S. Faine. 1982. The role of macrophages in the protection of mice against leptospirosis: In vitro and in vivo studies. Pathol. 14:463–468.

Vinh, T., S. Faine, and B. Adler. 1984. Adhesion of leptospires to mouse fibroblasts (L929) and its enhancement by specific antibody. J. Med. Microbiol. 18:73–85.

Vinh, T., B. Adler, and S. Faine. 1986. Ultrastructure and chemical composition of lipopolysaccharide extracted from Leptospira interrogans serovar copenhageni. J. Gen. Microbiol. 132:103–109.

Vinh, T., M.-H. Shi, B. Adler, and S. Faine. 1989. Characterization and taxonomic significance of lipopolysaccharides of Leptospira interrogans serovar hardjo. J. Gen. Microbiol. 135:2663–2673.

Wang, B., J. Sullivan, G. W. Sullivan, and G. L. Mandell. 1984. Interaction of leptospires with human polymorphonuclear neutrophils. Infect. Immun. 44:459–464.

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. Int. J. Syst. Bacteriol. 37:463–464.

Weil, A. 1886. Ueber eine eigenthümliche, mit Milztumor, Icterus und Nephritis einhergehende, acute Infectionskrankheit. Deutsch. Arch. Klin. Med. 39:209.

Werts, C., R. I. Tapping, J. C. Mathison, T. H. Chuang, V. Kravchenko, I. Saint Girons, D. A. Haake, P. J. Godowski, F. Hayashi, A. Ozinsky, D. M. Underhill, C. J. Kirschning, H. Wagner, A. Aderem, P. S. Tobias, and R. J. Ulevitch. 2001. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nature Immunol. 2:346–352.

Westfall, H. N., N. W. Charon, and Peterson. 1983. Multiple pathways for isoleucine biosynthesis in the spirochete Leptospira. J. Bacteriol. 154:846–853.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.

Woodward, M. J., and J. S. Redstone. 1994. Deoxynucleotide sequence conservation of the endoflagellin subunit protein gene, flaB, within the genus Leptospira. Vet. Microbiol. 40:239–251.

Yanagihara, Y., K. Kamisango, K. Takeda, I. Mifuchi, and I. Azuma. 1983. Identification of 4-o-methylmannose in cell wall polysachharide of Leptospira. Microbiol. Immunol. 27:711–715.

Yanagihara, Y., S. Kobayashi, and I. Mifuchi. 1984a. Sugar synthesis in Leptospira. I: Presence of glucosephosphate isomerase. Microbiol. Immunol. 28:189–196.

Yanagihara, Y., S. Kobayashi, and I. Mifuchi. 1984b. Sugar synthesis in Leptospira. II: Presence of glyoxalate cycle enzymes. Microbiol. Immunol. 28:529–534.

Yasuda, P. H., A. G. Steigerwalt, K. R. Sulzer, A. F. Kaufmann, F. Rogers, and D. J. Brenner. 1987. Deoxyribonucleic acid relatedness between serogroups and serovars in the family Leptospiraceae with proposals for seven new Leptospira species. Int. J. Syst. Bacteriol. 37:407–415.

Younes-Ibrahim, M., P. Burth, M. V. Faria, B. Buffin-Meyer, S. Marsy, C. Barlet-Bas, L. Cheval, and A. Doucet. 1995. Inhibition of Na,K-ATPase by an endotoxin extracted from Leptospira interrogans: a possible mechanism for the physiopathology of leptospirosis. C. R. Acad. Sci. (Paris) 318:619–625.

Yuri, K., Y. Takamoto, M. Okada, T. Hiramune, N. Kikuchi, and R. Yanagawa. 1993. Chemotaxis of leptospires to hemoglobin in relation to virulence. Infect. Immun. 61:2270–2272.

Zaki, S. R., and W.-J. Shieh. 1996. Leptospirosis associated with outbreak of acute febrile illness and pulmonary haemorrhage, Nicaragua, 1995: The Epidemic Working Group at Ministry of Health in Nicaragua. Lancet 347:535–536.

Zeigler, J. A., and W. VanEseltine. 1975. Isolation and chemical characterization of outer envelope of Leptospira pomona. Can. J. Microbiol. 21:1102–1112.

Zeilstra-Ryalls, J., O. Fayer, and C. Georgopoulos. 1991. The universally conserved GroE (Hsp60) chaperonins. Ann. Rev. Microbiol. 45:301–325.

Zuber, U., and W. Schumann. 1994. CIRCE, a novel heat-shock element involved in regulation of heat shock operon dnaK of Bacillus subtilis. J. Bacteriol. 176:1359–1363.

Zuerner, R. L. 1991. Physical map of chromosomal and plasmid DNA comprising the genome of Leptospira interrogans. Nucl. Acids Res. 19:4857–4860.

Zuerner, R. L., J. L. Herrmann, and I. Saint Girons. 1993. Comparison of genetic maps for two Leptospira interrogans serovars provides evidence for two chromosomes

and intraspecies heterogeneity. J. Bacteriol. 175:5445–5451.

Zuerner, R. L. 1994. Nucleotide sequence analysis of IS1533 from Leptospira borgpetersenii: identification and expression of two IS-encoded proteins. Plasmid 31:1–11.

Zuerner, R. L., D. Alt, and C. A. Bolin. 1995. IS1533-based PCR assay for identification of Leptospira interrogans sensu lato serovars. J. Clin. Microbiol. 33:3284–3290.

Zuerner, R. L., and C. A. Bolin. 1997. Differentiation of Leptospira interrogans isolates by IS1500 hybridization and PCR assays. J. Clin. Microbiol. 35:2612–2617.

Zuerner, R., D. Haake, B. Adler, and R. Segers. 2000. Technological advances in the molecular biology of Leptospira. J. Molec. Microbiol. Biotechnol. 2:455–462.

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CHAPTER 4.5

Termite Gut Spirochetes

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Introduction

There are few habitats on earth in which spirochetes are such prominent members of the microbial community as in the hindgut of termites. This was first documented over a century ago by Joseph Leidy (Leidy, 1874–1881; Leidy, 1877), who was struck by their abundance in hindgut contents of the eastern subterranean termite, *Termes* (now *Reticulitermes*) *flavipes*. In some termites, spirochetes account for up to 50% of all prokaryotes in the hindgut (Paster et al., 1996), and even casual phase contrast microscopic observation of hindgut contents usually reveals about a dozen different morphological types, distinguishable on the bases of cell size, wavelength and amplitude, or pitch (Fig. 1).

Their size range is striking: from cells measuring only 0.1 μm in diameter × 2.5 μm in length (i.e., at or near the limit of resolution of light microscopy) to many as large as 1.5 μm × 100 μm (Hollande and Gharagozlou, 1967; Bermudes et al., 1988; Paster et al., 1996). Thus, it is not surprising that termites were long ago recommended as an excellent source of spirochetes (and other diverse pro- and eukaryotic microbes) for classroom demonstrations (Beckwith and Light, 1927). Electron microscopy has revealed additional intriguing morphological features of some of the spirochetes, including the presence of crenulations (folds) and a sillon (deep groove) in the outer membrane, bundles of periplasmic flagella, cytoplasmic tubules, and tube-like polar appendages (Bermudes et al., 1988; Radek et al., 1992). Spirochetes are also fairly abundant in hindguts of *Cryptocercus punctulatus* (Grimstone, 1963; Wier et al., 2000), a wood-eating cockroach that, by most analyses, is closely related to termites phylogenetically (Nalepa and Bandi, 2000). However, not much work has been done on spirochetes of *C. punctulatus*, perhaps owing to the somewhat limited distribution of this insect (Cleveland et al., 1934).

In hindgut contents, spirochetes occur as individual, free-swimming cells within the gut fluid. In so-called "lower termites" (families Kalotermitidae, Mastotermitidae, Rhinotermitidae, Serritermitidae and Termopsidae), they also occur as epibionts attached end-on to the plasma membrane of flagellate protozoa, which are also abundant in such termites. Depending on the particular protozoan, the attached spirochetes may be uniformly distributed over the surface or localized in specific regions (Kirby, 1941; Ball, 1969; Radek et al., 1992; Radek et al., 1996; Rösel et al., 1996). Attachment appears to be facilitated by a structural modification of the protozoan plasma membrane and/or the end of the spirochetal cell (Cleveland and Grimstone, 1964; Bloodgood et al., 1974; Smith and Arnott, 1974; Smith et al., 1975a; Smith et al., 1975b; Bloodgood and Fitzharris, 1976; Radek et al., 1992; Radek et al., 1996). A spectacular consequence of such attachment is seen for *Mixotricha paradoxa*, a protozoan whose locomotion is conferred by a vestment of about 100,000 spirochetes, each inserted into the posterior face of bracket-like structures formed by evaginations of the plasma membrane (Cleveland and Grimstone, 1964). Although located primarily in the hindgut proper, spirochetes have also been observed among microvilli of the "mixed segment" of higher termites (family Termitidae; Bignell et al., 1983). The mixed segment is a relatively short region of the gut lying at the juncture of the midgut and hindgut: it is lined partially by microvilli of the midgut and partially by the cuticular lining of the hindgut.

The abundance of spirochetes in guts of healthy, vigorous termites showing no outward signs of disease implied that they were probably not pathogenic and might even benefit the termites. Evidence consistent with the latter possibility was obtained by Eutick and coworkers, who observed that elimination of spirochetes from the gut of the higher termite, *Nasutitermes exitiosus*, by treatment with metronidazole or by exposure to pure oxygen was accompanied by a rapid decrease in termite survival (Eutick et al., 1978). Although from such experiments it is not possible to conclude that spirochetes were the

Fig. 1. A wood-eating termite, *Reticulitermes flavipes* (top), is positioned next to a gut extracted from a separate individual (middle). Vertical white arrowhead indicates the juncture between the hindgut (left) and tubular midgut (right). Bottom is a phase contrast micrograph of diluted gut contents obtained by needle puncture of the bulbous "paunch" region of the hindgut at the point shown (circle). Numerous spirochetes of various size and shape (white arrows) are seen among protozoa (P), a few nonspirochete prokaryotes (black arrows), and a not-yet-digested wood particle (W). Note also spirochetes attached end-on to the surface of the protozoan (red arrow).

only members of the gut microbiota that were eliminated, most of the microscopically observable nonspirochetal microbes appeared to remain after such treatments, suggesting that spirochetes contributed in some way to termite vitality. Unfortunately, the nature of their putative contribution(s) remained obscure, as none had yet been isolated and studied in pure culture.

Ghidini and Archetti (1939) obtained an enrichment of what appeared to be spirochetes and other motile microbes from guts of *Reticulitermes lucifugus* by allowing the organisms to migrate as a band into the lateral arm of a culture tube filled with a medium containing Ringer's solution (composition not stated), 5% heat-inactivated rabbit serum, and 0.2–0.8% agar. However, although the spirochetes retained motility for several days, it is not clear that they actually grew in the medium or in subcultures made from the migrating band of microbes. No electron microscopy was done, but light micrographs included in their paper suggest that the spiral and undulate forms were indeed spirochetes. Most of the other published work on termite gut spirochetes over the past hundred years dealt with their occurrence in various termite species, their morphological diversity and ultrastructure, and their associations with hindgut protozoa, and over the years an assortment of new genus and species names were proposed for various types, largely on the basis of morphological properties. Early work on termite gut spirochetes has been reviewed elsewhere (Breznak, 1973; Breznak, 1984; Margulis and Hinkle, 1992).

In recent years, our understanding of termite gut spirochetes has taken a giant step forward. The nucleotide sequences of several hundred 16S rRNAs have been determined for spirochetes from a variety of termites by using cultivation-independent, molecular biological approaches. This has helped to clarify their phylogenetic diversity and their natural relationships to each other and to other spirochetes. Three years ago, the first pure cultures were obtained (Leadbetter et al., 1999). Yet even in the short time that such strains have been available, research on them has already revealed metabolic properties heretofore unrecognized in spirochetes (e.g., H_2/CO_2-acetogenesis and N_2 fixation) and, from that, some of the ways in which they contribute to termite nutrition. This chapter will describe a simple procedure (suitable for classroom use) for observing termite gut spirochetes, as well as methods and media that were used to isolate the first pure cultures. It will also summarize our knowledge of their phylogeny and our recently enlightened, but still limited, understanding of their physiology and biochemistry.

Microscopic Observation

Termite gut spirochetes are easily observed by light microscopy in diluted hindgut contents, liberated by puncturing the hindgut region of an extracted gut and allowing the contents to ooze into a drop or two of buffered salts solution on a microscope slide, which is then covered with a coverslip (Fig. 1). Termites can be obtained from a biological supply house (e.g., Carolina Science and Math, http://www.carolina.com) or collected from nature.

Extraction of termite guts is done by holding the insect by the thorax with one pair of forceps, and with another pair of fine-tipped forceps, grasping the extreme posterior portion (anus) perpendicular to the long body axis and pulling smoothly outward. The gut will often tear at or

near the foregut-midgut juncture so that only the hindgut, with a variable length of tubular midgut, is obtained. By chilling termites beforehand in a Petri dish held on crushed ice, they become almost completely immobile and easier to grasp with the forceps. Use of a dissecting microscope facilitates the degutting procedure, but is not absolutely necessary. The extracted hindgut is then placed on a glass slide and immediately covered with a small amount of anoxic buffered salts solution prior to being punctured with a needle or with the sharp tip of the same forceps used to remove the gut. One satisfactory buffered salts solution, BSS (below), is based on the major inorganic salts determined to be present in hindgut fluid of *Zootermopsis angusticollis* (Yamin, 1978).

"Worker" termites (i.e., externally undifferentiated larvae), which are typically the most abundant members of a colony, have the most robust, microbe-packed guts and are the best specimens to use for this procedure. For relatively small guts 1–2 µl in volume (e.g., those from subterranean termites such as *Reticulitermes* or *Coptotermes* species), it is not absolutely necessary to remove the guts or gut fragments prior to application of the coverslip. In fact, as most of the spirochetes appear to be sensitive to oxygen, the respiratory activity of the remaining gut tissue will help create and maintain anoxia. For relatively large termites (e.g., the western United States dampwood termite, *Zootermopsis angusticollis*), it is usually necessary to remove the gut from the slide after liberation of hindgut contents to allow the coverslip to lie flat. For extended observation, the edges of the coverslip can be sealed with petroleum jelly or nail polish to retard evaporation. Spirochetes in such preparations often retain their motility for several hours. They are best viewed by using phase contrast or darkfield microscopy at 400–1000X final magnification. However, many can be seen with brightfield illumination at 100–200X by using inexpensive student microscopes. If preparations are made from lower termites, the coverslip should be applied gently and without forceful compression so as to retain the morphological integrity and motility of the flagellate protozoa, which are also interesting to observe.

Phylogeny

The first insights into the phylogeny of termite gut spirochetes came from analysis of spirochetal 16S rRNA-encoding genes (16S rDNAs) cloned after polymerase chain reaction (PCR) amplification from termite gut DNA. The nucleotide sequence of the first clone so obtained, clone MDS1 from the Australian termite *Mastotermes darwiniensis* (family Mastotermitidae), grouped within the genus *Treponema*, but it was not closely related to any known treponeme (Berchtold et al., 1994). This report was soon followed by reports of numerous additional spirochete 16S rDNA clones from *M. darwiniensis* (Berchtold and Köig, 1996), as well as from *Nasutitermes lujae* (family Termitidae) (Paster et al., 1996), *Reticulitermes speratus* (family Rhinotermitidae; Ohkuma and Kudo, 1996a) and *Cryptotermes domesticus* (family Kalotermitidae; Ohkuma and Kudo, 1998), and all of those clones grouped within the genus *Treponema* as well. As part of some of these investigations, fluorescent, 16S rRNA-targeted oligonucleotide probes were designed on the basis of the nucleotide sequences of the rDNA clones, and the probes were shown to react with hindgut spirochetes (Berchtold and Köig, 1996; Paster et al., 1996), thereby confirming the spirochetal origin of the cloned rDNAs.

In 1999, Lilburn and coworkers analyzed nearly 300 spirochetal 16S rDNA clones obtained from termites representing five of the seven termite families (Lilburn et al., 1999). The emergent picture was that although all clones could be grouped in the genus *Treponema*, none were closely related (i.e., all bore ≤91% sequence similarity) to any known treponeme or to any 16S rDNA clone derived from not-yet-cultured treponemes. Moreover, the clones were remarkably diverse at what could be considered the "species-level," a result consistent with the morphological diversity of spirochetes seen in termite guts. Conservative estimates implied that individual termite species such as *Reticulitermes flavipes* contain as many as 21 different species of *Treponema*. However, phylotype rank-abundance plots analyzed by methods used for macroecological systems (Tokeshi, 1993) implied that the distribution of clone types in *R. flavipes* was quite equitable. This may suggest either that the termite gut ecosystem is not in equilibrium or that there exists in the gut an assortment of niches capable of being filled by different species of treponemes. The data also revealed the existence of at least two major phylogenetic groups of treponemes: one (the traditional treponemes) consisting of all currently known isolates of *Treponema* from animals other than termites, as well as a large number of spirochetal 16S rDNA clones from the human gingival crevice and from digital dermatitis lesions of cattle, but containing a minority of termite gut clones; and another, termed the "termite cluster," containing the vast majority of termite gut clones (Fig. 2), as well as the free-living spirochetes *Spirochaeta stenostrepta* and the thermophile *S. caldaria*.

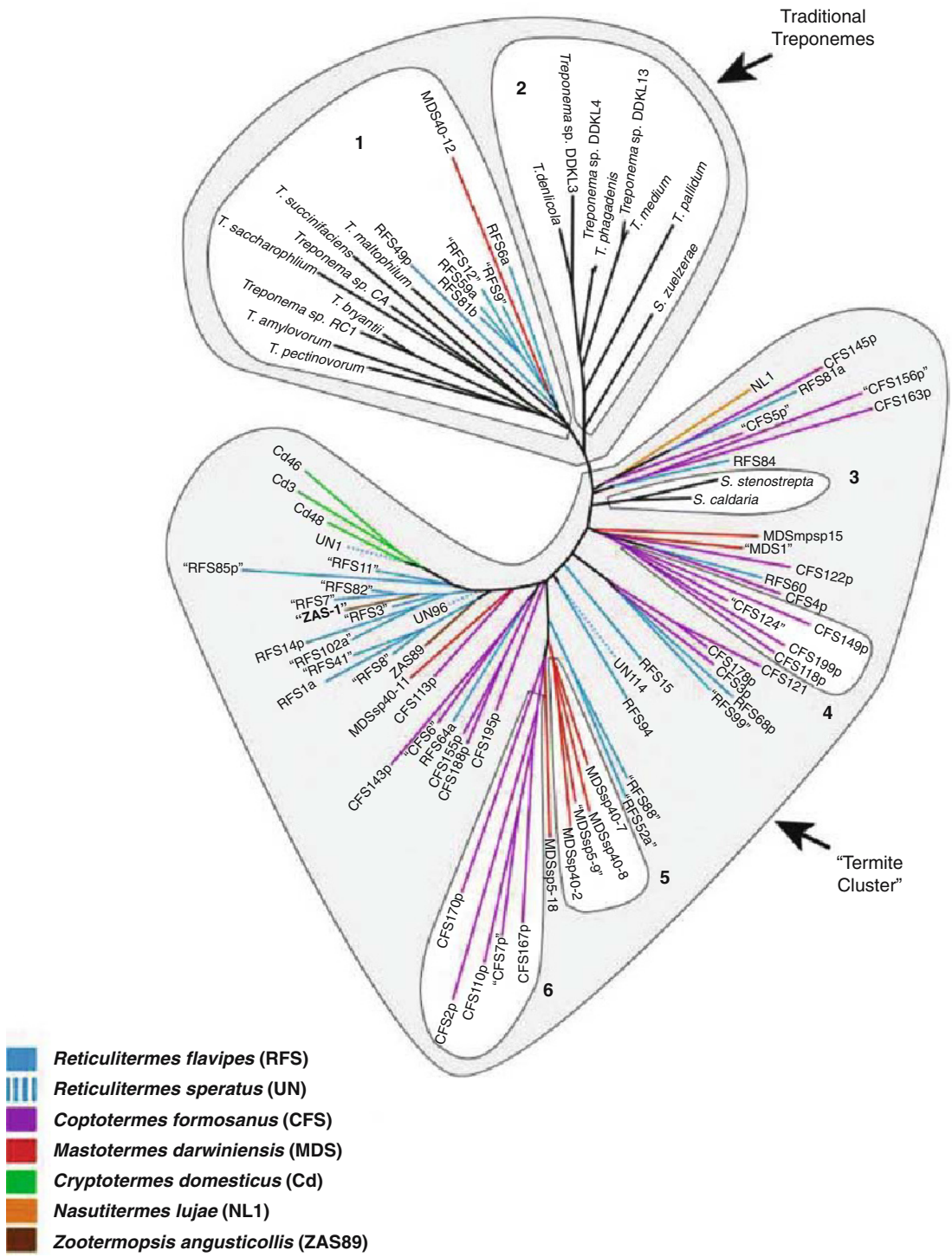

Fig. 2. A radial phylogenetic tree based on 16S rRNA sequence analysis of individual termite gut spirochete phylotypes or (in quotation marks) groups of closely related phylotypes color-coded to the termite species (bottom left) from which they were obtained. The latter are representatives from five of the seven known termite families. Branches in black were used for known species of *Treponema* and *Spirochaeta* and for treponemal phylotypes inferred from cloned 16S rDNAs. Within the two major groupings (the Traditional Treponemes and the "Termite Cluster" of treponemes), subgroups 1 and 2 represent treponemal subgroups defined by Paster et al. (1991); subgroup 3 represents the *Spirochaeta* subgroup; and subgroups 4, 5 and 6 represent closely related phylotypes from *C. formosanus* and *M. darwiniensis*. *Leptospira noguchii* (not shown) was used as the outgroup. A scale bar is not included, because some of the branch lengths were distorted during construction. Modified from Lilburn et al. (1999), which should be consulted for further details.

(The latter two, as well as *S. zuelzerae*, were named before their 16S rRNA sequences were known. Inasmuch as they were free-living anaerobes, they were assigned to the genus *Spirochaeta*. However, they group within the genus *Treponema* on the basis of their 16S rRNA sequences; Paster et al., 1991.)

Four nucleotide signatures were identified that almost perfectly distinguished members of the "termite cluster" from other treponemes. These were (*Escherichia coli* numbering): 289-GC-311; A at 812; and an inserted nucleotide at 1273. The first hints of possible coevolution between termites and their hindgut spirochetes were also seen, i.e., some subgroups of clones derived from a particular termite species were more closely related to each other than to clones derived from other termite species (subgroups 4, 5 and 6; Lilburn et al., 1999; see Fig. 2). However, there were also numerous exceptions (Lilburn et al., 1999). This was observed by Ohkuma and coworkers as well, who concluded that patterns of evolution between spirochetes and termites were far from simple (Ohkuma et al., 1999a). Nevertheless, it was becoming apparent that the roughly 2000 species of termites on earth constituted an enormous reservoir of novel treponemal diversity.

Isolation

Background and General Procedures

In 1999, the first stable and transferable enrichment cultures of termite gut spirochetes were established from hindgut contents of *Zootermopsis angusticollis* (family Termopsidae), and from them the first pure cultures of termite gut spirochetes were obtained: *Treponema* sp. strains ZAS-1 (Fig. 3a, b and c) and ZAS-2 (Leadbetter et al., 1999).

A third strain, ZAS-9 (Fig. 3d and e) was subsequently isolated by direct inoculation of hindgut contents into an agar dilution series (Lilburn et al., 2001).

The composition of the original enrichment and isolation media and improved growth media for routine cultivation of ZAS strains is given below. However, it is worth relating some characteristics of the initial enrichment cultures, as well as the methods used to monitor their progress and to isolate spirochetes from them, as this may aid future efforts. One factor that probably contributed to the success of initial enrichments was the inclusion of rifamycin and fosfomycin in media (two drugs to which spirochetes are intrinsically resistant; Stanton and Canale-Parola, 1979; Wyss et al., 1996). Another was supplementation of media with nutrient broth and pre-fermented, pH neutralized, clarified rumen fluid as a source of growth factors. Pre-fermentation of rumen fluid was done in an effort to minimize the amount of readily fer-

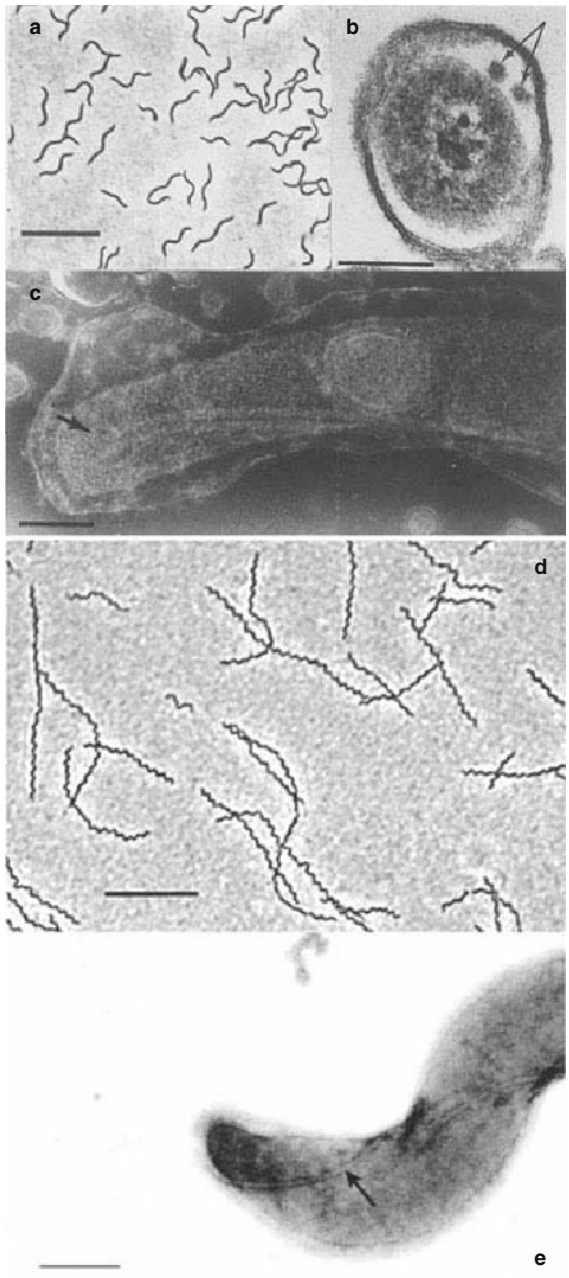

Fig. 3. Morphology of termite gut *Treponema* strain ZAS-1 (a, b and c), which is similar to strain ZAS-2 (not shown) and strain ZAS-9 (d and e). Arrows point to periplasmic flagella. Marker bars correspond to 10 μm (a and d; phase contrast micrographs) and 0.1 μm (c, d and e; electron micrographs). Panels a, b and c are from Leadbetter et al. (1999); panels d and e were provided by J. R. Graber. All were used by permission. Reprinted with permission from Leadbetter et al., *Science* 283, 686, Fig. 1. Copyright 1999 American Association for the Advancement of Science.

mentable carbohydrate present and hence discourage overgrowth of spirochetes by undesired fermentative forms (a problem that plagued many previous isolation attempts in our laboratory even when rifamycin was used to suppress nonspirochetal bacteria). A third factor was the use of an anoxic headspace composed of 80% H_2/20% CO_2 (a mixture that later proved to be an energy source for some of the isolates), along with the inclusion of bromoethanesulfonate in the medium to inhibit H_2-utilizing methanogens (Oremland, 1988). A fourth factor was patience. Successful enrichments took a long time to develop (10–12 weeks at room temperature) and even then did not result in much turbidity (maximum $OD_{600nm} \approx 0.1$; 18 mm light path). Enrichments were periodically checked for growth by viewing the rubber-stoppered, anoxic culture tubes or bottles when held near a diffusely sunlit window in an otherwise unlit room. The elongated, slender nature of spirochetal cells tended to impart a convective or silky appearance to the liquid medium when swirled, and so cultures were qualitatively examined for this property. Samples of culture fluid were also monitored microscopically, by using darkfield or phase contrast optics at 100X or 400X magnification. Periodic microscopy of incipient enrichment cultures exhibiting little or no visible turbidity was useful in evaluating which of a variety of test media formulations supported the longest retention of spirochete motility and morphological integrity and an increase in spirochete biomass. The latter was seen as an increase in the length of cells and the appearance of division stages. Microscopic examination also enabled an estimation of the number of spirochetes relative to nonspirochetal microbes and revealed when spirochete growth and motility were slowing, thereby prompting subculture into fresh medium.

It was noticed that growth of spirochetes in successful enrichments was accompanied by consumption of H_2 and CO_2 from the headspace and production of near stoichiometric amounts of acetate, suggesting that some of the spirochetes might be capable of performing H_2/CO_2-acetogenesis (i.e., $4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$ [$\Delta G^{o\prime} = -105$ kJ per mol acetate]), an assumption that later proved to be correct (see below). Consumption (or production) of headspace gas in rubber-stoppered culture vessels is easily measured by using graduated glass syringes, whose matched barrel and plunger surfaces are lubricated with a thin application of Sigmacote (Sigma-Aldrich, St. Louis, MO) so as to be gas tight but slide easily. These are fitted with a sterile, disposable filter unit (0.22 μm pore diameter) and a 25-gauge needle. The syringe is flushed several times with the sterile anoxic gas of choice and left half-filled on first insertion through the rubber stopper, whereupon the pressure is allowed to return to 1 atmosphere by allowing the plunger to glide to rest. If the cells use the headspace gas as a substrate, a syringe filled with that gas will simultaneously replenish the amount consumed. The size of syringe used depends on the volume of the headspace in the vessel and the extent of gas production or consumption. In general, the syringes used have a capacity of 10–20% of the headspace being measured. Syringe methods for the isolation and manipulation of anaerobes are described by Hungate (1969).

In such enrichments, spirochetes eventually grew to outnumber other bacteria by about 50 : 1, and from these the first pure cultures (*Treponema* strains ZAS-1 and ZAS-2) were isolated by performing dilution series in rubber stoppered tubes of the same medium solidified with agar. It was later found that spirochetes could be isolated directly from hindgut contents in agar dilution series without prior enrichment (*Treponema* strain ZAS-9). Agar tubes should be incubated upside down (mouth side down) to allow the water exuded from the agar to accumulate on the rubber stopper, from which it can be periodically removed by using a sterile syringe and needle. This will keep the surface of the agar relatively dry, facilitating subsequent picking of subsurface colonies for isolation of pure cultures. Sterile syringes can also be used to monitor gas consumption and production within the tubes, as described above. The general procedure for performing agar dilution series (which can be done by using rubber stoppered tubes and sterile, O_2-free gas-flushed syringes and needles) and for picking subsurface colonies with a drawn out Pasteur pipette is described by Hungate (1969) and by Widdel and Bak (see Gram-Negative Mesophilic Sulfate-Reducing Bacteria in the second edition).

In agar dilution series incubated at room temperature, colonies of spirochetes took 2–3 months to develop. Isolated colonies were spherical, and their diameter (1–3 mm) increased with time. They were semi-transparent and difficult to see and were best observed in diffuse light (as described above). Colonies of strains ZAS-1 and ZAS-2 growing under an atmosphere of 80% H_2/20% CO_2 were larger near the meniscus owing to their use of inwardly diffusing H_2 as an energy source. By contrast, colonies of strain ZAS-9 (which produces H_2) were accompanied by gas bubbles or cracks in the agar gel.

In pure culture, the growth rate and yield of isolated strains were greatly improved by replacing the rumen fluid and nutrient broth components of the isolation medium with yeast autolysate and by supplementation of the medium with additional cofactors (2YACo

medium and 4YACo medium, below) and, for strain ZAS-9 (which is not an H_2/CO_2-acetogen), by provision of a mixture of disaccharides.

Nine additional "ZAS" strains have since been isolated in our laboratory from *Z. angusticollis*, and six "RFS" strains have been isolated from *R. flavipes* (J. A. Breznak, unpublished data). However, these 15 strains still grow very poorly and hence have not yet been studied in detail. By contrast, we have been unsuccessful in establishing enrichment cultures or performing direct isolation of spirochetes from hindgut contents of the Formosan subterranean termite, *Coptotermes formosanus*, which is in the same family (Rhinotermitidae) as *R. flavipes*. Clearly, much remains to be learned about the nutritional and physiological idiosyncrasies of termite gut spirochetes.

Media for Observation, Enrichment, Isolation and Cultivation

For routine microscopic examination of termite hindgut contents, the following medium is recommended.

Buffered Salts Solution (BSS; Breznak and Switzer, 1986)

K_2HPO_4	2.5 g
KH_2PO_4	1.0 g
KCl	1.6 g
NaCl	1.4 g
Distilled water	999 ml

Adjust to pH 7.2; deoxygenate by boiling and cooling under 100% N_2; add deoxygenated 1 M dithiothreitol to a final concentration of 1 mM. Store in a butyl rubber-stoppered tube or serum bottle under 100% N_2.

Use the following for enrichment and isolation of termite gut *Treponema* strains ZAS-1 and ZAS-2.

Modified JM-4 Medium (Leadbetter et al., 1999)

NaCl	1.0 g
KCl	0.5 g
$MgCl_26H_2O$	0.4 g
$CaCl_22H_2O$	0.1 g
NH_4Cl	10.3 g
KH_2PO_4	0.2 g
Na_2SO_4	0.15 g
Nutrient broth (Difco)	2.0 g
Bromoethane sulfonate (BES), Na salt	10.0 g
Bovine rumen fluid (below)	170 ml
0.1 M 3-*N*-[morpholino]propanesulfonate (MOPS), pH 7.2	100 ml
Distilled water	686 ml
1.0 M $NaHCO_3$ (below)	69 ml
Trace element mixture with EDTA (below)	1 ml
Selenite-tungstate solution (below)	1 ml
Six-vitamin solution (below)	1 ml
Vitamin B_{12} Solution (below)	1 ml
Rifamycin-fosfomycin solution (below)	10 ml

The medium is prepared by using procedures similar to those described for cultivation of other anaerobic microbes (see Gram-Negative Mesophilic Sulfate-Reducing Bacteria in the second edition). All components except for the last six solutions are combined, autoclaved, and cooled under a gas phase of 80% N_2/20% CO_2, after which the other ingredients are added, and the pH is adjusted to 7.2–7.3. The headspace is then changed to 80% H_2/20% CO_2 for dispensing into culture tubes or larger vessels, which are subsequently sealed with butyl rubber stoppers. Prior to inoculation, the medium is reduced by adding 100 mM dithiothreitol (separately sterilized under 100% N_2) to a final concentration of 1 mM. Resazurin (0.1 mg per liter, final concentration) may be incorporated into the medium (if desired) as a redox indicator. Solid media are prepared by incorporating agarose (Gibco/Invitrogen, Carlsbad, CA) at a final concentration of 8.0–10.0 grams per liter.

Bovine Rumen Fluid

This is obtained from a fistulated, forage-fed dairy cow. Large particles are removed by wringing the fluid through two layers of cheesecloth immediately after collection and the expressed liquid is pooled in an Erlenmeyer flask, whereupon it is left to ferment overnight at 37°C in a water bath in a vented fume hood. The fermented liquid is adjusted to pH 7.0 with NaOH, clarified by centrifugation, and autoclaved under 100% N_2.

1.0 M $NaHCO_3$, Trace Element Mixture with EDTA, Selenite-Tungstate Solution and Vitamin B_{12} Solution

For composition, see Widdel and Bak, Gram-Negative Mesophilic Sulfate-Reducing Bacteria, in the second edition.

Six-Vitamin Solution (Widdel and Pfennig, 1981)

4-Aminobenzoic acid	4 mg
Nicotinic acid	10 mg
Calcium-D(+)-pantothenate	5 mg
Pyridoxine HCl	15 mg
Thiamine HCl	10 mg
Biotin	1 mg
Distilled water	80 ml

While being stirred, the mixture is neutralized by dropwise addition of 0.1 M NaOH, which facilitates solubilization of all constituents. The solution is then brought up to 100 ml with distilled water, filter-sterilized, and stored at 4°C.

Rifamycin-Fosfomycin Solution

Rifamycin SV (Na salt)	1.0 g
Fosfomycin	1.0 g
Distilled water	100 ml

This solution is filter-sterilized and stored at 4°C.

The following media are also for cultivation of *Treponema* strains ZAS-1 and ZAS-2.

2YACo and 4YACo Media (Leadbetter et al., 1999)

These are similar to modified JM-4 medium (above), except rifamycin-fosfomycin solution is omitted, and rumen fluid and nutrient broth are replaced in

2YACo medium by 140 ml of distilled water, 20 ml of yeast autolysate (below) and 20 ml of cofactor solution (below), and in

4YACo medium by 120 ml of distilled water, 40 ml of yeast autolysate and 20 ml of cofactor solution.

Yeast Autolysate Solution

> Preparation is similar to that described for inclusion in the OTI medium of Smibert (Miller et al., 1992). Fifty-six grams of dry baker's yeast (Red Star Yeast and Products, Milwaukee, WI) are suspended in 200 ml of distilled water in an Erlenmeyer flask and allowed to autolyse for 24 h at 56°C with occasional swirling of the contents. Cell debris is then removed by centrifugation, and the supernatant liquid is neutralized with 5 N NaOH, filter-sterilized, and stored under 100% N_2 at 4°C.

Cofactor Solution

> This includes a mixture of cofactors used in a defined medium for cultivating oral anaerobes (Wyss, 1992) and contains (mg per 100 ml of stock solution): thiamine pyrophosphate, 250; pyridoxal HCl and pyridoxal phosphate, 25 each; calcium folinic acid, β-NAD, coenzyme A and FAD, 5 each; nicotinamide, 2.5; folic acid, 0.25; riboflavin, 0.05; and 10 ml of hemin (650 μg/ml in 10 mM NaOH). It is filter-sterilized and stored under 100% N_2 at 4°C.

The following is recommended for the cultivation of *Treponema* strain ZAS-9.

SYACo Medium

> The composition is identical to that of 2YACo medium, except that it includes a mixture of disaccharides (cellobiose, maltose, sucrose and trehalose, 5 mM each; added from a 50X, filter-sterilized stock solution under 100% N_2) and is incubated under a headspace of 80% N_2/20% CO_2.

For studies of nitrogen fixation, cells were grown in homologous media (above), except that tungstate was omitted from the selenite-tungstate solution; $Na_2MoO_42H_2O$ present in the trace element mixture with EDTA was increased from 36 mg (equiv. to 0.15 μM in the complete medium) to 1200 mg (5 μM); and exogenous NH_4Cl was omitted for derepression of nitrogenase (Lilburn et al., 2001).

Properties of Isolated Strains

Treponema strains ZAS-1 and ZAS-2 are similar in morphology and size (0.2 μm × 3–7 μm), and both have two periplasmic flagella, each inserted at opposite ends and overlapping for most of the length of the cell (Fig. 3a, b and c). Both are homoacetogens capable of H_2/CO_2-acetogenesis, and cell extracts of both possess CO dehydrogenase, formate dehydrogenase and hydrogenase activities (Leadbetter et al., 1999), implying that H_2/CO_2-acetogenesis probably occurs via the Wood/Ljungdahl (acetyl CoA) pathway (Drake, 1994).

In 4YACo medium under a headspace of 80% H_2/20% CO_2 at 30°C, ZAS-1 grows with a doubling time of 23–24 h and achieves cell densities of 1.4×10^9 cells/ml, whereas ZAS-2 grows with a doubling time of about 48 h and achieves cell densities of about 3×10^8 cells/ml. However, although both strains consume H_2 during growth in 4YACo medium, the growth of ZAS-2 is strictly dependent on the presence of H_2 in the headspace, whereas ZAS-1 grows equally well in the absence of H_2 by using as yet unknown constituents of yeast autolysate as fermentable substrates. Little or no growth of either strain occurs if the cofactor solution or yeast autolysate is omitted from the medium, and for as yet unknown reasons, yeast autolysate prepared in the laboratory could not be replaced by a variety of commercial yeast extracts and autolysates (Leadbetter et al., 1999).

Treponema strain ZAS-9 is easily distinguished from strains ZAS-1 and ZAS-2 morphologically (Fig. 3d). Cells of ZAS-9 are longer (0.2 μm × 10–15 μm) and appear more tightly coiled, i.e., their wavelength or cell body pitch (1.2 μm) is substantially shorter than that of ZAS-1 and ZAS-2 (3.8 μm). Nevertheless, like ZAS-1 and ZAS-2, ZAS-9 also possesses two periplasmic flagella (Fig. 3e). ZAS-9 also differs from ZAS-1 and ZAS-2 physiologically. It is not a homoacetogen, but produces a mixture of acetate, ethanol and hydrogen (and presumably also CO_2) during sugar fermentation, and it is incapable of growth on $H_2 + CO_2$. During growth in SYACo medium at 30°C, strain ZAS-9 exhibits a doubling time of 46 h and attains a density of about 7×10^8 cell/ml (Graber and Breznak, 2000). When grown under nitrogen-fixing conditions (above), ZAS-9 exhibits N_2-dependent growth, and the levels of nitrogenase activity (1.2 μg N_2 fixed per h per mg protein; estimated by acetylene reduction) are much greater than those exhibited by strains ZAS-1 and ZAS-2 (<0.1 μg N_2 fixed per h per mg protein). Fixation of $^{15}N_2$ by ZAS-9 was demonstrated (Lilburn et al., 2001). Conditions have not yet been established to demonstrate N_2-dependent growth by strains ZAS-1 and ZAS-2.

Treponema strains ZAS-1, ZAS-2 and ZAS-9 all group within the "termite cluster" of treponemes (Fig. 4).

Ecology

Acetogenesis

The production of acetate by termite gut spirochetes, either by homoacetogenesis or heteroacetogenesis, implies an important role for them in the nutrition of termites, which use microbially produced acetate as a major carbon and energy source. However, the performance of H_2/CO_2-acetogenesis by termite gut spirochetes, although offered as a speculation nearly 25 years earlier (Breznak, 1973), was still surprising because this mode of metabolism had never been described in spirochetes. Nevertheless, this met-

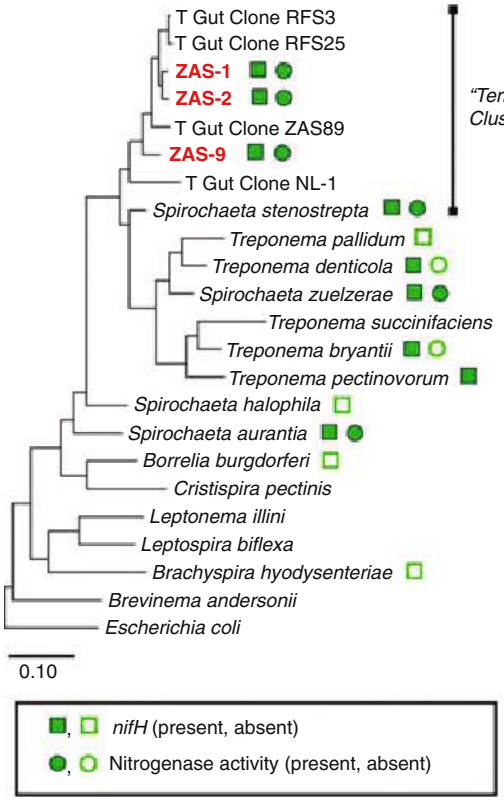

Fig. 4. Phylogeny of selected spirochetes and termite gut spirochete 16S rDNA clones (T Gut Clone) showing the distribution of *nifH* and nitrogenase activity within this phylum. The tree was inferred by maximum likelihood analysis of 16S rDNAs from isolated termite gut *Treponema* strains ZAS-1, ZAS-2 and ZAS-9 (red), from other representative host-associated and free-living spirochetes, and from cloned 16S rDNA genes obtained by PCR amplification from gut contents of the termites *Zootermopsis angusticollis* (prefix ZAS), *Reticulitermes flavipes* (RFS) and *Nasutitermes lujae* (NL). The homologous sequence from *E. coli* was used as the outgroup. Scale bar represents units of evolutionary distance and is based on sequence divergence. Absence of a symbol next to the species name indicates that the spirochete was not examined for the property. Modified from Lilburn et al. (2001).

abolic property helps explain the enigmatic dominance of H_2/CO_2-acetogenesis over H_2/CO_2-methanogenesis in the hindgut of many wood-feeding termites (Breznak, 1994). Microelectrode-determined, radial profiles of H_2 gradients in hindguts of wood-feeding termites such as *R. flavipes* had revealed that the highest concentrations of H_2 (up to 50,000 parts per million by volume [ppmv]) occurred in the luminal region (Ebert and Brune, 1997), being produced there largely by the protozoa in "lower" termites (such as *R. flavipes*) and by prokaryotes in "higher" termites. This is also the region of the hindgut in which spirochetes were most abundant. By contrast, H_2-consuming methanogens (and most other non-spirochetal prokaryotes) in *R. flavipes* were situated on or near the hindgut epithelium (Leadbetter and Breznak, 1996), where H_2 concentrations were lowest (ca. 3,000 ppmv). Thus, it appears that the dominance of H_2/CO_2-acetogenesis reflects, in large part, the spatial separation of the H_2-consuming populations, with H_2/CO_2-acetogenic spirochetes consuming most of the H_2 at its source of production and wall-associated methanogens using what H_2 is left over. That wall-associated methanogens are indeed limited for H_2 was shown by the fact that externally supplied H_2 stimulated methane emission (Ebert and Brune, 1997; Messer and Lee, 1989), but it had virtually no effect on in situ rates of acetogenesis from CO_2 (Tholen and Brune, 2000). The importance of microbial spatial relationships on the functioning of the termite gut microbiota has recently been discussed in detail (Brune and Friedrich, 2000).

The ability of spirochetes to conserve the energy of H_2 oxidation suggests that attachment of spirochetes to the surface of hindgut protozoa probably reflects a strategy used by some of them to remain close to major sites of H_2 production. Recent elegant experiments with fluorescent, rRNA-targeted oligonucleotide probes suggest that attachment to protozoa is limited to distinct phylogenetic types of spirochetes (Iida et al., 2000). However, the fact that strain ZAS-9 produces, but does not consume, H_2 implies that interspecies transfer of H_2 between spirochetes is probably also occurring in the luminal region of termite hindguts.

Nitrogen Fixation

N_2 fixation by termite gut microbes has been known for many years (Breznak, 2000), and in light of the typically carbon-rich but nitrogen-poor diet of termites, it is not surprising that N_2 fixation contributes as much as 60% of the N in some termite colonies (Tayasu et al., 1994). However, N_2-fixing microbes from termites were not well-represented in culture, and those that had been obtained *M Citrobacter freundii* (French et al., 1976), *Enterobacter* (now *Pantoea*) *agglomerans* (Potrikus and Breznak, 1977) and *Desulfovibrio* sp. (Kuhnigk et al., 1996)—were of uncertain quantitative significance to the process occurring in vivo. Indeed, surveys of *nifH* (nitrogenase Fe-protein-encoding gene) homologues present in termite guts indicated that the diversity of N_2-fixing organisms was far greater than that inferred by using cultivation-based methods, with most of such *nifH* homologues not readily attributable to known microbial taxa (Ohkuma et al., 1996b; Ohkuma et al., 1999b).

Recent examination of ZAS-strains revealed that each possessed two homologues of *nifH* and each exhibited nitrogenase activity, with ZAS-9 exhibiting the greatest specific activity, ca. 100-fold greater than that of ZAS-1 and ZAS-2 (Lilburn et al., 2001). Of particular interest was the fact that the deduced NifH amino acid sequences of several spirochetes, including ZAS-strains, were identical or nearly identical to various NifHs previously observed in termite guts, including NifHs known to be expressed in vivo (Noda et al., 1999), suggesting that the latter were likely to be of spirochete origin. Estimates of the potential contribution of spirochetes to the N_2-fixing activity exhibited by live termites indicated that it could be significant and account for all or a substantial fraction of N_2 fixed by various termite species (Lilburn et al., 2001).

Inasmuch as N_2 fixation was another property hitherto unknown in spirochetes, a survey was made for the presence of *nifH* and nitrogenase activity in other members of this phylum. So far, these traits are found to be restricted to certain species of *Treponema* and *Spirochaeta*; however, not all spirochetes that possessed *nifH* could be shown to fix N_2 (Lilburn et al., 2001; Fig. 4).

Conclusion

Our understanding of termite gut spirochetes, and in particular their role in termite nutrition, has come a long way in a relatively short time. It is now clear that spirochetes contribute to the carbon, nitrogen and energy requirements of termites via acetogenesis and N_2 fixation, and this is probably why their elimination from guts reduces the life span of termites. However, in light of the phylogenetic diversity of spirochetes in termites, it seems almost certain that the few strains now in culture offer but an introductory glimpse of the physiological diversity of the group as a whole, to be fully appreciated only when more representatives are coaxed into culture and better culture conditions are developed for those strains whose growth is still poor. In the meantime, metabolic properties elucidated with the already studied strains in vitro must now be evaluated in vivo. For example, what is the spirochete-specific contribution to H_2/CO_2-acetogenesis and N_2 fixation occurring in vivo? Which phylogenetic groups of spirochetes are most important in these processes? What properties of spirochetes (or of the termite gut itself) enable them to become such a prominent component of the microbiota? Hopefully, creative and converging experimentation will soon provide answers to these intriguing questions.

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Literature Cited

Ball, G. H. 1969. Organisms living on and in protozoa. *In:* T.-T. Chen (Ed.) Research in Protozoology. Pergamon Press. New York, NY. 565–718.

Beckwith, T. D., and S. F. Light. 1927. The spirals within the termite gut for class use. Science 66:656–657.

Berchtold, M., W. Ludwig, and H. Köig. 1994. 16S rDNA sequence and phylogenetic position of an uncultivated spirochete from the hindgut of the termite Mastotermes darwiniensis Froggatt. FEMS Microbiol. Lett. 123:269–273.

Berchtold, M., and H. Köig. 1996. Phylogenetic analysis and in situ identification of uncultivated spirochetes from the hindgut of the termite Mastotermes darwiniensis. Syst. Appl. Microbiol. 19:66–73.

Bermudes, D., D. Chase, and L. Margulis. 1988. Morphology as a basis for taxonomy of large spirochetes symbiotic in wood-eating cockroaches and termites: Pillotina gen. nov., nom. rev.; Pillotina calotermitidis sp. nov., nom. rev.; Diplocalyx gen. nov., nom. rev.; Diplocalyx calotermitidis sp. nov., nom. rev.; Hollandinagen. nov., nom. rev.; Hollandina pterotermitidis sp. nov., nom. rev.; and Clevelandina reticulitermitidis gen. nov., sp. nov. Int. J. Syst. Bacteriol. 38:291–302.

Bignell, D. E., H. Oskarsson, J. M. Anderson, and P. Ineson. 1983. Structure, microbial associations and function of the so-called "mixed segment" of the gut in two soil-feeding termites, Procubitermes aburiensis and Cubitermes severus (Termitidae, Termitinae). J. Zool. Lond. 201:445–480.

Bloodgood, R. A., K. R. Miller, T. P. Fitzharris, J. R. McIntosh. 1974. The ultrastructure of Pyrsonympha and its associated microorganisms. J. Morphol. 143:77–106.

Bloodgood, R. A., and T. P. Fitzharris. 1976. Specific associations of prokaryotes with symbiotic flagellate protozoa from the hindgut of the termite Reticulitermes and the wood-eating roack Cryptocercus. Cytobios 17:103–122.

Breznak, J. A. 1973. Biology of nonpathogenic, host-associated spirochetes. CRC Crit. Rev. Microbiol. 2:457–489.

Breznak, J. A. 1984. Hindgut spirochetes of termites and Cryptocercus punctulatus. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 67–70.

Breznak, J. A., and J. M. Switzer. 1986. Acetate synthesis from H_2 plus CO_2 by termite gut microbes. Appl. Environ. Microbiol. 52:623–630.

Breznak, J. A. 1994. Acetogenesis from carbon dioxide in termite guts. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 303–330.

Breznak, J. A. 2000. Ecology of prokaryotic microbes in the guts of wood- and litter-feeding termites. *In:* T. Abe, D. E. Bignell, and M. Higashi (Eds.) Termites: Evolution, Sociality, Symbiosis, Ecology. Kluwer Academic Publishers. Dordrecht, The Netherlands. 209–231.

Brune, A., and M. Friedrich. 2000. Microecology of the termite gut: Structure and function on a microscale. Curr. Opin. Microbiol. 3:263–269.

Cleveland, L. R., S. R. Hall, E. P. Sanders, and J. Collier. 1934. The wood-feeding roach Cryptocercus, its protozoa, and the symbiosis between protozoa and roach. Memoirs Am. Acad. Arts Sci. 17:187–342.

Cleveland, L. R., and A. V. Grimstone. 1964. The fine structure of the flagellate Mixotricha paradoxa and its associated micro-organisms. Proc. R. Soc. London (Series B) 159:668–686.

Drake, H. L. 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA "Wood/Ljungdahl" pathway: Past and current perspectives. *In:* H. L. Drake (Ed.) Acetogenesis. Chapman and Hall. New York, NY. 3–60.

Ebert, A., and A. Brune. 1997. Hydrogen concentration profiles at the oxic-anoxic interface: A microsensor study of the hindgut of the wood-feeding lower termite Reticulitermes flavipes (Kollar). Appl. Environ. Microbiol. 63:4039–4046.

Eutick, M. L., P. Veivers, R. W. O'Brien, and M. Slaytor. 1978. Dependence of the higher termite, Nasutitermes exitiosus and the lower termite, Coptotermes lacteus on their gut flora. J. Insect Physiol. 24:363–368.

French, J. R. J., G. L. Turner, and J. F. Bradbury. 1976. Nitrogen fixation by bacteria from the hindgut of termites. J. Gen. Microbiol. 95:202–206.

Ghidini, G. M., and I. Archetti. 1939. Studi sulle termiti. 2: Le spirochete presenti in Reticulitermes lucifugus Rossi. Rivista di Biologia Coloniale 2:125–140.

Graber, J., and J. A. Breznak. 2000. Abstr. Am. Soc. Microbiol.

Grimstone, A. V. 1963. A note on the fine structure of a spirochaete. Quart. J. Microsc. Sci. 104:145–153.

Hollande, A. C., and I. Gharagozlou. 1967. Morphologie infrastructurale de Pillotina calotermitidis nov. gen., nov. sp., spirochaetale de l'intestin de Calotermes praecox. C.R. Acad. Sci. (Paris) 265:1309–1312.

Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes. *In:* J. R. Norris and D. W. Ribbons (Eds.) Methods in Microbiology. Academic Press. New York, NY. 117–132.

Iida, T., M. Ohkuma, K. Ohtoko, and T. Kudo. 2000. Symbiotic spirochetes in the termite hindgut: Phylogenetic identification of ectosymbiotic spirochetes of oxymonad protists. FEMS Microbiol. Ecol. 34:17–26.

Kirby, H. J. 1941. Organisms living on and in protozoa. *In:* G. N. Calkins and F. M. Summers (Eds.) Protozoa in Biological Research. Columbia University Press. New York, NY. 1009–1113.

Kuhnigk, T., J. Branke, D. Krekeler, H. Cypionka, H. Konig. 1996. A feasible role of sulfate-reducing bacteria in the termite gut. Syst. Appl. Microbiol. 19:139–149.

Leadbetter, J. R., and J. A. Breznak. 1996. Physiological ecology of Methanobrevibacter cuticularis sp. nov. and Methanobrevibacter curvatus sp. nov., isolated from the hindgut of the termite Reticulitermes flavipes. Appl. Environ. Microbiol. 62:3620–3631.

Leadbetter, J. R., T. M. Schmidt, J. R. Graber, and J. A. Breznak. 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283:686–689.

Leidy, J. 1874–1881. The parasites of the termites. J. Acad. Nat. Sci. (Phila.) 8:425–447.

Leidy, J. 1877. On the intestinal parasites of Termes flavipes. Proc. Acad. Nat. Sci. (Phila.) 29:146–149.

Lilburn, T. G., T. M. Schmidt, and J. A. Breznak. 1999. Phylogenetic diversity of termite gut spirochaetes. Environ. Microbiol. 1:331–345.

Lilburn, T. G., K. S. Kim, N. E. Ostrom, K. R. Byzek, J. R. Leadbetter, and J. A. Breznak. 2001. Nitrogen fixation by symbiotic and free-living spirochetes. Science 292:2495–2498.

Margulis, L., and G. Hinkle. 1992. Large symbiotic spirochetes: Clevelandina, Cristispira, Diplocalyx, Hollandina, and Pillotina. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed.. Springer-Verlag. New York, NY. 3965–3978.

Miller, J. N., R. M. Smibert, and J. R. Norris. 1992. The genus Treponema. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 3537–3559.

Messer AC, Lee MJ. 1989. Effect of chemical treatments on methane emission by the hindgut microbiota in the termite Zootermopsis angusticollis. Microb Ecol 18:275–284.

Nalepa, C. A., and C. Bandi. 2000. Characterizing the ancestors: Paedomorphosis and termite evolution. *In:* T. Abe, D. E. Bignell, and M. Higashi (Eds.) Termites: Evolution, Sociality, Symbioses, Ecology. Kluwer Academic. Dordrecht, The Netherlands. 53–75.

Noda, S., M. Ohkuma, R. Usami, K. Horikoshi, and T. Kudo. 1999. Culture-independent characterization of a gene responsible for nitrogen fixation in the symbiotic microbial community in the gut of the termite Neotermes koshunensis. Appl. Environ. Microbiol. 65:4935–4942.

Ohkuma, M., and T. Kudo. 1996a. Phylogenetic diversity of the intestinal bacterial community in the termite Reticulitermes speratus. Appl. Environ. Microbiol. 62:461–468.

Ohkuma, M., S. Noda, R. Usami, K. Horikoshi, and T. Kudo. 1996b. Diversity of nitrogen fixation genes in the symbiotic intestinal microflora of the termite Reticulitermes speratus. Appl. Environ. Microbiol. 62:2747–2752.

Ohkuma, M., and T. Kudo. 1998. Phylogenetic analysis of the symbiotic intestinal microflora of the termite Cryptotermes domesticus. FEMS Microbiol. Lett. 164:389–395.

Ohkuma, M., T. Iida, and T. Kudo. 1999a. Phylogenetic relationships of symbiotic spirochetes in the gut of diverse termites. FEMS Microbiol. Lett. 181:123–129.

Ohkuma, M., S. Noda, and T. Kudo. 1999b. Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. Appl. Environ. Microbiol. 65:4926–4934.

Oremland, R. S. 1988. Biogeochemistry of methanogenic bacteria. *In:* A. J. B. Zehnder (Ed.) Biology of Anaerobic Microorganisms. John Wiley. New York, NY. 641–705.

Paster, B. J. et al. 1991. Phylogenetic analysis of the spirochetes. J. Bacteriol. 173:6101–6109.

Paster, B. J., F. E. Dewhirst, S. M. Cooke, V. Fussing, L. K. Poulsen, and J. A. Breznak. 1996. Phylogeny of not-yet-cultured spirochetes from termite guts. Appl. Environ. Microbiol. 62:347–352.

Potrikus, C. J., and J. A. Breznak. 1977. Nitrogen-fixing Enterobacter agglomerans isolated from guts of wood-eating termites. Appl. Environ. Microbiol. 33:392–399.

Radek, R., K. Hausmann, and A. Breunig. 1992. Ectobiotic and endocytobiotic bacteria associated with the termite flagellate Joenia annectens. Acta Protozool. 31:93–107.

Radek, R., J. Rösel, and K. Hausmann. 1996. Light and electron microscopic study of the bacterial adhesion to termite flagellates applying lectin cytochemistry. Protoplasma 193:105–122.

Rösel, J., R. Radek, and K. Hausmann. 1996. Ultrastructure of the trichomonad flagellate Stephanonympha nelumbium. J. Euk. Microbiol. 43:505–511.

Smith, H. E., and H. J. Arnott. 1974. Epi- and endobiotic bacteria associated with Pyrsonympha vertens, a symbiotic protozoan of the termite Reticulitermes flavipes. Trans. Am. Microsci. Soc. 93:180–194.

Smith, H. E., H. E. Buhse Jr., and S. J. Stamler. 1975a. Possible formation and development of spirochaete attachment sites found on the surface of symbiotic polymastigote flagellates of the termite Reticulitermes flavipes. Biosystems 7:374–379.

Smith, H. E., S. J. Stamler, and H. E. Buhse. 1975b. A scanning electron microscope survey of the surface features of polymastigote flagellates from Reticulitermes flavipes. Trans. Am. Microsci. Soc. 94:401–410.

Stanton, T. B., and E. Canale-Parola. 1979. Enumeration and selective isolation of rumen spirochetes. Appl. Environ. Microbiol. 38:965–973.

Tayasu, I., A. Sugimoto, E. Wada, and T. Abe. 1994. Xylophagous termites depending on atmospheric nitrogen. Naturwissenschaften 81:229–231.

Tholen, A., and A. Brune. 2000. Impact of oxygen on metabolic fluxes and in situ rates of reductive acetogenesis in the hindgut of the wood-feeding termite Reticulitermes flavipes. Environ. Microbiol. 2:436–449.

Tokeshi, M. 1993. Species abundance patterns and comunity structure. Adv. Ecol. Res. 24:111–186.

Widdel, F., and N. Pfennig. 1981. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of Desulfobacter postgatei gen. nov., sp. nov. Arch. Microbiol. 129:395–400.

Wier, A., J. Ashen, and L. Margulis. 2000. Canaleparolina darwiniensis, gen. nov., and other pillotinaceous spirochetes from insects. Int. Microbiol. 3:213–223.

Wyss, C. 1992. Growth of Porphyromonas gingivalis, Treponema denticola, T. pectinovorum, T. socranskii, and T. vincentii in a chemically defined medium. J. Clin. Microbiol. 30:2225–2229.

Wyss, C., B. K. Choi, P. Schupbach, B. Guggenheim, and U. B. Göbel. 1996. Treponema maltophilum sp. nov., a small oral spirochete isolated from human periodontal lesions. Int. J. Syst. Bacteriol. 46:745–752.

Yamin, M. A. 1978. Axenic cultivation of the cellulolytic flagellate Trichomitopsis termopsidis (Cleveland) from the termite Zootermopsis. J. Protozool. 25:535–538.

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CHAPTER 4.6

The Genus *Brachyspira*

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Phylogeny and Taxonomy

Brachyspira is the sole genus assigned to the proposed Family "Brachyspiraceae" in the Order Spirochaetales (Paster and Dewhirst, 2000). The Order Spirochaetales contains all spirochetes. The spirochetes represent a monophyletic lineage and a major early branch in eubacterial evolution (Paster et al., 1984; Paster and Dewhirst, 2000). *Brachyspira* cells share with other spirochetes several properties that distinguish them from other bacteria. These include a helical cell shape (Fig. 1) and a cell ultrastructure that features internal periplasmic flagella, 16S rDNA sequences with spirochete signature nucleotide bases, and a natural resistance to the antibiotic rifampin (Paster and Dewhirst, 1997; Paster and Dewhirst, 2000). *Brachyspira* species are readily differentiated from other spirochete genera based on comparisons of their 16S rDNA sequences (Paster et al., 1991; Paster and Dewhirst, 1997).

General Characteristics

Brachyspira species have been isolated from animal or human intestinal contents, feces-contaminated habitats (e.g., duck ponds), and human blood. They are anaerobic bacteria but are aerotolerant due, at least in part, to high NADH oxidase activity. They use soluble sugars as carbon and energy sources. Growing cells of *Brachyspira* species (*B. aalborgi* has not been investigated) consume low concentrations of oxygen via NADH oxidase and produce acetate, butyrate, H_2 and CO_2 from glucose. Some species also produce ethanol. *Brachyspira* DNAs have a low G+C content (24.5–26.7 mol%; undetermined for *B. aalborgi*). *Brachyspira* species have high 16S rDNA sequence similarities with each other. *Brachyspira* strains have been assigned to eight electrophoretic type (ET) groups based on multilocus enzyme electrophoresis (MEE) analysis. The MEE groups correspond to seven species as defined from DNA homology studies and one currently uncharacterized provisional species ("*B. canis*"). Uncharacterized intestinal spirochetes of chickens are likely to represent a new *Brachyspira* species, "*B. pulli*" (Gabe et al., 1998; Stephens and Hampson, 2001). It has been recommended that uncharacterized/uncultured spirochetes from the human intestine be named "B. christiani" (Jensen et al., 2001).[1]

Brachyspira Species

There are seven recognized species of the genus *Brachyspira* (Table 1). The species *B. hyodysenteriae*, *B. pilosicoli*, *B. alvinipulli* and *B. intermedia* have been shown to cause disease when inoculated as pure cultures into their healthy, natural hosts. The type strain of each species is available from the American Type Culture Collection (ATCC; Table 1) and these strains should be included in studies of *Brachyspira* species.

History of *Brachyspira* Taxonomy

In the past 25 years, there have been several taxonomic changes for spirochetes now assigned to the genus *Brachyspira*. Initially, the designation "*Treponema hyodysenteriae*" was applied to both pathogenic (strongly hemolytic) and nonpathogenic (weakly hemolytic) strains of intestinal spirochetes from swine. By analysis of DNA-DNA relative reassociation, Miao and colleagues found that these pathogenic and nonpathogenic strains share only 28% sequence homology (Miao et al., 1978). This low sequence homology led to the reclassification of the nonpathogenic strains as a new species, *Treponema innocens* (Kinyon and Harris, 1979). In 1991, Stanton and colleagues proposed a reclassification of *T. hyodysenteriae* and *T. innocens* to a new genus "*Serpula*" based on 16S rRNA sequence analysis, DNA-DNA relative reassoci-

[1]The naming of uncharacterized/uncultured brachyspires is scientifically imprudent, even as a temporary precedent to proper taxonomic studies. It can become detrimental to future scientific communication. The practice is not a substitute for phenotypic and genotypic analyses of these marvelous spirochetes and should be avoided.

ation (S1 nuclease method), protein electrophoretic profiles, and genomic DNA restriction endonuclease analysis (Stanton et al., 1991). The genus name *Serpula* was then changed to "*Serpulina*" after it was determined that *Serpula* had prior use as a name for a genus of fungi (Stanton, 1992). Most recently, Ochiai et al. (1997) proposed the unification of the genera *Serpulina* and *Brachyspira*. The genus name *Brachyspira* was first given to a human intestinal isolate, *Brachypsira aalborgi*, in 1982 (Hovind-Hougen et al., 1982). Owing to the use of the genus name *Brachyspira* prior to the genus designation *Serpulina*, this proposed change is consistent with international taxonomic rules governing bacterial nomenclature and should be followed. Unfortunately, at the same time, the action has raised *Brachyspira aalborgi* to the status of type species of the genus *Brachyspira*. Unlike strains of the other *Brachyspira* species (especially *hyodysenteriae* and *pilosicoli*), only strain 513A^T of *B. aalborgi* has undergone a very limited characterization (Hovind-Hougen et al., 1982; Ochiai et al., 1997). More extensive biochemical, physiological and genetic investigations of this species are clearly needed. *Brachyspira aalborgi* is currently considered a commensal species of the human intestinal microbiota. Finally, in older literature references, the species *B. pilosicoli* was given the provisional designation "*Anguillina coli*" (Lee et al., 1993b; Park et al., 1995).

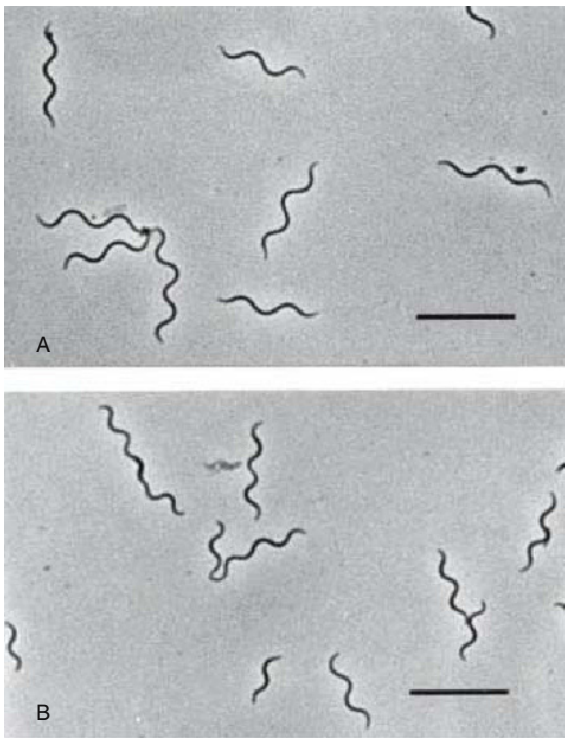

Fig. 1. A) *Brachyspira alvinipulli* C1^T cells and B) *B. hyodysenteriae* B78^T cells. Phase contrast photomicrographs of wet mount preparations. Marker bars = 10 μm. From Stanton et al. (1998), with permission.

Multilocus Enzyme Electrophoresis (MEE) Analysis

MEE analysis is a useful molecular typing technique for differentiating, identifying and determining phylogenetic relationships of taxonomically related bacteria and for determining their phylogenetic relationships (Selander et al., 1986). Based on MEE analysis of 15 constitutive enzymes, Hampson and colleagues (Lee et al., 1993a; Lee et al., 1993b; Lee et al., 1993c; Lee and Hampson, 1994; Swayne et al., 1995; Trott et al., 1996a) have assembled hundreds of intestinal *Brachyspira* strains into MEE groups (Stanton et al., 1996; Duhamel et al., 1998). The DNA homologies between strains in different MEE groups range from 21 to 64% and the homologies between strains within the same MEE group are 78% or greater (Stanton et al., 1991; Stanton et al., 1997b; Stanton et al., 1998; Trott et al., 1996c). The recommended phylogenetic definition of a species includes strains with 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m (Wayne et al., 1987). Thus, the MEE technique can be used for the presumptive identification of *Brachyspira* species and for predicting new species. The single best "stand alone" technique for confirmed identification of a new

Table 1. *Brachyspira* species, pathogenicity, and animal hosts.

Species	Type strain	ATCC number	Intestinal origin	Demonstrated pathogenicity (animal)[a]
B. hyodysenteriae	B78^T	27164	Swine and rheas	Yes (swine)
B. innocens	B256^T	29796	Swine	No
B. intermedia	PWS/A^T	51140	Swine and chickens	Yes (chickens)
B. pilosicoli	P43/6/78^T	51139	Swine, birds, dogs, humans, and nonhuman primates	Yes (swine) Yes (chickens)
B. murdochii	56-150^T	51254	Swine and rats	No
B. aalborgi	513A^T	43994	Humans	No
B. alvinipulli	C1^T	51933	Chickens	Yes (chickens)

[a]Pure cultures of either the type strain or other strains cause disease when inoculated into normal, healthy host animals.

Brachyspira species is DNA-DNA relative reassociation using the S1 nuclease method (Crosa et al., 1973; Grimont et al., 1980).

16S rDNA Sequence Analyses

Comparative analysis of 16S rDNA (rrs gene) sequences has played an essential role in establishing a phylogeny-based classification of the spirochetes (Paster and Dewhirst, 2000). Based on 16S rDNA sequence comparisons, the *Brachyspira* represent a distinct line in spirochete evolution (Paster et al., 1991; Stanton et al., 1991; Paster and Dewhirst, 1997). Consequently, 16S rDNA sequence determinations are important for classifying these enteric bacteria as spirochetes and for identifying them as members of the genus *Brachyspira* (Olsen et al., 2000).

Partial *rrs* sequences have been obtained for intestinal spirochetes from various animal hosts and humans (Hookey et al., 1994; Fellstrom et al., 1995; Pettersson et al., 1996; Stanton et al., 1996; De Smet et al., 1998; Kraaz et al., 2000). Intestinal spirochete dendrograms based on 16S rDNA sequence comparisons correlate with the genetic groupings based on MEE analysis (Stanton et al., 1996).

In view of the substantial *rrs* gene similarity among *Brachyspira* species (Table 2), it is recommended that new species be designated only after their phylogenetic relationships with known *Brachyspira* species are confirmed by additional techniques (Stanton et al., 1996). One additional method is MEE analysis (MEE analysis). Another method, the conventional "gold standard" technique for identification of bacterial species, is DNA sequence homology estimation (Wayne et al., 1987). For estimating DNA sequence homology, DNA-DNA relative reassociation using the S1 nuclease method with ΔT_m evaluation is recommended (Crosa et al., 1973; Grimont et al., 1980).

16S rDNA of Uncultured *B. aalborgi*-like Species

Recent studies suggest there are uncharacterized *Brachyspira* species phylogenetically more closely related to *B. aalborgi* than to *B. hyodysenteriae* (Pettersson et al., 2000). *Rrs* genes were amplified from unknown bacteria within colonic biopsy samples of two human patients. Seventeen spirochetal-like sequences resembled those of *B. aalborgi*. Three clusters of sequences could be differentiated. One contained the two cultivated strains of *B. aalborgi*. Although only 0.5–1.3% of the 16S rDNA sequence is different between clusters, this same level of sequence difference exists between different *Brachyspira* species (Table 2). Thus, the strains more than likely represent one or two new *Brachyspira* species (Pettersson et al., 2000).

By contrast to these studies analyzing almost complete 16S rRNA sequences of intestinal spirochetes, other investigators have relied on sequences of short sections (i.e. 433 bp and 241bp) of 16S rRNA genes for the identification of *Brachyspira aalborgi* strains (Koteish et al., 2003; Munshi et al., 2003). The highly conserved nature of the16S rRNA sequences of known *Brachyspira* species and the potential diversity of human intestinal spirochetes make these identifications, at best, presumptive. Important in determining the significance of intestinal spirochetes to human health will be the characterization of the HIS-specific spirochetes in pure culture.

23S rDNA Sequence Analysis

Approximately 2,470-bp of the 23S rDNA genes of *B. hyodysenteriae*, *B. innocens*, *B. intermedia*, *B. murdochii* and *B. pilosicoli* strains have been sequenced (Leser et al., 1997). The 23S rDNA sequences are highly (96.8–99.8%) similar. A phylogenetic tree based on sequence comparisons is consistent with 16S rDNA-based and MEE-based comparisons of these species. Various *Brachyspira* species can be differentiated by 23S rDNA-targeted polymerase chain reaction (PCR) assays (Leser et al., 1997) or by PCR-restriction fragment length polymorphism (RFLP) analysis of 23S rRNA genes (Barcellos et al., 2000).

Habitat

Brachyspira species colonize the lower intestinal tracts (ceca and colons) of animals and humans. *Brachyspira pilosicoli*, *B. aalborgi*, *B. hyodysenteriae* and *B. alvinipulli* have been observed in close physical proximity to epithelial tissues lining the intestinal tract. Intestinal mucus secreted by goblet cells is likely to be important both as a physical matrix and as chemical substrate for these spirochetes in their microhabitats. Various cell traits are considered or have been demonstrated to be important for *Brachyspira* to colonize the intestinal tract (see Ecology; Disease).

Cells of *B. pilosicoli*, a species isolated from various animal hosts, and *B. aalborgi*, a species so far isolated only from humans, can colonize intestinal mucosal surfaces by attaching to enterocytes (Barrett, 1997; Swayne and McLaren, 1997; Taylor and Trott, 1997; Mikosza et al., 1999; Jensen et al., 2000). One end of the spirochete cell attaches to an epithelial cell and the other end extends away from the surface of the epithelial cell (Fig. 2). Densely packed,

Table 2. *Brachyspira* species characteristics.

Species	Hemolysis type	Growth rate[a]	Cell size (μm)	Flagella per cell	Number of ETs	DNA G+C	16S rDNA signature[b]	16S rDNA sequence similarity[c]		Indole production	Hippurete hydrolysis
								B. aal. 513A^T	*B. hyo* B78^T		
B. aalborgi[d]	Weak	NR 7–14 d	2–6 × 0.2	8	N/A	NR	No	100	96.5	–	+
B. hyodysenteriae	Strong	3–5 h 3–4 d	7–9 × 0.3–0.4	22–28	29	25.9	No	96.5	100	+/–	
B. intermedia	Weak	2–4 h 3–5 d	8–10 × 0.35–0.45	24–28	7	25	No	96.0	99.1	+	+
B. innocens	Weak	3–5 h 3–5 d	7–9 × 0.3–0.4	20–26	10	26	No	96.5	99.5	–	
B. pilosicoli	Weak	1–2 h 3–4 d	5–7 × 0.23–0.3	8–12	33	24.6	Yes	96.4	98.5	+/–	+/(–)
B. murdochii	Weak	2–4 h 3–5	5–8 × 0.3–0.4	22–26	10	27	No	95.9	98.5	–	–
B. alvinipulli[d]	Weak	3–5 h 3–5 d	8–11 × 0.2–0.35	22–30	N/A	24.6	No	95.7	98.4	–	+

Symbols and abbreviations: ET, electrophoretic types; +, positive; –, negative; (–), indole not produced or hippurate not hydrolyzed; NR, not reported; and N/A, not applicable.

[a]Population doubling time in culture broth and days for colony development on agar-containing media.

[b]A unique signature sequence of nucleotides was identified within the 16S rDNA of *B. pilosicoli* and has been used to design specific polymerase chain reaction (PCR) tests for this species (Park et al., 1995; Fellstrom et al., 1997).

[c]16S rDNA values based on type strains except for *B. murdochii* 155–20. Based on GenBank sequences: Z22781, U14930, U23033, U14920, U14927, U22838, and U23030.

[d]Only type strains 513A^T and C1^T of *B. aalborgi* and *B. alvinipulli*, respectively, have been extensively characterized.

Information from Harris et al. (1972); Hovind-Hougen et al. (1982); Kinyon and Harris (1979); Stanton et al. (Stanton et al., 1991; Stanton et al., 1996; Stanton et al., 1997b; Stanton et al., 1998); Fellstrom et al. (Fellstrom et al., 1995; Fellstrom et al., 1997; Fellstrom et al., 1999); McLaren et al. (1997); Trott et al. (Trott et al., 1996a; Trott et al., 1996b; Trott et al., 1997); DeSmet et al. (1998); Duhamel et al. (1998); and Kraaz et al. (2000).

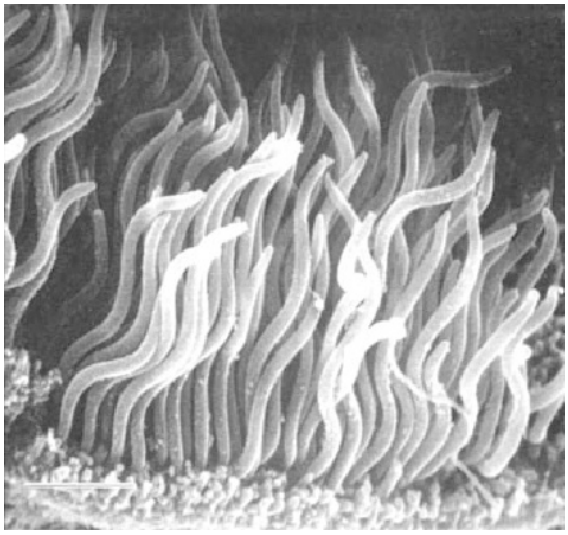

Fig. 2. Scanning electron micrograph of the colonic mucosa of a pig with colitis showing the typical appearance of intestinal spirochetosis. Epithelial cells are extensively colonized by spirochetes attached by one end to the luminal surface of the cells. Marker bar = 2 μm. From Sellwood and Bland (1997), with permission.

parallel arrays of spirochetes in close proximity form on the epithelial surface in this way. The density of spirochetes in these assemblages is estimated to be 20–80 spirochete cells per enterocyte (Stanton, 1997a). Although end-on attachment is a characteristic feature of *B. aalborgi* and *B. pilosicoli* colonization, it may not be a sine qua non for colonization and disease (Thomson et al., 1997; Jensen et al., 2000).

End-on attachment to intestinal tissues by densely packed, parallel arrays of *B. pilosicoli* or *B. aalborgi* cells creates the appearance of a "false brush border" on mucosal epithelial cells. This appearance of colonizing spirochetes has been commonly referred to as "intestinal spirochetosis" by histologists (Duhamel, 1997a; Swayne and McLaren, 1997). Intestinal spirochetosis has been observed in tissue sections both of healthy animals and humans and of individuals with intestinal disorders. Takeuchi and Zeller (1972) estimated that unidentified spirochetes attached to rhesus monkey colonic mucosa were present at a density of 1,800 bacteria·mm^{-2}. So far, *B. pilosicoli* and *B. aalborgi* are the only characterized spirochetes associated with intestinal spirochetosis.

Attachment to intestinal tissues is not a colonization feature of *B. hyodysenteriae*, the agent of swine dysentery, or of *B. alvinipulli*, a chicken pathogen. In the early stages of swine dysentery, *B. hyodysenteriae* cells first colonize along the intestinal epithelium of the swine cecum and colon and then among epithelial cells and within goblet cells (Glock et al., 1974; Kubo et al., 1979; Kennedy et al., 1988). As the disease progresses, lesions appear in the mucosa at sites of spirochete colonization, and host blood passes into the intestinal lumen through the lesions (Glock et al., 1974; Kennedy and Strafuss, 1977; Kubo et al., 1979). *Brachyspira alvinipulli* strain C1^T cells colonize chicken ceca and colons by irregular cell aggregates adjacent to intestinal epithelial cells and within villus crypts (Swayne et al., 1995; Swayne and McLaren, 1997; Stanton et al., 1998).

Microhabitats have yet to be extensively studied for *B. innocens*, *B. intermedia*, and *B. murdochii*.

Isolation

Physical Isolation Methods

In the first studies to identify *B. hyodysenteriae* as the etiologic agent of swine dysentery, the spirochete was isolated from dysenteric pigs after filtering colon contents through a 0.65 μm filter (Taylor and Alexander, 1971) and colonic epithelial tissue extracts through a series of filters of decreasing pore size (Harris et al., 1972b). The filtrates were inoculated onto agar media and incubated anaerobically to obtain isolated colonies.

Another physical isolation method takes advantage of bacterial motility to separate *Brachyspira* strains from nonmotile intestinal bacteria (Olson, 1996). Sterile scalpels are used to cut parallel lines in a selective agar medium containing spectinomycin (400 μg/ml). Intestinal samples are inoculated as streaks in the center of the Petri plates and perpendicular to the cut lines. The motile *B. hyodysenteriae* cells (strongly hemolytic) and cells of other intestinal spirochetes (weakly or nonhemolytic) migrate along the lines. The spirochetes can be isolated from agar medium samples taken at the ends of the cut lines and away from the original inoculation site.

Selective Culture Media

Brachyspira hyodysenteriae and other *Brachyspira* species can be isolated by inoculating intestinal contents or tissues onto solid agar culture media containing antibiotics selective for the growth of those spirochetes (Table 3). Achacha and Messier (1992) compared various selective media and found BJ medium (Table 3) with five antibiotics provided the highest rate of isolation of *B. hyodysenteriae* from feces of experimentally infected swine (130 of 145 samples tested). They also reported the encouraging observation that pig feces extract could be eliminated from

Table 3. *Brachyspira* culture media.

A) Selective isolation media

Medium	Medium components	Target species	References
TSB(S)	Trypticase-soy agar + 5% bovine blood + spectinomycin (400)	*B. hyodysenteriae*	Songer et al., 1976 Taylor et al., 1980
TSB(SCV)	Tryptone-soya agar + 10% sheep blood + spectinomycin (400) + colistin (25) + vancomycin (25)	*B. hyodysenteriae*	Jenkinson and Winger, 1981
BJ	Trypticase-soy agar + 5% bovine blood + 5% swine faces extract + spectinomycin (400) + spiramycin (25) + rifampin (12.5) + vancomycin (6.2) + colistin (6.2)	*B. hyodysenteriae*	Kunkle and Kinyon, 1988
TSB(SP)	Trypticase-soy agar + 10% bovine blood + spectinomycin (400) + polymyxin (5)	*B. aalborgi*	Kraaz, 2000 Hovind-Hougen et al., 1982
BB(SR)	Blood agar modified medium + 7% equine blood + spectinomycin (400) + rifampin (30)	*B. hyodysenteriae*	Calderaro et al., 2001

B) Liquid media

Medium	Medium components	References
BHIS broth (general purpose growth)	Brain-heart infusion broth (contains glucose); 10% calf serum (heat-treated 56°C, 30 min); 0.1% L-cysteine-HCl; 0.0001% resazurin	Stanton and Cornell, 1987b
HS broth (for identifying growth substrates)	Heart infusion broth; 2–10% calf serum (heart treated 56°C, 30 min); L-cysteine-HCl; 0.0001% resazurin	Stanton and Lebo, 1988
Kunkle's broth (autoclavable medium)	Trypticase soy broth; 0.5% glucose; 0.2% $NaHCO_3$; 0.05% L-cysteine-HCl; 1.0% yeast extract; and 2 of 3 of the following: fetal bovine serum, cholesterol, or swine facal extract	Kunkle et al., 1986

Values in table are final concentration (v/v or w/v). Antibiotic concentrations expressed as μg/ml of medium.

the medium with essentially no effect on isolation success (128 of 145 samples tested).

Brachyspira species other than *B. hyodysenteriae* can be isolated from swine, other animals, and humans by using selective culture media similar, if not identical, to those used for *B. hyodysenteriae* (Barrett, 1997; Jensen, 1997; Table 3). A note of caution is that antibiotics used to select for *B. hyodysenteriae* growth may select against growth of strains of certain *Brachyspira* species, such as *B. aalborgi* (Kraaz et al., 2000) and *B. pilosicoli* (Trott et al., 1996b). Selective culture media to directly isolate *B. aalborgi* from human feces have been described (Brooke et al., 2003; Calderaro et al., 2003). The media appear promising but so far have been used successfully for samples from only two patients.

Sample Handling, Inoculation, and Incubation Conditions

Brachyspira cells are aerotolerant anaerobes. *Brachyspira hyodysenteriae* strain B204 cells can withstand exposure to air for two or more hours (Stanton et al., 1999b). Nevertheless, whenever possible, intestinal contents or tissue samples should be protected from air exposure (for example, placed in anaerobic culture broth) and maintained on wet ice for transport to the laboratory. Swab samples of feces should be kept moist in a transport medium.

Brachyspira colonies can be isolated from intestinal samples by standard streaking methods using sterile inoculating loops to dilute bacterial cells across agar plate surfaces. Alter-

natively, serial tenfold dilutions of the samples can be made in tubes containing prereduced (oxygen-free) sterile basal culture broth (e.g., trypticase soy or brain heart infusion) and by spreading samples across the surface of agar plates. The latter technique, of course, enables viable colony forming units (CFU) in the original sample to be estimated.

Owing to their aerotolerant nature, preliminary steps in the isolation of known *Brachyspira* species (streaking agar plates and diluting samples) can be carried out on the lab bench (in an air atmosphere). Inoculated agar plates are incubated under anaerobic conditions by placing them within an anaerobic glove box or within anaerobic Gas-Pak jars. To further reduce exposure of *Brachyspira* cells to oxygen, intestinal samples can be transferred into an anaerobic glove box and all subsequent processing of samples carried out within the anaerobic atmosphere.

Brachyspira pilosicoli from Blood

Brachyspira pilosicoli strains have been isolated from the blood of critically ill human patients without the use of selective agar medium (Trott et al., 1997b). Cells of the spirochete were cultured from blood samples inoculated into Hemoline blood culture medium (bioMerieux, Lyon, France), BioArgos Sanofi diagnostic anaerobic medium (Pasteur Institute, Paris, France) and ESP automated blood culture media (Becton Dickinson Difco, Detroit, Michigan, United States). A study comparing various commercial blood culture systems indicated that prolonged culture incubation (5–14 days) is necessary to detect *B. pilosicoli* cells (Brooke et al., 2000).

Alternative Isolation Techniques

With few exceptions, isolation techniques for *Brachyspira* have revolved around selective culture media containing antibiotics and developed to clinically detect *B. hyodysenteriae*. These methods have led to the successful isolation of seven different *Brachyspira* species. It seems likely that the use of alternative sources of inocula, the modification of medium components and incubation conditions, and the incorporation of physical separation techniques will yield additional *Brachyspira* species.

Identification of Species

Various phenotypic characteristics and PCR based tests are useful for identifying known *Brachyspira* species (Table 2). "Strong" hemolysis of *B. hyodysenteriae* colonies and a unique signature sequence in the 16S rDNA of *B. pilosicoli* are often used in clinical and epidemiological studies to identify those pathogens.

Colony Hemolysis

Brachyspira hyodysenteriae is the only recognized *Brachyspira* species that is "strongly hemolytic" (Table 2). Colonies of *B. hyosysenteriae* form zones of β-hemolysis on trypticase soy-blood agar plates. Diagnosis of the disease swine dysentery is based on culturing of spirochete-like bacteria forming strongly hemolytic colonies and on histological evidence of spirochete-induced lesions in the ceca and colons of dysenteric swine. Well-isolated colonies on freshly prepared blood agar plates should be examined to assess hemolysis type of an intestinal spirochete. Control cultures of *Brachyspira*-specific type strains should be included for hemolysis comparisons.

Cell Ultrastructure

The number of flagella per cell has been commonly used in spirochete taxonomy and identification. *Brachyspira* species can be divided into two groups based on flagellar numbers and cell size (Table 2). Larger size species, such as *B. hyodysenteriae*, *B. innocens*, *B. intermedia*, *B. murdochii* and *B. alvinipulli*, have 20–30 flagella per cell. *Brachyspira pilosicoli* and *B. aalborgi* cells are shorter in length and have 8–12 flagella per cell. As with other spirochetes, the flagella attach in roughly equal numbers at each end of the cell (Fig. 3) and wind around the spirochete cell between the protoplasmic cylinder and outer sheath, and their free ends overlap in the cell center. Accurate estimates of flagellar numbers can be obtained by transmission electron microscopy of bacteria suspended in distilled water and negatively stained (e.g., 2% phosphotungstic acid, pH 7.0). Both flagella and flagellar attachment sites at the ends of the cells can be counted (Fig. 3).

Biochemical Tests

Biochemical tests for indole production (Finegold and Martin, 1982), hippurate hydrolysis (Smibert and Krieg, 1981), and commercial assay kits, such as API-ZYM (Hunter and Wood, 1979; Fellstrom et al., 1999; Stanton et al., 1998), have been used to characterize *Brachyspira* species (Table 2). For *B. aalborgi* and *B. alvinipulli*, only a limited number of strains have been examined.

Attempts have been made to classify porcine *Brachyspira* isolates based on hemolysis pattern, indole production, hippurate hydrolysis, colorimetric tests for sugar hydrolyzing enzymes, and pathogenicity for swine (Fellstrom et al., 1995;

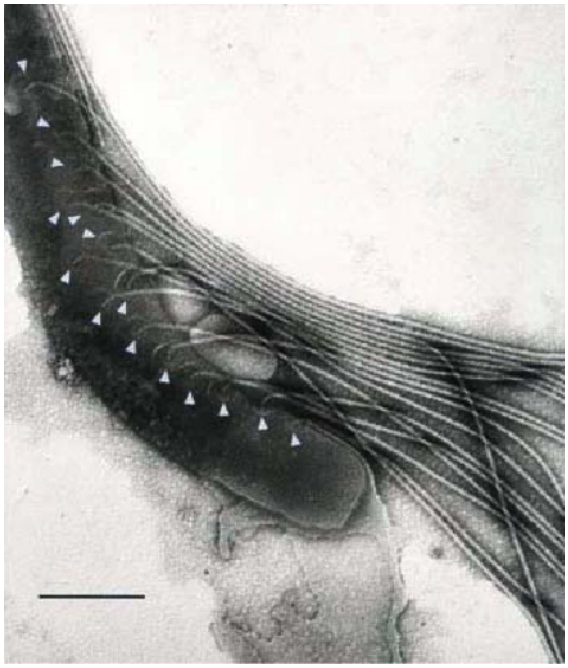

Fig. 3. Electron micrograph of one end of *B. alvinipulli* $C1^T$ cell negatively stained with 2% phosphotungstic acid (pH 7.0). Disrupted outer sheath enables insertion sites of 15 periplasmic flagella to be seen (white arrowheads). Marker bar = 0.25 μm. From Stanton et al. (1998), with permission.

Fellstrom et al., 1997). Unfortunately, porcine strains with properties different from the expected test results have been identified (Fellstrom et al., 1997; Fellstrom et al., 1999). Additionally, the biochemical classification of *Brachyspira* species does not work when non-porcine strains are included (De Smet et al., 1998; Stanton et al., 1998). Many biochemical tests, such as the hydrolysis of a certain color-yielding substrate, rely on one or a few encoding genes and therefore do not provide an extensive phylogenetic basis for taxonomy.

Species-specific PCR-based Assays

PCR assays have been described for the specific detection of porcine strains of *B. hyodysenteriae* (Elder et al., 1994; Harel and Forget, 1995; Leser et al., 1997; Atyeo et al., 1998; Atyeo et al., 1999), *B. pilosicoli* (Park et al., 1995; Fellstrom et al., 1997; Leser et al., 1997; Muniappa et al., 1997; Atyeo et al., 1998), and *B. intermedia* (Leser et al., 1997). A restriction fragment length polymorphism (RFLP)-PCR assay targeting 16S rDNA was developed to differentiate the type strains of seven *Brachyspira* species (Stanton et al., 1998). Unfortunately, clinical isolates of weakly hemolytic spirochetes, *Brachyspira* stains, have restriction patterns that do not match those of the type strains (T. B. Stanton, unpublished observation). In addition, PCR assays targetting NADH oxidase and 16S rRNA genes have been developed to differentiate *Brachyspira* species and used to detect *B. aalborgi* and *B. pilosicoli* in human biopsy specimens (Atyeo et al., 1999; Mikosza et al., 1999; Mikosza et al., 2001). Also, PCR assays targeting 23S rDNA were developed to distinguish porcine strains of *B. hyodysenteriae*, *B. intermedia* (group II) and *B. pilosicoli* (group IV) (Leser et al., 1997). RFLP-PCR assays targeting genes encoding NADH oxidase or 23S rRNA have been developed to differentitate *Brachyspira* species (Barcellos et al., 2000; Rohde et al., 2002).

Investigations with additional strains of known *Brachyspira* species and partially characterized intestinal spirochete isolates ("*B. canis*") are needed to determine whether these assays or any PCR-based assays will suffice as an individual test for identifying *Brachyspira* species. At this time, a likely to be reliable approach for species detection and identification would incorporate a combination of PCR-based tests for more than one gene (Mikosza et al., 1999; La et al., 2003).

Establishing New *Brachyspira* Species

Based on investigations of known *Brachyspira* species (see Phylogeny and Taxonomy), a new *Brachyspira* species should be characterized by 16S rDNA sequence analysis or/and MEE analysis (preferably both) of multiple isolates. Isolates (one or more strains) forming a unique 16S rDNA branch or MEE electrophoretic type should be compared by DNA sequence homology (S1 nuclease method with ΔT_m estimates) with closely related *Brachyspira* species. Finally, the ultrastructural, cultural, and biochemical characteristics of a species help define its lifestyle and facilitate additional research. These should be determined.

Preservation and Transport of *Brachyspira* Cultures

Short-term Stock Cultures

Broth cultures of most *Brachyspira* species remain viable when stored at 5°C for short time periods of 1–2 weeks. These cultures can be used as "working" stock cultures to inoculate fresh cultures for experiments. Refrigerated cultures should be in the exponential phase of growth (for many strains, 2–4 × 10^8 cells/ml, direct microscope counts). Importantly, avoid exposing cultures to air. The junction between the culture

tube mouth and the rubber stopper corking the tube should be firmly sealed with stretchable yellow plastic tape (3M Scotch Brand #471) before storage. These working stock cultures should be transferred every two weeks. Long-term in vitro passage of strains (with possible loss of virulence) should be avoided by starting fresh working stock cultures from long-term stock cultures every several months.

Agar plate cultures of *B. hyodysenteriae* can be stored at room temperature in an anaerobic atmosphere for at least a week. The plates should be sealed (Parafilm, American National Can Inc.) to prevent desiccation. A slowly growing spirochete identified as *B. aalborgi* remained viable when kept at room temperature in an anaerobic jar for over 3 months (Kraaz et al., 2000).

Long-term Stock Cultures

For long-term storage of *Brachyspira* strains, the following method is recommended. *Brachyspira* cultures in the exponential phase of growth in broth media ($2–4 \times 10^8$ cells/ml) are harvested by centrifugation (15 min, 5,000*g*). The pelleted bacteria are resuspended at 50–100 times their original concentration in fresh sterile broth medium containing dimethyl sulfoxide (DMSO), 10% (v/v, final concentration). The concentrated cell suspension is dispensed into Nunc cryovials (0.5–1.0 ml/vial). The sealed vials are placed upright in a beaker containing enough 95% ethanol to equal the fluid level of the suspensions (and well below the tops of the cryovials). The ethanol bath is intended to provide a more uniform rate of freezing of the cells and, with the DMSO as a cryoprotectant, prevent ice crystals from damaging the bacteria. The beaker is placed in an ultracold freezer (–75°C). After 24 h, the frozen stock cultures are transferred to storage boxes in the freezer. After 7–10 d, one of the cryovials should be examined to insure recovery of contaminant-free *Brachyspira* cells. *Brachyspira* cells have remained viable in frozen stocks prepared in this way for over 15 years (T. B. Stanton, unpublished observations). Completely thawed stocks should not be re-frozen. However, it is often possible to subculture without thawing the frozen stocks, by scraping the surface of a frozen cell suspension with a sterile inoculation loop and inoculating fresh media with the ice scrapings. *Brachyspira hyodysenteriae* cultures also have been preserved by lyophilization (Stanton and Lebo, 1988).

Shipping Stock Cultures

Brachyspira strains can be successfully transported through the mail as frozen cell stocks on dry ice. Alternatively, agar plate cultures will survive shipment by express mail delivery, without the need for wet or dry ice. Within an anaerobic chamber, agar plates with visible colony growth are sealed shut with flexible film (Parafilm). Within the chamber, the plates are then double-sealed in two plastic bags (smaller bag within larger bag) using a heat seal device (Seal-a-Meal, Dazey Corp., Industrial Airport, KS), such as used for DNA-DNA membrane blot hybridizations, and placed in appropriate shipping containers. For overnight delivery, stoppered broth cultures can be sealed with plastic tape and mailed in special shipping containers. Appropriate government and private carrier regulations governing the packaging and shipping of bacterial cultures must be followed.

Physiology

Culture Broth Media

Various anaerobic, nutritionally complex broth media have been described for *Brachyspira* species (Stanton, 1997a). Brain heart infusion with serum (BHIS) broth and Kunkle's broth are commonly used for routine growth of *Brachyspira hyodysenteriae* (Table 3) (Kunkle et al., 1986; Stanton and Lebo, 1988). Both media support high growth yields of this species (10^9 to 4×10^9 cells/ml, direct microscope counts). In BHIS broth, the population doubling times for type strains of *B. hyodysenteriae*, *B. innocens*, *B. intermedia*, *B. murdochii* and *B. alvinipulli* are 2–4 hours (Table 3). *Brachyspira pilosicoli* P43/6/78^T has a doubling time of 1–2 hours. Incubation temperatures of 38–40°C (the swine body temperature is 39°C) are used for *Brachyspira*, and stirring of broth cultures (with magnetic stir bars) is important for optimum growth.

Heart infusion with serum (HS) broth for *Brachyspira* species (Table 3) requires added carbohydrates to support optimum growth yields of *Brachyspira* species. Consequently, HS broth is useful for identifying growth substrates (Table 4) and metabolic end products. Since animal serum contains glucose, a minimal serum concentration, i.e., one not limiting for growth, should be used in studies to identify growth substrates and products.

NT broth, a serum-free medium ultrafiltered (10,000 MW cutoff filter) to remove high MW proteins, supports *B. hyodysenteriae* growth (Humphrey et al., 1997). Cholesterol, an essential growth requirement for the spirochete, is added to the medium in place of serum. NT broth cultures of *B. hyodysenteriae* are used to purify the generalized transducing bacteriophage VSH-1 (Humphrey et al., 1997). (See Genet-

Table 4. *Brachyspira* growth substrates.[a]

Compound	*B. hyodysenteriae* B78^T	*B. innocens* B256^T	*B. intermedia* PWS/A^T	*B. pilosicoli* P43/6/78^T	*B. murdochii* 56/150^T	*B. alvinipulli* C-1^T
None	+	+	+	+	+	+
D-Glucose	++++	++++	++++	++++	++++	++++
D-Fructose	++++	++++	++++	++++	+++	++
Sucrose	++++	+++	++++	++++	+++	NG
D-Trehalose	++++	+++	++++	++++	+++	NG
D-Galactose	++	++	++	++	NG	NG
D-Mannose	++	+++	NG	+++	++	++++
N-Acetyl-D-glucosamine	++++	++++	++++	++++	++++	++++
D-Glucosamine	+++	+++	NG	+++	NG	++++
D-Maltose	+	+++	+++	++	++	+++
D-Cellobiose	NG	+++	NG	++++	++++	NT
L-Fucose	NG	++	++	++	++	NG
Pyruvate	++	++	++	++	++	NG
D-Ribose	NG	NG	NG	++++	NG	NG
Lactose	+	NT	NG	NT	+	NG

Symbols: +, background growth (no added substrate); ++, low growth, final cell yields approximately 2- to 4-fold greater than background; +++, intermediate growth, final cell yields 4- to 10-fold greater than background; and ++++, optimum growth, final cell yields >10-fold background levels.

Abbreviations: NG, no detectable growth above background (medium without added substrate); and NT, substrate not tested.

[a]Based on growth studies with *B. hyodysenteriae* cultures in HS broth by Stanton and Lebo (1988); Trott et al. (1996); and Stanton et al. (Stanton et al., 1997b; Stanton et al., 1998). In those studies, additional substrates were found not to support growth of any species. Values in table refer to relative cell yields.

ics.) NT broth also may be useful for identifying and purifying extracellular (protein) products of *Brachyspira* spp.

The oxidation-reduction potential of broth cultures may be a controlling factor for *B. hyodysenteriae* growth (Stanton, 1997a). The spirochete can be difficult to culture in stringently prepared anaerobic broth media unless a small amount of air is introduced into the culture oxygen-free atmosphere (Stanton and Lebo, 1988). A simple method is to inject a volume of sterile air through the rubber stopper sealing a culture vessel so that the culture atmosphere contains 1% oxygen (e.g., 1% O_2:99% N_2). *Brachyspira hyodysenteriae* cells have been successfully cultured beneath an air atmosphere in large (i.e., 12 liter) volumes in a fermentor (Stanton and Jensen, 1993a; Mikosza et al., 1999). Alternatively, chemical reducing agents such as L-cysteine can be left out of the medium. *Brachyspira* species will not grow in tubes of media that have become oxidized (i.e., in which the resazurin indicator dye has become colored due to air exposure).

Fermentation and Growth Substrates

Potential fermentation substrates of *Brachyspira* spp. and unidentified intestinal spirochetes added to broth or solid agar media are identified as bona fide substrates when lower medium pH values (compared to medium without added substrate) result from spirochete growth (Kinyon and Harris, 1979; Jones et al., 1986; Tompkins et al., 1986; Ochiai et al., 1997). Fructose, galactose, glucose, lactose, maltose, mannose and trehalose are fermentation substrates for *B. aalborgi* 513A^T (Ochiai et al., 1997).

The sensitivity of medium pH changes for detecting *Brachyspira* growth substrates is questionable. Furthermore, fermentation substrates may not always correspond to carbon and energy sources used by bacteria for growth. A more definitive method for identifying growth substrates uses a culture medium, such as HS broth, in which *Brachyspira* growth is limited unless a carbon/energy source is added (Table 4).

In HS broth, *B. hyodysenteriae* B78^T, *B. innocens* B256^T, *B. intermedia* PWS/A^T, *B. murdochii* 56/150^T, *B. pilosicoli* P43/6/78^T and *B. alvinipulli* C1^T use for growth various monosaccharides, disaccharides, the trisaccharide trehalose, and amino sugars (Table 4). Other strains of these species use similar growth substrates (Stanton and Lebo, 1988; Trott et al., 1996b). None of the tested *Brachyspira* strains use polysaccharides such as cellulose, hog gastric mucin, pectin, glycogen and various other tested compounds. Growth substrates for *B. aalborgi* have not been identified in similar experiments.

Brachyspira hyodysenteriae and *B. innocens* strains contain a sucrase activity that increases 3- to 10-fold when sucrose is added to cultures (Jensen and Stanton, 1994). The sucrase is the

first inducible enzymatic activity to be identified for *Brachyspira* species.

Cholesterol and Phospholipids

Brachyspira hyodysenteriae requires cholesterol and phospholipid for growth (Lemcke and Burrows, 1980; Stanton and Cornell, 1987b; Stanton, 1997a). These requirements can be met by supplementing media with either cholesterol-phosphatidylcholine or erythrocyte membranes (Stanton and Cornell, 1987b). Cholesterol is likely required for outer membrane biosynthesis based on the following observations. Nutrient amounts of cholesterol (8–26 nmoles/ml culture broth) are sufficient for growth (Lemcke and Burrows, 1980; Stanton and Cornell, 1987b). *Brachyspira hyodysenteriae* cells incorporate radiolabel from [4-^{14}C] cholesterol into cell membrane fractions (Stanton, 1987a). Cholesterol preferentially locates within *B. hyodysenteriae* outer membranes (Plaza et al., 1997). The sterol requirement is unusual for a nonmycoplasma subgroup of bacterial species.

Brachyspira hyodysenteriae cellular fatty acids are distinct from those of *Borrelia* and *Leptospira* spirochetes (Livesley et al., 1993). *Brachyspira hyodysenteriae* and *B. innocens* cellular phospholipids and glycolipids were found to contain acyl (fatty acids with ester linkage) and alkenyl (unsaturated alcohol with ether linkage) side chains (Matthews et al., 1980a; Matthews et al., 1980b; Matthews and Kinyon, 1984). The culture medium for these spirochetes did not contain these same lipids, an indication that the bacteria have some capacity for fatty acid and lipid biosynthesis.

Intermediary Metabolism

Glucose and Pyruvate Metabolism Pathways of glucose and pyruvate metabolism have been analyzed extensively only for *B. hyodysenteriae* (Stanton, 1989; Stanton, 1997a). The endproducts of glucose metabolism by growing cells of numerous *Brachyspira* species are the same as those of *B. hyodysenteriae*—acetate, butyrate, H_2 and CO_2—suggesting these species have similar catabolic routes (Stanton and Lebo, 1988; Stanton, 1989; Trott et al., 1996d; Stanton et al., 1997b; Stanton et al., 1998).

Brachyspira hyodysenteriae uses the Embden-Meyerhof-Parnas (EMP) pathway for converting glucose to pyruvate (Fig. 4). Pyruvate is catabolized by a clostridial-type clastic reaction to acetyl-CoA, H_2 and CO_2. Acetyl-CoA is converted to either acetate or butyrate via a branched fermentation pathway. The ATP-yielding mechanisms are substrate-level phosphorylation reactions mediated by phosphoglycerate kinase and pyruvate kinase in the EMP pathway and by acetate kinase converting acetyl phosphate to acetate (Fig. 4).

NADH-H$^+$ Oxidation Reactions *Brachyspira hyodysenteriae* cells are versatile when it comes to NADH-H$^+$ oxidation reactions (Fig. 4). This versatility could enhance the metabolic fitness of the spirochete in its animal host (Thauer et al., 1977; Stanton, 1997a). The NADH-H$^+$ produced during glycolysis can be recycled or oxidized to NAD$^+$ by 3-hydroxybutyryl-CoA dehydrogenase and butyryl-CoA dehydrogenase (butyrate pathway), by NADH-ferredoxin oxidoreductase plus hydrogenase, and by NADH oxidase (Fig. 4).

Various *Brachyspira* species produce more H_2 than CO_2, indicative of the NADH-ferredoxin oxidoreductase reaction. Ethanol is produced in cultures of *B. pilosicoli*, *B. alvinipulli*, *B. murdochii*, *B. intermedia* and *B. innocens* (Stanton and Lebo, 1988; Stanton, 1989; Trott et al., 1996c; Stanton et al., 1997b; Stanton et al., 1998) and is likely formed from acetyl-CoA by the enzymes acetaldehyde dehydrogenase and alcohol dehydrogenase (Fig. 4).

NADH oxidase is widely distributed among *Brachyspira* species (Atyeo et al., 1999; Stanton et al., 1995). The *B. hyodysenteriae* NADH oxidase is a water-forming, FAD-linked enzyme (Stanton and Jensen, 1993b) and its gene (*nox*) has been cloned (Stanton and Sellwood, 1999a). *Nox*-defective mutant strains of *B. hyodysenteriae* are sensitive to oxygen (Ecology) and lose virulence (Stanton et al., 1999b; see Virulence).

Iron Metabolism

Several observations suggest that specific iron uptake mechanisms are present and are important for *Brachyspira* growth in animal hosts. *Brachyspira hyodysenteriae* cells grow in broth containing an iron chelator, 2,2′-dipyridyl, and increase the expression of three unidentified high molecular mass proteins, >200, 134, and 109 kDa (Li et al., 1995). Catechol and hydroxamate do not enhance *B. hyodysenteriae* growth in iron-depleted medium, suggesting the spirochete does not use these common bacterial siderophores to bind and take up iron. Dugourd and coworkers have identified a *B. hyodysenteriae* genome locus, designated *bit* ("*Brachyspira* iron transport"), encoding six proteins that are likely to form an iron ATP-binding transport system (Dugourd et al., 1999). BitD has ATP-binding motifs. BitA, BitB and BitC are lipoproteins with iron binding properties. BitE and BitF resemble membrane permeases. All of the Bit proteins are smaller in molecular mass (28–42 kDa) than are previously described iron-regulated proteins (Li et al., 1995).

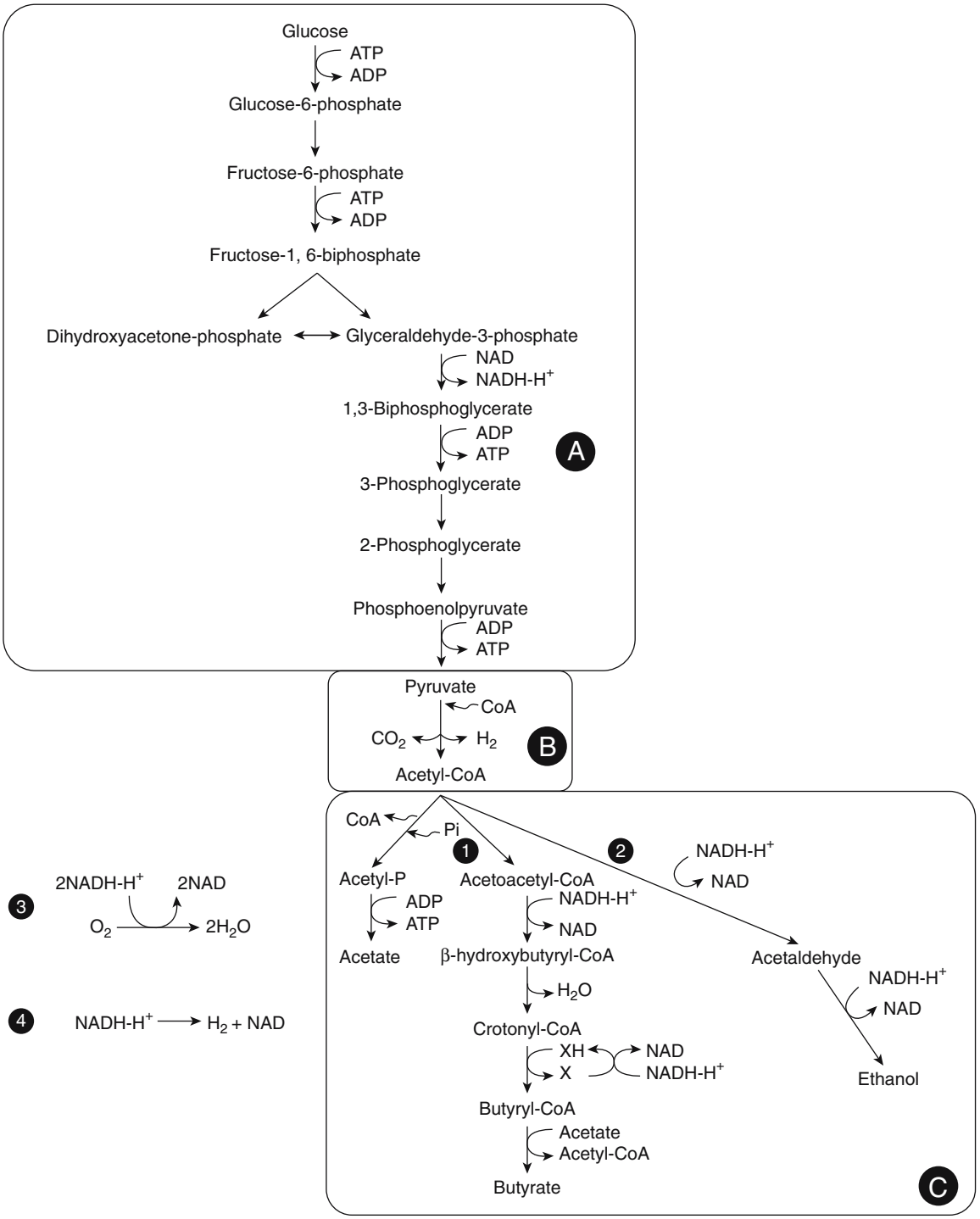

Fig. 4. Glucose metabolism by *Brachyspira* species. A) Glucose is converted to pyruvate by the Embden-Meyerhof-Parnas pathway. B) Pyruvate is metabolized by a clostridial-type clastic reaction. C) Acetyl-CoA is a branch point for pathways leading to acetate, butyrate, or ethanol. (Note: the pathway to ethanol has not been demonstrated.) NADH-H$^+$ is oxidized in the pathways to butyrate (1) and ethanol (2) and by two alternative mechanisms: NADH oxidase (3) and NADH-ferredoxin oxidoreductase coupled to hydrogenase (4) (Stanton, 1997a).

Genetics

Brachyspira Genes

Brachyspira genetics is at an early, promising stage of development (van der Zeijst and ter Huurne, 1997; Zuerner, 1997; Hardham and Rosey, 2000). Only a limited number of *Brachyspira* genes have been cloned and expressed as recombinant proteins (Table 5). *Brachyspira* genome libraries can be created by using *E. coli*-based vectors, primarily lambda phages and plasmids. An expression vector has been created that enables posttranslational processing of *B. hyodysenteriae* lipoprotein BlpA in *E. coli* and the incorporation of the protein into the *E. coli* outer membrane (Cullen et al., 2003). Cosmid cloning of *B. hyodysenteriae* DNA has generally been inefficient for unknown reasons (van der Zeijst and ter Huurne, 1997). Nearly complete 16S rRNA and 23S rRNA gene sequences are known for all *Brachyspira* species (Table 2) and are used for both taxonomic and clinical detection applications (See Phylogeny and Taxonomy and Identification).

Genome Properties

Unfortunately, no Brachyspira genome has yet been sequenced. A physical map of the *B. hyodysenteriae* B78^T chromosome is based on restriction enzyme fragments of the chromosome separated by pulsed field gel electrophoresis (PFGE) (Zuerner and Stanton, 1994). The *B. hyodysenteriae* chromosome is circular and has 3.2 Megabase pairs (Mbp), and the map (restriction fragment) locations are known for many *B. hyodysenteriae* genes (Zuerner and Stanton, 1994; Hsu et al., 2001; Zuerner et al., 2004). *Brachyspira pilosicoli* P43/6/78^T has a circular chromosome approximately 2.4 Mbp (Zuerner et al., 2004) and thus carrying substantially fewer genes than the *B. hyodysenteriae* chromosome. Rothkamp et al. (2002) recently cloned a putative *B. hyodysenteriae* phosphotransferase operon that was absent from *B. pilosicoli* and other *Brachyspira* species. Differences in gene content, organization, and regulation are likely to reflect differences in host range and disease capacities among the brachyspires. The physical and genetic maps of the genomes of *B. hyodysenteriae*, *Leptospira interrogans*, *Borrelia species*, *Treponema pallidum* and *T. denticola* reveal broad diversity among these spirochetes in terms of chromosomal conformation (linear and circular), chromosomal numbers (1–2), size (0.95–4.9 Mbp), and number and arrangement of rRNA genes on the chromosome (Zuerner, 1997).

Plasmids and Extrachromosomal DNA

Plasmids and extrachromosomal DNAs have been reported for various *Brachyspira* species (Combs et al., 1989; Turner and Sellwood, 1997; Cattani et al., 1998). Unfortunately, there is little information about these nucleic acids beyond their properties in electrophoretic gels. Plasmid DNA was not detected during the construction of a genomic map for *B. hyodysenteriae* B78^T (Zuerner and Stanton, 1994). In some reports (Combs et al., 1992; Turner and Sellwood, 1997), the characteristics of the plasmid/extrachromosomal DNAs resemble those of bacteriophage VSH-1 (virus of *Serpulina hyodysenteriae*; see Genetics). Some of the extrachromosomal DNAs could be DNA from VSH-1-like bacteriophage particles spontaneously produced in *Brachyspira* cultures.

Genetic Techniques

MUTAGENESIS The first isolation of a *B. hyodysenteriae* strain (mutated at a specific locus, *tlyA*) was reported in 1992 (ter Huurne et al., 1992b). The cloned *tlyA* gene, conferring a hemolytic phenotype in *E. coli* cells, was inactivated in vitro by inserting a gene encoding kanamycin resistance at a *BglII* site in the *tlyA* gene. This construct was then introduced as a plasmid into *B. hyodysenteriae* cells by electroporation. Mutant cells in which the kanamycin-resistant *tlyA* "knockout mutation" had undergone allelic exchange with the wild-type *tlyA* gene were selected by plating the bacteria on media containing kanamycin (ter Huurne et al., 1992b). With some further modifications, including the incorporation of another antibiotic selection marker (chloramphenicol), this technique has been used by other investigators to create *B. hyodysenteriae* strains with specific mutations in genes for flagellar proteins (Rosey et al., 1995; Li et al., 2000) and NADH oxidase (Stanton et al., 1999b).

The allelic exchange technique enables investigators to "custom design" *B. hyodysenteriae* strains, that is, to derive mutant strains that are isogenic to their progenitor strains, except for single genetic loci. Such strains are essential in determining bacterial virulence traits (See Disease).

Brachyspira hyodysenteriae mutant strains can be produced by exposure to UV light. Using UV mutagenesis, coumermycin A1-resistant *B. hyodysenteriae* strains with identifiable mutations in their *gyrB* (DNA gyrase subunit B) genes have been generated (Stanton et al., 2001). In addition to the kanamycin resistance and chloramphenicol resistance markers used for allelic exchange

Table 5. *Brachyspira* genes encoding identifiable proteins.

Gene (species)	Identified/putative gene product	GenBank Accession no.	Comments	Reference(s)
gyrA (Bh)	DNA Gyrase A subunit	N/A	Partial gene sequence	Zuemer and Stanton, 1994
gyrB (Bh)	DNA Gyrase B subunit	AF288224	Not contiguous with *gyrB*	Stanton et al., 2001
tlyA, B, C (Bh)	Genes conferring hemolytic activity on *E. coli*	A31492 X73140 X73141	*B. hyodysanteriae* hemolysins?	Hsu et al., 2001 Muir et al., 1992 ter Huurne et al., 1992 ter Huurne et al., 1994
hlyA (Bh) *hlyA* (Bp)	Bh gene conferring hemolytic activity on *E. coli*	U94886	Putative amino acid sequence matches Bh hemolysin sequence	Hsu et al., 2001 Zuerner et al., 2004
nox (all 7 current species)	NADH oxidase (water-forming)	U19610, AF060800 to AF060816	*Bh* protection from oxidative stress	Atyeo et al., 1999 Stanton and Sellwood, 1999a
smpA (Bh)	Outer membrane protein	X68401	Prolipoprotein; unique to some *Bh* strains	Sellwood et al., 1995 Turner et al., 1995
bmpB (several species)	Outer membrane protein	Patent limited	Prolipoprotein; immunogenic	Lee et al., 2000
flaA *flaB1, B2, B3 (Bh)*	Flagellar structural proteins	X63006 L34686 X63513 AF241832	Important in colonization and virulence	Gabe et al., 1995 Koopman et al., 1993 Li et al., 2000 Rosey et al., 1996 Koopman et al., 1992
*vsp*A-D (Bh) *vsp*E-H (Bh)	VspH immunoreactive surface protein	AF012102 AY027775	Two cluster multigene family	Gabe et al., 1998 McCarman et al., 1999 McCaman et al., 2003
vsh (Bh)	Capsid proteins of bacteriophage-like agent VSH-1	U90539	Putative amino acid sequences match protein sequences	T. B. Stanton et al., personal communication
mglB (Bp)	MglB homolog; immunogenic	AF200741	Putative glu-gal recognition protein (transport/chemotaxis)	Zhang et al., 2000
bitA-F (Bh)	BitA-F	U75349	Multigene system for iron transport	Dugourd et al., 1999
Unnamed (*Bp*)			Putative pyruvate oxidoreductase	Rayment et al., 1998
Unidentified (*Bh*)	Antigenic proteins	N/A	Uncharacterized genes and proteins; some protective	Boyden et al., 1989
tufA *rpsJ (Bh)*	Elongation factor Rb protein S10	U51635	N/A	T. B. Stanton, unpublished observation
blp genes G, F, E, A (Bh)	Immunoreactive lipoprotein BlpA	AY158073	Multigene family	Cullen et al., 2003
*bmp*C (Bp)	BmpC surface protein	AY363613	Membrane vesicle lipoprotein	Trott et al., 2004

Abbreviations: VSH, virus of *Brachyspira* (*Serpulina*) *hyodysenteriae*; Bh, *B. hyodysenteriae*; Bp, *B. pilosicoli*; N/A, not applicable.

mutagenesis, coumermycin resistance is a third antibiotic selection marker for genetic manipulations of *Brachsypira*. Some *B. hyodysenteriae* strains are resistant to the macrolide antibiotic tylosin, owing to a nucleotide base change in the 23S rDNA gene (Karlsson et al., 1999). Tylosin resistance can also be used as a selection marker for gene transfers in this species (T. B. Stanton, unpublished observations).

Bacteriophages and Generalized Transduction (VSH-1) When cultures are treated with mitomycin C, both *B. hyodysenteriae* and *B. innocens* cells lyse and release bacteriophage-like particles whose morphology is similar to, but smaller than, λ phage virions of *E. coli* (Humphrey et al., 1995). These virions resemble spontaneously appearing phages first detected by electron microscopy of *B. hyodysenteriae* cultures (Ritchie et al., 1978). A bacteriophage was purified from mitomycin C-treated *B. hyodysenteriae* cultures and named "VSH-1." The VSH-1 particles package random, 7.5-kB linear fragments of host DNA (Humphrey et al., 1997). The sequences of genes encoding several VSH-1 capsid head proteins have been deposited in GenBank (Table 5).

The significance of VSH-1 to *B. hyodysenteriae* genetics lies in its role as a gene vector. Purified from cultures of a chloramphenicol-resistant *B. hyodysenteriae* strain A203 (Δ*flaA1* 593:762::*cat*), VSH-1 virions will transfer chloramphenicol resistance to *B. hyodysenteriae* strain A216 (Δ*nox* 438-760::*kan*) (Humphrey et al., 1997). This is the first example of natural gene transfer for a spirochete and indicates that VSH-1 behaves like a generalized transducing bacteriophage. More significantly, it is not necessary to use purified VSH-1 particles for gene exchange. Coumermycin-resistant *gyrB* genes are transferred by spontaneously produced VSH-1 between two *B. hyodysenteriae* strains when the strains are cocultured (Stanton et al., 2001).

VSH-1 may also have ecological significance. Based on MEE analysis of 231 *B. hyodysenteriae* strains, Trott et al. (1997c) concluded that substantial genetic recombination had shaped the overall population structure of this spirochete. Virions of VSH-1 are logical vehicles for cell-to-cell gene transfer among *B. hyodysenteriae* strains.

Bacteriophages have also been detected in both mitomycin C-treated and untreated cultures of weakly hemolytic intestinal brachyspires from humans and animals (Calderaro et al., 1998a; Calderaro et al., 1998b; Stanton et al., 2003). These bacteriophages should be examined for gene transduction capabilities similar to those of VSH-1.

Ecology

General Concepts

Brachyspira natural habitats are animal intestinal tracts. The species *B. hyodysenteriae*, *B. pilosicoli*, *B. aalborgi* and *B. alvinipulli* colonize the mucosal epithelial surfaces of the cecum and colon (See Habitat). *Brachyspira pilosicoli* and *B. aalborgi* have been observed attached to intestinal enterocytes (Fig. 2) whereas *B. hyodysenteriae* and *B. alvinipulli* colonize over, among, and perhaps within intestinal epithelial cells.

To be successful intestinal colonizers, all *Brachyspira* species must survive passage between hosts, reach suitable intestinal sites, establish dividing cell populations at those sites, and persist at least long enough to allow exiting cells to colonize other hosts. Additionally, the pathogenic *Brachyspira* species inflict damage on host tissues. *Brachyspira* ecology, knowledge of the interactions of these spirochetes with the living and nonliving components of their environment, has arisen from investigations of virulence-associated traits of the pathogenic species (Disease). Nevertheless, some of these virulence-associated traits are undoubtedly also colonization factors shared by nonpathogenic *Brachyspira* species. These colonization traits are discussed below.

Chemotaxis and Motility

The intestinal mucosal epithelium is covered with a layer of mucus, a barrier to bacterial colonization (Savage, 1980; Forstner et al., 1984). *Brachyspira* species, whether or not they attach to the underlying epithelial cells, transit and populate this mucus blanket. The ability to swim in environments of high viscosity, such as mucous gels, and the ability to be attracted to mucus components or compounds diffusing from the underlying tissues are likely to be important adaptations for *Brachyspira* spirochetes (Canale-Parola, 1978; Holt, 1978; Kennedy et al., 1988; Milner and Sellwood, 1994; Kennedy and Yancey, 1996). *Brachyspira hyodysenteriae* cells are highly motile within mucus samples taken from dysenteric pigs and do not attach to intestinal epithelial cells (Kennedy et al., 1988). Cells of the pathogen are commonly observed in mucus-filled crypts of Lieberkuhn and within mucigen droplets of mucus-secreting goblet cells (Glock et al., 1974; Kennedy et al., 1988). In chemotaxis assays, *B. hyodysenteriae* cells are attracted to gastric mucin, the structural glycoprotein of mucus (Kennedy et al., 1988; Milner and Sellwood, 1994). Fucose and L-serine, chemical components of mucin, are strong chemoattractants (Kennedy and Yancey, 1996).

Oxygen Metabolism, Oxidative Stress, NADH Oxidase

Those *Brachyspira* species that have been studied are aerotolerant anaerobes. *Brachyspira hyodysenteriae* cells will grow in sealed culture vessels containing an initial atmosphere of 1% O_2 : 99% N_2 and will consume oxygen (Stanton and Lebo, 1988). Strains of *Brachyspira hyodysenteriae* and of other *Brachyspira* species contain the enzyme NADH oxidase at high levels of specific activity (Stanton et al., 1995). The *B. hyodysenteriae* NADH oxidase is a soluble, FAD-dependent, monomeric protein with a molecular mass of 47–48 kDa (Stanton and Jensen, 1993b).

NADH oxidase is considered to be a mechanism by which *B. hyodysenteriae* cells cope with oxygen in their native microhabitats, i.e., among the oxygen-respiring mucosal tissues of the swine intestinal tract (Stanton, 1997a; Stanton et al., 1999b). *Brachyspira hyodysenteriae nox*-deficient mutant strains are 100- to 10,000-fold more sensitive to oxygen exposure than are cells of an isogenic, wild-type strain (Stanton et al., 1999b). The mutant stains also are attenuated in virulence (See Disease). Protection from oxidative stress is an important factor in colonization of intestinal mucosal surfaces by *B. hyodysenteriae* cells. NADH oxidases are involved in protecting pathogenic *Streptococcus* species from oxidative stress and in achieving virulence (Gibson et al., 2000; Yu et al., 2001). *Brachyspira hyodysenteriae* has additional enzymes for protection against oxidative stress, namely, NADH peroxidase, superoxide dismutase, and catalase (Jensen and Stanton, 1993b; Stanton et al., 1999b).

Intestinal spirochete strains representing all seven *Brachyspira* species (Table 1) have NADH oxidase genes and the tested strains also contain significant NADH oxidase activity (Stanton et al., 1995; Atyeo et al., 1999). Neither swine intestinal spirochete *Treponema succinifaciens* 6091 nor bovine rumen spirochete *T. bryantii* RUS-1 has detectable NADH oxidase (Stanton et al., 1995).

Attachment to Tissues and Intestinal Colonization

Brachyspira pilosicoli and *B. aalborgi* spirochetes attach by one end to mature intestinal cells. Colonization in this manner leads to dense parallel assemblages of spirochetes (Fig. 2). In histological examinations of sectioned intestinal tissues, these assemblages give the appearance of a false brush border. This histological phenomenon, commonly referred to as "intestinal spirochetosis," is observed for both healthy and diseased humans and non-avian animal species (Taylor and Trott, 1997). It should be noted that the term "avian intestinal spirochetosis" (AIS) has been given to intestinal disorders of poultry associated with spirochetes regardless of whether the spirochetes are attached or not attached to intestinal tissues (Swayne and McLaren, 1997; Swayne, 2002).

For so elegant an example of prokaryotic-eukaryotic cell interactions, the mechanisms and significance of *Brachyspira* end-on attachments are disappointingly understudied. The polar attachment of *B. pilosicoli* cells to cecal enterocytes in one-day-old chicks currently provides the most practical experimental model for these investigations (Trott et al., 1995; Muniappa et al., 1998). Lattice-like structure (Sellwood and Bland, 1997) and surface, ring-like structures (Muniappa et al., 1998) observed at the tips of *B. pilosicoli* cells may be involved in attachment to the enterocytes.

Intestinal spirochetosis occurs in tissues from both healthy animals and animals with intestinal disorders (Takeuchi and Zeller, 1972; Neutra, 1980; Ruane et al., 1989; Barrett, 1997; Duhamel et al., 1997b). In swine this appearance is associated with *B. pilosicoli*-induced colitis (Taylor and Trott, 1997; Hampson and Trott, 1999b). In humans both *B. pilosicoli* and *B. aalborgi* can be detected in rectal biopsy samples where intestinal spirochetosis is identified (Hovind-Hougen, 1982; Trivett-Moore, 1998; Mikosza, 1999; Kraaz, 2000; Mikosza, 2001). Fluorescent oligonucleotide probes can be used to identify *Brachyspira* species among swine intestinal tissues (Jensen et al., 2000). This fluorescent in situ hybridization (FISH) technology will undoubtedly be important for the confirmed identification of spirochetes attached to cecal and colonic tissues.

Brachyspira aalborgi and *B. pilosicoli* may not be the only spirochete species capable of end-on attachment to enterocytes. Intestinal spirochetes attached to the rectal mucosa of humans (Neutra, 1980) and rhesus monkeys (Takeuchi and Zeller, 1972) are larger in cell diameter (0.5 μm) than cells of *B. aalborgi* (0.2 μm) and *B. pilosicoli* (0.3 μm) (Sellwood and Bland, 1997). *Brachyspira*-like spirochetes also colonize by end-on attachment to the cecal mucosal tissues of guinea pigs with typhlitis (Vanrobaeys et al., 1998). Other than *B. aalborgi* and *B. pilosicoli*, none of these attached spirochetes has been isolated.

Survival Outside the Host

An important aspect of the transmission of any host-associated bacterium is the ability to survive outside the host species. *Brachyspira hyodysenteriae* cells at concentrations sufficient to induce swine dysentery will survive up to a week in

lagoon water used to clean swine buildings (Olson, 1995). *Brachyspira* pilosicoli cells survive in lake water for 66 days at 4°C (Oxberry et al., 1998). In laboratory studies, *B. hyodysenteriae* cells remain viable in diluted feces stored at 5°C for up to 60 days (Chia and Taylor, 1978). Pure cultures of *B. hyodysenteriae* and *B. pilosicoli* added at high concentrations (>10^9 cfu/gm) to swine feces or a 10:90 mix of feces and soil and stored at 10°C remain viable for 78–210 days (Boye et al., 2001). Environmental factors such as humidity and temperature undoubtedly affect survival. *Brachyspira* bacterial traits involved in survival have not been identified.

Epidemiology

Subspecies Identification

Serotype Analysis *Brachyspira hyodysenteriae* strains can be subdivided into serotypes based on the immunological reactivities of lipo-oligosaccharides (LOS) in hot water-phenol extracts of whole cells (Baum and Joens, 1979; Li et al., 1991). A more elaborate system of immunological identification assigns *B. hyodysenteriae* strains to serogroups and subdivides the groups into serovars (Hampson et al., 1989; Hampson et al., 1997; Lau and Hampson, 1992). This system has not been widely adopted because serogroups are not entirely consistent with genetic groupings based on MEE analysis (Lee et al., 1993a; Trott et al., 1997c). Enzyme-linked immunosorbent assay (ELISA) methods based on antibodies to LOS have been used to detect animal herd exposure to *B. hyodysenteriae* strains (Joens et al., 1982; Wright et al., 1989).

Differentiation Based on Gene and Genome Differences Several methods for differentiating strains of uncharacterized intestinal spirochetes and strains within the species *B. hyodysenteriae* or *B. pilosicoli* take advantage of DNA sequence differences. Sequence differences have been directly detected through restriction endonuclease analyses of genomic DNAs either with or without hybridization with specific gene probes (Combs et al., 1992; ter Huurne et al., 1992a; Koopman et al., 1993a; Harel et al., 1994; Atyeo et al., 1996; Rayment et al., 1997; Fellstrom et al., 1999).

Multilocus enzyme electrophoresis (MEE) analysis is useful for determining genetic relationships among intestinal spirochetes at the species level for taxonomy. The technique can also be used to differentiate *B. hyodysenteriae* or *B. pilosicoli* at the subspecies level for epidemiological purposes (Lymbery et al., 1990; Oxberry et al., 1998; Trott et al., 1998).

Brachyspira Population Structure

Trott and colleagues investigated the genetic diversity of 231 *B. hyodysenteriae* isolates by MEE (Trott et al., 1997c). The electrophoretic profiles of 12 out of 15 tested enzymes were used to identify 50 different electrophoretic types among the isolates. Based on the findings, *B. hyodysenteriae* appears as a genetically diverse species with an epidemic population structure. Substantial genetic recombination likely has shaped the population structure of the species. Generalized gene transduction by VSH-1 (see Genetics) could play a role in this natural genetic recombination (Trott et al., 1997c; Stanton et al., 2001). *Brachyspira hyodysenteriae* population structure and epidemic clones may result from disease control measures and animal management practices, including antibiotic use (Trott et al., 1997c; Fellstrom et al., 1999).

Based on both MEE and PFGE methods, population analyses of *B. pilosicoli* from indigenous peoples of villages in Papua, New Guinea, suggest a recombinant structure also for that spirochete (Trott et al., 1998). Thus far, assays to differentiate *B. pilosicoli* strains originating from different host species indicate a high diversity of genotypes for this spirochete, making it difficult to conclude that zoonotic transmission of the pathogen to humans occurs (Rayment et al., 1997; Trott et al., 1997c), although this remains a possibility.

Disease

Brachyspira hyodysenteriae, *B. pilosicoli*, *B. intermedia* and *B. alvinipulli* cause intestinal disease when inoculated into their normal, healthy host animals (Table 1). *Brachyspira hyodysenteriae*, the etiologic agent of swine dysentery, has been investigated for several decades, whereas the other two species were identified and have been studied only over the last few years. Thus, most disease-related research on *Brachyspira* has focused on *B. hyodysenteriae*.

Several recent publications extensively describe *Brachyspira* diseases from the viewpoint of host manifestations, clinical detection methods, therapies, and experimental models (Barrett, 1997; Galvin et al., 1997; Hampson et al., 1997; Swayne and McLaren, 1997; Taylor and Trott, 1997; Hampson and Trott, 1999b; Harris et al., 1999; Swayne, 2002; Stephens and Hampson, 2001). Published research papers from the recurrent International Conference on Colonic Spirochaetes also provide topical information on these subjects.

Swine Dysentery (*B. hyodysenteriae*)

Swine dysentery (bloody scours or black scours) is a severe intestinal disease that affects piglets, primarily in the postweaning stage of growth (8–14 weeks after birth). The disease has been reported worldwide in every major pig producing country. A typical sign of the disease is profuse bleeding into the large bowel lumen through lesions induced by *B. hyodysenteriae* cells. Afflicted animals pass loose stools containing blood and mucus and microscopically visible spirochetes. These are presumptive signs of the disease. Culturing and identifying *B. hyodysenteriae* cells, along with histopathological observations, provide conclusive evidence of swine dysentery. Up to 90–100% of a herd can become infected, and without effective treatment, 20–30% of infected animals may die. Economic losses result from death, poor weight gain/feed efficiency, and medication expenses. Swine management strategies, including segregation by age and prophylactic administration of antibiotics, are believed responsible for a reduction in swine dysentery in the United States in recent years.

Swine dysentery is easily produced by feeding or intragastrically inoculating normal swine with *B. hyodysenteriae* cultures (Kinyon et al., 1977; Kennedy et al., 1988; Stanton and Jensen, 1993a). However, the type strain B78^T of *B. hyodysenteriae* is weakly virulent and should not be used in experimental infections (Jensen and Stanton, 1993a).

Various approaches have been used to develop whole cell or cell subunit-based vaccines for swine dysentery (summarized in Lee et al., 2000). One commercial vaccine for swine dysentery is based on pepsin-digested *B. hyodysenteriae* cells (Intervet, Akzo Nobel, DeSoto, KS). The immunological properties of the vaccine are now being examined (Waters et al., 2000).

Brachyspira hyodysenteriae has a limited host range. Swine are the common, but not the exclusive, hosts. Strains of the spirochete also have been isolated from juvenile rheas with a severe necrotizing typhlitis (Sagartz et al., 1992; Jensen et al., 1996; Buckles et al., 1997). Mice (Joens and Glock, 1979; Nibbelink and Wannemuehler, 1992; ter Huurne et al., 1992b; Rosey et al., 1996) and one-day-old chicks (Sueyoshi and Adachi, 1990) have been used in experimental infections. Nevertheless, nuances or inconsistencies are associated with the use of these surrogate animal models (Jensen and Stanton, 1993a; Achacha et al., 1996). For this reason, conclusions regarding *B. hyodysenteriae* pathogenesis based on mouse or one-day-old chick models should be confirmed through the use of swine infections.

Brachyspira hyodysenteriae Virulence Determinants

Several cell properties are putative or demonstrated virulence-associated traits of *B. hyodysenteriae*. They include the following: LOS in a mouse model (Nuessen et al., 1982; Nuessen et al., 1983; Greer and Wannemuehler, 1989; Nibbelink and Wannemuehler, 1991); hemolysin/hemolytic activity (Saheb et al., 1980; Saheb et al., 1981; Kent et al., 1988; Lysons et al., 1991; ter Huurne et al., 1992b; ter Huurne et al., 1994; Hutto and Wannemuehler, 1999; Hsu et al., 2001); chemotaxis/motility (Glock et al., 1974; Kennedy et al., 1988; Milner and Sellwood, 1994; Kennedy and Yancey, 1996); oxygen metabolism/NADH oxidase (Jensen and Stanton, 1993b; Stanton and Jensen, 1993b; Stanton, 1997a; Stanton et al., 1999b); and immunoreactive membrane proteins VspH and BlpA with a potential for antigenic variation (Cullen et al., 2003; Gabe et al., 1998; McCaman et al., 2003). The ability to create strains with specific gene mutations provides direct evidence of a link between virulence and NADH oxidase activity, motility/flagella (see Ecology), and hemolytic activity. Mutant strains with specific deletions of *nox* (Stanton et al., 1999b), *flaA* or *flaB* (Kennedy et al., 1997), or *tlyA* (Hyatt et al., 1994; Joens, 1997) are avirulent for swine compared to their isogenic wild-type counterparts.

Though *tlyA*, *tlyB*, and *tlyC* were considered to be hemolysin genes of *B. hyodysenteriae* (Muir et al., 1992; ter Huurne et al., 1992b; ter Huurne et al., 1994), a recent study raises questions about whether the proteins encoded by these genes are true hemolysins (Hsu et al., 2001). In that study, a protein with hemolytic activity was purified from *B. hyodysenteriae* cells and its gene (*hlyA*) was cloned in *E. coli*. The recombinant protein has hemolytic and biochemical activities similar to those of the native purified hemolysin. These properties are different from those of the previously described TlyA-C proteins. The latter proteins, obtained by random "shotgun" cloning of *B. hyodysenteriae* DNA into *E. coli* with selection for hemolytic colonies, are possibly *B. hyodysenteriae* regulatory proteins that induce synthesis of known or cryptic hemolysins in *E. coli* cells. Alternatively they could be "pseudo"-hemolytic proteins, the result of being overexpressed from high copy plasmids in *E. coli*. Interestingly, although its identity as a true hemolysin has been questioned, the *tlyA* gene product is essential for virulence (ter Huurne et al., 1992b; Hyatt et al., 1994). Its role in virulence should be examined further.

Spirochetal Colitis and Spirochetal Diarrhea (*B. pilosicoli*)

Spirochetal colitis caused by *Brachyspira pilosicoli* is a mild to moderate diarrheal disease of swine, birds, and possibly humans (Swayne and McLaren, 1997; Taylor and Trott, 1997; Hampson and Trott, 1999b). Watery, mucoid diarrhea and a reduction of growth rate of affected animals are common clinical signs. Spirochetal colitis of swine resembles a mild case or a very early stage of swine dysentery. The disease can be experimentally produced by inoculating pure cultures of *B. pilosicoli* into normal swine (Taylor et al., 1980; Trott et al., 1996c; Thomson et al., 1997).

Evidence that *B. pilosicoli* is a pathogen of humans is circumstantial but multifarious. *Brachyspira pilosicoli* strains have been isolated from humans, including some (homosexual males or those living in developing countries) with intestinal disorders and who are immunocompromised (Barrett, 1997; Trott et al., 1997a; Trott et al., 1997b). Human strains are virulent for healthy piglets (Trott et al., 1996b). A human volunteer became colonized after drinking cultures of *B. pilosicoli* strain Wes B (Oxberry et al., 1998). Finally, *B. pilosicoli* has been isolated from the blood of human patients (Trott et al., 1997b). The significance and the capacity of *B. pilosicoli* cells to leave the intestinal tract and circulate throughout the host's body have not been sufficiently investigated.

Virulence factors of *B. pilosicoli* are unknown. By virtue of their location, outer membrane proteins undoubtedly mediate interactions between spirochete cells and their environment and are likely involved in host colonization and virulence (Trott et al., 2001; Trott et al., 2004). Cell motility, chemotaxis and spirochete end-on attachment to host tissues (see Ecology) are likely to be associated with colonization of the intestinal tract and therefore important for pathogenesis. In the absence of gross lesions, extensive colonization of intestinal tissues by *B. pilosicoli* cells with associated damage to microvilli could interfere with intestinal absorptive processes and lead to diarrhea (Gad et al., 1977; Taylor and Trott, 1997).

Human Intestinal Spirochetosis (*B. pilosicoli*, *B. aalborgi*, *Brachyspira* spp.)

There is no doubt that human colonic and rectal mucosae can be colonized by dense arrays of spirochetes (Barrett, 1997; Harland and Lee, 1967; Korner and Gebbers, 2003). The colonization is visible upon histological examination of intestinal biopsies and is known as human intestinal spirochetosis (HIS). There are unanswered questions, however, about the clinical significance of HIS and about the identities of the colonizing spirochetes. The prevalence of HIS appears greater among humans with poor living standards and among immunocompromised patients, for example HIV patients, than among healthy humans. HIS has been associated with intestinal disorders but also has been observed in healthy humans (reviewed in Barrett, 1997; Korner and Gebbers, 2003). Spirochetes in human feces and/or in association with HIS biopsies have been reported to be strains of *B. pilosicoli*, *B. aalborgi*, and unknown *Brachyspira* species (Brooke et al., 2003; Hovind-Hougen et al., 1982; Jensen et al., 2001; Mikosza et al., 2001). This species diversity could explain differences in clinical symptoms in humans. An alternative explanation is that HIS strains belonging to the same *Brachyspira* species may differ in pathogenic properties. The patient's physiologic, immune response, and non-spirochete microbiota could also be factors in determining whether the colonizing spirochetes are commensals or pathogens. Progress in understanding HIS would undoubtedly benefit from advances in understanding the diversity and unique properties of human spirochete species.

Avian Spirochete Intestinal Diseases

Avian diarrheal diseases can have various *Brachyspira* species as etiological agents (Swayne and McLaren, 1997; Swayne, 2002; Stephens and Hampson, 2001, 2004). In addition to *B. pilosicoli* and *B. hyodysenteriae* (see Disease), *B. alvinipulli* $C1^T$, isolated from a diarrheic chicken, is a chicken enteropathogen. The $C1^T$ cells colonize the ceca of 1-day-old chicks and 14-month-old hens and produce mild typhlitis with discolored and watery cecal contents (Swayne et al., 1995). *Brachyspira alvinipulli* has been isolated only from poultry and resembles an enteropathogenic spirochete earlier described by Davelaar et al. (1986).

Many studies of avian intestinal infections associated with spirochetes were made before *Brachyspira* species had been characterized and taxonomically established (Davelaar et al., 1986; Griffiths et al., 1987; Dwars et al., 1989). *Brachyspira intermedia* appears to be another common avian enteropathgen, inasmuch as spirochetes of that species have been isolated from birds with moderate intestinal colitis (Swayne and McLaren, 1997; Stephens and Hampson, 2001; Swayne, 2002). One-day-old chicks inoculated with pure cultures of an intestinal spirochete strain 1380, later identified by MEE analysis as a strain of *B. intermedia* (Swayne and McLaren, 1997), shed watery feces containing spirochetes and had detectable reductions in body weight compared to control birds (Dwars

et al., 1992). Avian *B. intermedia* strain HB60 isolated from a hen with diarrhea causes reduced egg production and watery feces when inoculated into healthy hens (Hampson and McLaren, 1999a). A *B. intermedia* strain, freshly isolated from a swine herd with diarrhea, was not pathogenic for swine (Jensen et al., 2000).

Virulence traits of *B. alvinipulli* and other avian enteropathogenic *Brachyspira* species have yet to be determined.

Brachyspira spp. Antimicrobial Resistance

International and national guidelines for evaluating and monitoring *Brachyspira* antimicrobial susceptibilities under standard culture conditions are not yet available. *B. hyodysenteriae* strains have been divided into sensitive and resistant groups based on their susceptibilities to tylosin, erythromycin, and clindamycin (Karlsson et al., 2003). Resistance to these antibiotics is associated with a 23S rRNA gene mutation (Karlsson et al., 1999). *B. pilosicoli* isolates from humans or swine were found to be resistant to tetracycline, clindamycin, and amoxicillin. All strains were susceptible to an amoxicillin-clavulanic acid combination, indirect evidence that a b-lactamase is involved in resistance to amoxicillin.

Applications

Among the spirochetes, *Brachyspira hyodysenteriae* stands out as a good, practical choice for studying spirochete genetics and for investigating basic biological mechanisms of spirochetes, including host-pathogen interactions. The cultural, nutritional, and metabolic properties of *B. hyodysenteriae* have been substantially characterized and a serum-free, low-protein culture medium has been described (See Physiology). *Brachyspira hyodysenteriae* is an anaerobic spirochete but is aerotolerant. An experimental disease model featuring swine, the natural animal host of *B. hyodysenteriae*, has been available for many years (See Disease). Most importantly, recent research has provided a basis for understanding and manipulating this spirochete at the gene level (See Genetics).

Brachyspira hyodysenteriae is currently being used as a model in studies of spirochete motility. Strains with single or double mutations in specific flagellar genes are being created and used to evaluate the role of specific flagellar proteins in flagellar ultrastructure and cell motility (Li et al., 2000; C. Li and N. W. Charon, personal communication).

Literature Cited

Achacha, M., and S. Messier. 1992. Comparison of six different culture media for isolation of Treponema hyodysenteriae. J. Clin. Microbiol. 30:249–251.

Achacha, M., S. Messier, and K. R. Mittal. 1996. Development of an experimental model allowing discrimination between virulent and avirulent isolates of Serpulina (Treponema) hyodysenteriae. Can. J. Vet. Res. 60:45–49.

Atyeo, R. F., S. L. Oxberry, and D. J. Hampson. 1996. Pulsed-field gel electrophoresis for sub-specific differentiation of Serpulina pilosicoli (formerly "Anguillina coli"). FEMS Microbiol. Lett. 141:77–81.

Atyeo, R. F., S. L. Oxberry, B. G. Combs, and D. J. Hampson. 1998. Development and evaluation of polymerase chain reaction tests as an aid to diagnosis of swine dysentery and intestinal spirochaetosis. Lett. Appl. Microbiol. 26:126–130.

Atyeo, R. F., T. B. Stanton, N. S. Jensen, D. S. Suriyaarachichi, and D. J. Hampson. 1999. Differentiation of Serpulina species by NADH oxidase gene (nox) sequence comparisons and nox-based polymerase chain reaction tests. Vet. Microbiol. 67:47–60.

Barcellos, D. E. S. N., de Useda, M., Ikuta, N., Lunge, V. R., Fonseca, A. S. K., Kader, I. I. T. A., and Duhamel, G. E. 2000. Identification of porcine intestinal spirochetes by PCR-restriction fragment length polymorphism analysis of ribosomal DNA encoding 23S rRNA. Vet. Microbiol. 75:189–198.

Barrett, S. P. 1997. Human intestinal spirochaetosis. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 243–265.

Baum, D. H., and L. A. Joens. 1979. Serotypes of beta-hemolytic Treponema hyodysenteriae. Infect. Immun. 25:792–796.

Boyden, D. A., F. G. Albert, and C. S. Robinson. 1989. Cloning and characterization of Treponema hyodysenteriae antigens and protection in a CF-1 mouse model by immunization with a cloned endoflagellar antigen. Infect. Immun. 57:3808–3815.

Boye, M., S. B. Baloda, T. D. Leser, and K. Moller. 2001. Survival of Brachyspira hyodysenteriae and B. pilosicoli in terrestrial microcosms. Vet. Microbiol. 81:33–40.

Brooke, C. J., K. R. Margawani, A. K. Pearson, T. V. Riley, I. D. Robertson, and D. J. Hampson. 2000. Evaluation of blood culture systems for detection of the intestinal spirochaete Brachyspira (Serpulina) pilosicoli in human blood. J. Med. Microbiol. 49:1031–1036.

Brooke, C. J., T. V. Riley, and D. J. Hampson. 2003. Evaluation of selective media for the isolation of Brachyspira aalborgi from human faeces. J Med Microbiol 52:509–513.

Buckles, E. L., K. A. Eaton, and D. E. Swayne. 1997. Cases of spirochete-associated necrotizing typhlitis in captive common rheas (Rhea americana). Avian Dis. 41:144–148.

Calderaro, A., G. Dettori, L. Collini, P. Ragni, R. Grillo, P. Cattani, G. Fadda, and C. Chezzi. 1998a. Bacteriophages induced from weakly beta-haemolytic human intestinal spirochaetes by mitomycin C. J. Basic Microbiol. 38:323–335.

Calderaro, A., G. Dettori, R. Grillo, P. Plaisant, G. Amalfitano, and C. Chezzi. 1998b. Search for bacteriophages spontaneously occurring in cultures of haemolytic intes-

tinal spirochaetes of human and animal origin. J. Basic Microbiol. 38:313–322.

Calderaro, A., G. Merialdi, S. Perini, P. Ragni, R. Guegan, G. Dettori, and C. Chezzi. 2001. A novel method for isolation of Brachyspira (Serpulina) hyodysenteriae from pigs with swine dysentery in Italy. Vet. Microbiol. 80:47–52.

Calderaro, A., V. Villanacci, M. Conter, P. Ragni, G. Piccolo, C. Zuelli, S. Bommezzadri, R. Guegan, C. Zambelli, F. Perandin, M. C. Arcangeletti, M. C. Medici, N. Manca, G. Dettori, and C. Chezzi. 2003. Rapid detection and identification of Brachyspira aalborgi from rectal biopsies and faeces of a patient. Res Microbiol 154:145–153.

Canale-Parola, E. 1978. Motility and chemotaxis of spirochetes. Ann. Rev. Microbiol. 32:69–99.

Cattani, P., G. Dettori, A. Calderaro, R. Grillo, G. Fadda, and C. Chezzi. 1998. Detection of extrachromosomal DNA in Italian isolates of weakly beta-haemolytic human intestinal spirochaetes. Microbiologica 21:241–248.

Chia, S. P., and D. J. Taylor. 1978. Factors affecting the survival of Treponema hyodysenteriae in dysenteric pig faeces. Vet. Rec. 103:68–70.

Combs, B., D. J. Hampson, J. R. L. Mhoma, and J. R. Buddle. 1989. Typing of Treponema hyodysenteriae by restriction endonuclease analysis. Vet. Microbiol. 19:351–359.

Combs, B. G., D. J. Hampson, and S. J. Harders. 1992. Typing of Australian isolates of Treponema hyodysenteriae by serology and by DNA restriction endonuclease analysis. Vet. Microbiol. 31:273–285.

Crosa, J. H., D. J. Brenner, and S. Falkow. 1973. Use of a single-strand specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. J. Bacteriol. 115:904–911.

Cullen, P. A., M. Lo, D. M. Bulach, S. J. Cordwell, and B. Adler. 2003. Construction and evaluation of a plasmid vector for the expression of recombinant lipoproteins in Escherichia coli. Plasmid 49:18–29.

Cullen, P. A., S. A. Coutts, S. J. Cordwell, D. M. Bulach, and B. Adler. 2003. Characterization of a locus encoding four paralogous outer membrane lipoproteins of Brachyspira hyodysenteriae. Microbes Infect 5:275–283.

Davelaar, F. G., H. F. Smit, K. Hovind-Hougen, R. M. Dwars, and P. C. van der Valk. 1986. Infectious typhlitis in chickens caused by spirochetes. Avian Pathol. 15:247–258.

De Smet, K. A., D. E. Worth, and S. P. Barrett. 1998. Variation amongst human isolates of Brachyspira (Serpulina) pilosicoli based on biochemical characterization and 16S rRNA gene sequencing. Int. J. Syst. Bacteriol. 48(4):1257–1263.

Dugourd, D., C. Martin, C. R. Rioux, M. Jacques, and J. Harel. 1999. Characterization of a periplasmic ATP-binding cassette iron import system of Brachyspira (Serpulina) hyodysenteriae. J. Bacteriol. 181:6948–6957.

Duhamel, G. E. 1997a. Intestinal spirochaetes in non-production animals. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 301–320.

Duhamel, G. E., R. O. Elder, N. Muniappa, M. R. Mathiesen, V. J. Wong, and R. P. Tarara. 1997b. Colonic spirochetal infections in nonhuman primates that were associated with Brachyspira aalborgi, Serpulina pilosicoli, and unclassified flagellated bacteria. Clin. Infect. Dis. 25:S186–S188.

Duhamel, G. E., D. J. Trott, N. Muniappa, M. R. Mathiesen, K. Tarasiuk, J. I. Lee, and D. J. Hampson. 1998. Canine intestinal spirochetes consist of Serpulina pilosicoli and a newly identified group provisionally designated "Serpulina canis" sp. nov. J. Clin. Microbiol. 36:2264–2270.

Dwars, R. M., H. F. Smit, F. G. Davelaar, and V. T. Veer. 1989. Incidence of spirochaetal infections in cases of intestinal disorder in chickens. Avian Pathol. 18:591–595.

Dwars, R. M., F. G. Davelaar, and H. F. Smit. 1992. Spirochaetosis in broilers. Avian Pathol. 21:261–273.

Elder, R. O., G. E. Duhamel, R. W. Schafer, M. R. Mathiesen, and M. Ramanathan. 1994. Rapid detection of Serpulina hyodysenteriae in diagnostic specimens by PCR. J. Clin. Microbiol. 32:1497–1502.

Fellstrom, C., B. Petterson, M. Uhlen, A. Gunnarsson, and K. E. Johansson. 1995. Phylogeny of Serpulina based on sequence analyses of the 16S rRNA gene and comparison with a scheme involving biochemical classification. Res. Vet. Sci. 59:5–9.

Fellstrom, C., B. Pettersson, J. Thomson, A. Gunnarsson, M. Persson, and K.-E. Johansson. 1997. Identification of Serpulina species associated with porcine colitis by biochemical analysis and PCR. J. Clin. Microbiol. 35:462–467.

Fellstrom, C., M. Karlsson, B. Pettersson, U. Zimmerman, A. Gunnarsson, and A. Aspan. 1999. Emended descriptions of indole negative and indole positive isolates of Brachyspira (Serpulina) hyodysenteriae. Vet. Microbiol. 70:225–238.

Finegold, S. M., and W. J. Martin. 1982. *In:* Bailey and Scott's Diagnostic Microbiology, 6th ed. The C.V. Mosby Company. St. Louis, MO. 668–669.

Forstner, G., P. Sherman, and J. Forstner. 1984. Mucus: Function and structure. *In:* E. C. Boedeker (Ed.) Attachment of Organisms to the Gut Mucosa. CRC Press. Boca Raton, FL. 13–21.

Gabe, J. D., R.-J. Chang, R. Slomiany, W. H. Andrews, and M. T. McCaman. 1995. Isolation of extracytoplasmic proteins from Serpulina hyodysenteriae (B204) and the molecular cloning of the flaB1 gene encoding a 38 kDa flagellar protein. Infect. Immun. 63:142.

Gabe, J. D., E. Dragon, R. J. Chang, and M. T. McCaman. 1998. Identification of a linked set of genes in Serpulina hyodysenteriae (B204) predicted to encode closely related 39-kilodalton extracytoplasmic proteins. J. Bacteriol. 180:444–448.

Gad, A., R. Willen, K. Furugard, R. Fors, and M. Hradsky. 1977. Intestinal spirochaetosis as a cause of longstanding diarrhoea. Uppsala J. Med. Sci. 82:49–54.

Galvin, J. E., D. L. Harris, and M. J. Wannemuehler. 1997. Prevention and control of intestinal spirochaetal disease: immunological and pharmacological mechanisms. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 343–374.

Gibson, C. M., T. C. Mallett, A. Claiborne, and M. G. Caparon. 2000. Contribution of NADH oxidase to aerobic metabolism of Streptococcus pyogenes. J. Bacteriol. 182:448–455.

Glock, R. D., D. L. Harris, and J. P. Kluge. 1974. Localization of spirochetes with the structural characteristics of Treponema hyodysenteriae in the lesions of swine dysentery. Infect. Immun. 9:167–178.

Greer, J. M., and M. J. Wannemuehler. 1989. Comparison of the biological responses induced by lipopolysaccharide and endotoxin of Treponema hyodysenteriae and Treponema innocens. Infect. Immun. 57:717–723.

Griffiths, I. B., B. W. Hunt, S. A. Lister, and M. H. Lamont. 1987. Retarded growth rate and delayed onset of egg

production associated with spirochaete infection in pullets. Vet. Rec. 121:35–37.

Grimont, P. A. D., M. Y. Popoff, F. Grimont, C. Coynault, and M. Lemelin. 1980. Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. Curr. Microbiol. 4:325–330.

Hampson, D.J., J.R. Mhoma, B. Combs, and J.R. Buddle. 1989. Proposed revisions to the serological typing system for Treponema hyodysenteriae. Epidemiol. Infect. 102: 75–84.

Hampson, D. J., R. F. Atyeo, and B. G. Combs. 1997. Swine dysentery. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 175–209.

Hampson, D. J., and A. J. McLaren. 1999a. Experimental infection of laying hens with Serpulina intermedia causes reduced egg production and increased faecal water content. Avian Path. 28:113–117.

Hampson, D. J., and D. J. Trott. 1999b. Spirochetal diarrhea/porcine intestinal spirochetosis. *In:* B. E. Straw, S. D'Allaire, W. L. Mengeling, and D. E. Taylor (Eds.) Diseases of Swine. Iowa State University Press. Ames, IA. 553–562.

Hardham, J. M., and E. L. Rosey. 2000. Antibiotic selective markers and spirochete genetics. J. Molec. Microbiol. Biotechnol. 2:425–432.

Harel, J., M. Belanger, C. Forget, and M. Jacques. 1994. Characterization of Serpulina hyodysenteriae isolates of serotypes 8 and 9 from Quebec by restriction endonuclease fingerprinting and ribotyping. Can. J. Vet. Res. 58:302–305.

Harel, J., and C. Forget. 1995. DNA probe and polymerase chain reaction precedure for the specific detection of Serpulina hyodysenteriae. Molec. Cell. Probes 9:111–119.

Harland, W.A. and F.D. Lee. 1967. Intestinal spirochaetosis. British Medical Journal 3:718–719.

Harris, D. L., R. D. Glock, C. R. Christensen, and J. M. Kinyon. 1972a. Swine dysentery. I: Inoculation of pigs with Treponema hyodysenteriae (new species) and reproduction of the disease. Vet. Med. 67:61–64.

Harris, D. L., J. M. Kinyon, M. T. Mullin, and R. D. Glock. 1972b. Isolation and propagation of spirochetes from the colon of swine dysentery affected pigs. Can. J. Comp. Med. 36:74–76.

Harris, D. L., D. J. Hampson, and R. D. Glock. 1999. Diseases of swine. *In:* B. E. Straw, S. D'Allaire, W. L. Mengeling, and D. E. Taylor (Eds.) Diseases of Swine. Iowa State University Press. Ames, IA. 579–600.

Holt, S. C. 1978. Anatomy and chemistry of spirochetes. Microbiol. Rev. 42:114–160.

Hookey, J. V., S. P. Barrett, C. S. Reed, and P. Barber. 1994. Phylogeny of human intestinal spirochaetes inferred from 16S rDNA sequence comparisons. FEMS Microbiol. Lett. 117:345–350.

Hovind-Hougen, K., A. Birch-Andersen, R. Henrik-Nielsen, M. Orholm, J.O. Pedersen, P. S. Teglbjaerg, and E. H. Thaysen. 1982. Intestinal spirochetosis:morphological characterization and cultivation of the spirochete Brachyspira aalborgi gen. nov., sp. nov. J. Clin. Microbiol. 16:1127–1136.

Hsu, T., D. L. Hutto, F. C. Minion, R. L. Zuerner, and M. J. Wannemuehler. 2001. Cloning of a beta-hemolysin gene of Brachyspira (Serpulina) hyodysenteriae and its expression in Escherichia coli. Infect. Immun. 69:706–711.

Humphrey, S. B., T. B. Stanton, and N. S. Jensen. 1995. Mitomycin C induction of bacteriophages from Serpulina hyodysenteriae and Serpulina innocens. FEMS Microbiol. Lett. 134:97–101.

Humphrey, S. B., T. B. Stanton, N. S. Jensen, and R. L. Zuerner. 1997. Purification and characterization of VSH-1, a generalized transducing bacteriophage of Serpulina hyodysenteriae. J. Bacteriol. 179:323–329.

Hunter, D., and T. Wood. 1979. An evaluation of the API ZYM system as a means of classifying spirochaetes associated with swine dysentery. Vet. Rec. 104:383–384.

Hutto, D. L., and M. J. Wannemuehler. 1999. A comparison of the morphologic effects of Serpulina hyodysenteriae or its beta-hemolysin on the murine cecal mucosa. Vet. Pathol. 36:412–422.

Hyatt, D. R., A. A. H. M. ter Huurne, B. A. M. van der Zeijst, and L. A. Joens. 1994. Reduced virulence of Serpulina hyodysenteriae hemolysin-negative mutants in pigs and their potential to protect pigs against challenge with a virulent strain. Infect. Immun. 62:2244–2248.

Jenkinson, S. R., and C. R. Winger. 1981. Selective medium for isolation of Treponema hyodysenteriae. Vet. Rec. 109:384–385.

Jensen, N. S., and T. B. Stanton. 1993a. Comparison of Serpulina hyodysenteriae B78, the type strain of the species, with other S. hyodysenteriae strains using enteropathogenicity studies and restriction fragment length polymorphism analysis. Vet. Microbiol. 36:221–231.

Jensen, N. S., and T. B. Stanton. 1993b. Production of a hydrogen peroxide-inducible catalase activity by Serpulina hyodysenteriae. *In:* Ann. Mtg. ASM 93rd. Atlanta, GA. Abstract D-175:126.

Jensen, N. S., and T. B. Stanton. 1994. Production of an inducible sucrase activity by Serpulina hyodysenteriae. Appl. Environ. Microbiol. 60:3429–3432.

Jensen, N. S., T. B. Stanton, and D. E. Swayne. 1996. Identification of the swine pathogen Serpulina hyodysenteriae in rheas (Rhea americana). Vet. Microbiol. 52:259–269.

Jensen, N. S. 1997. Detection, identification, and subspecific differentiation of intestinal spirochaetes. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 323–341.

Jensen, T. K., K. Moller, M. Boye, T. D. Leser, and S. E. Jorsal. 2000. Scanning electron microscopy and fluorescent in situ hybridization of experimental Brachyspira (Serpulina) pilosicoli infection in growing pigs. Vet. Pathol. 37:22–32.

Jensen, T. K., M. Boye, P. Ahrens, B. Korsager, P. S. Teglbjaerg, C.F. Lindboe, and K. Moller. 2001. Diagnostic examination of human intestinal spirochetosis by fluorescent in situ hybridization for Brachyspira aalborgi, Brachyspira pilosicoli, and other species of the genus Brachyspira (Serpulina). J. Clin. Microbiol. 39:4111–4118.

Joens, L. A., and R. D. Glock. 1979. Experimental infection in mice with Treponema hyodysenteriae. Infect. Immun. 25:757–760.

Joens, L. A., N. A. Nord, J. M. Kinyon, and I. T. Egan. 1982. Enzyme-linked immunosorbent assay for detection of antibody to Treponema hyodysenteriae antigens. J. Clin. Microbiol. 15:249–252.

Joens, L. A. 1997. Virulence factors associated with Serpulina hyodysenteriae. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 151–172.

Jones, M. J., J. N. Miller, and W. L. George. 1986. Microbiological and biochemical characterization of spirochetes isolated from the feces of homosexual males. J. Clin. Microbiol. 24:1071–1074.

Karlsson, M., C. Fellstrom, M. U. Heldtander, K. E. Johansson, and A. Franklin. 1999. Genetic basis of macrolide and lincosamide resistance in Brachyspira (Serpulina) hyodysenteriae. FEMS Microbiol. Lett. 172:255–260.

Karlsson, M., C. Fellstrom, A. Gunnarsson, A. Landen, and A. Franklin. 2003. Antimicrobial susceptibility testing of porcine Brachyspira (Serpulina) species isolates. J. Clin. Microbiol. 41:2596–2604.

Kennedy, G. A., and A. C. Strafuss. 1977. Scanning electron microscopy of the lesions of swine dysentery. Proceedings Workshop on Biomedical Applications–Scanning Electron Microscopy Applications in Medical Microbiology. IIT Research Institute. Chicago, IL. 283–290.

Kennedy, M. J., D. K. Rosnick, R. G. Ulrich, and R. J. Yancey Jr. 1988. Association of Treponema hyodysenteriae with porcine intestinal mucosa. J. Gen. Microbiol. 134:1565–1576.

Kennedy, M. J., and R. J. Yancey Jr. 1996. Motility and chemotaxis in Serpulina hyodysenteriae. Vet. Microbiol. 49:21–30.

Kennedy, M. J., E. L. Rosey, and R. J. Yancey Jr. 1997. Characterization of flaA- and flaB- mutants of Serpulina hyodysenteriae: Both flagellin subunits, FlaA and FlaB, are necessary for full motility and intestinal colonization. FEMS Microbiol. Lett. 153:119–128.

Kent, K. A., R. M. Lemcke, and R. J. Lysons. 1988. Production, purification, and molecuar weight determination of the haemolysin of Treponema hyodysenteriae. J. Med. Microbiol. 27:215–224.

Kinyon, J. M., D. L. Harris, and R. D. Glock. 1977. Enteropathogenicity of various isolates of Treponema hyodysenteriae. Infect. Immun. 15:638–646.

Kinyon, J. M., and D. L. Harris. 1979. Treponema innocens, a new species of intestinal bacteria, and emended description of the type strain of Treponema hyodysenteriae Harris et al. Int. J. Syst. Bacteriol. 29:102–109.

Koopman, M. B., O. S. de Leeuw, B. M. van der Zeijst, and J. G. Kusters. 1992. Cloning and DNA sequence analysis of a Serpulina (Treponema) hyodysenteriae gene encoding a periplasmic flagellar sheath protein. Infect. Immun. 60:2920–2925.

Koopman, M. B. H., E. Baats, O. S. de Leeuw, B. A. M. van der Zeijst, and J. G. Kusters. 1993a. Molecular analysis of a flagellar core protein gene of Serpulina (Treponema) hyodysenteriae. J. Gen. Microbiol. 139:1701–1706.

Koopman, M. B. H., A. Kasbohrer, G. Beckmann, B. A. M. van der Zeijst, and J. G. Kusters. 1993b. Genetic similarity of intestinal spirochetes from humans and various animal species. J. Clin. Microbiol. 31:711–716.

Korner, M. and J. O. Gebbers. 2003. Clinical significance of human intestinal spirochetosis—a morphologic approach. Infection 31:341–349.

Koteish, A., R. Kannangai, S.C. Abraham, and M. Torbenson. 2003. Colonic spirochetosis in children and adults. Am. J. Clin. Pathol. 120:828–832.

Kraaz, W., B. Pettersson, U. Thunberg, L. Engstrand, and C. Fellstrom. 2000. Brachyspira aalborgi infection diagnosed by culture and 16S ribosomal DNA sequencing using human colonic biopsy specimens. J. Clin. Microbiol. 38:3555–3560.

Kubo, M., M. Nakagawa, M. Kashiwazaki, and S. Konno. 1979. Pathological observation on experimental swine dysentery. Natl. Inst. Anim. Health Quart. 19:83–90.

Kunkle, R. A., D. L. Harris, and J. M. Kinyon. 1986. Autoclaved liquid medium for propagation of Treponema hyodysenteriae. J. Clin. Microbiol. 24:669–671.

Kunkle, R. A., and J. M. Kinyon. 1988. Improved selective medium for the isolation of Treponema hyoydsenteriae. J. Clin. Microbiol. 26:2357–2360.

La, T., N. D. Phillips, and D. J. Hampson. 2003. Development of a duplex PCR assay for detection of Brachyspira hyodysenteriae and Brachyspira pilosicoli in pig feces. J. Clin. Microbiol. 41:3372–3375.

Lau, T. T., and D. J. Hampson. 1992. The serological grouping system for Serpulina (Treponema) hyodysenteriae. Epidemiol. Infect. 109:255–263.

Lee, J. I., D. J. Hampson, B. G. Combs, and A. J. Lymbery. 1993a. Genetic relationships between isolates of Serpulina (Treponema) hyodysenteriae, and comparison of methods for their subspecific differentiation. Vet. Microbiol. 34:35–46.

Lee, J. I., D. J. Hampson, A. J. Lymbery, and S. J. Harders. 1993b. The porcine intestinal spirochaetes: identification of new genetic groups. Vet. Microbiol. 34:273–285.

Lee, J. I., A. J. McLaren, A. J. Lymbery, and D. J. Hampson. 1993c. Human intestinal spirochetes are distinct from Serpulina hyodysenteriae. J. Clin. Microbiol. 31:16–21.

Lee, J. I., and D. J. Hampson. 1994. Genetic characterisation of intestinal spirochaetes and their association with disease. J. Med. Microbiol. 40:365–371.

Lee, B. J., T. La, A. S. Mikosza, and D. J. Hampson. 2000. Identification of the gene encoding BmpB, a 30 kDa outer envelope lipoprotein of Brachyspira (Serpulina) hyodysenteriae, and immunogenicity of recombinant BmpB in mice and pigs. Vet. Microbiol. 76:245–257.

Lemcke, R. M., and M. R. Burrows. 1980. Sterol requirement for the growth of Treponema hyodysenteriae. J. Gen. Microbiol. 116:539–543.

Leser, T. D., K. Moller, T. K. Jensen, and S. E. Jorsal. 1997. Specific detection of Serpulina hyodysenteriae and potentially pathogenic weakly beta-haemolytic porcine intestinal spirochetes by polymerase chain reaction targeting 23S rDNA. Molec. Cell Probes 11:363–372.

Li, Z. S., M. Belanger, and M. Jacques. 1991. Serotyping of Canadian isolates of Treponema hyodysenteriae and description of two new serotypes. J. Clin. Microbiol. 29:2794–2797.

Li, Z., B. Foiry, and M. Jacques. 1995. Growth of Serpulina (Treponema) hyodysenteriae under iron-restricted conditions. Can. J. Vet. Res. 59:149–153.

Li, C., L. Corum, D. Morgan, E. L. Rosey, T. B. Stanton, and N. W. Charon. 2000. The spirochete FlaA periplasmic flagellar sheath protein impacts flagellar helicity. J. Bacteriol. 182:6698–6706.

Livesley, M. A., I. P. Thompson, M. J. Bailey, and P. A. Nuttall. 1993. Comparison of the fatty acid profiles of Borrelia, Serpulina, and Leptospira species. J. Gen. Microbiol. 139:889–895.

Lymbery, A. J., D. J. Hampson, R. M. Hopkins, B. Combs, and J. R. L. Mhoma. 1990. Multilocus enzyme electrophoresis for identification and typing of Treponema hyodysenteriae and related spirochaetes. Vet. Microbiol. 22:89–99.

Lysons, R. J., K. A. Kent, A. P. Bland, R. Sellwood, W. F. Robinson, and A. J. Frost. 1991. A cytotoxic haemolysin from Treponema hyodysenteriae—a probable virulence

determinant in swine dysentery. J. Med. Microbiol. 34:97–102.

Matthews, H. M., T.-K. Yang, and H. M. Jenkin. 1980a. Alk-1-enyl ether phospholipids (plasmalogens) and glycolipids of Treponema hyodysenteriae. Biochim. Biophys. Acta 618:273–281.

Matthews, H. M., T.-K. Yang, and H. M. Jenkin. 1980b. Treponema innocens lipids and further description of an unusual galactolipid of Treponema hyodysenteriae. J. Bacteriol. 143:1151–1155.

Matthews, H. M., and J. M. Kinyon. 1984. Cellular lipid comparisons between strains of Treponema hyodysenteriae and Treponema innocens. Int. J. Syst. Bacteriol. 34:160–165.

McCaman, M. T., K. Auer, W. Foley, and J. D. Gabe. 1999. Sequence characterization of two new members of a multi-gene family in Serpulina hyodysenteriae (B204) with homology to a 39 kDa surface exposed protein: vspC and D. Vet. Microbiol. 68:273–83.

McCaman, M. T., K. Auer, W. Foley, and J. D. Gabe. 2003. Brachyspira hyodysenteriae contains eight linked gene copies related to an expressed 39-kDa surface protein. Microbes Infect 5:1–6.

McLaren, A. J., D. J. Trott, D. E. Swayne, S. L. Oxberry, and D. J. Hampson. 1997. Genetic and phenotypic characterization of intestinal spirochetes colonizing chickens and allocation of known pathogenic isolates to three distinct genetic groups. J. Clin. Microbiol. 35:412–417.

Miao, R. M., A. H. Fieldsteel, and D. L. Harris. 1978. Genetics of Treponema: characterization of Treponema hyodysenteriae and its relationship to Treponema pallidum. Infect. Immun. 22:736–739.

Mikosza, A. S., T. La, C. J. Brooke, C. F. Lindboe, P. B. Ward, R. G. Heine, J. G. Guccion, W. B. de Boer, and D. J. Hampson. 1999. PCR amplification from fixed tissue indicates frequent involvement of Brachyspira aalborgi in human intestinal spirochetosis. J. Clin. Microbiol. 37:2093–2098.

Mikosza, A. S., T. La, W. B. de Boer, and D. J. Hampson. 2001. Comparative prevalences of Brachyspira aalborgi and Brachyspira (Serpulina) pilosicoli as etiologic agents of histologically identified intestinal spirochetosis in Australia. J. Clin. Microbiol. 39:347–350.

Mikosza, A.S., T. La, K.R. Margawani, C.J. Brooke, and D.J. Hampson. 2001. PCR detection of Brachyspira aalborgi and Brachyspira pilosicoli in human faeces. FEMS Microbiol. Lett. 197:167–170.

Milner, J. A., and R. Sellwood. 1994. Chemotactic response to mucin by Serpulina hyodysenteriae and other porcine spirochetes: Potential role in intestinal colonization. Infect. Immun. 62:4095–4099.

Muir, S., M. B. H. Koopman, S. J. Libby, L. A. Joens, F. Heffron, and J. G. Kusters. 1992. Cloning and expression of a Serpula (Treponema) hyodysenteriae hemolysin gene. Infect. Immun. 60:529–535.

Muniappa, N., M. R. Mathiesen, and G. E. Duhamel. 1997. Laboratory identification and enteropathogenicity testing of Serpulina pilosicoli associated with porcine colonic spirochetosis. J. Vet. Diagn. Invest. 9:165–171.

Muniappa, N., M. R. Ramanathan, R. P. Tarara, R. B. Westerman, M. R. Mathiesen, and G. E. Duhamel. 1998. Attachment of human and rhesus Serpulina pilosicoli to cultured cells and comparison with a chick infection model. J. Spiroch.Tick-borne Dis. 5:44–53.

Munshi, M. A., N. M. Taylor, A. S. Mikosza, P. B. Spencer, and D. J. Hampson. 2003. Detection by PCR and isolation assays of the anaerobic intestinal spirochete Brachyspira aalborgi from the feces of captive nonhuman primates. J. Clin. Microbiol. 41:1187–1191.

Neutra, M. R. 1980. Prokaryotic-eukaryotic cell junctions: attachment of spirochetes and flagellated bacteria to primate large intestinal cells. J. Ultrastruct. Res. 70:186–203.

Nibbelink, S. K., and M. J. Wannemuehler. 1991. Susceptibility of inbred mouse strains to infection with Serpula (Treponema) hyodysenteriae. Infect. Immun. 59:3111–3118.

Nibbelink, S. K., and M. J. Wannemuehler. 1992. An enhanced murine model for studies of Serpulina (Treponema) hyodysenteriae pathogenesis. Infect. Immun. 60:3433–3436.

Nuessen, M. E., J. R. Birmingham, and L. A. Joens. 1982. Biological activity of a lipopolysaccharide extracted from Treponema hyodysenteriae. Infect. Immun. 37: 138–142.

Nuessen, M. E., L. A. Joens, and R. D. Glock. 1983. Involvement of lipopolysaccharide in the pathogenicity of Treponema hyodysenteriae. J. Immunol. 131:997–999.

Ochiai, S., Y. Adachi, and K. Mori. 1997. Unification of the genera Serpulina and Brachyspira, and proposals of Brachyspira hyodysenteriae comb. nov., Brachyspira innocens comb. nov. and Brachyspira pilosicoli comb. nov. Microbiol. Immunol. 41:445–452.

Olsen, I., B. J. Paster, and F. E. Dewhirst. 2000. Taxonomy of spirochetes. Anaerobe 6:39–57.

Olson, L. D. 1995. Survival of Serpulina hyodysenteriae in an effluent lagoon. J. Am. Vet. Med. Assoc. 207:1470–1472.

Olson, L. D. 1996. Enhanced isolation of Serpulina hyodysenteriae by using sliced agar media. J. Clin. Microbiol. 34:2937–2941.

Oxberry, S. L., D. J. Trott, and D. J. Hampson. 1998. Serpulina pilosicoli, waterbirds and water: potential sources of infection for humans and other animals. Epidemiol. Infect. 121:219–225.

Park, N. Y., C. Y. Chung, A. J. McLaren, R. F. Atyeo, and D. J. Hampson. 1995. Polymerase chain reaction for identification of human and porcine spirochaetes recovered from cases of intestinal spirochaetosis. FEMS Microbiol. Lett. 125:225–230.

Paster, B. J., E. Stackebrandt, R. B. Hespell, C. M. Hahn, and C. R. Woese. 1984. The phylogeny of the spirochetes. Syst. Appl. Microbiol. 5:337–351.

Paster, B. J., F. E. Dewhirst, W. G. Weisburg, L. A. Tordoff, G. J. Fraser, R. B. Hespell, T. B. Stanton, L. Zablen, L. Mandelco, and C. R. Woese. 1991. Phylogenetic analysis of the spirochetes. J. Bacteriol. 173:6101–6109.

Paster, B. J., and F. E. Dewhirst. 1997. Taxonomy and phylogeny of intestinal spirochaetes. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes of Domestic Animals and Humans. CAB International. New York, NY. 47–61.

Paster, B. J., and F. E. Dewhirst. 2000. Phylogenetic foundation of spirochetes. J. Molec. Microbiol. Biotechnol. 2:341–344.

Pettersson, B., C. Fellstrom, A. Andersson, M. Uhlen, A. Gunnarsson, and K. E. Johansson. 1996. The phylogeny of intestinal porcine spirochetes (Serpulina species) based on sequence analysis of the 16S rRNA gene. J. Bacteriol. 178:4189–4199.

Pettersson, B., M. Wang, C. Fellstrom, M. Uhlen, G. Molin, B. Jeppsson, and S. Ahrne. 2000. Phylogenetic evidence

for novel and genetically different intestinal spirochetes resembling Brachyspira aalborgi in the mucosa of the human colon as revealed by 16S rDNA analysis. Syst. Appl. Microbiol. 23:355–363.

Plaza, H., T. R. Whelchel, S. F. Garczynski, E. W. Howerth, and F. C. Gherardini. 1997. Purified outer membranes of Serpulina hyodysenteriae contain cholesterol. J. Bacteriol. 179:5414–5421.

Rayment, S. J., S. P. Barrett, and M. A. Livesley. 1997. Subspecific differentiation of intestinal spirochaete isolates by macrorestriction fragment profiling. Microbiology 143:2923–2929.

Rayment, S. J., B. J. Lee, D. J. Hampson, and M. A. Livesley. 1998. Identification of a gene sequence encoding a putative pyruvate oxidoreductase in Serpulina pilosicoli. FEMS Microbiol. Lett. 166:121–126.

Ritchie, A. E., I. M. Robinson, L. A. Joens, and J. M. Kinyon. 1978. A bacteriophage for Treponema hyodysenteriae. Vet. Rec. 102:34–35.

Rohde, J., A. Rothkamp, and G. F. Gerlach. 2002. Differentiation of porcine Brachyspira species by a novel nox PCR-based restriction fragment length polymorphism analysis. J. Clin. Microbiol. 40:2598–2600.

Rosey, E. L., M. J. Kennedy, D. K. Petrella, R. G. Ulrich, and R. J. Yancey. 1995. Inactivation of Serpulina hyodysenteriae flaA1 and flaB1 periplasmic flagellar genes by electroporation-mediated allelic exchange. J. Bacteriol. 177:5959–5970.

Rosey, E. L., M. J. Kennedy, and R. J. Yancey. 1996. Dual flaA1 flaB1 mutant of Serpulina hyodysenteriae expressing periplasmic flagella is severely attenuated in a murine model of swine dysentery. Infect. Immun. 64:4154–4162.

Rothkamp, A., B. Strommenger, and G.F. Gerlach. 2002. Identification of Brachyspira hyodysenteriae-specific DNA fragments using representational difference analysis. FEMS Microbiol. Lett. 210:173–179.

Ruane, P. J., M. M. Nakata, J. F. Reinhardt, and W. L. George. 1989. Spirochete-like organisms in the human gastrointestinal tract. Rev. Infect. Dis. 11:184–196.

Sagartz, J. E., D. E. Swayne, K. A. Eaton, J. R. Hayes, K. D. Amass, R. Wack, and L. Kramer. 1992. Necrotizing typhlocolitis associated with a spirochete in rheas (Rhea americana). Avian Dis. 36:282–289.

Saheb, S. A., L. Massicotte, and B. Picard. 1980. Purification and characterization of Treponema hyodysenteriae hemolysin. Biochimie 62:779–785.

Saheb, S. A., N. Daigneauly-Sylvestre, and B. Picard. 1981. Comparative study of the hemolysins of Treponema hyodysenteriae and Treponema innocens. Curr. Microbiol. 5:87–90.

Savage, D. C. 1980. Colonization by and survival of pathogenic bacteia on intestinal mucosal surfaces. *In:* G. Bitton and K. C. Marshall (Eds.) Adsorption of Microorganisms to Surfaces. John Wiley. New York, NY. 175–206.

Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam. 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. 51:873–884.

Sellwood, R., F. Walton, W. Thomas, M. R. Burrows, and J. Chesham. 1995. Expression of the SmpA outer membrane lipoprotein of Serpulina hyodysenteriae strain P18A in vivo. Vet. Microbiol. 44:25–35.

Sellwood, R., and A. P. Bland. 1997. Ultrastructure of intestinal spirochaetes. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 109–149.

Smibert, R. M., and N. R. Krieg. 1981. General characterization. *In:* Manual of Methods for General Bacteriology. American Society for Microbiology. Washington DC, 409–443.

Songer, J. G., J. M. Kinyon, and D. L. Harris. 1976. Selective medium for isolation of Treponema hyodysenteriae. J. Clin. Microbiol. 4:57–60.

Stanton, T. B. 1987a. Cholesterol metabolism by Treponema hyodysenteriae. Infect. Immun. 55:309–313.

Stanton, T. B., and C. P. Cornell. 1987b. Erythrocytes as a source of essential lipids for Treponema hyodysenteriae. Infect. Immun. 55:304–308.

Stanton, T. B., and D. F. Lebo. 1988. Treponema hyodysenteriae growth under various culture conditions. Vet. Microbiol. 18:177–190.

Stanton, T. B. 1989. Glucose metabolism and NADH recycling by Treponema hyodysenteriae, the agent of swine dysentery. Appl. Environ. Microbiol. 55:2365–2371.

Stanton, T. B., N. S. Jensen, T. A. Casey, L. A. Tordoff, F. E. Dewhirst, and B. J. Paster. 1991. Reclassification of Treponema hyodysenteriae and Treponema innocens in a new genus, Serpula gen. nov., as Serpula hyodysenteriae comb. nov. and Serpula innocens comb. nov. Int. J. Syst. Bacteriol. 41:50–58.

Stanton, T. B. 1992. Proposal to change the genus designation Serpula to Serpulina gen.nov. containing the species Serpulina hyodysenteriae comb.nov. and Serpulina innocens comb. nov. Int. J. Syst. Bacteriol. 42:189–190.

Stanton, T. B., and N. S. Jensen. 1993a. Monitoring experimental swine dysentery: rectal swab blood test and Serpulina (Treponema) hyodysenteriae detection. Vet. Microbiol. 34:389–396.

Stanton, T. B., and N. S. Jensen. 1993b. Purification and characterization of NADH oxidase from Serpulina (Treponema) hyodysenteriae. J. Bacteriol. 175:2980–2987.

Stanton, T. B., B. L. Hanzelka, and N. S. Jensen. 1995. Survey of intestinal spirochaetes for NADH oxidase by gene probe and by enzyme assay. Microb. Ecol. Health Dis. 8:93–100.

Stanton, T. B., D. J. Trott, J. I. Lee, A. J. McLaren, D. J. Hampson, B. J. Paster, and N. S. Jensen. 1996. Differentiation of intestinal spirochaetes by multilocus enzyme electrophoresis analysis and 16S rRNA sequence comparisons. FEMS Microbiol. Lett. 136:181–186.

Stanton, T. B. 1997a. Physiology of ruminal and intestinal spirochaetes. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 7–45.

Stanton, T. B., E. Fournie-Amazouz, D. Postic, D. J. Trott, P. A. Grimont, G. Baranton, D. J. Hampson, and I. Saint Girons. 1997b. Recognition of two new species of intestinal spirochetes: Serpulina intermedia sp. nov. and Serpulina murdochii sp. nov. Int. J. Syst. Bacteriol. 47:1007–1012.

Stanton, T. B., D. Postic, and N. S. Jensen. 1998. Serpulina alvinipulli sp. nov., a new Serpulina species that is enteropathogenic for chickens. Int. J. Syst. Bacteriol. 48 Pt 3:669–676.

Stanton, T. B., and R. Sellwood. 1999a. Cloning and characteristics of a gene encoding NADH oxidase, a major mechanism for oxygen metabolism by the anaerobic

spirochete, Brachyspira (Serpulina) hyodysenteriae. Anaerobe 5:539–546.

Stanton, T. B., E. L. Rosey, M. J. Kennedy, N. S. Jensen, and B. T. Bosworth. 1999b. Isolation, oxygen sensitivity, and virulence of NADH oxidase mutants of the anaerobic spirochete Brachyspira (Serpulina) hyodysenteriae, etiologic agent of swine dysentery. Appl. Environ. Microbiol. 65:5028–5034.

Stanton, T. B., E. G. Matson, and S. B. Humphrey. 2001. Brachyspira (Serpulina) hyodysenteriae gyrB mutants and interstrain transfer of coumermycin A(1) resistance. Appl. Environ. Microbiol. 67:2037–2043.

Stanton, T. B., M. G. Thompson, S. B. Humphrey, and R. L. Zuerner. 2003. Detection of bacteriophage VSH-1 svp38 gene in Brachyspira spirochetes. FEMS Microbiol Lett 224:225–229.

Stephens, C. P., and D. J. Hampson. 2001. Intestinal spirochete infections of chickens: a review of disease associations, epidemiology and control. Anim. Health Res. Rev. 2:83–91.

Stephens, C. P. and D. J. Hampson. 2002. Experimental infection of broiler breeder hens with the intestinal spirochaete Brachyspira (Serpulina) pilosicoli causes reduced egg production. Avian Pathol 31:169–175.

Sueyoshi, M., and Y. Adachi. 1990. Diarrhea induced by Treponema hyodysenteriae: a young chick cecal model for swine dysentery. Infect. Immun. 58:3348–3362.

Swayne, D. E., K. A. Eaton, J. Stoutenburg, D. J. Trott, D. J. Hampson, and N. S. Jensen. 1995. Identification of a new intestinal spirochete with pathogenicity for chickens. Infect. Immun. 63:430–436.

Swayne, D. E., and A. J. McLaren. 1997. Avian intestinal spirochaetes and avian intestinal spirochaetosis. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 267–300.

Swayne, D. E. 2002. Avian intestinal spirochetosis. *In:* Y. M. Saif, H. J. Barnes, A. M. Fadly, J. R. Glisson, L. R. McDougald, and D. E. Swayne (Eds.) Diseases of Poultry, 11th ed. Iowa State University Press. Ames, IA.

Takeuchi, A., and J. A. Zeller. 1972. Scanning electron microscopic observations on the surface of the normal and spirochete–infested colonic mucosa of the rhesus monkey. J. Ultra. Res. 40:313–324.

Taylor, D. J., and T. J. L. Alexander. 1971. The production of dysentery in swine by feeding cultures containing a spirochaete. Br. Vet. J. 127:58–61.

Taylor, D. J., J. R. Simmons, and H. M. Laird. 1980. Production of diarrhoea and dysentery in pigs by feeding pure cultures of a spirochaete differing from Treponema hyodysenteriae. Vet. Rec. 106:326–332.

Taylor, D. J., and D. J. Trott. 1997. Porcine intestinal spirochaetosis and spirochaetal colitis. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 211–241.

ter Huurne, A. A. H. M., M. van Houten, M. B. H. Koopman, B. A. M. van der Zeijst, and W. Gaastra. 1992a. Characterization of Dutch porcine Serpulina (Treponema) isolates by restriction endonuclease analysis and DNA hybridization. J. Gen. Microbiol. 138:1929–1934.

ter Huurne, A. A. H. M., M. van Houten, S. Muir, J. G. Kusters, B. A. M. van der Zeijst, and W. Gaastra. 1992b. Inactivation of a Serpula (Treponema) hyodysenteriae hemolysin gene by homologous recombination: importance of this hemolysin in pathogenesis of S. hyodysenteriae in mice. FEMS Microbiol. Lett. 92:109–114.

ter Huurne, A. A. H. M., S. Muir, M. van Houten, B. A. M. van der Zeijst, W. Gaastra, and J. G. Kusters. 1994. Characterization of three putative Serpulina hyodysenteriae hemolysins. Microb. Pathog. 16:269–282.

Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41:100–180.

Thomson, J. R., W. J. Smith, B. P. Murray, and S. McOrist. 1997. Pathogenicity of three strains of Serpulina pilosicoli in pigs with a naturally acquired intestinal flora. Infect. Immun. 65:3693–3700.

Tompkins, D. S., S. J. Foulkes, P. G. R. Godwin, and A. P. West. 1986. Isolation and characterisation of intestinal spirochaetes. J. Clin. Path. 39:535–541.

Trivett-Moore, N. L., Gilbert, G. L., Law, C. L. H., Trott, D. J. and Hampson, D. J. 1998. J. Clin. Microbiol. 36: 262–265.

Trott, D. J., A. J. McLaren, and D. J. Hampson. 1995. Pathogenicity of human and porcine intestinal spirochetes in one-day-old specific-pathogen-free chicks: An animal model of intestinal spirochetosis. Infect. Immun. 63:3705–3710.

Trott, D. J., R. F. Atyeo, J. I. Lee, D. A. S. Swayne, J. W. Stoutenberg, and D. J. Hampson. 1996a. Genetic relatedness amongst intestinal spirochaetes isolated from rats and birds. Lett. Appl. Microbiol. 23:431–436.

Trott, D. J., C. R. Huxtable, and D. J. Hampson. 1996b. Experimental infection of newly weaned pigs with human and porcine strains of Serpulina pilosicoli. Infect. Immun. 64:4648–4654.

Trott, D. J., T. B. Stanton, N. S. Jensen, G. E. Duhamel, J. L. Johnson, and D. J. Hampson. 1996c. Serpulina pilosicoli sp nov; the agent of porcine intestinal spirochetosis. Int. J. Syst. Bacteriol. 46:206–215.

Trott, D. J., T. B. Stanton, N. S. Jensen, and D. J. Hampson. 1996d. Phenotypic characteristics of Serpulina pilosicoli the agent of intestinal spirochaetosis. FEMS Microbiol. Lett. 142:209–214.

Trott, D. J., B. G. Combs, A. S. J. Mikosza, S. L. Oxberry, I. D. Robertson, M. Passey, J. Taime, R. Sehuko, M. P. Alpers, and D. J. Hampson. 1997a. The prevalence of Serpulina pilosicoli in humans and domestic animals in the Eastern Highlands of Papna New Guinea. Epidemiol. Infect. 119:369–379.

Trott, D. J., N. S. Jensen, I. Saintgirons, S. L. Oxberry, T. B. Stanton, D. Lindquist, and D. J. Hampson. 1997b. Identification and characterization of Serpulina pilosicoli isolates recovered from the blood of critically ill patients. J. Clin. Microbiol. 35:482–485.

Trott, D. J., S. L. Oxberry, and D. J. Hampson. 1997c. Evidence for Serpulina hyodysenteriae being recombinant, with an epidemic population structure. Microbiology 143:3357–3365.

Trott, D. J., A. S. J. Mikosza, B. G. Combs, S. L. Oxberry, and D. J. Hampson. 1998. Population genetic analysis of Serpulina pilosicoli and its molecular epidemiology in villages in the Eastern Highlands of Papua New Guinea. Int. J. Syst. Bacteriol. 48:659–668.

Trott, D. J., D. P. Alt, R. L. Zuerner, M. J. Wannemuehler, and T. B. Stanton. 2001. The search for Brachyspira outer membrane proteins that interact with the host. Anim. Health Res. Rev. 2, pp:19–30.

Trott, D. J., D. P. Alt, R. L. Zuerner, D. M. Bulach, M. J. Wannemuehler, J. Stasko, K. M. Townsend, and T. B.

Stanton. 2004. Identification of an outer membrane lipoprotein (BmpC) in Brachyspira pilosicoli membrane vesicles obtained by osmotic lysis and sucrose density ultracentrifugation. Microbiology in press.

Turner, A. K., R. Atyeo, R. Sellwood, and D. Hampson. 1995. Distribution of the smpA gene from Serpulina hyodysenteriae among intestinal spirochaetes. Microbiology 141:2041–2046.

Turner, A. K., and R. Sellwood. 1997. Extracellular DNA from Serpulina hyodysenteriae consists of 6.5 kbp random fragments of chrosomal DNA. FEMS Microbiol. Lett. 150:75–80.

van der Zeijst, B. A. and A. A. H. M. ter Huurne. 1997. Molecular biology of intestinal spirochaetes. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 91–108.

Vanrobaeys, M., P. De Herdt, R. Ducatelle, L. A. Devriese, G. Charlier, and F. Haesebrouck. 1998. Typhlitis caused by intestinal Serpulina-like bacteria in domestic guinea pigs (Cavia porcellus). J. Clin. Microbiol. 36:690–694.

Waters, W. R., B. A. Pesch, R. Hontecillas, R. E. Sacco, F. A. Zuckermann, and M. J. Wannemuehler. 2000. Cellular immune responses of pigs induced by vaccination with either a whole cell sonciate or pepsin-digested Brachyspira (Serpulina) hyodysenteriae bacterin. Vaccine 18:711–719.

Wayne, L. G., D. Brenner, R. R. Colwell, P. A. D. Grimond, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Truper. 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. Int. J. Syst. Bacteriol. 37:463–464.

Wright, J. C., G. R. Wilt, R. B. Reed, and T. A. Powe. 1989. Use of an enzyme-linked immunosorbent assay for detection of Treponema hyodysenteriae infection in swine. J. Clin. Microbiol. 27:411–416.

Yu, J., A. P. Bryant, A. Marra, M. A. Lonetto, K. A. Ingraham, A. F. Chalker, D. J. Holmes, D. Holden, M. Rosenberg, and D. McDevitt. 2001. Characterization of the Streptococcus pneumoniae NADH oxidase that is required for infection. Microbiology 147:431–438.

Zhang, P., X. Cheng, and G. E. Duhamel. 2000. Cloning and DNA sequence analysis of an immunogenic glucose-galactose MglB lipoprotein homologue from Brachyspira pilosicoli, the agent of colonic spirochetosis. Infect. Immun. 68:4559–4565.

Zuerner, R. L., and T. B. Stanton. 1994. Physical and genetic map of the Serpulina hyodysenteriae $B78^T$ chromosome. J. Bacteriol. 176:1087–1092.

Zuerner, R. L. 1997. Genetic organization in spirochaetes. *In:* D. J. Hampson and T. B. Stanton (Eds.) Intestinal Spirochaetes in Domestic Animals and Humans. CAB International. New York, NY. 63–89.

PART 5

Chlorobiaceae

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CHAPTER 5.1

The Family Chlorobiaceae

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Introduction

Green sulfur bacteria (*Chlorobiaceae*) represent a phylogenetically coherent and isolated group within the domain Bacteria (see "Phylogeny"). As a distinct cytological feature, *Chlorobiaceae*-contain special light-harvesting complexes, so-called chlorosomes which harbor bacteriochlorophylls and carotenoids. Furthermore, green sulfur bacteria differ from most other phototrophic organisms with respect to the chemical structure of the antenna bacteriochlorophylls. Similar antenna complexes have only been found in the phylogenetically distant bacterial family *Chloroflexaceae*.

Green sulfur bacteria are obligate photolithotrophs and are similar to the *Chloroflexaceae*, *Chromatiaceae*, and phototrophic species of the α- and β-proteobacteria, such that water cannot serve as electron donating substrate of the photosynthetic reaction center and molecular oxygen is not generated during anoxygenic photosynthesis. Instead, the electrons which are ultimately required for the assimilatory reduction of CO_2 originate from sulfur compounds with low redox potential or from molecular hydrogen (see The Phototrophic Way of Life in Volume 2).

Because of their limited physiological flexibility, the ecological niche of green sulfur bacteria is rather narrow. All known species of the green sulfur bacteria are typical aquatic microorganisms and occur where light reaches anoxic water layers of lakes or littoral sediments. In some of these ecosystems green sulfur bacteria play a key role in the transformations of carbon and sulfur compounds. Another phenotypic trait of ecological significance is the adaptation to low light intensities. Compared to any other phototrophic microorganism, green sulfur bacteria are able to colonize the lowermost water or sediment layers in oxic/anoxic ecosystems (see "Ecology").

The cells of most species of green sulfur bacteria are morphologically rather inconspicuous members of natural bacterial communities (see "Identification"). Exceptions are the phototrophic consortia, stable associations of green sulfur bacteria with chemotrophic bacteria which are found in many freshwater habitats. At the present state of knowledge, these phototrophic consortia represent the most highly evolved symbiosis in the prokaryotic world.

Phylogeny

Relatedness to Other Bacterial Phyla

Based on a recent phylogenetic analysis (Overmann and Tuschak, 1997b), green sulfur bacteria as a group are phylogenetically isolated within the eubacterial radiation. The comparison of 16S rRNA gene sequences thus fully confirmed earlier results of ribosomal oligonucleotid cataloguing (Gibson et al., 1985; Woese et al., 1985). The closest relative presently in pure culture is *Cytophaga* sp. strain BD1-15 (77.9% similarity, Knuc = 0.26). Green sulfur bacteria appear to be the sister taxon of the *Bacteroides*/*Flavobacterium* phylum (Stackebrandt et al., 1996), which is also indicated by sequence homology of the *recA* gene of both groups (Gruber et al., 1998b).

Green sulfur bacteria harbor chlorosomes which contain bacteriochlorophylls *c*, *d*, or *e* as accessory pigments. Chlorosomes and the bacteriochlorophylls *c* or *d* are also present in the multicellular filamentous gliding bacteria of the family *Chloroflexaceae*. Furthermore, the CsmA proteins of the chlorosome envelope are homologous in *Chlorobiaceae* and *Chloroflexaceae*. The occurrence of chlorosomes in such distantly related bacterial groups could indicate a lateral transfer of the genetic information coding for this unique photosynthetic light-harvesting structure (Stackebrandt et al., 1996).

On a more detailed level, however, differences exist between the molecular architecture of the photosynthetic apparatus in green sulfur bacteria and *Chloroflexaceae*. The photosynthetic unit of green sulfur bacteria contains ten times more antenna pigments as the one in *Chloroflexus* contains (Olson, 1998) and the amino sequences of the chlorosome proteins of both groups exhibit a rather low degree of homology (Blankenship et al., 1995). The antenna bacteriochlorophylls in

green sulfur bacteria are esterified with the alcohol farnesol, those of *Chloroflexus* with stearol instead. Furthermore, the water-soluble trimeric FMO-protein which in green sulfur bacteria links the chlorosome to the reaction center is absent from the photosynthetic apparatus of *Chloroflexus*. Even more significantly, the structure of the reaction center is conspicuously different for both phylogenetic groups. Whereas the reaction center of green sulfur bacteria belongs to the iron-sulfur-type, the quinone-type is found in *Chloroflexus* (see The Phototrophic Way of Life in Volume 2). These considerable differences support the phylogenetic divergence between green sulfur bacteria and *Chloroflexaceae*.

Compared to the photosynthetic *Proteobacteria*, the *Heliobacteriaceae*, and the *Cyanobacteria*, all green sulfur bacteria sequenced to date branch shallow within the phylogenetic tree of eubacteria (Stackebrandt et al., 1996), suggesting a more recent radiation. This could indicate either a lateral transfer of genes for chlorosome formation to a single predecessor containing a Fe-S-type reaction center or that the more deeply branching green sulfur bacteria have not been isolated so far. Several 16S rRNA sequences cloned from environmental samples exhibit a significantly higher sequence similarity than that of members of the *Bacteroides*/*Flavobacterium* phylum (76.3–87% similarity; Overmann and Tuschak, 1997b). Because these sequences originate from uncultured bacteria in geothermal springs (Kopczynski et al., 1994; Ward et al., 1992) or soil samples (Maidak et al., 1994), closer relatives of the green sulfur bacteria must actually exist in a variety of habitats and still await isolation and characterization.

Diversity within the Family Chlorobiaceae

Green sulfur bacteria can be considered as a family of genetically related species. The similarity of the 16S rRNA gene of all species except one is >90.1% ($K_{nuc} < 0.11$). Only *Chloroherpeton thalassium* is more distantly related (85.5 to 87% similarity) and thus may represent an isolated branch within the radiation of green sulfur bacteria.

Presently, the genera and species of green sulfur bacteria are distinguished according to cell morphology, motility, ability to form gas vesicles, and pigment composition (Pfennig, 1989; see "Taxonomy"). The results of the 16S rRNA sequence analysis indicate that most of these phenotypic traits are of little phylogenetic significance. Some species belonging to different genera are phylogenetically closely related (e.g. *Prosthecochloris aestuarii* with *Chlorobium vibrioforme*). The three different species of the genus *Pelodictyon* do not form a coherent cluster within the green sulfur bacterial radiation. Furthermore, considerable genetic diversity has been discovered within the species *Chlorobium limicola* or the species *Chl. vibrioforme*; different strains of the same species can exhibit as much as 8% sequence divergence of the 16S rRNA gene (Figueras et al., 1997; Overmann and Tuschak, 1997b). The mol% G+C of *Chl. limicola* strains varies between 49 and 58.1 mol% and total genome size ranges from 1,435 to 3,342 kb (Méndez-Alvarez et al., 1996). Consequently, the genera and species of the green sulfur bacteria have to be redefined. However, because only a limited number of 16S rRNA gene sequences are presently available, additional strains need to be isolated and analyzed before a consistent new classification system can be established.

Taxonomy

Green sulfur bacteria are presently divided into 5 genera and 14 species. As all of the species share most of the physiological capacities, the six genera *Ancalochloris*, *Chlorobium*, *Chloroherpeton*, *Clathrochloris*, *Pelodictyon*, and *Prosthecochloris* are presently distinguished based on cell morphology, motility, and ability to form gas vesicles. Species are classified according to their morphology and pigment composition (Table 1).

A recent analysis of the phylogenetic relationships between the different species of green sulfur bacteria (Diversity) within the family *Chlorobiaceae* has revealed that most of the phenotypic traits used in the classification of this family have no taxonomic implications. Because the current taxonomy of green sulfur bacteria is based on these latter characteristics, it is not congruent with the phylogenetic tree. However, ternary fission has been observed only for the two closely related *Pelodictyon clathratiforme* and *Pld. phaeoclathratiforme*. Other traits of phylogenetic significance might be the filamentous morphology and gliding motility which are only found in the phylogenetically rather isolated *Chloroherpeton thalassium*. Interestingly, closely related strains seem to have similar salt requirements for growth (Overmann, 1999a). Although only few data are available, the fatty acid composition may similarly reflect the phylogenetic relations. For example, three phylogenetically closely related saltwater strains (*Chlorobium phaeovibrioides* DSM 269T, *Chl. vibrioforme* DSM 262, and *Pelodictyon luteolum* DSM 273T) contain relatively low amounts of the fatty acid myristate but high amounts of palmitate and palmitoleate (Kenyon and Gray, 1974; Knudsen et al., 1982; Imhoff, 1988).

Table 1. Phenotypic characteristics of the known species of green sulfur bacteria.

Species	Shape	Size (µm)	Cell aggregates	Gas vesicles	Color of cell suspension	Antenna BChl	Major carotenoid	GC content (mol%)	Salt requirement	Vitamin B_{12} requirement	Type or neotype strain
Ancalochloris perfihevii	Sphere	0.5–2.0	Microcolonies	+	Green	n.d.	n.d.	n.d.	–	+	—
Chlorobium chlorovibrioides	Vibrio	0.3–0.l4 × 0.7–1.4	Spirals	–	Green	*cord*	chl	54	+	–	DSM 1370
Chlorobium limicola	Rod	0.7–1.1 × 0.9–1.5	Chains	–	Green	*cord*	chl	51.0–58.1	–	–	DSM 245
Chlorobium phaeobacteroides	Rod	0.6–0.8 × 1.3–2.7	Chains	–	Brown	*e*	irt	49.0–50.0	–	+	DSM 266
Chlorobium phaeovibrioides	Vibrio	0.3–0.4 × 0.7–1.4	Spirals	–	Brown	*e*	irt	52.0–53.0	+	+	DSM 269
Chlorobium vibrioforme	Vibrio	0.5–0.7 × 1.0–1.2	Spirals	–	Green	*cord*	chl	52.0–57.1	+	–	DSM 260
Chloroherpeton thalassium	Filamentous rod	10 × 8–30	—	+	Green	*cord*	γ-car	45–48.2	+	+	ATCC 35110
"Clathrochloris sulfarica"	Rod	1.0–1.5 × ??	Trellis-shaped	+	Green	*cord*	n.d.	n.d.	–	n.d.	—
Pelodictyon clathratiforme	Rod	0.7–1.2 × 1.5–2.5	Nets	+	Green	*cord*	chl	48.5	–	+	—
Pelodictyon luteolum	Ovoid	0.6–0.9 × 1.2–2.0	Clumps, spheres	+	Green	*cord*	chl	53.5–58.1	+	–	DSM 273
Pelodictyon phaeoclathratiforme	Rod	0.8–1.1 × 1.5–3.0	Nets	+	Brown	*e*	irt	47.9	–	–	DSM 5477
Pelodictyon phaeum	Vibrio	0.6–0.9 × 1.0–2.0	Spirals	+	Brown	*e*	irt	n.d.	+	+	DSM 728
Prosthecochloris aestuarii	Sphere with prosthecae	0.5–0.7 × 1.0–1.2	Chains	–	Green	*cord*	chl	52.0–56.0	+	+	DSM 271
Prosthecochloris phaeoasteroidea	Sphere with prosthecae	0.3–0.6 × 0.5–0.8	Chains	–	Brown	*e*	irt	52.2	+	–	DSM 1378

Abreviations: +, present; –, absent; n.d., not determined; BChl, bacteriochlorophyll; chl, chlorobactene; irt, isorenieratene; car, carotene.
Adapted from Trüper and Pfennig, 1992.

Habitat

Green sulfur bacteria are found in various types of aquatic habitats like the pelagial of lakes or lagoons, bottom layers of bacterial mats in intertidal sediments or bacterial mats in hot springs. As a result of their limited physiological capacities, these bacteria can only grow in the narrow zone of overlap between the opposing gradients of light and sulfide. In lakes and lagoons this zone extends over 0.5 to 2 m and occurs at a depth of 2 to 20 m. Layers of *Prosthecochloris aestuarii* in marine sediments are only 1 mm thick and are found at about 5 mm depth (Pierson et al., 1987). In contrast to their purple counterparts, the *Chromatiaceae*, macrocopically visible accumulations of green sulfur bacteria are observed less frequently. Nonetheless, *Chlorobium* species can be enriched using anoxic mud and sediment samples from a wide variety of freshwater and marine environments (Trüper and Pfennig, 1992). This indicates that small populations of green sulfur bacteria are able to survive in a wide range of habitats.

Inland Lakes and Lagoons

Blooms of green sulfur bacteria occur at the chemocline of thermally stratified or meromictic lakes. For example, *Chlorobium limicola* dominates the green layer in the chemocline of Wadolek Lake (Poland) (Czeczuga, 1968). *Chlorobium phaeobacteroides* and *Chlorobium phaeovibrioides* thrive in the chemocline of fjords (Pfennig, 1968), meromictic lakes (Trüper and Genovese, 1968; Culver and Brunskill, 1969), or tropical lagoons (Caumette, 1984). Frequently, such planktonic habitats are predominantly colonized by those species which are capable of forming gas vesicles, like *Ancalochloris perfilievii* (Gorlenko and Lebedeva, 1971b), *Pelodictyon luteolum* (Gorlenko and Lebedeva, 1971b), *Pelodictyon phaeum* (Gorlenko et al., 1973), or *Pelodictyon phaeoclathratiforme* (Overmann and Tilzer, 1989b). A complete list and a description of the various habitats were recently compiled by van Gemerden and Mas, 1995.

Black Sea

Today, the Black Sea represents the largest anoxic water mass on Earth. The chemocline is positioned at a depth of 68–98 m and harbors the deepest extant assemblage of green sulfur bacteria known so far, albeit at a very low biomass density (0.94 ng bacteriochlorophyll $\cdot$ L^{-1}) (Repeta et al., 1989). A strain of *Chlorobium phaeobacteroides* isolated from chemocline samples exhibited outstanding low-light adapatation (see Physiology). This is an impressive example of the capacity of brown *Chlorobium* species to survive at incredibly low light intensities. The Black Sea may serve as a model ecosystem for past anoxic states of large ocean basins (Green Sulfur Bacteria during Geological Time Scales).

Marine Sediments

Only in a few marine sediments green sulfur bacteria have been found to develop profusely. In their fully developed state some multilayered microbial mats ("Farbstreifensandwatt") contain a bottom layer of green sulfur bacterial cells belonging to the species *Prosthecochloris aestuarii* and *Chloroherpeton thalassium* (Gibson et al., 1984; Nicholson et al., 1987).

Sulfur Springs

The first reports of natural accumulations of green sulfur bacteria are from Szafer, 1910, and Streszewski, 1913, who observed layers of green bacteria in the effluents of sulfur springs in Poland. Subsequently, dense accumulations of thermophilic green sulfur bacteria were described for sulfur springs in New Zealand, where green sulfur bacteria form 3 mm-thick, unlaminated mats on the bottom substrate and thrive at sulfide concentrations above 50 µM, pH values between 6.0 and 4.5, and temperatures between 55° and 45°C (Castenholz et al., 1990). From the latter habitat, the thermophilic *Chlorobium tepidum* has been isolated (Wahlund et al., 1991).

Isolation

Direct Isolation

During enrichment of green sulfur bacteria in defined liquid media, those species which dominate in natural samples may be entirely outcompeted by the rare species. Consequently, samples in which green sulfur bacterial cells of unknown morphology or phylotype have been detected by microscopy or molecular methods (see "Identification") must be inoculated directly in deep-agar dilution series. Medium GSB is used and supplemented with 3 mM sodium acetate, which serves as an additional carbon source and therefore yields much larger colonies. The tubes are incubated at 20–50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of fluorescent light in order to provide the least selective conditions. At least in one case (*Pld. phaeoclathratiforme* DSM 5477^T) colony formation in the agar tubes is critically dependent on the addition of sodium dithionite (final concentration, 200 µM) to the agar medium (Overmann and Pfennig, 1989a).

Pure cultures are obtained by repeated application of the deep-agar dilution method. A

3.3% (w/v) suspension of Difco Bacto agar is prepared and washed five times with distilled water. After boiling (or autoclaving for 10 min), 3-ml aliquots of the water agar are dispensed into test tubes which are stoppered with cotton plugs and autoclaved. The agar is kept molten in a water bath at 60°C, 6-ml aliquots of perwarmed (42°C) mineral medium GSB are added to each tube, and the complete molten agar medium kept at 42°C. After inoculation with 0.05 to 0.5 ml of the sample, serial dilutions of 8 tubes are made in 1 : 10 dilution steps. Afterwards, all tubes are transferred to a cold water bath in order to harden the agar plugs, and each tube is flushed with a mixture of N_2/CO_2 (90/10) and sealed with a butyl rubber stopper. The media have to be preincubated in the dark for at least 12 h before incubation in the light. After the growth of green sulfur bacteria, well-separated lens-shaped green or brown colonies are removed with sterile Pasteur pipettes and—if pure—transferred to liquid medium. Alternatively, a colony is resuspended in 0.5 ml of medium and used as inoculum for subsequent deep-agar dilution series until a pure cultures is obtained.

Selective Enrichment

Green sulfur bacteria can be obtained from a wide variety of anoxic aquatic habitats if water, mud, or sediment samples are used as inoculum for selective enrichment cultures. Medium GSB should be used at pH values between 6.6 and 6.9, but not above 7.0. High light intensities (50–200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, daylight fluorescent tubes) and temperatures (30–35°C) select for green *Chlorobium* and *Prosthecochloris* species (Trüper, 1970a, Matheron and Baulaigue, 1972). Many typical gas vacuole-containing freshwater species, like *Pelodictyon clathratiforme*, *Pld. phaeoclathratiforme*, and *Ancalochloris perfilievii*, compete successfully only at low sulfide concentrations (≤2 mM) and low temperatures (≤20°C). For the enrichment of green sulfur bacteria from samples containing large numbers of purple sulfur bacteria, cool white fluorescent light should be used at low intensities (0.5–5 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Using this technique, cultures of green sulfur bacteria can be obtained even from samples in which green sulfur bacteria represent only 0.001% of the total number of phototrophic cells (Overmann et al., 1991a). The phototrophic consortium "Chlorochromatium aggregatum" can be enriched from anoxic lake sediment samples using the mineral medium GSB supplemented with 2 mM 2-oxoglutarate. It is mandatory to lower the sulfide concentration to 300 µM sulfide in order to suppress growth of free-living green sulfur bacteria. The pH of the mineral medium should be set to 7.2 and for illumination, daylight fluorescent tubes and a light intensity of 2–5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ should be employed. Under these conditions, highly enriched cultures of intact consortia can be maintained in the laboratory (Fröstl and Overmann, 1998).

Identification

Phenotypically, the genera and species of green sulfur bacteria are distinguished based on their morphology and pigment composition (see "Taxonomy"). It has to be kept in mind, however, that both morphological traits have no phylogenetic significance (see "Phylogeny"). Depending on the species, cells are spherical, ovoid, or curved-shaped. All species multiply by binary fission and are immotile (Table 1). Cells of *Chloroherpeton thalassium* are long unicellular filaments, highly flexible, and motile by gliding. During stationary phase, strains of rod-shaped *Chlorobium* spp. may form long chains of almost spherical cells, and strains with vibrioid morphology can form coils of C-shaped cells. Ternary fission occurs in *Pelodictyon clathratiforme* and *Pld. phaeoclathratiforme* and leads to the formation of large three-dimensional nets. Fig. 1 shows the different morphologies which occur among the known species of green sulfur bacteria. The cells stain Gram-negative.

Depending on their pigment composition, cultures of green sulfur bacteria are either green- or brown-colored and thus macroscopically can resemble those of purple sulfur bacteria. In contrast to the latter, however, the pigments dominating in green sulfur bacteria are bacteriochlorophylls *c*, *d* or *e*. In intact cells, the long wavelength absorption peaks of these bacteriochlorophylls (BChl) are positioned at 745–755 nm (BChl *c*), 715–745 nm (BChl *d*), and 710–725 nm (BChl *e*) (Fig. 2) (Gloe et al., 1975) and can be used to rapidly differentiate between green sulfur bacteria and other photosynthetic prokaryotes like *Chromatiaceae*, *Ectothiorhodospiraceae*, and *cyanobacteria*. When recording the absorption spectra of living cell suspensions, light scattering can be decreased by the addition of 5 g sucrose to 3.7 ml of culture fluid and by using a spectrophotometer equipped with an end-on photomultiplier (Pfennig and Trüper, 1992). The scattering can further be decreased by a disintegration of the cells, passing them once through a french press. Control experiments have demonstrated that no change in the position of the absorption peaks of bacterial pigments occurs if the lysates are scanned directly after french press treatment (Glaeser and Overmann, 1999).

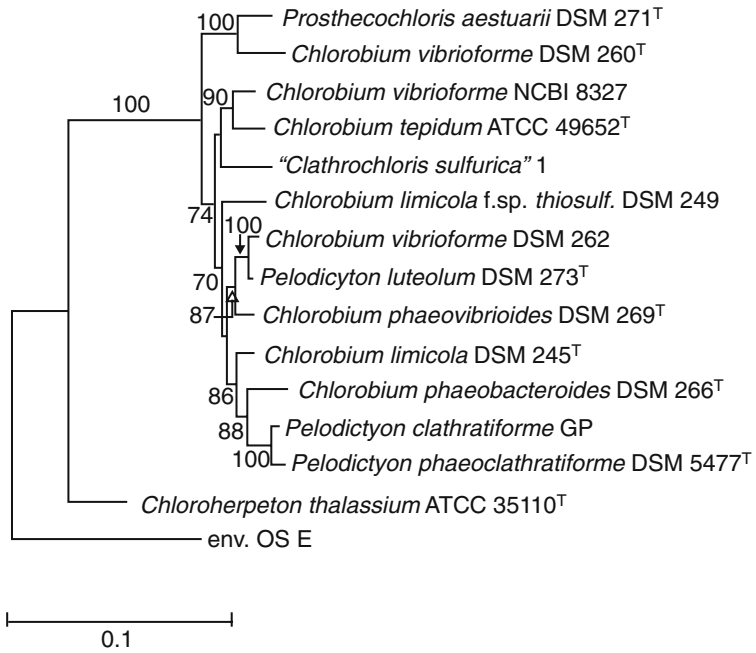

Fig. 1. Phylogenetic tree of the green sulfur bacteria. Construction was done as described by Overmann and Tuschak (1997b), using the most closely related 16S rRNA gene sequence available as the outgroup. Numbers at nodes indicate bootstrap values for each node out of 100 bootstrap resamplings, only values <50 are given. Bar, branch length for 0.1 substitutions per base.

Certain features of green sulfur bacteria can be observed by simple light microscopy. Due to the high intracellular concentrations of antenna pigments, bright field microscopy is often sufficient to discern even individual cells of green sulfur bacteria from accompanying chemotrophic cells in natural samples. During oxidation of sulfide, droplets consisting mainly of zero valence sulfur form outside of the cells and become microscopically visible as highly refractile sulfur globules. In phase contrast, these globules appear yellowish and are surrounded by a pronounced dark fringe (Fig. 1A). Strains which are capable of gas vesicle formation may be devoid of gas vesicles at the high light intensities routinely employed during laboratory incubations. At least in one strain (*Pelodictyon phaeoclathratiforme* DSM 5477^T), gas vesicle formation is induced exclusively by transfer of cultures to very low light intensities (<5 µmol·m^{-2}·s^{-1}, fluorescent light).

An important diagnostic feature of green sulfur bacteria is the presence of chlorosomes, although these structures are not exclusively found in this group but also are found in *Chloroflexaceae*. Chlorosomes can be detected by electron microscopy either in ultrathin sections (see The Phototrophic Way of Life in Volume 2) or by negative staining.

Meanwhile, a fluorescently labelled oligonucleotide probe with a high specificity for green sulfur bacteria has become available (Tuschak et al., 1999, Fig. 6) and permits a rapid and unambigous identification of members of this family in complex microbial communities. In order to obtain previously unkown 16S rRNA gene sequences of green sulfur bacteria in natural samples, an oligonucleotide primer pair has been developed for the selective amplification of these sequences by PCR. Different sequences can then be separated based on their different melting behaviour in denaturing gradient gel electrophoresis (DGGE) and the resulting DNA bands excised from the gel and sequenced (Overmann et al., 1999b). Using this technique, a number of new sequence types of green sulfur bacteria were detected in natural samples.

For a physiological characterization, a set of 40 different carbon substrates is routinely employed (Overmann and Pfennig, 1989a) with the standard medium GSB. Only pyruvate, acetate, and propionate are photoassimilated by the strains tested so far.

Future descriptions of new species or genera have to be based on pure cultures and not only should contain detailed information about cell morphology (color, size, shape, motility, mode of division, presence of gas vesicles, slime capsules, sheath), the occurrence of cell aggregates, the ultrastructure, and photosynthetic pigments, but also must contain the full 16S rRNA gene sequence and the GC content. The sulfide tolerance, salinity requirements, optimum growth temperature and pH, vitamin requirements, the sulfur compounds used as electron-donating substrates, and the carbon sources utilized should be indicated. A description of typical habitats is also necessary. At the present state of knowledge, the creation of a new species only appears to be justified if a new isolate shows a considerable

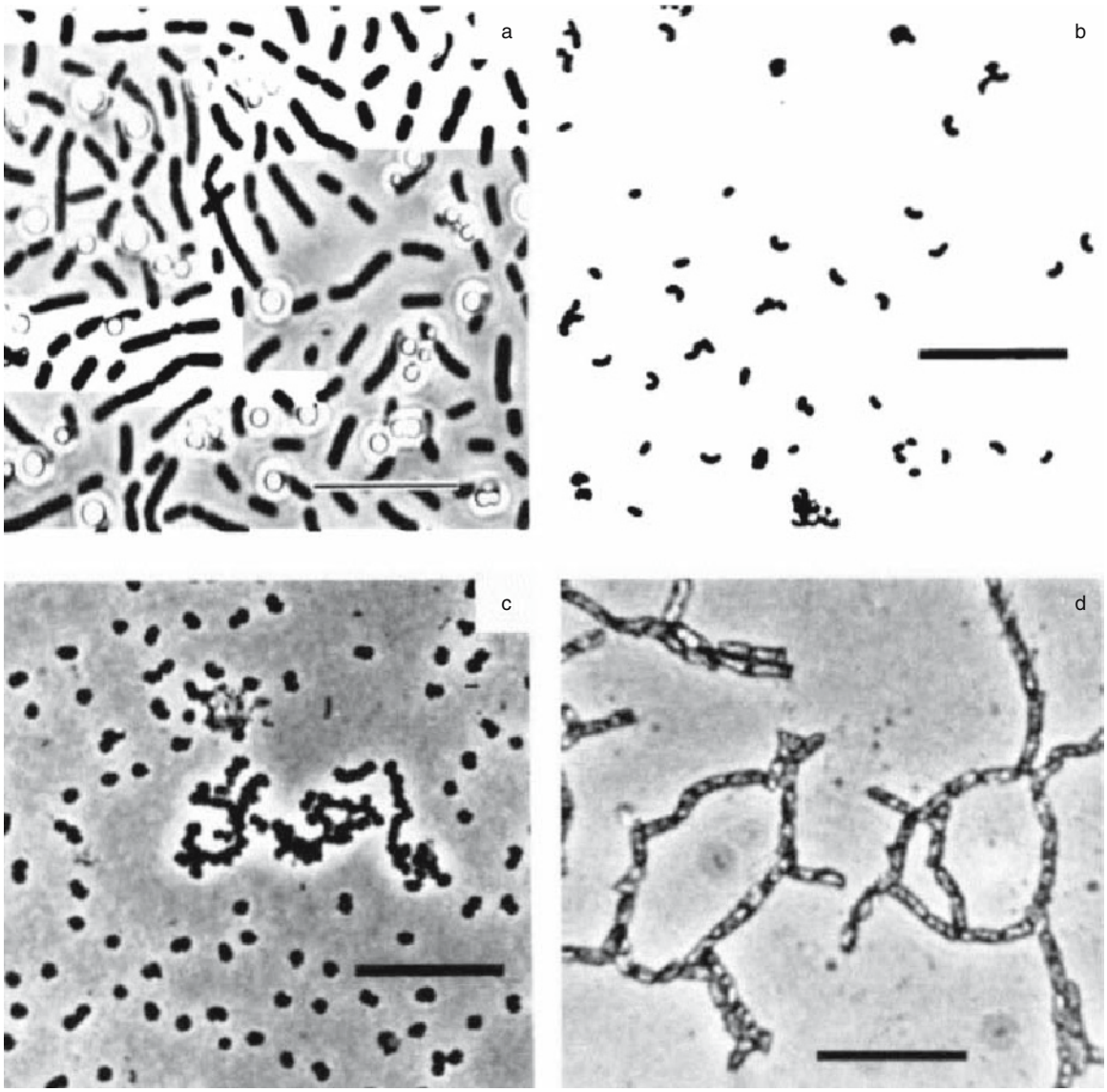

Fig. 2. Morphology of the Chlorobiaceae (phase contrast micrographs). Bars 10 µm. (a) *Chlorobium limicola*. (b) *Chlorobium phaeovibrioides*. (c) *Prosthecochloris aestuarii*. (d) *Pelodictyon clathratiforme*. (Courtesy of Trüper and Pfennig, 1992.)

sequence divergence to all known strains of green sulfur bacteria and if the strain exhibits distinctly different physiological or morphological properties.

Screening PCR-amplified dispersed repetitive DNA sequences (ERIC-fingerprinting) is a rapid method which is well suited to distinguish between strains with very similar or identical 16S rRNA gene sequences (Overmann and Tuschak, 1997b).

A type strain must be designated and deposited in one of the recognized national type culture collections, preferably ATCC, DSM, NCBI, or NHTC, as required by the International Code of Nomenclature of Bacteria (Lapage et al., 1975).

Cultivation

The defined medium for green sulfur bacteria, medium GSB, is prepared anoxically as described for the sulfate-reducing bacteria and contains per liter of distilled water:

	For freshwater strains For marine strains
KH_2PO_4	0.30 g 0.30 g
NH_4Cl	0.34 g 0.34 g
KCl	0.34 g 0.34 g
$CaCl_2 \cdot 2H_2O$	0.15 g 0.15 g

NaCl	—
	20.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
	3.0 g

After the medium has been autoclaved and cooled under an atmosphere of 90% N_2/10% CO_2, aliquots of the following stock solution are added aseptically:

Stock solution	Added per l of medium GSB
10% (w/w) $NaHCO_3$, saturated with CO_2	15 ml
1 M $Na_2S \cdot 9H_2O$ (autoclaved under N_2)	2.5 ml
2 mg vitamin B_{12} in 100 ml H_2O	1 ml
Trace element solution SL 10*	1 ml

*SL 10 contains per liter of distilled water: 25% HCl, 10 ml; FeSO4 · 7H2O, 2.0g; CoCl2 · 6H2O, 190 mg; MnCl2 · 4H2O, 100 mg; ZnCl2, 70 mg; Na2MoO4 · 2H2O, 36 mg; NiCl2 · 6H2O, 24 mg; H3BO3, 6 mg; and CuCl2 · 2H2O, 2 mg. The FeSO4 is dissolved in HCl, and distilled water is then added, followed by the other components.

The final pH of the medium is adjusted to 6.7–6.8.

The concentration of sulfide initially present in medium GSB yields only a limited amount of cell biomass. After oxidation of sulfide and sulfur, cells stop growing and—depending on the strain—are rapidly damaged and eventually die. Therefore the cultures have to be replenished with sterile, neutralized sulfide solution which is prepared in a serum vial by neutralizing a stirred sodium sulfide solution (60 mM) under a N_2 atmosphere with 2M sulfuric acid to a pH of about 7.2. A convenient device for the preparation and long time storage of neutralized sulfide solution is described by Siefert and Pfennig, 1984.

In addition to the liquid medium, a new medium suitable for the growth of green sulfur bacteria on agar plates has been designed (Wahlund and Madigan, 1995).

Preservation

Just after depletion of sulfide and sulfur, most stock cultures can be stored for 3–4 months in the dark at 4°C. Long term preservation is done in nitrogen. Dense cell suspensions of liquid cultures are supplemented with DMSO (final concentration, 5% v/v) as a protective agent and filled into 2-ml plastic ampules, sealed, and freeze-stored. Some *Chlorobium* strains are successfully preserved by lyophilization.

Physiology

The photosynthetic apparatus of green sulfur bacteria has a particular molecular organization (Olson, 1998). Light is absorbed in unique structures, the chlorosomes. These ovoid organelles are 70 to 180 nm long and 30 to 60 nm wide and are attached to the reaction centers in the cytoplasmic membrane. Chlorosomes of the green sulfur bacteria contain bacteriochlorophylls *c*, *d*, or *e*, small amounts of bacteriochlorophyll a and three isoprenoid quinones (1′-oxomenaquinone-7, a derivative thereof, and menaquinone-7). The chlorosome envelope has a width of 2–3 nm and consists of monogalactosyl diglyceride and several polypeptides. Up to 10 different polypeptides can be present (Chung et al., 1994). Compared to the light-harvesting complexes in photosynthetic species of the α-Proteobacteria, β-Proteobacteria, *Heliobacteriaceae*, and *cyanobacteria*, the protein content of chlorosomes is tenfold lower such that the chlorosome proteins cannot serve as a scaffold for the attachment of the antenna bacteriochlorophylls. The functional role of the polypeptides CsmA, CsmB, and CsmC is not well understood. CsmA represents a major fraction of chlorosome proteins. Relatively abundant is also CsmB, which seems to be a component of the chlorosome envelope (Chung and Bryant, 1996) and may be a constituent of the chlorosome baseplate (Højrup et al., 1991). As indicated by insertional inactivation studies, CsmA is required for viability, whereas cells are capable of growing phototrophically (albeit slower than the wild type) in the absence of CsmC (Chung et al., 1998). The current molecular model assumes that antenna bacteriochlorophylls are tightly packed in a highly aggregated, crystalline state and form the (5–10 nm diameter) rod elements which can be visualized by electron microscopy inside the chlorosomes. Thus chlorosomes are unique in that their structure and function seem to be determined by pigment-pigment interactions (Blankenship et al., 1995) rather than by pigment-protein-interactions (*Chromatiaceae*).

Unique to the green sulfur bacteria, a small water-soluble protein is located between the chlorosome and the photosynthetic reaction center. This so-called Fenna-Matthews-Olson FMO-protein is trimeric, contains bacteriochlorophyll *a*, and is assumed to pass excitation energy from the chlorosome to the reaction center.

The antenna pigments of green sulfur bacteria are the bacteriochlorophylls *c*, *d*, or *e* and carotenoids. Green-colored strains contain bacteriochlorophyll c or d and the carotenoid chlorobactene, and brown-colored strains contain bacteriochlorophyll e and the carotenoids isorenieratene and b-isorenieratene. In the genus *Chloroherpeton*, γ-carotene is the main carotenoid. The absorption maxima of isorenieratene and β-isorenieratene are located at 505–520 nm. Intracellular concentration of carotenoids in

brown-colored strains of green sulfur bacteria are up to 4 times higher than those of their green-colored counterparts (Montesinos et al., 1983). This fact is of significance for the competition between the green and brown-colored species of *Chlorobiaceae* and between *Chlorobiaceae* and *Chromatiaceae* (see Interactions between Different Groups of Phototrophic Sulfur Bacteria). The majority of antenna bacteriochlorophyll molecules are farnesol esters. Up to 15 different bacteriochlorophyll homologues occur within the cells of a single strain of green sulfur bacteria (Borrego and Garcia-Gil, 1994) and differ with respect to the aliphatic side chains which are attached to the tetrapyrrole moity. A higher degree of alkylation (with mainly isobutyl or neopentyl side chains) occurs during growth at low as opposed to high light intensities (methyl, ethyl, n-propyl side chains) (Huster and Smith, 1990). Higher alkylation leads to a red-shift of the position of the absorption maximum (van Noort et al., 1997).

The transfer of excitation energy to the photosynthetic reaction center results in the formation of low redox potential electron carriers which in turn react readily with molecular oxygen to form harmful activated oxygen species. Under oxidizing conditions, energy transfer from chlorosomes to the reaction center decreases significantly due to the quenching by chlorobium quinone which decreases photooxidative damage (Frigaard et al., 1997). Similarly, the formation of oxidized bacteriochlorophyll *c* radicals under oxidizing conditions seems to decrease the energy transfer (van Noort et al., 1997).

Because each chlorosome contains about 10,000 molecules of bacteriochlorophyll and is energetically connected to only 5 to 10 reaction centers within the cytoplasmic membrane (Amesz, 1991), the size of the photosynthetic antennae of green sulfur bacteria exceeds that of *Chromatiaceae* by one order of magnitude. The large size of their antennae is one prerequisite for the extreme low light adaptation of the green sulfur bacteria (see Ecology; Black Sea). Biosynthesis of bacteriochlorophylls is regulated by light intensity. Upon a shift to growth-limiting light intensities, an increase in the size (Fuhrmann et al., 1993) and the cellular number of chlorosomes occurs (Broch-Due et al., 1978; Holt et al., 1966) and the cell-specific bacteriochlorophyll content rises by a factor of up to four (Overmann et al., 1992). This adaptation increasing the light-harvesting capacity of the cells of green sulfur bacteria. Experiments with axenic biofilms of *Prosthecochloris aestuarii* have provided indirect evidence of chromatic adapatation of this species. The ratio of bacteriochlorophyll *a*: bacteriochlorophyll *c* seems to increase at lower light intensities and higher fractions of infrared light (Pringault et al., 1998).

Green sulfur bacteria require about one-fourth of the light intensity of the purple sulfur bacteria in order to attain comparable growth rates (Biebl and Pfennig, 1978). Most strains of green sulfur bacteria reach light saturation of growth at 5–10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of fluorescent light (Bergstein et al., 1979; Overmann et al., 1991b; Overmann et al., 1992) and are inhibited at intensities above 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The strain *Chlorobium phaeobacterioides* MN1 which was isolated from the chemocline of the Black Sea exhibited the most pronounced low light adaptation known for any phototrophic microorganism so far (Overmann et al., 1992).

Most strains of green sulfur bacteria require vitamin B_{12} for growth (Pfennig and Lippert, 1966). In the absence of this vitamin the specific bacteriochlorophyll content of the cells is drastically reduced and cells are incapable of forming chlorosomes (Fuhrmann et al., 1993).

Photosynthesis: Reaction Center

The photosynthetic reaction centers are located in the cytoplasmic membrane. They are of the iron-sulfur-type and thus resemble photosystem I of the oxygenic phototrophs and the reaction center of *Heliobacteriaceae* (Olson, 1998), but differ from the quinone-type of *Chromatiaceae*, *Ectothiorhodospiraceae*, and phototrophic members of the α-Proteobacteria and β-Proteobacteria. The reaction center contains a highly symmetric dimer of bacteriochlorophyll *a*, the primary donor P840, which receives excitation energy from the chlorosomes via the FMO-protein and additional bacteriochlorophyll *a* molecules in the reaction center.

Upon excitation, the reduced P840 becomes a powerful reductant [$E_0'(P840^+/P840^*) \approx -1200$ mV] and the electrons are transferred by several cofactors, a chlorophyll *a* isomer (A_0, $BChl_{663}$), a menaquinone-7 (A_1, Kjaer et al., 1998), and three iron-sulfur centers (F_x, F_A, F_B) towards the cytoplasmic side of the membrane. Because of the low redox potential of F_A and F_B ($E_0' \approx -540$ mV), the electrons can be directly transferred to soluble ferredoxin ($E_0' = -410$ mV). Ferredoxin is the electron donor in the reductive tricarboxylic acid cycle. Furthermore, NAD^+ can be directly reduced by ferredoxin. Therefore a reverse electron flow for NAD^+ reduction is not necessary, which partially explains the low energy requirements of green sulfur bacteria. Cyclic photophosphorylation in the green sulfur bacteria proceeds like in *Chromatiaceae* (Brune, 1989; Drews, 1989).

Because the standard redox potential of the photosynthetic reaction center in its oxidized

state is +240 mV, it cannot oxidize water to O_2. Instead, sulfide (S^0/HS^-, $E_0' = -270$ mV), sulfur/polysulfide-sulfur, in some cases thiosulfate (see sulfur metabolism) or—in most species (Lippert and Pfennig, 1969)—molecular hydrogen serves as electron donor of anoxygenic photosynthesis. Uptake hydrogenases appear to be membrane-bound and probably contain nickel and iron-sulfur clusters as do other uptake hydrogenases (Gogotov, 1986). Recently, a *Chlorobium* strain has been isolated which is capable of anoxygenic photosynthesis with ferrous iron ($Fe(OH)_3/Fe^{2+}$, $E_0' = -236$ mV) as electron donor for photosynthesis. In noncyclic electron flow, electrons removed by reduction of ferredoxin are replaced by external electrons transferred from sulfide via menaquinone or soluble cytochrome c_{555} and a membrane-bound monoheme cytochrome c_{551} on the periplasmic side of the membrane (Meyer and Donohue, 1995). As mobile intramembraneous hydrogen carriers, menaquinones replace the ubiquinones of purple bacteria.

Because of the considerably shorter span in redox potential, absorption of only about one photon is required per electron transferred to CO_2. Fixation of 1 mol C in green sulfur bacteria requires ~4 (3.3–4.5) mol photons (Brune, 1989); thus the quantum yield (mol C assimilated: mol quanta absorbed = 0.25) is twice as high as that of purple sulfur bacteria (0.12) or oxygenic phototrophic organisms (maximum, 0.125).

Sulfur Metabolism

Sulfide and elemental sulfur are utilized by all known isolates of green sulfur bacteria, whereas thiosulfate can serve as electron donor in only a few strains (*Chlorobium limicola* f.sp. thiosulfatophilum DSM 249, *Chl. vibrioforme* f.sp. thiosulfatophilum DSM 265, *Chl. tepidum* ATCC 49652^T, *Pelodictyon phaeoclathratiforme* DSM 5477^T). A soluble homodimeric cytochrome c_{551} is involved in thiosulfate oxidation of *Chlorobium limicola* f.sp. thiosulfatophilum DSM 249 (Klarskov et al., 1998). *Chlorobium limicola* f.sp. thiosulfatophilum and *Chl. vibrioforme* f.sp. thiosulfatophilum are also capable of sulfur disproportionation in the absence of CO_2 (Paschinger et al., 1974, Trüper et al., 1988). Only *Chl. vibrioforme* f.sp. thiosulfatophilum forms extracellular sulfur globules from thiosulfate (Steinmetz and Fischer, 1982). Tetrathionate ($S_4O_6^{2-}$) is utilized as electron donor by *Chlorobium limicola* f.sp. thiosulfatophilum and *Chl. vibrioforme* f.sp. thiosulfatophilum (Brune, 1989).

Oxidation of sulfide results in the formation of sulfur globules (zero valence sulfur). After depletion of sulfide, zero valence sulfur is further oxidized to sulfate. *Chlorobium limicola* f.sp. thiosulfatophilum and *Chl. vibrioforme* f.sp. thiosulfatophilum form thiosulfate simultaneously to elemental sulfur (Trüper, 1992), and the thiosulfate formation from sulfide is catalyzed by flavocytochrome *c* (Fischer, 1984). None of the green sulfur bacteria presently in pure culture are known to use sulfite as photosynthetic electron donor and sulfite can even inhibit the growth of *Prosthecochloris aestuarii* (Pringault et al., 1998). Sulfate formation in *Chlorobium* is mediated by adenylylsulfate reductase (Trüper and Peck, 1970b; Kirchhoff and Trüper, 1974), followed by cleavage of adenylylsulfate into sulfate and ADP or ATP by the enzyme ADP sulfurylase in *Chl. vibrioforme* (Trüper and Fischer, 1982; Khanna and Nicholas, 1983; Bias and Trüper, 1987) or ATP sulfurylase in *Chl. limicola* (Bias and Trüper, 1987), respectively.

The affinity for sulfide of the green sulfur bacteria is one order of magnitude higher than that of *Chromatiaceae* (van Gemerden and Mas, 1995). Growth is inhibited at concentrations of 5–8 mM (Bergstein et al., 1979, Overmann and Pfennig, 1989a, Overmann, 1999a). Green sulfur bacteria lack assimilatory sulfate reduction and require a reduced sulfur source during growth with molecular hydrogen as electron donating substrate (Lippert and Pfennig, 1969).

Carbon Metabolism

In contrast to *Chromatiaceae* with which they share the anoxygenic phototrophic type of metabolism, all known strains of green sulfur bacteria grow exclusively photolithoautotrophically. Carbon dioxide is fixed by the reductive tricarboxylic acid cycle (Fuchs et al., 1980). The key enzymes of the cycle are the ferredoxin-dependent a-oxoglutarate and pyruvate synthetases and an ATP-dependent citrate lyase. The latter is inhibited by oxaloacetate. All other enzymatic steps of the tricarboxylic acid cycle are reversible. Compared to the Calvin cycle, less ATP are required per molecule CO_2 fixed (5 ATP for one molecule glyceraldehyde-3-phosphate [Brune, 1989] as opposed to 9 ATP in the *Chromatiaceae*), which, together with the large size of their photosynthetic antennae, explains the low light adaptation of the family.

The reductive tricarboxylic acid cycle provides the cells with precursors for the biosynthesis of cell compounds. As the enzyme α-oxoglutarate dehydrogenase is not present and the tricarboxylic acid cycle can only function in the reductive direction, organic carbon compounds cannot be oxidized to yield CO_2 and reducing power for biosynthetic purposes. In the presence of inorganic reductants and CO_2, however, green sulfur bacteria are potentially mixotrophic and use simple organic carbon compounds (acetate, propionate, or pyruvate) for biomass formation.

Acetate is incorporated by reductive carboxylation, which requires the reducing equivalents from sulfide and involves a reduced ferredoxin. In the presence of acetate, the biomass yield per mol sulfide is increased about threefold, accordingly (Bergstein et al., 1979). In green sulfur bacteria, the affinity for acetate is one order of magnitude lower than that in the *Chromatiaceae* (van Gemerden and Mas, 1995).

The enzymes involved in the CO_2-fixation in the reverse tricarboxylic acid cycle discriminate only slightly against heavy carbon isotopes as compared to the enzymes of the Calvin cycle (Sirevåg et al., 1977b). This fact is of prime significance for paleoecological investigations of marine sediments and sedimentary rocks, where molecular fossils of green sulfur bacteria indicate past anoxia of certain ocean basins (Green Sulfur Bacteria during Geological Time Scales).

In the presence of excess carbon substrates and light energy, but limiting amounts of inorganic nutrients like ammonium and phosphate, green sulfur bacteria synthesize glycogen which is deposited intracellularly as non membrane-bound granules. During incubation in the dark, polyglucose is oxidized and excretion of its degradation products acetate, propionate, caproate, and succinate occurs (Sirevåg and Ormerod, 1977a).

Nitrogen Metabolism

Although the best nitrogen source for growth is the ammonium ion, the majority of strains of the green sulfur bacteria are capable of nitrogen fixation (Hallenbeck, 1987; Vignais et al., 1985).

Genetics

The size of the total genome in strains of the genus *Chlorobium* varies between 1,435 and 3,342 kilo base pairs (Méndez-Alvarez et al., 1996). Some 34 genes of green sulfur bacteria, mostly from *Chlorobium vibrioforme*, have been sequenced (Shiozawa, 1995). Aside from the 16S rRNA genes, most of these genes code for enzymes and polypeptides involved in photosynthesis.

Only one gene for the large reaction center polypetide PscA has been detected, which corresponds to the homodimeric structure of the reaction center of green sulfur bacteria. As expected, *pscA* shows sequence homology with the genes of Fe-S-type reaction centers in other photosynthetic bacteria. The polypeptide which contains the two FeS-cofactors FA and FB of the reaction center is coded by *pscB* which together with *pscA* forms a bicistronic unit. The gene of the cytochrome subunit of the reaction center, *pscC*, does not exhibit significant similarities with other genes coding for cytochrome subunits.

Of the genes coding the chlorosome polypeptides, *csmC* and *csmA* form the *csmCA* operon, whereas *csmB* is transcribed as a monocistron (Shiozawa, 1995).

Another operon contains three genes encoding enzymes of bacteriochlorophyll synthesis, *hemA* (glutamyl-tRNA reductase; Majumdar and Wyche, 1997), *hemC* (coding porphobilinogen deaminase; Majumdar and Wyche, 1997), and *hemD* (uroporphyrinogen III synthase). As a special feature, the 3′-region following the termination codon of each of the genes contains dA/dT-rich DNA stretches. In addition, *hem A* is followed by a potential adenylation signal and represents the first example for the occurrence of this typical eukaryotic feature in a bacterial genome (Shiozawa, 1995).

The limited data concerning the transcription start signals show that transcripts contain sequence motifs that are quite similar to the *Escherichia coli* σ^{70} consenus promotor sequence (TTGACA-17bp-TATAAT) (Chung and Bryant, 1996). *Chlorobium tepidum* contains only one group 1 sigma factor of the σ^{70} family (Gruber and Bryant, 1998a). In the same species, an AT-rich spacer region occurs between the –35 and –10 recognition sites in the promoter regions of several genes (Gruber and Bryant, 1998a) and may act as an additional promotor element. Transcription of the genes in green sulfur bacteria in most cases seems to be terminated by rho-independent stemloop structures. Characteristically, stemloops often begin with a stretch of 6 or 7 deoxyadenosines and with 4–7 deoxythymidines (Chung and Bryant, 1996; Gruber and Bryant, 1998a).

Chlorobium limicola strain 8327 exhibits a natural competence for intrastrain genetic transformation with chromosomal DNA (Ormerod, 1988). However, DNA extracts of this strain did not transform other *Chlorobium* strains. Similarly, transfer of an endogeneous plasmid which confers the ability to utilize thiosulfate has been demonstrated for *Chlorobium limicola* (Méndez-Alvarez et al., 1994). However, foreign DNA has been successfully introduced into *Chlorobium vibrioforme* by electroporation and was integrated into the genome by homologous recombination (Kjaerulff et al., 1994). A conjugation system for genetic studies of the thermophilic *Chlorobium tepidum* strains WT2321 and Nal-1 has been established which permits genetic analysis of gene expression and regulation directly within a green sulfur bacterium (Wahlund and Madigan, 1995). Two broad-host-range IncQ group plasmids can be transferred by conjugation from *E. coli* to *Chl. tepidum*. The latter species is especially well-suited for genetic

studies because of its short generation time of 2 h. As another prerequisite for molecular genetic studies, a new medium for the growth of green sulfur bacteria on agar plates has been designed (Wahlund and Madigan, 1995).

Ecology

Limiting Factors

Accumulations of green sulfur bacteria are positioned in the narrow zone of overlap between the opposing gradients of light and sulfide. In general, anoxygenic photosynthesis is limited by the availability of light (van Gemerden and Mas, 1995). In lakes, the light intensity available for anoxygenic photosynthesis is determined by the absorption of light in the water layers above the chemocline. Morphology and physicochemical factors like the density differences between water layers and wind-induced water currents determine the vertical position of the chemocline. Absorption of light in oxic water layers is high in eutrophic lakes and mediated by phytoplankton, whereas in dystrophic lakes light absorption is mainly due to the high concentrations of humic substances (Parkin and Brock, 1980). In general, the biomass density of green sulfur bacteria is inversely correlated with their depth in the environment and the thickness of the bacterial layer and reaches up to 120,000 μg bacteriochlorophyll·L^{-1} in benthic microbial mats (1 mm thin; Pierson et al., 1987), an average of 600 μg bacteriochlorophyll·L^{-1} in lakes, but only 0.94 μg bacteriochlorophyll·L^{-1} in the deepest anoxygenic phototrophic community which extends from 68 to 98 m depth in the Black Sea (Repeta et al., 1989). However, the physicochemical environment of green sulfur bacteria may be more dynamic not only in microbial mats but also in some lakes. For example, temporary exposure of the phototrophic bacteria to higher light intensities occurs during the daily lift up of the chemocline by internal seiches in Lake Kinneret and may explain the growth of *Chlorobium phaeobacteroides* in the chemocline despite of the prevailing very low light intensities (Bergstein et al., 1979).

The major factor determining the species composition of green sulfur bacteria in natural habitats is the spectral composition of underwater light. In lakes where the chemocline is located at greater depths (≥9 m) and in eutrophic lakes, the selective absorption of light by phytoplankton and water results in a progressive narrowing of the spectrum such that only light in the blue-green to green wavelength range is available for anoxygenic photosynthesis. The brown-colored forms of green sulfur bacteria which contain high intracellular concentrations of the light-harvesting carotenoids isorenieratene and β-isorenieratene have a selective advantage under these conditions (Montesinos et al., 1983). In contrast, green-colored species dominate underneath populations of purple sulfur bacteria (Interactions between Different Groups of Phototrophic Bacteria) or in dystrophic lakes (Parkin and Brock, 1980), where light of the blue (450 nm) or red (700 nm) wavelength range prevails, respectively.

Gas vesicle formation occurs in six of the fourteen described species. Green sulfur bacteria in pelagic microbial communities frequently contain gas vesicles (Caldwell and Tiedje, 1975; Clark and Walsby, 1978). In *Pelodictyon phaeoclathratiforme* DSM 5477^T, gas vesicle formation is induced exclusively at low light intensities (Overmann et al., 1991a). Given the low light intensities in most of their habitats, the growth rates of green sulfur bacteria are low. Gas vesicles are of selective advantage because they reduce the buoyant density of the cells and thereby minimize the loss of cells by sedimentation into dark bottom layers. However, unlike cyanobacteria, diurnal migrations by means of rapid changes in cellular gas vesicle content have not been observed so far for the green sulfur bacteria (Parkin and Brock, 1981).

Interactions between Different Groups of Phototrophic Sulfur Bacteria

Green sulfur bacteria and *Chromatiaceae* are both photolithoautotrophic and employ reduced sulfur compounds as electron donating substrates for anoxygenic photosynthesis. However, green sulfur bacteria are capable of using significantly lower light intensities for photosynthetic growth (see Physiology), and their maintenance energy requirements are significantly lower than those of the *Chromatiaceae* (Veldhuis and van Gemerden, 1986; Overmann et al., 1992). Unlike many *Chromatiaceae*, green sulfur bacteria cannot grow chemotrophically with molecular oxygen as the terminal electron acceptor and cannot use organic carbon compounds as photosynthetic electron donors. Because of these differences in their ecological niches, green sulfur bacteria often thrive below layers of *Chromatiaceae* (Caldwell and Tiedje, 1975; Gorlenko and Kuznetsov, 1971a; Overmann et al., 1998); however, both groups show some overlap. Light penetrating the layers of purple sulfur bacteria has its maximum intensity at 420–450 nm, coinciding with the short wavelength absorption maximum of bacteriochlorophylls *c* and *d* (see "Identification"). As a consequence, green-colored forms of the green sulfur bacteria accompany *Chromati-*

aceae in the natural environment (Montesinos et al., 1983). Although absorption of light by *Chromatiaceae* in upper water layers limits the light energy which can be harvested by green sulfur bacteria below, the photooxidation of sulfide by the low-light adapted green sulfur bacteria in turn may limit the diffusive flux of sulfid into the layers of purple sulfur bacteria.

However, syntrophic interactions also occur between the two groups of phototrophic sulfur bacteria, as exemplified by cocultures of *Chlorobium limicola* and *Chromatium vinosum*. These positive interactions are based on the oxidation of sulfide to zero valence sulfur by the green sulfur bacterium and the subsequent oxidation of sulfur to sulfate by the purple sulfur bacterial partner (van Gemerden and Mas, 1995). At limiting sulfide concentrations, *Chl. limicola* has a selective advantage due to its higher affinity (see Physiology), but the oxidation of the generated sulfur is inhibited in the presence of sulfide. The extracellular sulfur formed by the green sulfur bacterium abiotically reacts with sulfide to form polysulfides, which then can be used instanteneously by *Chr. vinosum*. Syntrophic interactions of this kind may be one major reason for the coexistence of purple and green sulfur bacteria, which is frequently observed in the natural habitat.

Interactions with Chemotrophic Bacteria

Stable associations between green sulfur bacteria and sulfur- or sulfate-reducing bacteria can be established in laboratory cocultures (Pfennig, 1980; Warthmann et al., 1992). As for the interactions between different groups of phototrophic sulfur bacteria such associations are based on the exchange of inorganic sulfur compounds. In this case, however, a closed sulfur cycle is established: sulfide is oxidized photosynthetically to sulfur or sulfate which in turn are utilized by the chemotrophic partner bacterium. Due to the extensive recycling of inorganic sulfur compounds, sulfur syntrophy can proceed even at trace concentrations of reduced sulfur compounds. Ultimately, the cycling of sulfur is fueled by the oxidation of organic carbon substrates and light.

One of the outstanding features of the green sulfur bacteria is their participation in highly specialized symbiotic associations with other bacteria, the phototrophic consortia. These morphologically conspicuous and yet still enigmatic structural associations of green sulfur bacteria and chemotrophic bacteria occur in the pelagial of many freshwater habitats. The regular structure of phototrophic consortia is unique in the microbial world such that they can be distinguished from other cell aggregates by light

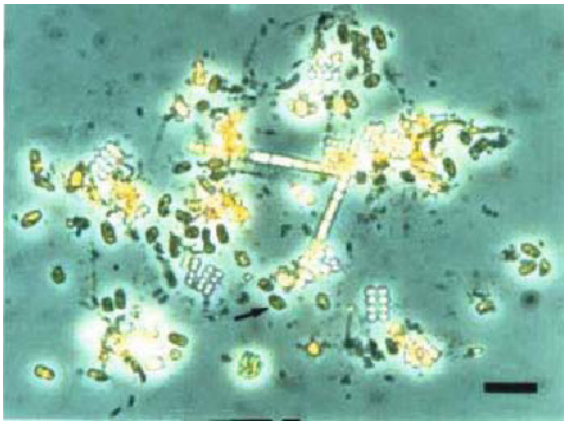

Fig. 3. Phase contrast photomicrograph of the bacterial community found in the chemocline of a little eutrophic hard-water lake (Lake Dagow; Overmann et al., 1998). A major fraction of phototrophic sulfur bacterial cells is found in the phototrophic consortium "*Pelochromatium roseum*" (arrow). In addition, *Thiopedia rosea* (flat sheaths of eight cells) and multicellular filaments of *Chloronema* spp. (center) are observed. Bar, 20 μm. (Courtesy of Jens Glaeser.)

microscopy (Fig. 3, 4). Presently, seven different types of phototrophic consortia are known (Fig. 5). In "*Chlorochromatium aggregatum*" (Lauterborn, 1915; *Chloronium mirabile*, Buder, 1914), "*C. glebulum*" (Skuja, 1957), "*C. lunatum*" (Abella et al., 1998), "*Pelochromatium roseum*" (Lauterborn, 1914), "*P. roseo-viride*" (Gorlenko and Kusnezow, 1972), and "*P. selenoides*" (Abella et al., 1998), the cells of green sulfur bacteria form a regular array around a central colorless bacterial cell (Fig. 6). "*C. aggregatum*" and "*P. roseum*" contain up to 24 epibionts, the other associations up to 69. Epibionts in "*Chlorochromatium*" are green, whereas those in "*Pelochromatium*" are brown-colored. "*Chloroplana vacuolata*" (Dubinina and Kusnezov, 1976) has a different structural arrangement and consists of parallel rows of alternating green and colorless bacteria. These platelets comprise up to 400 green cells and are immotile. "*Cylindrogloea bacterifera*" is an association of a colorless filamentous bacterium covered with a slime layer in which green sulfur bacteria are embedded (Perfiliev, 1914; Skuja, 1957). According to bacterial nomenclature, all of the above binominal names are without taxonomic significance and are therefore given in quotation marks only (Trüper and Pfennig, 1971).

The number of epibionts per central bacterium in "*C. aggregatum*" and "*P. roseum*" is rather constant and all epibionts have the same size. This indicates that all epibionts and the central bacterium must divide synchronously (Overmann et al., 1998). "*C. aggregatum*" accumulates at 740 nm in the light, corresponding to the wavelength of the absorption maximum of

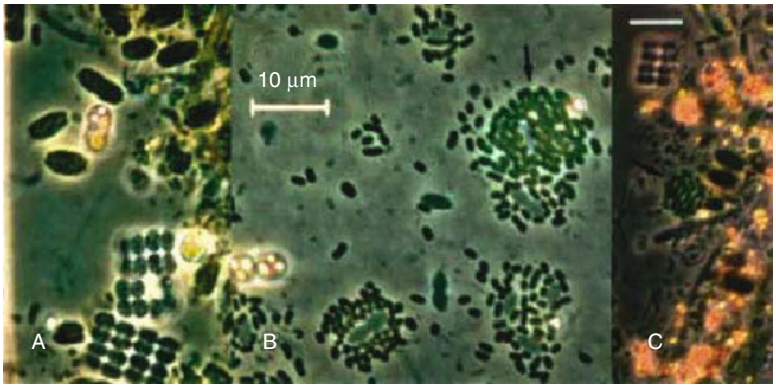

Fig. 4. Phase contrast photomicrographs of samples from the chemocline of a small eutrophic hardwater lake (Lake Dagow; Overmann et al., 1998). A) Untreated sample (bar is the same as for B). B) Same sample, but squash preparation of several "*Pelochromatium roseum*" consortia and one green consortium (arrow; note that gas vacuoles can be discerned in some of the epibiont cells). C) Sample containing globular green consortia (arrow) in which epibionts are arranged in loose unordered fashion as compared to "*P. roseum.*" Bars, 10 µm (taken from Overmann et al., 1998).

the bacteriochlorophyll present in the epibionts. As only the colorless central bacterium is flagellated, a rapid signal transduction must therefore occur between the light-sensitive epibionts and the central bacterium. Based on their 16S rRNA gene sequences, the epibionts in "*Chlorochromatium aggregatum*," "*C. glebulum*," and "*Pelochromatium roseum*" are phylogenetically different from all known phylotypes of green sulfur bacteria. Phototrophic consortia therefore seem to be highly specialized symbiotic associations. In symbiosis, green sulfur bacteria attain the ecologically significant capacity to respond to different light intensity, as do the motile *Chromatiaceae* (Pfennig, 1980).

Interestingly, "*C. aggregatum*" exhibits a chemotaxis toward 2-oxoglutarate, citrate, sulfide, and thiosulfate, while the typical carbon substrates of green sulfur bacteria (acetate, propionate, pyruvate) do not act as chemoattractants (Fröstl and Overmann, 1998). It has been suggested that the central bacterium of the phototrophic consortia is a sulfate- or sulfur-reducing bacterium (Kuznetsov, 1977; Pfennig, 1980). Based on the results of in situ-hybridization with group-specific oligonucleotides probes, the central bacterium does not belong to the δ-subgroup of the *Proteobacteria* and therefore does not represent a classical sulfate- or sulfur-reducing bacterium.

An antagonistic relationship of green sulfur bacteria has been described with the Gram-negative bacterium *Stenotrophomonas maltophilia*. This chemotrophic bacterium causes growth inhibition zones on cell lawns of several *Chlorobium* species (Nogales et al., 1997), in which green sulfur bacterial cells are lyzed and form ghosts. This anatagonism is non-obligatory and not limited to green sulfur bacteria as the hosts.

Ecological Significance

Green sulfur bacteria can reach high biomass densities (up to 4000 µg bacteriochlorophyll·L^{-1}, van Gemerden and Mas, 1995) in some lakes and contribute up to 83% of total primary production (Culver and Brunskill, 1969). Because anoxygenic photosynthesis is indirectly fueled by carbon that has previously been fixed by oxygenic phototrophs, the production of green sulfur bacteria represents a net biomass input only in those aquatic ecosystems where the anaerobic food chain and thus sulfide production are driven by the degradation of allochthonous organic carbon (Overmann, 1997a). Exceptions are geothermal springs, in which the sulfide is of geological origin and anoxygenic photosynthesis represents true primary production.

Green Sulfur Bacteria during Geological Time Scales

With the exception of the Black Sea, the habitats of green sulfur bacteria known today represent rather small bodies of water, mainly stratified lakes, coastal lagoons or fjords. However, isorenieratene and its degradation products have been discovered in Devonian sedimentary rocks (Hartgers et al., 1994), Miocene evaporites (Kohnen et al., 1992) and eastern Mediterranean sapropel sediments of the Pliocene (Passier et al., 1999) which originally were located at the bottom of much larger oceanic basins. Isorenieratene seems to occur solely in green sulfur bacteria. These bacteria fix CO_2 in the reverse

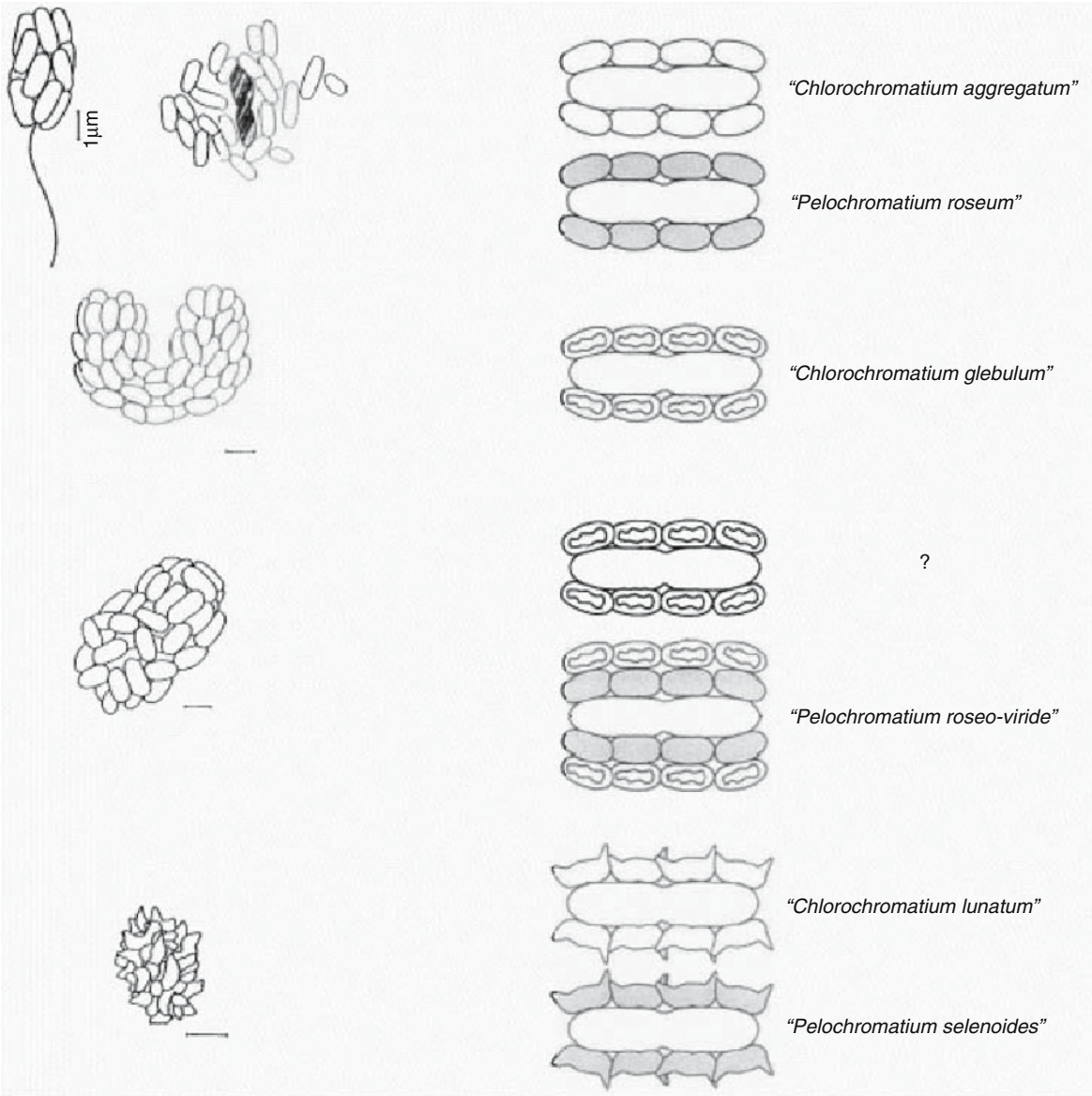

Fig. 5. Schematic representation of 7 different morphological types of motile phototrophic consortia. Left part depicts light microscopic appearance of the different types. Bar is 1 µm. In the right part of the figure, the central cell represents the unknown chemotrophic bacterium. White epibiont cells indicate green-colored, shaded epibiont cells the brown-colored forms of green sulfur bacteria. Irregular central area indicates the presence of gas vesicles in epibionts. One morphological type has yet to be described (question mark). (Modified after Pfennig, 1980, and Abella et al., 1998.)

tricarboxylic acid cycle which leads to organic carbon anomalously enriched in ^{13}C. Indeed isorenieratene and its degradation products are enriched by 12–24% in ^{13}C in the geological samples. Consequently, these molecular fossils have been taken as evidence that a large portion of the water column in major oceanic basins contained sulfide and extended into the photic zone. The Black Sea may represent a model ecosystem for such euxinic (restricted circulation leading to oxygen depletion) states of larger ocean basins during geological time scales. Anoxia of the bottom photic zone even seems to have occurred in the open North Atlantic Ocean during the Cenoman/Turon (i.e. up to 100 Mio years ago) (Sinninghe Damsté and Köster, 1998).

Applications

Green sulfur bacteria may be used for the removal of obnoxious smells of the toxic hydrogen sulfide during anaerobic waste treatment (Kobayashi et al., 1983). Cork and Cusanovich

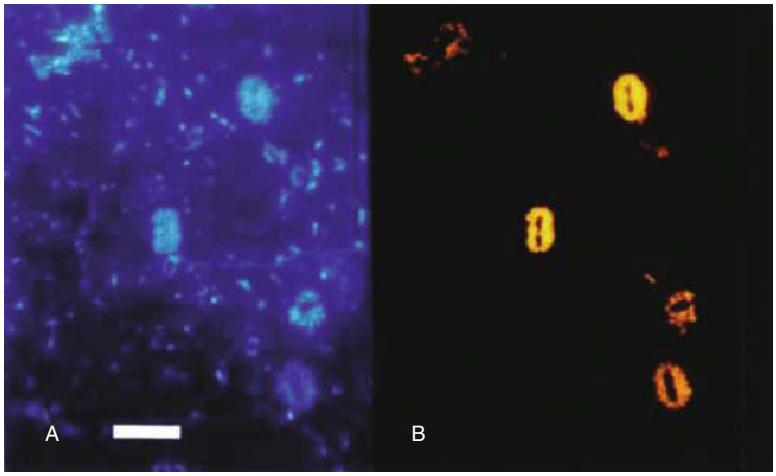

Fig. 6. Bacterial cells from the chemocline of a small eutrophic hard-water lake (Lake Dagow; Overmann et al., 1998) concentrated on a polycarbonate filter, stained with DAPI and hybridized with Cy 3-labeled probe GSB-532. A) DAPI fluorescence. B) Fluorescence of Cy 3-labeled probe GSB-532, showing the phylogenetic affiliation of the epibionts of "*Pelochromatium roseum*" with the Chlorobiaceae. Bar, 10 µm.

(1979) presented a process for the continuous disposal of sulfate by a mutual bacterial system consisting of *Desulfovibrio desulfuricans* and *Chlorobium limicola*. A system has been developed for the formation of elemental sulfur and organic carbon (biomass) from acid gas (containing H_2S and CO_2) which employs *Chl. limicola* as a biocatalyst (Cork, 1982a; Cork and Ma, 1982b; Cork et al., 1983; Cork et al., 1985). This represents an alternative to the Claus desulfuration process used in cleaning sour natural gases.

Literature Cited

Abella, C. A., X. P. Cristina, A. Martinez, I. Pibernat, and X. Vila. 1998. Two motile phototrophic consortia: "Chlorochromatium lunatum" and "Pelochromatium selenoides." Arch. Microbiol. 169:452–459.

Amesz, J. 1991. Green photosynthetic bacteria and heliobacteria. *In:* L. L. Barton and J. M. Shively (Eds.) Variations in Autotrophic Life. Academic Press. London, 99–119.

Bergstein, T., Y. Henis, and B. Z. Cavari. 1979. Investigations on the photosynthetic sulfur bacterium Chlorobium phaeobacteroides causing seasonal blooms in Lake Kinneret. Can. J. Microbiol. 25:999–1007.

Bias, U., and H. G. Trüper. 1987. Species specific release of sulfate from adenylyl sulfate by ATP sulfurylase or ADP sulfurylase in the green sulfur bacteria Chlorobium limicola and Chlorobium vibrioforme. Arch. Microbiol. 147:406–410.

Biebl, H., and N. Pfennig. 1978. Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. Arch. Microbiol. 117:9–16.

Blankenship, R. E., J. M. Olson, and M. Miller. 1995. Antenna complexes from green photosynthetic bacteria. *In:* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (Eds.) Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers. Dordrecht, Boston, London, 399–435.

Borrego, C. M., and L. J. Garcia-Gil. 1994. Separation of bacteriochlorophyll homologues from green photosynthetic sulfur bacteria by reversed-phase HPLC. Photosynth. Res. 41:157–163.

Broch-Due, M., J. G. Ormerod, and B. S. Fjerdingen. 1978. Effect of light intensity on vesicle formation in Chlorobium. Arch. Microbiol. 116:269–274.

Brune, D. C. 1989. Sulfur oxidation by phototrophic bacteria. Biochem. Biophys. Acta 975:189–221.

Buder, J. 1914. Chloronium mirabile. Berichte Dtsch. Bot. Ges. 31:80–97.

Caldwell, D. E., and J. M. Tiedje. 1975. The structure of anaerobic bacterial communities in the hypolimnia of several Michigan lakes. Can. J. Microbiol. 21:377–385.

Castenholz, R. W., J. Bauld, and B. B. Jørgensen. 1990. Anoxygenic microbial mats of hot springs: thermophilic Chlorobium sp. FEMS Microbiol. Ecol. 74:325–336.

Caumette, P. 1984. Distribution and characterization of phototrophic bacteria isolated from the water of Bietri Bay (Ebrie Lagoon, Ivory Coast). Can. J. Microbiol. 30:273–284.

Chung, S., G. Frank, H. Zuber, and D. A. Bryant. 1994. Genes encoding two chlorosome components from the green sulfur bacteria Chlorobium vibrioforme strain 8327D and Chlorobium tepidum. Photosynth. Res. 41:261–275.

Chung, S., and D. A. Bryant. 1996. Characterization of csmB genes, encoding a 7.5-kDa protein of the chlorosome envelope, from the green sulfur bacteria Chlorobium vibrioforme 8327D and Chlorobium tepidum. Arch. Microbiol. 166:234–244.

Chung, S., G. Shen, J. Ormerod, and D. A. Bryant. 1998. Insertional inactivation studies of the csmA and csmC genes of the green sulfur bacterium Chlorobium vibrioforme 8327: The chlorosome protein CsmA is required for viability but CsmC is dispensable. FEMS Microbiol. Lett. 164:353–361.

Clark, A. E., and A. E. Walsby. 1978. The occurrence of gas-vacuolate bacteria in lakes. Arch. Microbiol. 118:223–228.

Cork, D. J., and M. A. Cusanovich. 1979. Continuous disposal of sulfate by a bacterial mutualism. Dev. Ind. Microbiol. 20:591–602.

Cork, D. J. 1982a. Acid waste gas bioconversion-an alternative to the Claus desulfurization process. Dev. Ind. Microbiol. 23:379–387.

Cork, D. J., and S. Ma. 1982b. Acid-gas bioconversion favors sulfur production. Biotech. Bioeng. 12:285–290.

Cork, D. J., R. Garunas, and A. Sajjad. 1983. Chlorobium limicola forma thiosulfatophilum: Biocatalyst in the production of sulfur and organic carbon from a gas stream

containing H_2S and CO_2. Appl. Environ. Microbiol. 45:913–918.

Cork, D. J., J. Mathers, A. Maka, and A. Srnak. 1985. Control of oxidative sulfur metabolism of Chlorobium limicola f. thiosulfatophilum. Appl. Environ. Microbiol. 49:269–272.

Culver, D. A., and G. J. Brunskill. 1969. Fayetteville Green Lake, New York. V: Studies of primary production and zooplankton in a meromictic marl lake. Limnol. Oceanogr. 14:862–873.

Czeczuga, B. 1968. Primary production of the green hydrosulfuric bacteria Chlorobium limicola Nads. (Chlorobacteriaceae). Photosynthetica 2:11–15.

Drews, G. 1989. Energy transduction in phototrophic bacteria. *In:* H. G. Schlegel and B. Bowien (Eds.) Autotrophic Bacteria. Science Tech and Springer. Madison, WI. 461–480.

Dubinina, G. A., and S. I. Kuznetsov. 1976. The ecological and morphological characteristica of microorganisms in Lesnaya Lamba (Karelia). Int. Rev. Ges. Hydrobiol. 61:1–19.

Figueras, J. B., L. J. Garcia-Gil, and C. A. Abella. 1997. Phylogeny of the genus Chlorobium based on 16S rDNA sequence. FEMS Microbiol. Lett. 152:

Fischer, U. 1984. Cytochromes and iron sulfur proteins in sulfur metabolism of phototrophic bacteria. *In:* A. Miller and B. Krebs (Eds.) Sulfur, Its Significance for Chemistry, for the Geo-, Bio- and Cosmosphere and Technology. Elsevier. Amsterdam, 383–407.

Frigaard, N.-U., S. Takaichi, M. Hirota, K. Shimada, and K. Matsuura. 1997. Quinones in chlorosomes of green sulfur bacteria and their role in the redox-dependent fluorescence studied in chlorosome-like bacteriochlorophyll c aggregates. Arch. Microbiol. 167:343–349.

Fröstl, J. M., and J. Overmann. 1998. Physiology and tactic response of the phototrophic consortium "Chlorochromatium aggregatum." Arch. Microbiol. 169:129–135.

Fuchs, G., E. Stupperich, and R. Jaenchen. 1980. Autotrophic CO_2 fixation in Chlorobium limicola: Evidence for the operation of a reductive tricarboxylic acid cycle in growing cells. Arch. Microbiol. 128:64–72.

Fuhrmann, S., J. Overmann, N. Pfennig, and U. Fischer. 1993. Influence of vitamin B12 and light on the formation of chlorosomes in green- and brown-colored Chlorobium species. Arch. Microbiol. 160:193–198.

Gibson, J., N. Pfennig, and J. B. Waterbury. 1984. Chloroherpeton thalassium gen. nov. et spec. nov., a nonfilamentous, flexing and gliding green sulfur bacterium. Arch. Microbiol. 138:96–101.

Gibson, J., W. Ludwig, W. Stackebrandt, and C. R. Woese. 1985. The phylogeny of green photosynthetic bacteria: Absence of a close relationship between Chlorobium and Chloroflexus. Syst. Appl. Microbiol. 6:152–156.

Glaeser, J., and J. Overmann. 1999. Selective enrichment and characterization of Roseospirillum parvum, gen. nov. and sp. nov., a new purple nonsulfur bacterium with unusual light absorption properties. Arch. Microbiol.

Gloe, A., N. Pfennig, H. Brockmann, and W. Trowitzsch. 1975. A new bacteriochlorophyll from brown-colored Chlorobiaceae. Arch. Microbiol. 102:103–109.

Gogotov, I. N. 1986. Hydrogenases of phototrophic microorganisms. Biochimie 68:181–187.

Gorlenko, V. M., and S. I. Kuznetsov. 1971a. Vertical distribution of photosynthetic bacteria in the Konoer Lake of the Mari ASSR. Microbiology 40:651–652.

Gorlenko, V. M., and E. V. Lebedeva. 1971b. New green sulphur bacteria with apophyses (in Russian, with English summary). Mikrobiologiya 40:1035–1039.

Gorlenko, V. M., and S. I. Kusnezow. 1972. Über die photosynthetisierenden Bakterien des Kononjer-Sees. Arch. Hydrobiol. 70:1–13.

Gorlenko, V. M., E. N. Chebotarev, and V. I. Kachalkin. 1973. Microbial processes of oxidation of hydrogen sulfide in the Repnoe lake (Slavonic lakes). Microbiology (English translation of Mikrobiologiya) 42:723–728.

Gruber, T. M., and D. A. Bryant. 1998a. Characterization of the group 1 and group 2 sigma factors of the green sulfur bacterium Chlorobium tepidum and the green nonsulfur bacterium Chloroflexus aurantiacus. Arch. Microbiol. 170:285–296.

Gruber, T. M., J. A. Eisen, K. Gish, and D. A. Bryant. 1998b. The phylogenetic relationships of Chlorobium tepidum and Chloroflexus aurantiacus based upon their RecA sequences. FEMS Microbiol. Lett. 162:53–60.

Hallenbeck, P. C. 1987. Molecular aspects of nitrogen fixation by photosynthetic prokaryotes. Crit. Rev. Microbiol. 14:1–48.

Hartgers, W. A., J. A. Sinninghe Damsté, A. G. Requejo, J. Allan, J. M. Hayes, and J. W. de Leeuw. 1994. Evidence for only minor contributions from bacteria to sedimentary organic carbon. Nature 369:224–227.

Holt, S. C., S. F. Conti, and R. C. Fuller. 1966. Effects of light intensity on the formation of the photochemical apparatus in the green bacterium Chloropseudomonas ethylicum. J. Bacteriol. 91:349–355.

Huster, M. S., and K. M. Smith. 1990. Biosynthetic studies of substituent homologation in bacteriochlorophylls c and d. Biochemistry 29:4348–4355.

Imhoff, J. F. 1988. Lipids, fatty acids and quinones in taxonomy and phylogeny of anoxygenic phototrophic bacteria. *In:* J. M. Olson, E. Stackebrandt, and H. G. Trüper (Eds.) Green Photosynthetic Bacteria. Plenum Publishing. New York, NY. 223–232.

Kenyon, C. N., and A. M. Gray. 1974. Preliminary analysis of lipids and fatty acids of green bacteria and Chloroflexus. J. Bacteriol. 120:131–138.

Khanna, S., and D. J. D. Nicholas. 1983. Substrate phosphorylation in Chlorobium vibrioforme f. sp. thiosulfatophilum. J. Gen. Microbiol. 129:1365–1370.

Kirchhoff, J., and H. G. Trüper. 1974. Adenylylsulfate reductase of Chlorobium limicola. Arch. Microbiol. 100:115–120.

Kjaer, B., N. U. Frigaard, F. Yang, B. Zybailov, M. Miller, J. H. Golbeck, and H. V. Scheller. 1998. Menaquinone-7 in the reaction center complex of the green sulfur bacterium Chlorobium vibrioforme functions as the electron acceptor A1. Biochemistry 37:3237–3242.

Kjaerulff, S., D. B. Diep, J. S. Okkels, H. V. Scheller, and J. G. Ormerod. 1994. Highly efficient integration of foreign DNA into the genome of the green sulfur bacterium, Chlorobium vibrioforme, by homologous recombination. Photosynth. Res. 41:277–283.

Klarskov, K., F. Verte, G. van Driessche, T. E. Meyer, M. A. Cusanovich, and J. van Beeumen. 1998. The primary structure of soluble cytochrome C-551 from the phototrophic green sulfur bacterium Chlorobium limicola, strain Tassajara, reveals a novel c-type cytochrome. Biochemistry 37:10555–10562.

Knudsen, E., E. Jantzen, K. Bryn, J. G. Ormerod, and R. Sirevåg. 1982. Quantitative and structural characteristics

of lipids in Chlorobium and Chloroflexus. Arch. Microbiol. 132:149–154.

Kobayashi, H. A., M. Stenstrom, and R. Mah. 1983. Use of photosynthetic bacteria for hydrogen sulfide removal from anaerobic waste treatment effluent. Water Res. 17:579–587.

Kohnen, M. E. L., S. Schouten, J. A. Sinninghe Damsté, J. W. de Leeuw, D. A. Merritt, and J. M. Hayes. 1992. Recognition of paleochemicals by a combined molecular sulfur and isotope geochemical approach. Science 256:358–362.

Kopczynski, E. D., M. M. Bateson, and D. M. Ward. 1994. Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. Appl. Environ. Microbiol. 60:746–748.

Kuznetsov, S. I. 1977. Trends in the development of ecological microbiology. Adv. Aquat. Microbiol. 1:1–48.

Lapage, S. P., P. H. A. Sneath, E. F. Lessel, V. B. D. Skerman, H. P. R. Seeliger, and W. A. Clark. 1975. *In:* S. P. Lapage, P. H. A. Sneath, E. F. Lessel, V. B. D. Skerman, H. P. R. Seeliger, and W. A. Clark (Eds.) International Code of Nomenclature of Bacteria. American Society of Microbiology. Washington DC.

Lauterborn, R. 1915. Die sapropelische Lebewelt. Verhandlungen der naturhistorisch-medizinischen Vereinigung zu Heidelberg, Neue Folge 13:395–481.

Lippert, K. D., and N. Pfennig. 1969. Die Verwertung von molekularem Wasserstoff durch Chlorobium thiosulfatophilum: Wachstum und CO_2-Fixierung. Arch. Microbiol. 65:29–47.

Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese. 1994. The ribosomal database project. Nucleic Acids Res. 22:3485–3487.

Majumdar, D., and J. H. Wyche. 1997. Molecular cloning and nucleotide sequence of the porphobilinogen deaminase gene, hemC, from Chlorobium vibrioforme. Curr. Microbiol. 34:258–263.

Matheron, R., and R. Baulaigue. 1972. Bactéries photosynthetique sulfureuses marines: Assimilation des substances organiques et minérales, et influence de la teneur en chlorure de sodium du milieu de culture sur leur développement. Arch. Mikrobiol. 86:291–304.

Méndez-Alvarez, S., V. Pavón, I. Esteve, R. Guerrero, and N. Gaju. 1994. Transformation of Chlorobium limicola by a plasmid that confers the ability to utilize thiosulfate. J. Bacteriol. 176:7395–7397.

Méndez-Alvarez, S., I. Esteve, R. Guerrero, and N. Gaju. 1996. Genomic analysis of different Chlorobium strains by pulsed-field gel electrophoresis and ribotyping. Int. J. Syst. Bacteriol. 46:1177–1179.

Meyer, T. E., and T. J. Donohue. 1995. Cytochromes, iron-sulfur, and copper proteins mediating electron transfer from cyt bc1 complex to photosynthetic reaction center complexes. *In:* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (Eds.) Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers. Dordrecht, Boston, London, 725–745.

Montesinos, E. R., R. Guerrero, C. Abella, and I. Esteve. 1983. Ecology and physiology of the competition for light between Chlorobium limicola and Chlorobium phaeobacteroides in natural habitats. Appl. Environ. Microbiol. 46:1007–1016.

Nicholson, J. A. M., J. F. Stolz, and B. K. Pierson. 1987. Structure of a microbial mat at Great Sippewissett Marsh, Cape Cod, Massachusetts. FEMS Microbiol. Ecol. 45:343–364.

Nogales, B., R. Guerrero, and I. Esteve. 1997. A heterotrophic bacterium inhibits growth of several species of the genus Chlorobium. Arch. Microbiol. 167:396–399.

Olson, J. M. 1998. Chlorophyll organization and function in green photosynthetic bacteria. Photochem. Photobiol. 67:61–75.

Ormerod, J. G. 1988. Natural genetic transformation in Chlorobium. *In:* J. M. Olson, E. Stackebrandt, and H. G. Trüper (Eds.) Green Photosynthetic Bacteria. Plenum Publishing. New York, NY. 315–319.

Overmann, J., and N. Pfennig. 1989a. Pelodictyon phaeoclathratiforme sp. nov., a new brown-colored member of the Chlorobiaceae forming net-like colonies. Arch. Microbiol. 152:401–406.

Overmann, J., and M. M. Tilzer. 1989b. Control of primary productivity and the significance of photosynthetic bacteria in a meromictic kettle lake, Mittlerer Buchensee, West Germany. Aquatic Sciences 51:261–278.

Overmann, J., S. Lehmann, and N. Pfennig. 1991a. Gas vesicle formation and buoyancy regulation in Pelodictyon phaeoclathratiforme (Green sulfur bacteria). Arch. Microbiol. 157:29–37.

Overmann, J., J. T. Beatty, K. J. Hall, N. Pfennig, and T. G. Northcote. 1991b. Characterization of a dense, purple sulfur bacterial layer in a meromictic salt lake. Limnol. Oceanogr. 36:846–859.

Overmann, J., H. Cypionka, N. Pfennig. 1992. An extremely low-light adapted phototrophic sulfur bacterium from the chemocline of the Black Sea. Limnol. Oceanogr. 37:150–155.

Overmann, J. 1997a. Mahoney Lake: A case study of the ecological significance of phototrophic sulfur bacteria. Adv. Microbiol. Ecol. 15:251–288.

Overmann, J., and C. Tuschak. 1997b. Phylogeny and molecular fingerprinting of green sulfur bacteria. Arch. Microbiol. 167:302–309.

Overmann, J., C. Tuschak, J. M. Fröstl, and H. Sass. 1998. The ecological niche of the consortium "Pelochromatium roseum." Arch. Microbiol. 169:120–128.

Overmann, J. 1999a. Green sulfur bacteria. *In:* G. M. Garrity, et al. (Eds.) Bergey's Manual of Systematic Bacteriology. Springer-Verlag. New York, NY.

Overmann, J., M. J. L. Coolen, and C. Tuschak. 1999b. Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments. Arch. Microbiol.

Parkin, T. B., and T. D. Brock. 1980. The effects of light quality on the growth of phototrophic bacteria in lakes. Arch. Microbiol. 125:19–27, 31–36.

Parkin, T. B., and T. D. Brock. 1981. The role of phototrophic bacteria in the sulfur cycle of a meromictic lake. Limnol. Oceanogr. 26:880–890.

Paschinger, H., J. Paschinger, and H. Gaffron. 1974. Photochemical disproportionation of sulfur into sulfide and sulfate by Chlorobium limicola forma thiosulfatophilum. Arch. Microbiol. 96:341–351.

Passier, H. F., H.-J. Bosch, I. A. Nijenhuis, L. J. Lourens, M. E. Böttcher, A. Leenders, J. A. Sinninghe Damsté, and G. J. de Lange. 1999. Sulphidic mediterranean surface waters during Pliocene sapropel formation. Nature 397:146–149.

Perfiliev, B. V. 1914. On the theory of symbiosis of Chlorochromatium aggregatum Lauterb. and Cylindrogloea bacterifera n.g. n.sp. J. Microbiol. Petrogr. 1:222–224.

Pfennig, N., and K. D. Lippert. 1966. Über das Vitamin B_{12}-Bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. 55:245–256.

Pfennig, N. 1968. Chlorobium phaeobacteroides nov. spec. und C. phaeovibrioides nov. spec., zwei neue Arten der grünen Schwefelbakterien. Arch. Mikrobiol. 63:224–226.

Pfennig, N. 1980. Syntrophic mixed cultures and symbiotic consortia with phototrophic bacteria: A review. *In:* G. Gottschalk, N. Pfennig, and Werner (Eds.) Anaerobes and Anaerobic Infections. Stuttgart and Fischer. New York, NY. 127–131.

Pfennig, N. 1989. Green sulfur bacteria. *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. C. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 1682–1697.

Pfennig, N., and H. G. Trüper. 1992. The family Chromatiace ae. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. 3200–3221.

Pierson, B., A. Oesterle, and G. L. Murphy. 1987. Pigments, light penetration, and photosynthetic activity in the multi-layered microbial mats of Great Sippewissett Salt Marsh, Massachusetts. FEMS Microbiol. Ecol. 45:365–376.

Pringault, O., M. Kühl, R. de Wit, and P. Caumette. 1998. Growth of green sulphur bacteria in experimental benthic oxygen, sulphide, pH and light gradients. Microbiology 144:1051–1061.

Repeta, D. J., D. J. Simpson, B. B. Jørgensen, and H. W. Jannasch. 1989. Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea. Nature (London) 342:69–72.

Rodriguez-Tomé, P., P. J. Stoehr, G. N. Cameron, and T. P. Flores. 1996. The European Bioinformatics Institute (EBI) databases. Nucleic Acids Res. 24:6–12.

Shiozawa, J. A. 1995. A foundation for the genetic analysis of green sulfur, green filamentous and Heliobacteria. *In:* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (Eds.) Anoxygenic Photosynthetic Bacteria. Kluwer. Dordrecht, 1159–1173.

Siefert, E., and N. Pfennig. 1984. Convenient method to prepare neutral sulfide solution for cultivation of phototrophic sulfur bacteria. Arch. Microbiol. 139:100–101.

Sinninghe Damsté, J. A., and J. Köster. 1998. A euxinic southern North Atlantic Ocean during the Cenomanian/Turonian oceanic anoxic event. Earth Planet Sci. Lett. 158:165–173.

Sirevåg, R., and J. G. Ormerod. 1977a. Synthesis, storage, and degradation of polyglucose in Chlorobium thiosulfatophilum. Arch. Mikrobiol. 111:239–244.

Sirevåg, R., B. B. Buchanan, J. A. Berry, and J. H. Troughton. 1977b. Mechanisms of CO_2 fixation in bacterial photosynthesis studied by carbon isotope fractionation technique. Arch. Microbiol. 112:35–38.

Skuja, H. 1957. Taxonomische und biologische Studien über das Phytoplankton schwedischeer Binnengewässer. Nova Acta Reg. Soc. Sci. Uppsala, Ser. IV 16:1–404.

Stackebrandt, E., F. A. Rainey, N. Ward-Rainey. 1996. Anoxygenic phototrophy across the phylogenetic spectrum: current understanding and future perspectives. Arch. Microbiol. 166:211–223.

Steinmetz, M., and U. Fischer. 1982. Cytochromes of the green sulfur bacterium Chlorobium vibrioforme f. thiosulfatophilum, purification, characterization and sulfur metabolism. Arch. Microbiol. 131:19–26.

Streszewski, B. 1913. Beitrag zur Kenntnis der Schwefelflora in der Umgebung von Krakau. Bulletin de L'Academie des Sciences de Cracovie Serie B:309–334.

Szafer, W. 1910. Zur Kenntnis der Schwefelflora in der Umgebung von Lemberg. Bulletin de L'Academie des Sciences de Cracovie Serie B:161–167.

Trüper, H. G., and S. Genovese. 1968. Characterization of photosynthetic sulfur bacteria causing red water in Lake Faro (Messina, Sicily). Limnol. Oceanogr. 13:225–232.

Trüper, H. G. 1970a. Culture and isolation of phototrophic sulfur bacteria from the marine environment. Helgoländer wiss Meeresunters 20:6–16.

Trüper, H. G., and H. D. Peck. 1970b. Formation of adenylylsulfate in photosynthetic bacteria. Arch. Mikrobiol. 73:125–142.

Trüper, H. G., and N. Pfennig. 1971. Family of phototrophic Green Sulfur Bacteria: Chlorobiaceae Copeland, the correct family name; rejection of Chlorobacterium Lauterborn; and the taxonomic situation of the consortium-forming species. Int. J. Syst. Bacteriol. 21:8–10.

Trüper, H. G., and U. Fischer. 1982. Anaerobic oxidation of sulphur compounds as electron donors for bacterial photosynthesis. Phil. Trans. R. Soc. B 298:529–542.

Trüper, H. G. 1984. Phototrophic bacteria and their sulfur metabolism. *In:* A. Müller and B. Krebs (Eds.) Studies in Inorganic Chemistry, Vol. 5: Sulfur, Its Significance for the Geo-, Bio-, and Cosmosphere and Technology. Elsevier. Amsterdam, 367–382.

Trüper, H. G., C. Lorenz, M. Schedel, and M. Steinmetz. 1988. Metabolism of thiosulfate in Chlorobium. *In:* J. M. Olson, J. G. Ormerod, J. Amesz, E. Stackebrandt, and H. G. Trüper (Eds.) Green Photosynthetic Bacteria. Plenum Press. New York, NY. 189–200.

Trüper, H. G., and N. Pfennig. 1992. The family Chlorobiaceae. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. Berlin, Heidelberg, New York, NY. 3583–3592.

Tuschak, C., J. Glaeser, and J. Overmann. 1999. Specific detection of green sulfur bacteria by in situ-hybridization with a fluorescently labeled oligonucleotide probe. Arch. Microbiol.

Van Gemerden, H., and J. Mas. 1995. Ecology of phototrophic sulfur bacteria. *In:* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (Eds.) Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers. Dordrecht, Boston, London, 49–85.

Van Noort, P. I., Y. Zhu, R. LoBrutto, and R. E. Blankenship. 1997. Redox effects on the excited-state lifetime in chlorosomes and bacteriochlorophyll c oligomers. Biophys. J. 72:316–325.

Veldhuis, M. J. W., and H. van Gemerden. 1986. Competition between purple and brown phototrophic bacteria in stratified lakes: sulfide, acetate, and light as limiting factors. FEMS Microbiol. Ecol. 38:31–38.

Vignais, P. M., A. Colbeau, J. C. Willison, and Y. Jouanneau. 1985. Hydrogenase, nitrogenase, and hydrogen metabolism in photosynthetic bacteria. Adv. Microb. Physiol. 26:155–234.

Wahlund, T. M., R. W. Woese, R. W. Castenholz, and M. T. Madigan. 1991. A thermophilic green sulfur bacterium

from New Zealand hot springs, Chlorobium tepidum sp. nov. Arch. Microbiol. 156:81–90.

Wahlund, T. M., and M. T. Madigan. 1995. Genetic transfer by conjugation in the thermophilic green sulfur bacterium Chlorobium tepidum. J. Bacteriol. 177:2583–2588.

Ward, D. M., M. M. Bateson, R. Weller, and A. L. Ruff-Roberts. 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. Adv. Microb. Ecol. 12:219–286.

Warthmann, R., H. Cypionka, and N. Pfennig. 1992. Photoproduction of H_2 and acetate by syntrophic cocultures of green sulfur bacteria and sulfur-reducing bacteria. Arch. Microbiol. 157:343–348.

Woese, C. R., E. Stackebrandt, T. J. Macke, and G. E. Fox. 1985. The phylogenetic definition of the major eubacterial taxa. Syst. Appl. Microbiol. 6:143–151.

PART 6

Bacteroides and Cytophaga Group

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CHAPTER 6.1

The Medically Important *Bacteroides* spp. in Health and Disease

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Introduction to the *Bacteroides*

The genus *Bacteroides* contains Gram-negative, nonsporeforming, nonmotile, anaerobic rods generally isolated from the gastrointestinal tract (GI-tract) of humans and animals. Up until about 12 years ago the genus was quite diverse and included nearly every organism that fit this generalized description. In 1989, however, the genus was formally restricted to include only those organisms that are closely related to the type species, *Bacteroides fragilis*, resulting in a group that is much more biochemically and genetically cohesive (Shah and Collins, 1989). This group is now comprised of what were previously *B. fragilis* subspecies plus a few additional newly recognized species. The genus includes *Bacteroides fragilis*, *B. thetaiotaomicron*, *B. ovatus*, *B. uniformis*, *B. vulgatus*, *B. distasonis*, *B. eggerthii*, *B. caccae*, *B. merdae* and *B. stercoris* (Shah and Collins, 1989).

This narrowed description of the *Bacteroides* has resulted in a genus in which all of the recognized species have some role in human or animal health. These organisms are generally isolated from human hosts as members of the normal microflora, and in fact *Bacteroides* spp. are the predominant organisms in the human colon. As members of the indigenous flora, they play a variety of roles that contribute to normal intestinal physiology and function. These include beneficial roles such as polysaccharide breakdown or nitrogen cycling, as well as other functions that may be disadvantageous such as the rapid deconjugation of bile acids or the production of mutagenic compounds (Macy, 1984; Salyers, 1984). Another important aspect of *Bacteroides* biology is their pathogenic potential. In this regard, several of the species including the type species, *B. fragilis*, are important opportunistic pathogens and the most frequently isolated organisms from anaerobic infections (Finegold and George, 1989). Thus it is clear that the *Bacteroides* spp. display a range of complex interrelationships with their animal hosts. It is the host-commensal or host-parasite interactions that define this group of organisms. In the following sections we will focus primarily on those characteristics that contribute to their successful colonization of mammals. Unfortunately, most serious research on the *Bacteroides* has focused on *B. fragilis* and *B. thetaiotaomicron* owing to their prominence in human infection and in the indigenous microflora. Much of the discussion in this chapter will necessarily describe the results of this work; however, these two organisms do in fact embody many of the features responsible for the success of the *Bacteroides* genus in its environment, and in this regard they have proven to be suitable model systems.

Phylogeny and Taxonomy

On the basis of the comparative analysis of 16S rRNA gene sequences, members of the genus *Bacteroides* form a coherent phylogenetic cluster within the *Cytophaga-Flexibacter-Bacteroides* (CFB) phylum (Paster et al., 1994; Fig. 1). According to the *Bergeys Manual of Systematic Bacteriology*, 2nd edition (Garrity, 2001) this bacterial phylum or group is referred to as the Bacteroidetes phylum (see the Bergeys website). The genus *Bacteroides* falls within the family Bacteroidaceae of the proposed Order "Bacteroidales" of the proposed Class "Bacteroidetes." Those species shown in the figure include cultivable and not-yet-cultivated species of *Bacteroides* in addition to representatives of closely related genera, such as *Porphyromonas*, *Prevotella* and misclassified species of *Bacteroides* (Olsen and Shah, 2001). It had been suggested previously that the two oral species, *B. heparinolyticus* and *B. zoogleoformans*, be members of the genus *Prevotella*; however, on the basis of 16S rRNA sequence analysis, it has been recommended these species be reinstated as true members of the *Bacteroides* (Olsen and Shah, 2001). Most of the known species and unclassified strains have been isolated from the human intestine or oral cavity; however, a few have been isolated from animal sources. For example, *B. acidifaciens* was isolated from the mouse cecum (Miyamoto

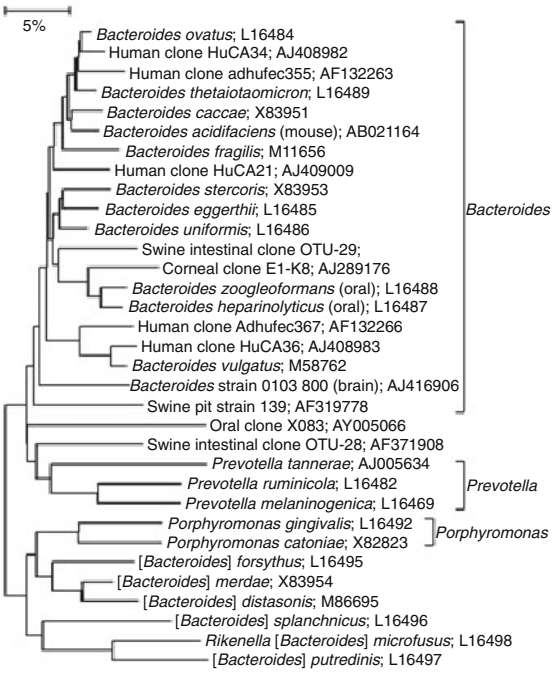

Fig. 1. Phylogeny of the genus *Bacteroides*. On the basis of 16S rRNA gene sequence comparisons, the phylogenetic position of known species, uncharacterized strains, and not-yet-cultivated phylotypes of *Bacteroides* is shown relative to other genera of family Bacteroidaceae. Misclassified species of *Bacteroides* are bracketed since they are clearly not true members of the genus. GenBank accession numbers for the 16S rRNA sequences of the species tested are shown. Similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor (1969). The neighbor-joining method (Saitou and Nei, 1987) was used for phylogenetic tree construction. TREECON, a software package for the Microsoft Windows environment, was used for the construction and drawing of evolutionary trees (Van de Peer and De Wachter, 1994). The scale bar represents a 5% difference in nucleotide sequence determined by taking the sum of all of the horizontal lines connecting two species. Vertical distance has no meaning.

and Itoh, 2000), and *B. ovatus* and *B. thetaiotaomicron* were isolated from the bovine rumen (GenBank accession numbers AF139524 and AF139525, respectively).

In addition to cultivable species of *Bacteroides*, novel phylotypes, i.e., species that have not yet been cultivated in vitro, were detected by phylogenetic analysis of 16S rRNA genes amplified from DNA isolated from the human intestine (Suau et al., 1999; Hold et al., 2002), the human oral cavity (Paster et al., 2001), and the swine intestine (Lesser et al., 2002). Most of these phylotypes, designated as clones, cluster together with other members of the genus (Fig. 1). Some phylotypes, e.g., oral clone X083 and swine clone OTU-28, fall just outside the cluster. Another culture-independent study demonstrated the presence of a *Bacteroides ovatus*-like phylotype from the cecum of chickens (not included in Fig. 1 since it was not a complete sequence; Zhu et al., 2002). Although many members of the CFB group have been recovered from the hindgut of the termite, no true *Bacteroides* have been detected thus far from that environment (Ohkuma et al., 2002).

Although the *Bacteroides* clearly form a distinct cluster on the basis of 16S rRNA sequences, early DNA homology studies established that there was only minimal DNA identity between the major species (Johnson, 1978a). For example, *B. fragilis* shares 21–36% identity with *B. thetaiotaomicron*, 13–22% with *B. ovatus*, 21% with *B. uniformis*, 18% with *B. eggerthii*, 5–10% with *B. vulgatus*, and 5% with *B. distasonis*. Similar ranges have been observed between the other species as well. Even with this DNA sequence variability, the morphological and general metabolic properties of the genus are relatively uniform as can be seen in these brief descriptions of the major species (Holdeman et al., 1986). *Bacteroides fragilis* is the type species; it is the most frequently isolated anaerobe from clinical specimens, and it also is found in feces. A variety of sugars are fermented, but polysaccharide digestion is limited. Glucose grown cultures yield cells that are 0.8–1.3 by 1.6–8.0 μm, are rounded at the ends, often occur in pairs, and frequently contain large vacuoles. On the surface of horse blood agar plates, colonies are 1–3 mm, circular, entire, low convex, and semiopaque, and hemolysis is rare. *Bacteroides fragilis* cells are very aerotolerant, growth is enhanced by 20% bile, and hemin is required for normal, optimal growth. *Bacteroides thetaiotaomicron* is more frequently isolated from feces than from clinical samples. A wide range of sugars is fermented, and this is one of the more versatile organisms with regard to polysaccharide digestion. Glucose-grown cells are pleomorphic, 0.7–1.1 by 1.3–8.0 μm. Cells are aerotolerant and surface colonies on blood agar plates are punctiform, circular, entire, convex, semiopaque, and not hemolytic. Hemin and bile stimulation are similar to *B. fragilis*. *Bacteroides vulgatus* will ferment a range of sugars but is restricted in its polysaccharide use, similar to *B. fragilis*. It produces slightly smaller cells in glucose broth. Colonies on blood agar plates are 1–2 mm, circular, entire, convex, semiopaque, and not hemolytic. Hemin is required and bile stimulates growth. *Bacteroides vulgatus* is one of the most frequent isolates from feces but rarely is found in clinical samples. *Bacteroides uniformis* is a frequent stool isolate, but it sometimes is found in clinical specimens. The organism is a versatile

sugar fermenter and will degrade a range of polysaccharides. Glucose-grown cells are somewhat more slender than *B. fragilis*, and colonies on the surface of blood agar plates are only 0.5–2.0 mm, circular, entire, low convex, translucent, and generally not hemolytic. Bile is not stimulatory, hemin is required, and cells are somewhat less aerotolerant. *Bacteroides ovatus* uses a wide range of sugars and it is the most versatile polysaccharide fermenter. *Bacterides ovatus* is rarely isolated from clinical samples but is a common inhabitant of the colon. Cells taken from glucose broth are generally more rounded than *B. fragilis* and only range in size from 0.6–0.8 by 1.6–5.0 μm. *Bacteroides ovatus* is not hemolytic on sheep blood and colonies are 0.5–1.0 mm, circular, entire, convex, and semiopaque. This organism requires hemin for normal growth and bile is stimulatory. *Bacteroides eggerthii* is not a common clinical isolate but is often found in feces. Hemin stimulates growth but to a much lesser extent than seen in other *Bacteroides* spp. Cells from broth cultures are 0.4–1.0 by 1.0–1.6 μm, and surface colonies on blood agar plates are punctiform, circular, entire, convex, translucent, smooth and not hemolytic. Finally, *B. distasonis* is one of the most common fecal isolates and an infrequent isolate from clinical specimens. *Bacteroides distasonis* does not utilize many plant polysaccharides, although it does have the ability to ferment a wide range of sugars. Twenty percent bile is highly stimulatory and hemin is required for optimal growth. Glucose-grown cells are 0.6–1.0 by 1.6–11.0 μm with rounded ends and occasional vacuoles. Strains are occasionally α-hemolytic on sheep blood agar plates, and colonies are pinpointed to 0.5 mm, circular, entire, convex and semiopaque.

Physiology and Metabolism

Nutritional Features

The *Bacteroides* have simple nutritional requirements that reflect their environment. Most of the species can be grown on a defined medium containing a fermentable carbohydrate, hemin, vitamin B_{12}, ammonia, carbon dioxide, and sulfide, all of which are plentiful in the colon in some form. As discussed in the section on Polysaccharide Utilization, a wide variety of mono-, di-, and polysaccharides serve as carbon and energy sources, and together with carbon dioxide, they provide all of the carbon requirements for the cell. Nitrogen requirements are minimal, with ammonia serving as the preferred nitrogen source for all of the species (Varel and Bryant, 1974). Ammonia is plentiful in the colon since it is a product of urea cycling and a byproduct of protein/amino acid catabolism. Amino acids, on the other hand, are not present at high concentrations in the colon, and they are not utilized as the sole source of nitrogen. It may be that amino acid transport is not sufficient to supply the needs for cellular nitrogen, or the yield of ammonia from amino acid catabolism is below levels the organisms can optimally assimilate. An apparent paradox is that *B. fragilis* has the ability to utilize a mixture of peptides for nitrogen; however, the peptides are not utilized as efficiently as ammonia. Even when provided at fivefold higher molar concentrations, they only support about 35–70% of the growth yield observed with ammonia. Likewise, proteins such as gelatin or trypticase only allow about 20% of the growth yields seen when *B. fragilis* is grown with ammonia. It is not known whether the other *Bacteroides* spp. can utilize peptides. No other nitrogen sources such as nitrate or urea can be utilized by these organisms (Varel and Bryant, 1974).

Sulfur sources for the *Bacteroides* are typically reduced compounds such as sulfide, cysteine, and, to a lesser extent, thioglycolate. Sulfate, sulfite, thiosulfate, β-mercaptoethanol, or methionine is not used (Varel and Bryant, 1974). The requirements for other inorganic ions has been examined, and the needs proved to be fairly typical with Na^+, K^+, PO_4^{-3}, Ca^{+2}, and Mg^{+2} being required for optimal growth of *B. fragilis* and *B. vulgatus* (Caldwell and Arcand, 1974). It was interesting to note that both *B. fragilis* and *B. thetaiotaomicron* displayed a requirement for Fe^{+2} above what was provided by hemin. With the possible exception of *B. ovatus*, the only vitamin required by the *Bacteroides* spp. is B_{12}. The requirement for B_{12} was substituted by methionine, but other potential methyl donors such as betaine, choline, or dimethylacetothetin were not effective replacements, suggesting a specific need for B_{12} in methionine biosynthesis. *Bacteroides ovatus* also required B_{12}, but the requirement was not replaced by methionine, and a strain of *B. distasonis* had a methionine requirement that was not replaced by B_{12} (Varel and Bryant, 1974).

All of the *Bacteroides* spp. require hemin for optimal growth. This requirement has been the subject of a number of studies, some of which have indicated an essential growth requirement for hemin (or closely related compounds), but the majority have shown that hemin greatly stimulates both growth rate and yield (Varel and Bryant, 1974; Sperry et al., 1977; Chen and Wolin, 1981; Fuller and Caldwell, 1982; Al-Jalili and Shah, 1988). Although there may be some strain-to-strain variation, *B. fragilis* and *B. vulgatus* both can substitute protoporphyrin, hematoporphyrin, and hemoglobin for hemin with little effect on growth, and catalase can par-

tially substitute for hemin (Fuller and Caldwell, 1982). Tetrapyrrole precursors were of little benefit and did not effectively replace hemin (Sperry et al., 1977; Fuller and Caldwell, 1982). In the absence of hemin, growth yields of *B. fragilis* are greatly reduced, but the organisms can be maintained in chemostats for weeks without added hemin. Cells grown in this manner were similar morphologically to hemin-grown cells, but they did lack the *b*-type cytochrome and there was a shift in fermentation products (Al-Jalili and Shah, 1988). Other reports have also linked the synthesis of a *b*-type cytochrome to the hemin requirement (Fuller and Caldwell, 1982). These studies have documented the importance of heme for the synthesis of cytochrome *b* and for optimal growth. More information on the specific role of cytochrome *b* in energy generation will be presented in the section on Sugar Catabolism and Fermentation Pathways. Hemin utilization mechanisms have not been elucidated in any *Bacteroides* spp. yet, but in *B. fragilis* there is a 44-kDa outer-membrane protein involved in the uptake. This protein is iron repressible, and blocking studies using a polyclonal antibody raised against this protein inhibited hemin-dependent growth (Otto et al., 1990). Thus there is evidence for a specific hemin uptake system that is at least in part iron responsive. This will be addressed further during a discussion of pathogenic mechanisms as they relate to iron uptake systems (see section on Iron Acquisition from Host Tissues in this Chapter). The last specific growth requirement is for CO_2, which primes the synthesis of oxaloacetate (the precursor for the fumarate used as an electron acceptor during energy generation; see section on Sugar Catabolism and Fermentation Pathways in this Chapter). In the absence of CO_2, long lag periods are experienced until the requirement can be met through internal generation of CO_2 during fermentation of carbohydrates (Caspari and Macy, 1983).

Energy Metabolism

Polysaccharide Utilization The *Bacteroides* are saccharolytic, and this directly reflects their environment, which is rich in complex polysaccharides derived from dietary sources (cellulose, hemicellulose, starch and pectin) and host secretions (chondroitin sulfate). Degradation of these compounds is an important characteristic of most *Bacteroides* spp. In fact, as a group this genus is extremely versatile in the range of substrates that can be utilized as carbon and energy sources. Polysaccharides commonly used by *Bacteroides* spp. are listed in Table 1 together with the enzymes and genes associated with their use (if known). The efficiency of polysaccharide digestion also is significant. Chemostat studies and batch cultures with *B. thetaiotaomicron* and with *B. ovatus* have indicated utilization of starch, guar gum, xylan, arabinogalactan, and chondroitin sulfate was greatest at low dilution rates, which mimic the slow transit times in the gut (Salyers et al., 1982; Salyers, 1984; Macfarlane et al., 1990; Macfarlane and Gibson, 1991b; Degnan et al., 1997a; Degnan et al., 1997b). Further, growth rates and growth yields are as good or better for the organisms grown with various polysaccharides when compared to their component sugars. These results suggest that the mechanisms of polysaccharide digestion are highly effective, having adapted to the varied substrates available in the intestinal tract ecosystem. Although the range of substrates is wide, there are several common properties that characterize their utilization by *Bacteroides*. That is, these are complex, highly regulated systems relying on a network of outer membrane proteins to bind and degrade the substrates, rather than secreted extracellular enzymes. Generally these systems are induced by their substrates and repressed by glucose or other readily utilizable sugars.

Very early on, it was found that high activities of various polysaccharidases were only possible when *Bacteroides* spp. cultures were grown with their particular substrate (Salyers et al., 1977b). Interestingly it also was shown that in the case of *B. thetaiotaomicron*, the outer membrane protein profiles changed markedly during growth with different polysaccharides (Kotarski et al., 1985). Since most of the polysaccharidases are cell associated, it has been reasoned that these outer membrane proteins are important for binding of substrates to aid their passage to the periplasm and cytoplasm. Starch utilization by *B. thetaiotaomicron* exemplifies this scenario; there are seven structural genes of which four (*susC*, *susD*, *susE* and *susF*) encode outer membrane proteins with a role in starch binding (Reeves et al., 1996; Reeves et al., 1997; Shipman et al., 2000; Cho and Salyers, 2001a). Other genes in this regulon encode for a soluble neopullulanase (*susA*), an outer membrane neopullulanase (*susG*), and an α-glucosidase (*susB*), all of which are important for the digestion of the starch molecule into oligosaccharides and then glucose. All of these *sus* genes are transcriptionally controlled by a regulator SusR in response to the presence of starch or maltose in the medium (D'elia and Salyers, 1996a; Cho et al., 2001b).

Information on starch utilization is less complete for other *Bacteroides* spp. Studies with *B. ovatus* growing in a chemostat with mixtures of different polysaccharides indicated that starch and pectin were preferentially utilized, suggesting these were the most important carbon/energy

Table 1. Utilization of polysaccharide substrates among *Bacteroides* spp.

Polysaccharide	Organism	Enzymes	Genes
Starch	*B. fragilis*, *B. thetaiotaomicron*, *B. ovatus*, *B. uniformis*, *B. eggerthii*, and *B. vulgatus*	pullulanase	*pull* U67061
		neopullulanase	*susA* U66897
		amylase	*osuD*
		α-glycosidase	*susB* U66897
		gluycoamylase/-glycosidase	
		β-amylase	
Cellulose Xylan	*Bacteroides* spp. *B. ovatus*, *B. eggerthii*, and *B. vulgatus*	ND	ND
		xylanase	*xyl* U04957
		xylosidase/arabinosidase	*xa* U04957
		arabinosidase I	*asdl* U15178
		arabinosidase II	*asdll* U15179
Pectin	*B. thetaiotaomicron*, *B. ovatus*, and *B. vulgatus*	polygalacturonic acid lyase	ND
		polygalacturonic acid hydrolase	
		pectate lyase	
		pectin esterase	
Dextran	*B. thetaiotaomicron*, *B. ovatus*, and *B. uniformis*	dextranase	ND
Arabinogalactan	*B. thetaiotaomicron*, *B. ovatus*, *B. uniformis*, and *B. vulgatus*	arabinogalactanase	ND
		galactosidase	
Guar gum	*B. uniformis*	galactomannanase	ND
		mannosidase	
		galactosidase	
Laminarin	*B. thetaiotaomicron*, *B. uniformis*, and *B. distasonis*	β-glucanase	ND
		β-glucosidase	
Chondroitin sulfate	*B. thetaiotaomicron* and *B. ovatus*	chondroitin lyase I	*csuA*
		chondroitin lyase II	*csuB* L42367
		chondro-4-sulfatase	*csuC*
		chondro-6-sulfatase	*csuD*
		β-glucuronidase	*csuE*
Hyaluronic acid	*B. thetaiotaomicron* and *B. ovatus*	chondroitin lyase I	*csuA*
		chondroitin lyase II	*csuB* L42367
Heparin sulfate	*B. thetaiotaomicron*, *B. ovatus*, and *B. eggerthii*	ND	ND

Abbreviation: ND, not determined.
From Salyers et al. (1977a, b), Salyers and Leedle (1983), Macfarlane and Cummings (1991), Cheng et al. (1995), D'elia and Salyers (1996b), and Whitehead (1995).

sources in their environment (Degnan et al., 1997a). Digestion of starch was best at lower dilution rates where the activities of amylase and α-glucosidase were highest. The amylase and α-glucosidase activities were soluble and could be resolved into three components, one of which was a 70-kDa protein dimer with dual amylase and α-glucosidase activities. Glucosamylase and β-amylase activities also were found in *B. ovatus*, but there has been little in the way of genetic analysis of the starch utilization genes. An examination of starch digestion by *B. vulgatus* showed that amylase and α-glucosidase activities were induced 20–40-fold when cultures were grown with maltose, amylose, or amylopectin and that both enzymes had the same molecular weight and pH optimum, suggesting that they are the same enzyme, perhaps similar to the *B. ovatus* enzyme (McCarthy et al., 1988). Finally, starch utilization in *B. fragilis* also seems to require some of the same outer membrane proteins as *B. thetaiotaomicron* requires. A starch or maltose inducible operon with homologs to *susC*, *susD* and *susF* and an α-amylase were recently shown to be involved in starch and glycogen utilization in *B. fragilis* (Smith, 2002). In addition, periplasmic α-glucosidase activity has been demonstrated previously in this organism (Berg et al., 1980). Thus at least superficially, starch utilization among these four *Bacteroides* spp. has some significant similarities.

Chondroitin sulfate utilization in *B. thetaiotaomicron* is the model for digestion of host-derived polysaccharides. This highly sulfated polymer of *N*-acetylgalactosamine-glucuronic acid dimers is degraded by an inducible set of enzymes located in the periplasm and cytoplasm (Salyers and O'Brien, 1980; Guthrie et al., 1985; Hwa and Salyers, 1992a). The enzymatic activities documented are two periplasmic chondroitin lyases (CsuA and CsuB), two cytoplasmic sulfatases (CsuC and CsuD), and a β-glucuronidase

(CsuE) in the cytoplasm. As seen with starch, there were several outer membrane proteins induced by growth with chondroitin sulfate and one of these, CsuF, appears to be involved in substrate binding and was required for growth on the substrate (Kotarski et al., 1985; Cheng et al., 1995b). The regulatory mechanism of this inducible system has not yet been elucidated (Hwa and Salyers, 1992b; Cheng and Salyers, 1995a). Chondroitin sulfate utilization in *B. ovatus* and an unnamed *Bacteroides* spp. also has been examined and found to be similar to *B. thetaiotaomicron*. That is, all three organisms produced two inducible, cell-associated chondroitin lyases with similar biochemical properties, and there were similar chondroitin sulfate inducible outer membrane proteins synthesized (Lipeski et al., 1986). The digestion of these host polysaccharides in the environment most likely requires that the organism degrade protein complexes to which the polysaccharides are covalently linked. The chondroitin sulfate present in proteoglycan was degraded by *B. thetaiotaomicron*, albeit at a slower rate than purified substrate (Kuritza and Salyers, 1983). The rate of utilization was not improved when proteoglycan was treated with protease, suggesting that this is not an important rate-limiting step in degradation.

Another important dietary polysaccharide is xylan, a component of hemicellulose, which is utilized by *B. ovatus*. Some initial genetic and biochemical studies on *B. ovatus* xylan degradation have been performed showing that the major enzymatic activities, xylanase, arabinosidase, and xylosidase, were induced 6- to 100-fold by growth on oatspelt xylan (Whitehead and Hespell, 1990). Two genes involved in xylan utilization were cloned and found to encode the xylanase activity (*xylI*) and a dual function xylosidase-arabinosidase enzyme (*xsa*). These genes were part of an inducible operon that probably includes an additional arabinosidase (Weaver et al., 1992; Whitehead, 1995). Interestingly, mutations in these genes did not abolish xylan utilization, although the rate was considerably slower. This finding, together with some additional analysis of the xylan breakdown products produced by the mutants, suggested that another set of enzymes may be responsible for removal of glucose and hexuronic acid residues from the xylan molecule, thus allowing for growth of the *xsa* and *xylI* mutants (Weaver et al., 1992). The presence of xylan-specific outer membrane proteins was not examined.

The examination of other polysaccharide degrading systems has been minimal, with only cursory studies documenting the presence of specific enzymatic activities. Pectin degradation in *B. thetaiotaomicron* was shown to require several activities, a polygalacturonic acid hydrolase and lyase, pectate lyase, and pectin esterase, all of which were inducible by pectin or polygalacturonate (McCarthy et al., 1985; Tierny et al., 1994). The genes encoding the *B. thetaiotaomicron* pectate lyase and pectin esterase have been cloned, but little in the way of genetic or further biochemical data is available on these or any of the other polysaccharide degrading activities (Tierny et al., 1994; Table 1). Inducible, cell-associated laminarinase and galactomannanase also have been documented in a variety of *Bacteroides* spp. (Salyers et al., 1977b; Salyers and Leedle, 1983). Overall, it seems likely that these polysaccharide digestion systems will use a strategy similar to the starch and chondroitin sulfate models in which outer membrane proteins mediate binding of the polymers for digestion by cell-associated enzymes.

Consistent with the range of polysaccharide substrates utilized is the need for a variety of glycosidase activities. In one study with *B. fragilis*, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, β-N-acetyl glucosaminidase, and α-fucosidase activities were identified and partially purified (Berg et al., 1980). In addition, a β-glucosidase capable of esculin hydrolysis has been cloned from *B. fragilis* and expressed in *Escherichia coli*, and the gene, *bglA*, was sequenced (GenBank accession number AF006658). An insertional mutant in *bglA*, however, was still able to hydrolyze esculin, suggesting the presence of at least one additional β-glucosidase in this organism (Smith, 2002). In fact, examination of the *B. fragilis* genome sequence does reveal a second β-glucosidase gene.

Sugar Catabolism and Fermentation Pathways Most studies on the utilization of sugars have focused on *B. fragilis* and *B. thetaiotaomicron*. This work has shown that there are distinct patterns of carbohydrate preferences, and in the case of *B. thetaiotaomicron*, mannose appears to play a central role in controlling sugar uptake (Hylemon et al., 1977; Degnan and Macfarlane, 1995). Glucose, galactose, arabinose and xylose can all be co-utilized in batch or chemostat cultures; however, their catabolism is rapidly inhibited by the addition of mannose but not glucose (Degnan and Macfarlane, 1995). This result suggests the presence of an active catabolite inhibition mechanism, and it is consistent with earlier work, which showed that glucose transport was strongly inhibited by the addition of mannose (Hylemon et al., 1977). In addition, other transport studies showed that the rates of hexose uptake were greatest in cells grown with glucose and lowest in mannose grown cells (Degnan and Macfarlane, 1995). Taken together,

these studies indicate that some form of catabolite repression operates in the *Bacteroides* spp., and although the mechanism is not known, the classic Enterobacteriaceae pathway does not operate in these organisms since they do not form cAMP (Hylemon and Phibbs, 1974). Induction mechanisms also may be involved in control of sugar catabolism. In *B. fragilis*, sucrose uptake and sucrase activity were induced 8- and 3-fold, respectively, in cultures grown with sucrose, and in *B. thetaiotaomicron* xylose, uptake was clearly induced by its substrate (Scholle et al., 1990; Degnan and Macfarlane, 1995).

The mechanisms of sugar transport have not been elucidated. It has been shown that *B. thetaiotaomicron* does not use a phosphoenolpyruvate:phosphotransferase for transport of glucose, mannose, galactose or xylose (Hylemon et al., 1977; Degnan and Macfarlane, 1995). Sugar transport is inhibited by a variety of compounds that interfere with the generation of proton gradients, electron transport processes, and substrate phosphorylation. The fact that the transport of different sugars was inhibited by different combinations of inhibitors suggests that several types of energy dependent carrier-mediated transport systems are involved in *B. thetaiotaomicron* sugar uptake (Degnan and Macfarlane, 1995). In *B. fragilis* grown under conditions of excess carbon/energy, the glucose taken up by the cell was stored in cytoplasmic glycogen granules, which could be rapidly degraded when glucose became limiting (Lindner et al., 1979). A similar finding was seen in *B. thetaiotaomicron* cells given [^{14}C]-glucose, -mannose, -galactose or -xylose, and it was suggested that the ability to form these storage polymers may provide a competitive advantage in the colon (Hylemon et al., 1977; Degnan and Macfarlane, 1995).

The catabolism of glucose to pyruvate generally occurs via the Embden-Meyerhof pathway. Pathway enzymes such as fructose 1,6,-diphosphate aldolase, phosphofructokinase, hexokinase, and glucose-6-phosphate isomerase have been demonstrated in *B. thetaiotaomicron* and *B. fragilis* (Macy et al., 1978; Caspari and Macy, 1983). In *B. fragilis*, both ATP and PPi-linked phosphofructokinase activity were observed. Although the PPi-linked activity was much higher than the ATP-linked activity, which of these has a more important role in glycolysis has not been resolved (Roberton and Glucina, 1982). Other observations from [^{14}C]-glucose labeling studies indicated that the labeled fermentation products in *B. fragilis* were consistent with Embden-Meyerhof degradation (Macy et al., 1978). There also is enzymatic evidence for the presence of the pentose pathway as well. Strains of *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus*, *B. vulgatus*, *B. distasonis* and *B. eggerthii* all have been shown to possess 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase, but the role of this pathway in *Bacteroides* catabolism has never been closely examined (Shah and Williams, 1982; Shah et al., 1987).

During growth with glucose, hemin and carbon dioxide, the major fermentation products produced by *Bacteroides* spp. include succinate, propionate and acetate, with lesser amounts of lactate, formate and malate (Macy et al., 1975; Macy et al., 1978; Holdeman et al., 1977; Caspari and Macy, 1983). The pathway leading to succinate and propionate in *B. fragilis* begins with formation of oxaloacetate by the carboxylation of phosphoenolpyruvate (PEP) in an ATP-generating reaction mediated by PEP carboxykinase (Macy et al., 1978; Caspari and Macy, 1983). *Bacteroides thetaiotaomicron* uses a similar pathway, but pyruvate carboxylase activity also has been demonstrated in these organisms (Pan and Imlay, 2001). Oxaloacetate is reduced to malate and then to fumarate by malate dehydrogenase and fumarate hydratase (Macy et al., 1978). The reduction of fumarate by fumarate reductase is one of the key reactions in *Bacteroides* energy metabolism. Fumarate is the terminal electron acceptor in a rudimentary electron transport system, which provides additional ATP as judged by studies on the molar growth yields of *B. fragilis* grown with and without hemin (Macy et al., 1975). On the basis of studies with different electron transport inhibitors, the flow of electrons likely involves reduction of a flavoprotein by reduced nicotinamide adenine dinucleotide (NADH) followed by quinone reduction and then reduction of cytochrome *b* (Macy et al., 1975; Harris and Reddy, 1977). The formation of succinate is then mediated by a membrane-bound fumarate reductase, which is specifically induced in the presence of hemin (Macy et al., 1975). In *B. fragilis*, hydrogen and formate also have been shown to be possible electron donors in this pathway; thus, potentially this is a branched electron transport system (Harris and Reddy, 1977). Fumarase- and NADH-linked fumarate reductase activities also have been demonstrated in *B. thetaiotaomicron* (Pan and Imlay, 2001).

Some succinate is metabolized further to propionate in a series of reactions that may or may not lead to further energy gains. One enzyme involved in propionate formation is methylmalonyl-CoA mutase, a B_{12}-dependent enzyme commonly found in succinate-propionate pathways (Macy et al., 1978). This enzyme activity has been demonstrated in *B. fragilis*, and B_{12}-dependent propionate formation has been shown in *B. fragilis* and *B. eggerthii* (Macy et al., 1978; Chen and

Wolin, 1981). Acetate formation in the *Bacteroides* has not been studied, but it is likely that reducing equivalents produced during the oxidation of pyruvate to acetyl-CoA will enter into the electron transport system.

Bacteroides spp. grown in the absence of hemin have a major shift in fermentation, with the major products being lactate, fumarate and acetate (Macy et al., 1975; Sperry et al., 1977; Al-Jalili and Shah, 1988). In *B. fragilis*, this shift reflects the lack of fumarate reductase and cytochrome *b*, which are required for succinate production. The result is fumarate accumulation, and pyruvate becomes a major electron sink from which lactate is formed by the action of an NAD-dependent lactate dehydrogenase (Macy et al., 1978).

Nitrogen Metabolism

Ammonia Assimilation The central features of *Bacteroides* nitrogen metabolism relate directly to their dependency on ammonia as the primary nitrogen source and their inability to utilize amino acids as a sole source of nitrogen. Rapid growth is dependent on the uptake and fixation of ammonia into glutamate. As found in other eubacterial species, the *Bacteroides* have two pathways for ammonia assimilation. First is the glutamine synthetase/glutamate synthase route that is often associated with growth under limiting ammonia conditions in organisms such as the Enterobacteriaceae. The activity of this pathway was established in *B. fragilis*, but the activity of glutamate synthase was very low (Yamamoto et al., 1984). In addition, on the basis of studies with the glutamine synthetase inhibitor, methionine sulfoximine, it was concluded that the pathway was not required for growth with low ammonia, since growth rates did not decrease in cells treated with the inhibitor, and glutamate dehydrogenase levels increased dramatically. The glutamine synthetase activity was highest under nitrogen limiting conditions, and another report using a reporter gene to assess glutamine synthetase transcription indicated that the enzyme was upregulated under nitrogen limiting conditions, thus indicating some role during nitrogen limitation (Yamamoto et al., 1984; Abratt et al., 1993). Clearly more defined genetic studies will be needed to elucidate the role of glutamine synthetase in ammonia assimilation.

The *B. fragilis* glutamine synthetase gene has been cloned and sequenced, but to date the glutamate synthase gene has not yet been identified (Southern et al., 1986; Hill et al., 1989). *Bacteroides thetaiotaomicron* also has glutamine synthetase activity; no pyridine nucleotide-linked glutamate synthase activity was detected in whole cell assays (Baggio and Morrision, 1996). The glutamine synthetase from *B. fragilis* is novel when compared to those of other eubacterial species. The subunit size is somewhat larger (82,827 Da), and the enzyme in its native form is a hexamer rather than the dodecamer (or octamer) structural arrangement seen in other organisms (Southern et al., 1986). Not surprisingly, phylogenetic analyses of glutamine synthetases have revealed that the *B. fragilis* enzyme was part of a novel group designated "GSIII" that also includes the enzymes of *Synechocystis*, *Butyrivibrio* and a few other non-Enterobacteriaceae species (Reyes and Florencio, 1994). The GSIII enzymes do not appear to be controlled by adenylylation, and they lack several other key structural features of the more common enzymes. Little information on glutamine synthetase is available for the other *Bacteroides* spp.

In their normal environment, the *Bacteroides* rarely encounter growth-limiting concentrations of ammonia. Thus these organisms may have evolved their primary strategy for ammonia assimilation on the basis of the second pathway, direct incorporation of ammonia into glutamate via glutamate dehydrogenase. Both *B. fragilis* and *B. thetaiotaomicron* possess two glutamate dehydrogenase enzymes. The enzyme encoded by *gdhA* has dual cofactor dependency, whereas the *gdhB* gene encodes for an NADH specific enzyme (Glass and Hylemon, 1980; Yamamoto et al., 1987a; Baggio and Morrison, 1996). The GdhA from *B. fragilis* and *B. thetaiotaomicron* has a subunit size of about 48,500 and the holoenzymes are about 300,000 Da, suggesting a hexameric structure (Gibson and Macfarlane, 1988a). The GdhA of both species also has greater activity with reduced nicotinamide adenine dinucleotide phosphate (NADPH) as opposed to NADH, but in *B. fragilis*, the K_m values for ammonia are 1.7 and 4.9 mM, which are much lower than the 5.0 and 15 mM values obtained for the *B. thetaiotaomicron* enzyme (Yamamoto et al., 1987a; Gibson and Macfarlane, 1988a). Thus in *B. fragilis*, GdhA is thought to be involved in ammonia incorporation during growth with low levels of ammonia. GdhA undergoes a rapid inactivation in response to exposure to high concentrations of ammonia during growth, indicating it can be rapidly shut down as the need for glutamate decreases (Yamamoto et al., 1987b; Saito et al., 1988). Cells shifted from media containing 50 mM ammonia to media with 1 mM ammonia had a fivefold increase in GdhA activity. Also, when cells are grown in the presence of high concentrations of peptide nitrogen, the activity of GdhA is low but increases if ammonia is added to the culture (Abrahams et al., 2001). There have been no transcriptional studies of *gdhA* in *B. fragilis*, but in *B. thetaiotaomicron*,

levels of message for different growth conditions did not change (Baggio and Morrision, 1996). This suggests that control of activity may be the primary mechanism used to regulate GdhA, and a candidate for this inactivation activity is encoded by the *gdhX* gene, which is co-transcribed with *gdhA* in *B. thetaiotaomicron*. Overall, however, the role for GdhA is not entirely clear because the growth of a *B. thetaiotaomicron* mutant lacking an intact *gdhA* gene was not affected on any medium tested (Baggio and Morrision, 1996). That is, there was no obvious phenotype for the *B. thetaiotaomicron gdhA* mutant, but similar studies have not yet been performed in *B. fragilis*.

The second glutamate dehydrogenase, GdhB, has been cloned from *B. fragilis* and was found to belong to the hexameric glutamate dehydrogenase Family I (Abrahams and Abratt, 1998). The structural gene would encode a peptide of 49,000 Da, and this peptide had greatest similarity to the NADH-specific Gdh from *P. gingivalis* and GdhA from *B. thetaiotaomicron*. In contrast to *gdhA*, there was significant induction of *gdhB* seen in Northern blots of cells grown in the presence of peptides (Abrahams and Abratt, 1998). There also may be some modification of GdhB activity in response to environmental conditions, since activity rapidly decreased when cells were removed from media containing high levels of peptides. Further, it was found that GdhB localized to the cell surface even though there was no obvious signal sequence (Abrahams et al., 2001). Taken together, it is thought that this enzyme may play a role during growth on proteins or peptides, perhaps during infection as well as in the intestinal tract.

In *B. fragilis* and *B. thetaiotaomicron*, there are two distinct glutamate dehydrogenases with different regulatory patterns, but little information is available for the other *Bacteroides* spp. However, one survey looking at electrophoretic mobility showed that an NADH-linked glutamate dehydrogenase activity migrated as a single protein species in *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus*, *B. vulgatus*, *B. eggerthii* and *B. distasonis* (Shah and Williams, 1982). Additionally in another survey, the *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus*, *B. vulgatus*, *B. uniformis* and *B. distasonis* Gdh enzymes were found to function with either NADPH or NADH (Glass and Hylemon, 1980). Taken together, the two results suggest that these are enzymes with dual cofactor specificity.

Protein Utilization The *Bacteroides* spp. have significant proteolytic capability, but they cannot use peptides as a sole carbon and energy source. They can however grow with peptides as the sole nitrogen source, and as mentioned above, GdhB may have a role in the assimilation of the nitrogen in glutamate-containing peptides. Proteolytic activity has been demonstrated in several *Bacteroides* spp. In one survey, protease activity (gelatinase) was found in *B. fragilis*, *B. distasonis* and *B. vulgatus* (Rudek and Haque, 1976). Approximately 50% of both *B. fragilis* and *B. thetaiotaomicron* were found to have elastase activity and a 100-kDa protein with fibrinogen-degrading activity was purified from *B. fragilis* (Chen et al., 1995; Mikamo et al., 1999). This protease appeared to be a serine-thiol protease that had activity against azocasein, casein, fibrinogen, gelatin and azocoll but not bovine serum albumin fibronectin, hemoglobin or collagen. Other species, *B. thetaiotaomicron*, *B. ovatus*, *B. uniformis*, *B. eggerthii* and *B. distasonis*, also had similar fibrinolytic activity, but the activities were reduced compared to those of *B. fragilis*. In another study on *Bacteroides* proteases, three separate activities with high levels of activity against trypsin and casein were found in *B. fragilis* (Gibson and Macfarlane, 1988b). The substrate profile was somewhat different in that gelatin and azocoll were not degraded and elastase activity was absent in this strain. The range observed on synthetic substrates was also very restricted (Gibson and Macfarlane, 1988a). Two of the *B. fragilis* proteases were endopeptidases (P2 and P3) with characteristics of serine-thiol proteases, and the third (P1) was an exopeptidase in the cysteine protease group. The P2 and P3 proteases were cell bound and associated with the outer membrane, while P1 was predominately associated with the periplasm (Gibson and Macfarlane, 1988a). The overall protease activity was higher in organisms grown in media with casein than in those from media with ammonia as the nitrogen source. In general, it appears that the substrate range of the GI-tract *Bacteroides* proteases is limited when compared to the proteolytic activities of related organisms such as *Prevotella ruminicola* or *P. gingivalis*. The *Bacteroides* proteases probably have a role in the utilization of nitrogen and turnover of protein in the gut, and a potential role in pathogenesis cannot be overlooked. More of the proteolytic capabilities important for growth in the intestinal tract or for pathogenesis are discussed in their relevant sections on Protease Activity in the Colon and on Proteases and Hydrolytic Enzymes in Pathogenesis.

Amino Acid Biosynthesis There are few studies of *Bacteroides* amino acid biosynthesis, but it is clear from the nutritional studies that with the possible exception of *B. ovatus*, all *Bacteroides* spp. can synthesize their amino acids de novo (Varel and Bryant, 1974). Glutamate is likely the primary source for α-amino groups of amino

acids, which are formed via transamination of the corresponding 2-oxoacid. As described above, glutamate dehydrogenase catalyzes the reductive amination of α-ketoglutarate in a typical reaction, but the synthesis of the precursor α-ketoglutarate does not occur by typical pathways. The major route of α-ketoglutarate synthesis occurs via a reductive carboxylation of succinate as shown by studies following the incorporation of [^{14}C]-succinate into glutamate. This α-ketoglutarate synthase activity has been demonstrated in *B. fragilis*, *B. vulgatus*, *B. uniformis* and *B. distasonis* (Allison et al., 1979; Allison et al., 1984). Reductive carboxylation also appears to be important in *B. fragilis* for the synthesis of leucine, isoleucine and phenylalanine, which can be derived from isovalerate, 2-methylbutyrate and phenylacetate, respectively (Allison et al., 1984). Thus the presence of these acid precursors in the external environment may be a competitive advantage by providing the carbon skeletons for biosynthesis. Recent studies also have documented the synthesis of α-ketoglutarate by a pathway that involves the oxidative branch of the Krebs cycle (Baughn and Malamy, 2002). In an important observation, it was shown that aconitase (GenBank accession number AF434843) and isocitrate dehydrogenase were present in *B. fragilis* and that this route of α-ketoglutarate synthesis was used during growth in the absence of hemin, inasmuch as succinate cannot be produced via the fumarate reductase reaction under these conditions (see section on Sugar Catabolism and Fermentation Pathways in this Chapter). This work and further inspection of the *B. fragilis* genome database indicate that most of the forward Krebs cycle enzymes are present, although it is not likely to function in the synthesis of fumarate from succinate (http://www.sanger.ac.uk/Projects/B_fragilis/).

During growth under conditions that do not provide the acids for reductive carboxylation, *B. fragilis* has the ability to synthesize the amino acids from glucose by typical pathways. In the case of leucine biosynthesis, two of the requisite enzymes have been demonstrated in *B. fragilis*, α-isopropylmalate synthase (*leuA*) and β-isopropylmalate dehydrogenase (*leuB*; Wiegel, 1981; Sarker et al., 1995a; Sarker et al., 1995b). The *B. fragilis leuB* gene was in fact cloned by complementation of the *leuB* mutation in *E. coli*, thus indicating functional, as well as sequence homology in this pathway. Interestingly, isopropylmalate synthase appears to be regulated such that its activity is lower in cells grown with isovalerate or leucine in the medium, thus supporting the notion that it may be advantageous to obtain this precursor from the environment when it is available. Nutritional studies provide insight into methionine biosynthesis. The vitamin B_{12} requirement for *B. fragilis*, *B. vulgatus*, *B. thetaiotaomicron* and *B. distasonis* could be replaced by methionine, suggesting that biosynthesis occurs via a B_{12}-dependent homocysteine-N5-methyltetrahydrofolate transmethylase (MetH, methionine synthase). A gene encoding a protein with 45% identity to MetH from *E. coli* was present in the B. fragilis unfinished genome database.

The completed genome sequence will be a powerful tool for better understanding the peculiarities of amino acid biosynthetic pathways in *Bacteroides* spp.

Aerotolerance and the Response to Oxidative Stress

Many studies have shown that anaerobic bacteria are not uniformly sensitive to oxygen, and their range of oxygen tolerance is broad (Morris, 1991). Among anaerobic bacteria, the *Bacteroides* spp. are some of the most aerotolerant species known. In addition, aerotolerance may prove to be an important virulence factor. This is supported by the fact that clinical isolates of *B. fragilis* are more resistant to oxygen exposure than fecal strains are. The former strains were able to survive in the presence of oxygen for 48–72 h, while the latter lost viability after 4 h of oxygen exposure (Tally et al., 1975; Rolfe et al., 1977). This suggests that there is an adaptive mechanism that may protect cells during an oxidative stress. In fact, *B. fragilis* is able to adapt and survive in tissues with oxygen tensions up to 6% during the initial steps of infection until adequate anaerobic conditions for growth are established (Rotstein et al., 1985; Park et al., 1992).

To survive in the more oxygenated environment outside the colon, *B. fragilis* must defend itself from the damage caused by reactive oxygen species (ROS). The formation of these ROS is an unavoidable consequence of aerobic exposure and can lead to macromolecule damage such as peroxidation of membrane lipids, oxidation of amino acids in proteins, the destruction of iron-sulfur clusters in enzymes, and DNA base modifications and strand breaks (Storz and Imlay, 1999). A few studies describe the mechanisms leading to oxidative damage in anaerobes. Studies comparing the oxidative DNA damage between the strictly anaerobic *Prevotella melaninogenica* and *B. fragilis* after exposure to O_2 or H_2O_2 have demonstrated that formation of 8-hydroxydeoxyguanosine (8OHdG, a DNA damage marker) was induced in *P. melaninogenica* but not in *B. fragilis* (Takeuchi et al., 1999). Furthermore, in vivo and in vitro studies have shown

that aeration caused a drastic reduction in the fumarase and pyruvate:ferredoxin oxidoreductase as well as oxidation of other iron-sulfur center-containing proteins in *B. thetaiotaomicron* (Pan and Imlay, 2001).

More information is available for enzymes that protect from ROS. Among *Bacteroides* spp., catalase activity is not universally present, although *B. fragilis* and *B. distasonis* are generally catalase positive while *B. thetaiotaomicron*, *B. ovatus* and *B. eggerthii* possess variable catalase activity (Wilkens et al., 1978). *Bacteroides vulgatus* and *B. uniformis* are catalase negative. A single catalase gene (*katB*) was found in *B. fragilis*, and hybridization analysis showed that *katB* had strong homology with all *B. fragilis* strains tested but not with other *Bacteroides* spp. except for *B. thetaiotaomicron*. Also, some rearrangements in the catalase gene region were observed between *B. fragilis* ATCC 2393 (homology group II) and the two homology group I strains ATCC 25285 and 638R (Rocha and Smith, 1995). Superoxide dismutase (Sod) activity was found in cell extracts of *Bacteroides* species. Isoelectric focusing has demonstrated that Sod occurs in different forms in the different species (Carlsson et al., 1977). Moreover, the frequency of Sod occurrence varies within the genera and among the species (Gregory et al., 1978). For example, Sod activity in some *B. fragilis* strains varies over a 15-fold range, while some strains of *B. vulgatus* have no detectable Sod activity. *Bacteroides fragilis* and *B. thetaiotaomicron* synthesize a single superoxide dismutase that exists as dimers of 42,000 and 46,000 Da, respectively (Gregory and Dapper, 1983; Pennington and Gregory, 1986). *Bacteroides fragilis* and *B. thetaiotaomicron* Sod enzymes are cambialistic metalloenzymes that contain Fe (FeSod) in anaerobic cells, and under aerobic conditions, Fe is replaced by Mn (MnSod; Gregory and Dapper, 1983; Pennington and Gregory, 1986). The variation in oxygen sensitivity among *Bacteroides* species might be attributed to the heterogeneous expression of catalases and superoxide dismutase among the different species.

Bacteroides cannot change to aerobic metabolism, and when shifted from an optimal anaerobic growth condition to an adverse aerobic environment, it will most likely "redirect" its energy resources and metabolism for protection and survival. Consistent with this, *B. fragilis* synthesizes two very similar and overlapping sets of approximately 28 new proteins following stress induction by either oxygen exposure or addition of hydrogen peroxide. Thus far, superoxide dismutase, alkyl hydroperoxide reductase (AhpCF), the nonspecific DNA-binding protein, Dps, catalase (KatB) and *nrdA* have been identified as important components of the oxidative stress response (Gregory and Dapper, 1983; Rocha et al., 2000; Smalley et al., 2002). Pretreatment with sublethal concentrations of hydrogen peroxide has been shown to induce catalase (*katB*) expression and protection against further exposure to high concentrations of hydrogen peroxide (Rocha et al., 1996). In addition, catalase was important (but it was not the only factor needed) to protect *B. fragilis* DNA against oxidative damage as determined by the levels of 8OHdG following treatment with H_2O_2 or O_2 (Takeuchi et al., 1999). *Bacteroides fragilis* mutants in *ahpCF* or *katB* have an increased sensitivity to the toxic and mutagenic effects of peroxides, which confirms the role of this inducible response in protection from oxidative stress (Rocha et al., 1996; Rocha and Smith, 1999).

The *B. fragilis* peroxide response is regulated by OxyR, which is a LysR-family transcriptional activator similar to that found in facultative organisms (Rocha et al., 2000). The OxyR protein regulates the transcription of *katB*, *ahpCF* and *dps* in response to H_2O_2 or O_2. A mutated OxyR with a glycine substitution for the aspartate at position 202 resulted in a permanently activated form of OxyR, which was responsible for the constitutive expression of *katB*, *ahpCF* and *dps* in the *B. fragilis* mutant strain. There also appears to be an oxygen responsive regulatory network that is independent of OxyR. This was suggested by transcriptional analysis of *dps*, where induction by H_2O_2 was lost in an *oxyR* mutant, but some induction still occurred during exposure to air (Rocha et al., 2000). In addition, recent results showed several other genes (*nrdA*, *sod* and *asdA*) that were induced only by O_2 and not by H_2O_2 (Rocha et al., 2000; Smalley et al., 2002).

Mechanisms other than classic "detoxification" also seem to help *B. fragilis* withstand long periods of oxygen exposure. One novel mechanism was the discovery of the *Bacteroides* aerotolerance operon (*batABCDE*). The *bat* genes were shown to be important for survival in the presence of oxygen and growth in vivo in model systems (Tang et al., 1999). The less aerotolerant *bat* mutants were unable to compete with the wildtype strain in an experimental mixed infection. This clearly demonstrated an association between the ability of *B. fragilis* to survive in the presence of oxygen and survival in host tissues. Bat proteins seem to form a complex involved in the generation or export of reducing power equivalents into the periplasm of *B. fragilis* (Tang et al., 1999), but no data are available yet to determine whether the *bat* operon are induced as part of the oxidative stress response.

Genetics

Genome Structure

During the past 15 years, understanding of *Bacteroides* genetics and the development of molecular tools have progressed sufficiently so that genetic manipulation of these organisms is now routine. Thus, there is a wealth of new information available and the promise of even more rapid advances as the genome sequencing projects for two *B. fragilis* strains (see http://www.sanger.ac.uk/Projects/B_fragilis/) near completion.

The G+C content of *Bacteroides* ranges from 39–48 mol%, averaging 44% for all species (Johnson, 1978a; Table 2). Genome sizes for the predominant species have been estimated by pulsed-field gel electrophoresis analysis of chromosomes digested with the rare-cutting restriction enzymes *Cru*I, *Not*I, and *Asc*I (Shaheduzzaman et al., 1997). As shown in Table 2, genome size varies from the smallest for *B. eggerthii* at 4.4 Mb to the largest, *B. ovatus* at 6.7 Mb. Overall the average size range of the *Bacteroides* genomes is similar to the median size of the known eubacterial genomes that are already or in the process of being sequenced (see TIGR in progress and TIGR complete websites). A chromosome map of one *B. fragilis* strain (homology group II strain YCH46) has been constructed for the enzymes *Not*I (16 sites), *Asc*I (7 sites), and *Ceu*I (6 sites; Kuwahara et al., 2002). The chromosome was circular and approximately 5.3 Mb. Several genetic markers, including *cfiA*, *glnA*, *recA*, *nanH* and six rRNA operons, were mapped and found to be scattered around the chromosome. In the case of *B. fragilis*, genome size (5.225 Mb) has been independently verified by the near completion (99.9%) of the genome sequence for the type strain, ATCC 25285. *Bacteroides* genomes are composed of a diverse array of plasmids, transposons and insertion sequence (IS) elements, as will be discussed in the section Genetic Elements, but evidence for a pathogenicity island(s) in strains containing the zinc metalloprotease toxin, fragilysin, is also present (Moncrief et al., 1998; Franco et al., 1999). The first of these is BfPAI, which is about 6 kb and 35 mol% G+C. BfPAI encodes two metalloprotease toxin genes, *bft1* and *mpII*, which share about 28% identity at the amino acid level. This pathogenicity island is located within a larger genetic element (≥12 kb; 49 mol% G+C), which shared some features associated with mobilizable *Bacteroides* plasmids. On the basis of their dissimilar mol% G+C composition (compared to the chromosomal values), it was suggested that these two genetic elements were obtained by horizontal transfer (Franco et al., 1999).

Table 2. Structural features of *Bacteroides* genomes.

Species	G+C (mol%)	Genome size (Mb)
B. fragilis	41–44	5.2
B. thetaiotaomicron	40–43	4.7
B. vulgatus	40–42	5.2
B. uniformis	45–48	4.8
B. ovatus	39–43	6.7
B. distasonis	43–45	4.8
B. eggerthii	44–46	4.4

From Johnson (1978), Holdeman et al. (1986), and Shaheduzzaman et al. (1997).

Genetic Elements

Additional genetic elements that make up the genomes of *Bacteroides* spp. are plasmids, IS elements, and transposons. Plasmids are found frequently in all of the *Bacteroides* spp., with surveys reporting 20–50% of clinical or fecal isolates containing plasmid DNA (Stiffler et al., 1974; Wallace et al., 1981; Callihan et al., 1983; Odelson et al., 1987; Soki et al., 1999). Multiple plasmids (each 2.7–80 kb) are not uncommonly found in a single strain. The majority of plasmids isolated from these organisms are small (<8 kb), cryptic plasmids that can be classified into one of just several homology groups, and most appear to contain mobilization genes that allow them to be transferred by conjugation (see Conjugation; Callihan et al., 1983; Smith et al., 1998). Although relatively rare, antibiotic-resistance plasmids also have been described in *Bacteroides* spp. These plasmids have been associated with transmissible resistance to macrolide-lincosamide-streptogramin drugs (MLS) and to 5-nitroimidazole drugs (reviewed in Smith et al., 1998). The MLS-resistance determinant, *ermF*, has been found on several unrelated plasmids, but in each case, it was located in closely related compound transposons (reviewed in Odelson et al., 1987, and Smith et al., 1998). The MLS-resistance plasmids were either self-transmissible (pBF4 and pBI136) or mobilizable (pBFTM10). The 5-nitroimidazole-resistance determinants (*nim*) have been observed on a wide variety of mobilizable plasmids. In contrast to the MLS-resistance plasmids, the *nim* genes were not part of compound transposons. Other than the MLS and *nim*-resistance plasmids, there are no other verified antibiotic-resistance plasmids in the *Bacteroides*.

Similar to other genera, the *Bacteroides* harbor a variety of IS elements that have been

found in the chromosome and on plasmids of several different species. Thus far about nine different IS elements have been documented, and these generally fall in the 1.1–1.6-kb range. The elements belong to several different IS families (e.g., IS*5*, IS*30*, IS*4* and IS*21*) that represent a range of genetic organizations and transposition mechanisms (reviewed in Smith et al., 1998). All of the *Bacteroides* IS elements identified to date were first found to be associated with antibiotic-resistance genes. In these cases, the IS elements were located directly adjacent to the resistance determinants and had outward firing promoters that controlled transcription of the resistance genes. IS elements have been shown to activate expression of *nim*, *ermF*, *cfiA* (metallo-β-lactamase) and *cepA* (β-lactamase) by acting as transcription promoters (Podglajen et al., 1992; Podglajen et al., 1994; Podglajen et al., 2001; Haggoud, et al., 1994; Rogers et al., 1994). Even though the IS elements are required for expression of many antibiotic-resistance genes, they are not frequently found associated with compound transposons. Thus far, only IS*4351* has been linked to compound transposons, and it is part of Tn*4351*, Tn*4400* and Tn*4551*, which are three plasmid-borne compound transposons encoding MLS-resistance. The structure of these transposons is similar to the IS elements in direct orientation, flanking the *erm* gene and one other antibiotic-resistance gene (Odelson et al., 1987; Smith et al., 1998). The compound transposons have been used as genetic tools for random mutagenesis of *B. fragilis* and *B. thetaiotaomicron* and other related genera from the oral cavity (Salyers et al., 2000). Recently, a modified version of Tn*4400* was constructed to increase transposition frequency and identification of mutants (Tang and Malamy, 2000).

The most common transposons found in *Bacteroides* spp. are conjugative transposons (CTn) and mobilizable transposons (MTn), both of which share common features and are highly transmissible by a conjugation-like mechanism (Salyers et al., 1995a; Salyers et al., 1995b). The CTns are generally large elements ranging in size from 50 kb to more that 150 kb. The common features of the CTns are that they encode tetracycline-resistance, mediated by the *tetQ* gene, and they are self-transmissible, encoding all of the genes for the conjugation apparatus. A recent survey that included all of the *Bacteroides* species showed that these elements are widespread, being found in 60–80% of recent isolates as compared to about 30% of isolates obtained prior to 1970 (Shoemaker et al., 2001). This dissemination can be accounted for by the broad host range of the CTns, which are able to transfer to all *Bacteroides* spp., to related genera (*Porphyromonas* and *Prevotella*), and to *E. coli*. The majority of CTns appear to be closely related, sharing significant DNA sequence homology to each other and to CTnDOT, which is the prototypic element (Shoemaker et al., 2001; Whittle et al., 2001). However, the structure of the CTns is somewhat variable, and the elements can be classified into several groups on the basis of restriction fragment length polymorphisms (RFLPs; Shoemaker et al., 2001). Also, a few CTns contain the *tetQ* gene but otherwise do not hybridize with CTnDOT, suggesting that other conjugative transposon classes are present in these organisms. The mechanism of CTn transposition appears similar to that of the well-studied Tn*916* except that transposition is not random and there are only a limited (5–10) number of CTn insertion sites in the *Bacteroides* chromosomes (Bedzyk et al., 1992; Cheng et al., 2000). Transposition is a several step process that requires the element to excise from the chromosome and then form a covalently closed circular intermediate. Next the transposon can either insert at a new location or be transferred to a new host where it will integrate upon entry. Both the integration and excision steps require a lambda-family integrase that makes staggered cuts 5 bp from the ends of the transposon to excise the element and then presumably produces similar cuts in the target site for integration (Cheng et al., 2000). Further characterization of the transposition mechanism is underway. Perhaps the most notable feature of the *Bacteroides* CTns is that their transfer and transposition are induced (100–1000-fold) by exposure to tetracycline (Privitera et al., 1979b; Salyers et al., 1995a). This induction is mediated by a signal transduction mechanism involving three regulatory genes (*rteA*, *rteB* and *rteC*) plus the resistance gene *tetQ* (Stevens et al., 1993). This regulatory system appears to control expression of the conjugation apparatus, but no specific targets of this control have yet been identified (Stevens et al., 1993). The RteA and RteB gene products have homology to classic two-component regulatory system sensors and regulators, and their roles in signal transduction are currently being worked out.

The last class of transposon frequently encountered in *Bacteroides* spp. is the novel mobilizable transposons (MTn). These elements can be transferred to a wide variety of bacterial genera, but they differ from the CTns in that they cannot self-transfer. Rather, they require a co-resident CTn or conjugative plasmid to provide the conjugation apparatus for transfer and thus are mobilized in *trans* by these conjugative elements. There have been five MTns studied in detail: NBU1 and NBU2 (from *B. uniformis*), Tn*4555* (from *B. vulgatus*), and Tn*4399* and

Tn*5520* (from *B. fragilis*; reviewed in Smith et al., 1998). Surveys using NBU1 or NBU2 as a probe in Southern hybridization assays have shown that MTns are found in virtually all *Bacteroides* spp. (Wang et al., 2000). These elements range in size from 4.7 to 12.1 kb, and all have the same basic genetic organization, containing at a minimum a lambda-family integrase, a mobilization gene(s) and an *oriT*. Among the MTns, there is considerable homology, but this appears to be due to their modular construction. That is, some elements share related integrase genes and others share similar mobilization genes but have unrelated integrases (Smith et al., 1998). It is thought that these elements may be important for antibiotic-resistance dissemination in *Bacteroides*, since two MTns have been shown to encode resistance determinants (Parker and Smith, 1993; Smith and Parker, 1993; Wang et al., 2000).

Mechanisms of Genetic Exchange

Conjugation First described in 1979 for the MLS-resistance plasmids and the CTns, conjugation is the most important genetic exchange mechanism in *Bacteroides* spp. and is responsible for the widespread dispersal of all genetic elements in this genus (Privitera et al., 1979a; Privitera et al., 1979b; Welch et al., 1979). Conjugation requires direct cell-to-cell contact of donor and recipient cells on a solid substrate, and there have been no reports of successful matings in liquid cultures. In contrast to many other organisms, self-transmissible plasmids such as pBF4 and pBI136 seem to be relatively rare; thus nearly all conjugation among the *Bacteroides* is driven by the CTns. The CTns are remarkable, self-transmissible elements that also have the ability to mobilize a diverse set of unlinked plasmids and MTns. In fact, mobilization does not appear to be specific, and essentially any element that has a *Bacteroides* spp. mobilization gene and *oriT* can be transferred by a CTn. As mentioned above, transfer and mobilization are highly inducible by pre-exposure to tetracycline, which increases conjugation frequencies by as much as 1000-fold (Salyers et al., 1995a; Salyers et al., 1995b). Control appears to be at the transcriptional level and depends on the same regulatory genes as found for transposition (Stevens et al., 1992; Stevens et al., 1993). This signal transduction pathway is very complex and there are likely other factors involved, but their identity remains to be determined. Recently the 19-kb conjugation region from CTnDOT was cloned and sequenced (GenBank accession number AF289050). There were 17 genes in the transfer region with a mol% G+C content of 48–60% (Li et al., 1995; Bonheyo et al., 2001a). All but one of the genes encoded apparently novel proteins that have never been seen in conjugation systems from other Gram-negative or Gram-positive bacteria. One gene, *traG*, encoded a protein that had homology to VirB4 or TrsE, which are proteins thought to be involved in the translocation of DNA across the mating pore. The TraG protein also had homology to BctA, which is required for conjugation of pBF4 (Morgan and Macrina, 1997; Bonheyo et al., 2001a; Bonheyo et al., 2001b).

Conjugation also has been the most successful technique used for the genetic manipulation of *Bacteroides* spp. Plasmids can be readily transferred into any *Bacteroides* spp. from *E. coli* using the IncP plasmid-based conjugation systems such as RK2, RP4, R751 or S17-1 (which contains RP4 in the chromosome) as first described by Guiney et al. (1984a). The main requirements are that the vectors contain an *oriT* recognized by RK2 and a selectable marker such as *ermF*, which can be expressed in a *Bacteroides* spp. host. A wide variety of vectors are currently available including suicide vectors for allelic exchange or insertional mutagenesis as well as replication-competent vectors for complementation or gene expression studies. All of these plasmids can be transferred at a relatively high frequency (10^{-3}–10^{-5} transconjugants per input donor cell) to recipient cells using standard mating protocols (Shoemaker et al., 1986; Smith et al., 1998; Salyers et al., 2000). The most common technique for conjugation experiments is to mix donor and recipient cells together and deposit them on a 0.45-μm filter that has been placed on an agar plate of suitable growth medium. An overnight incubation then is done under anaerobic conditions if the donor cells are *Bacteroides* spp., but in cases where *E. coli* donor cells are being used (for recombinant plasmids), aerobic incubatation of the mating plate is recommended for optimal transfer frequency (Shoemaker et al., 1986; Salyers et al., 2000). Following overnight incubation, the cells are washed off the filter and plated on selective media.

Transformation Although there are no known natural genetic transformation systems in the *Bacteroides*, both chemically induced competence and electroporation have been successfully used for the introduction of plasmid DNA into these organisms (Smith, 1985; Smith et al., 1990). Electroporation has proven more effective than chemical methods of induction, but for both methods, transformation has been documented only for strains of *B. fragilis*. Further, the utility of transformation as a genetic tool has been limited by low transformation frequencies when us-

ing plasmid DNA isolated from *E. coli*. Typical transformation frequencies are 10^4–10^6 transformants per μg of plasmid DNA for *B. fragilis* strains transformed with plasmid DNA isolated from *B. fragilis*. Frequencies are usually about 1/10 these values when plasmid DNA was from *B. uniformis*, *B. thetaiotaomicron* or *B. ovatus* and 1/1000 when the DNA was of *E. coli* origin (Smith et al., 1990). The strong inhibition of transformation most likely is due to the effective restriction/modification systems present in *Bacteroides* spp. There have been reports of restriction enzymes from *B. fragilis*, *B. distasonis*, *B. caccae* and *B. vulgatus*, and the recognition sequences for these can be viewed at the rebase website (rebase.neb.com/rebase/rebase.html). Enzymes with 4- , 5- or 6-bp cleavage sites have been identified and the enzymes have been shown to produce flush ends or ends with 5′ overhangs (Murakami et al., 1990; Murakami et al., 1991; Reinecke and Morgan, 1991; Azeddoug et al., 1992). Thus far the only methylase activity demonstrated has been for *Bfr*BI (ATG^CAT) from *B. fragilis* (Azeddoug et al., 1992). The significance of these restriction systems is apparent from inspection of the DNA sequence from two MTns, Tn*4555* and NBU1. These elements (12.1 and 10.3 kb, respectively) harbor only one *Bfa*I (C^TAG) site, two *Bfr*BI sites, and no *Bfr*I (C^TTAAG) sites. Selection against the *Bacteroides* endonuclease recognition sites is obvious since (for example, in the case of Tn*4555*) one would predict on the basis of the nucleotide composition of the element that there would be 46 *Bfa*I sites (Tribble et al., 1999).

Bacteriophage The last potential mechanism of genetic exchange is transduction mediated by bacteriophage. There have been numerous reports on the isolation of bacteriophage capable of growth on all of the *Bacteroides* spp. except *B. vulgatus* (Keller and Traub, 1974; Booth et al., 1979; Cooper et al., 1984; Kory and Booth, 1986). These phage are easily isolated, usually from sewage effluent, and in one extensive survey, 68 bacteriophage representing four morphological types were isolated (Booth et al., 1979). Each of the phage had a narrow host range, infecting only *Bacteroides* species from which it was originally isolated. This specificity has been used to devise a phage typing scheme to differentiate *B. thetaiotaomicron* and *B. ovatus* (Cooper et al., 1984). There is one report of successful transfection of a *Bacteroides* spp. in which cells of *B. thetaiotaomicron* were transfected with DNA isolated from phage β_1, a hexagonal headed DNA bacteriophage (Burt and Woods, 1977). Transfection required Ca^{+2} treatment of cells obtained from any stage of growth, and efficiency was 5.5×10^8 PFU per μg of DNA. In these studies and in an earlier work with a *B. fragilis* phage, an unusual pseudolysogeny or phage-carrier state was demonstrated in which phage and bacteria existed in equilibrium, but a true lysogen was not produced. Despite the widespread presence of phage, numerous attempts have failed to demonstrate any form of transduction in *Bacteroides* spp. (Booth et al., 1979).

Ecology and Habitat

Features of the Gastrointestinal Tract Environment

The *Bacteroides* spp. are obligate host-associated organisms most commonly found in the gastrointestinal tract of humans and other mammals. All of the *Bacteroides* spp. also have the potential to be opportunistic pathogens when translocated to a normally sterile body site; thus they can survive outside of their normal habitat in a mammalian host. Of the two major alimentary tract architectures of mammals, those with a hindgut fermentation generally seem to be preferred, although *Bacteroides* spp. have occasionally been isolated from the rumen of sheep or cattle (Holdeman et al., 1977; Holdeman et al., 1986; Macy, 1984; Wood et al., 1998). In hindgut habitats, *Bacteroides* spp. are found most frequently distal to the stomach where the gastric secretions have been diluted and transit times begin to decrease. The primary areas of colonization begin in the distal small intestine towards the terminal ileum and are maximal in the colon (Finegold et al., 1983). The intestinal tract should be considered as an open ecosystem in which nutrients enter at one end and bacterial cells and waste exit from the other end. This ecosystem, especially the large intestine, is a highly competitive environment in which some 500 species of bacteria compete for the limiting nutrient supply. A variety of other environmental factors also limit or influence the growth of microorganisms in this habitat, but these are for the most part stable. Temperature is maintained at a relatively high level in mammals, and this leads to high metabolic activity and rapid fermentation rates of the flora. The pH of the colon varies along its tract from slightly acid along the ascending colon to neutrality or slightly above in the descending colon (Fallingborg, 1999). These values are thought to reflect the relative carbohydrate fermentation activity, which is highest as fresh material enters the cecum, slows in the transverse colon as substrates become limiting, and then finally switches to a protein fermentation as carbohydrate is exhausted (Macfarlane and Cummings,

1991a). Gas composition and redox potential of the colon also are the result of the high level of microbial activity. This is an anaerobic environment with oxygen at less than 12 mmHg (1.5%) and carbon dioxide levels of up to 84 mmHg (5–10%; Bornside et al., 1976). Other gases present in the environment include hydrogen and methane, both of which are products of metabolism in a complex anaerobic ecosystem. In such highly reducing conditions, the redox potential for the intestinal tract usually runs in the range of –150 to –350 mV.

Distribution of *Bacteroides* spp. in the Colon as a Function of Space and Time

Flora of the Adult Intestinal Tract In rodents, *Bacteroides* spp. colonize the upper gastrointestinal tract including the stomach and small intestine, reaching numbers of 10^9 and 10^6 per g wet wt., respectively, but in humans, *Bacteroides* spp. are not routinely isolated from either of these locations (Tannock, 1977; Finegold et al., 1983; Bernhardt and Knoke, 1989). Progressing down the tract toward the distal small intestine, there is an increase in the numbers of *Bacteroides* spp. until the ileum, where the composition of the flora begins to take on characteristics typical of the colonic flora with numbers of *Bacteroides* spp. reaching 10^6 per g wet wt. However, this flora and that of the cecum generally have fewer numbers and a lesser percentage of *Bacteroides* than are encountered further down the tract (Marteau et al., 2001). The large intestine is the primary site of *Bacteroides* colonization, in which these organisms can account for ca. 30% of the total microbial population in humans as determined by direct culture methods (Finegold et al., 1974; Moore and Holdeman, 1974; Holdeman et al., 1976; Duerden, 1980). Similarly, using an oligonucleotide hybridization approach to detect 16S rRNA, *Bacteroides*-specific probes were found to account for 37% of the total bacterial rRNA in fecal samples, thus upholding the findings from direct culture methods (Sghir et al., 2000).

Each of the *Bacteroides* spp. is isolated from the colon in high numbers (10^9–10^{11} per g dry wt), but there is considerable variation from person to person and there seems to be an important contribution of diet and culture on the distribution of *Bacteroides* spp. (Table 3). *Bacteroides thetaiotaomicron* and *B. vulgatus* are generally considered to be the most numerous species in the colon, sometimes accounting for more than 20% of the total flora; however, variable isolation, even of these numerous species, is observed from individuals on certain diets (e.g., Japanese diet with no beef) (Finegold et al., 1974; Moore and Holdeman, 1974; Holdeman et al., 1976; Duerden, 1980). Next *B. distasonis* and *B. eggerthii* appear to be important residents of most flora, consistently isolated from up to 64% of subjects. In some cases though, the data are limited since earlier studies on fecal flora often did not recognize *B. eggerthii*, and *B. thetaiotaomicron* was a compilation of several species. The last two species for which there are sufficient data, *B. ovatus* and *B. fragilis*, are the least common of the *Bacteroides* intestinal isolates from subjects on most diets, but in one study, *B. fragilis* was the most common *Bacteroides* spp. (8.7% of flora) isolated from fecal samples of rural native Africans (Moore and Moore, 1995). It is interesting to note here that even though *B. fragilis* generally is a small percentage of the total microbial population in the colon, it is by far the most frequently isolated species from clinical specimens (Finegold and George, 1989). Finally, there has been considerable discussion and study on the effect of diet on the composition of the colonic microflora and in general the results tend

Table 3. Presence of *Bacteroides* spp. in the fecal flora of humans and animals.

	Count/g dry wt (mean)	Human flora Percent of total flora (range)	Percent of positive samples (range)	House pets	Swine	Cattle	Sheep	Poultry	Horse	Rodent
B. thetaiotaomicron	4.5×10^{10}	1.0–8.9	67–100	X	X	X	X	X	X	X
B. vulgatus	4.2×10^{10}	7.5–13.1	47–100	X	X	X	X	X	0	0
B. distasonis	2.9×10^{10}	1.3–6.0	36–64	X	X	X	X	X	0	0
B. eggerthii	2.8×10^{10}	nd	2.1–5.0	nd	nd	nd	nd	nd	nd	nd
B. fragilis	2.3×10^{10}	0.3–8.7	17–68	0	X	0	X	0	0	0
B. ovatus	0.8×10^{10}	0.1–2.6	17–44	0	0	0	0	X	0	0

Abbreviations: X, organism is present; 0, organism not present; and nd, analysis not done.
From Finegold et al. (1974, 1983), Moore and Holdeman (1974), Holdeman et al. (1976), Robinson et al. (1981), Macy (1984), Moore et al. (1987), and Moore and Moore (1995).

to show that while some differences do exist, there is a remarkable similarity in the makeup of the flora, with each of the *Bacteroides* spp. comprising 0.1–12% of the flora and *B. vulgatus* being ranked as the first or second most common fecal isolate (Finegold et al., 1974; Moore and Holdeman, 1974; Holdeman et al., 1976; Duerden, 1980; Finegold et al., 1983; Moore and Moore, 1995). Only through continued study of the flora will it be possible to determine the significance of these variations.

Colonization of the Infant Intestinal Tract At birth the human colon is sterile, but within hours colonization begins and proceeds as a predictable succession of bacterial populations. Colonization occurs in several phases and can be influenced by diet, i.e., whether the infant is breast-fed or bottle-fed. Initially, colonization is by the facultative anaerobes, *Enterobacteriaceae* and enterococci, which apparently provide the anoxic environment suitable for the next phase of colonization by *Bifidobacterium* spp., followed closely by the appearance of the *Clostridium* and *Bacteroides* by the end of the first week (Long and Swenson, 1977; Cooperstock and Zeed, 1983). In bottle-fed infants, the anaerobic populations continue to develop and the levels of facultative species are maintained. *Bacteroides fragilis*, *B. vulgatus* and *B. ovatus* are among the most common of the *Bacteroides* spp. being isolated from 20–75% of samples (Cooperstock and Zeed, 1983; Mevissen-Verhage et al., 1987). In contrast, in breast-fed infants, the *Bacteroides*, *Clostridium* and the facultative species all decline or disappear after about the first week and the *Bifidobacterium* spp. predominate. Once dietary supplements are introduced to the infants, the clostridia, bacteroides, and other facultative and obligate anaerobes appear in higher numbers. After weaning, the flora begins to take on characteristics of the adult as the facultative species decline, and by two years of age, the normal flora is well established (Cooperstock and Zeed, 1983).

Bacteroides spp. in Animals The intestinal tracts of many animals also are a source of *Bacteroides* spp. (summarized in Table 3), but their concentration is generally much lower. Commonly, *B. vulgatus* and *B. distasonis* are associated with house pets, and *B. thetaiotaomicron* is a common inhabitant of the rodent gastrointestinal tract (Tannock, 1977; Macy, 1984; Kreader, 1995; Wang et al., 1996). A notable member of the swine intestinal microflora is *B. uniformis*, which has been consistently isolated at a level of 1–3% of the flora (Robinson et al., 1981; Moore et al., 1987). *Bacteroides fragilis* also has been a common isolate from sick and healthy lambs, calves, and other livestock where it is often associated with a diarrheal disease of young animals (Border et al., 1985; Myers et al., 1985; Myers and Shoop, 1987).

Indicators of Fecal Pollution Even though *Bacteroides* spp. are associated with animals, the numbers are low, and it has been suggested that the *Bacteroides* might be useful indicators of human fecal pollution, since they do not replicate in the environment and they are highly correlated with their human hosts. Direct culture methods were originally used to show that *Bacteroides* spp. rapidly died off in water samples and that animal feces were not a significant source of contamination (Allsop and Stickler, 1984; Allsop and Stickler, 1985). Recently two new approaches have been used for the detection of these organisms in environmental samples. First is the enumeration of *B. fragilis* bacteriophages in which standard strains are used to propagate phage from water samples (Tartera and Jofre, 1987; Tartera et al., 1989; Cornax et al., 1990). The idea is that the phage could only originate from recent human contamination, since the *Bacteroides* do not grow (and hence could not support phage replication) outside of the human. Using this approach, it has been shown that phage can be found in about 10% of human fecal samples, but they were not found in the feces of other animals. Samples from sewage and recently contaminated river water also contained the phage, but none were found in slaughterhouse effluents or waters contaminated by wildlife. Similarly, a strategy based on the use of polymerase chain reaction (PCR) to screen for the presence of the 16S rRNA gene has shown promise (Kreader, 1995; Kreader, 1998). In this report, results similar to direct culture methods were obtained. Only 7–11% of nonhuman fecal samples were positive for the *Bacteroides* spp. probes, whereas nearly 80% of the human samples were positive. Taken together, the three methods have resulted in similar data and suggest that use of *Bacteroides* spp. as pollution indicators warrants further study.

Role of *Bacteroides* spp. in Colon Physiology

As the predominant microbial group in the large intestine, *Bacteroides* spp. are thought to contribute to a wide array of normal physiological functions in the intestinal tract. Some of the most significant activities in which *Bacteroides* spp. directly participate are polysaccharide digestion, nitrogen cycling, bile acid metabolism, and vari-

ous other transformation reactions including the production of fecal mutagens.

Polysaccharide Digestion The fermentation of polysaccharides is a major activity of the large intestine. It has been estimated that 50–70 g of substrate enter the colon and that the end products (short chain fatty acids) of this fermentation can meet about 7–10% of the human daily energy allowance (Smith and Bryant, 1979). The two major sources of fermentable substrate are dietary plant polysaccharides and host-derived polysaccharides. The plant-derived carbohydrates are primarily in the form of resistant starch that has escaped digestion in the upper gastrointestinal tract and dietary fiber composed of cellulose, hemicellulose, arabinogalactans, xylans and pectins that are not digested by the host (Macfarlane and Cummings, 1991a). Carbohydrate sources from the host come primarily from intestinal tract secretions and include mucins and mucopolysaccharides such as chondroitin sulfates. As discussed in the section Metabolism, the *Bacteroides* spp. are metabolically versatile with a wide array of polysaccharide-degrading enzymes, which make them well suited to compete for these substrates (Table 1). In one study, DNA probes used to enumerate the predominant polysaccharide-degrading *Bacteroides* spp. from feces resulted in data that were for the most part consistent with previous direct culture counts (Kuritza et al., 1986a). The somewhat less versatile organisms, *B. vulgatus* and *B. uniformis*, were found at slightly higher levels ($1.2–3.0 \times 10^{10}$) than *B. thetaiotaomicron* (1×10^{10}), which can use a wider array of host and plant polysaccharides. *Bacteroides ovatus* was below the level of detection ($<2 \times 10^{9}$). Work with ex-germ-free mice suggests that utilization of the host-derived polysaccharides may be less important than dietary sources for colonization, at least on standard mouse chow diets. In one study, *B. thetaiotaomicron* mutants lacking the ability to utilize chondroitin sulfate were not outcompeted by wildtype strains in the intestinal tract unless the mutations caused the inability to utilize *N*-acetylgalactosamine, one of the component sugars of chondroitin sulfate (Salyers et al., 1988). Further, there was no evidence for the expression of proteins in the chondroitin sulfate utilization pathway during growth of *B. thetaiotaomicron* in the intestine. Another study with *B. thetaiotaomicron* suggested that perhaps these organisms have maximized their ability to utilize small quantities of a variety of carbohydrate sources as a strategy for survival in the gut (Salyers and Pajeau, 1989). In this work, mutants lacking the ability to utilize starch or polygalacturonic acid were not outcompeted by the wild-type strains. In contrast, mutations causing a defect in utilization of the component sugars of these polysaccharides did in fact result in a colonization defect. The concept was supported by studies on the expression of disaccharidases from *B. ovatus* growing in the cecum of ex-germ-free mice (Valentine and Salyers, 1992). The authors found that disaccharidase activities were not induced as would be expected during growth on specific polysaccharides such as xylan or arabinogalactan. Rather, the level of activity of the four disaccharidases seemed to be low, suggesting that a mixture of substrates might be present in the environment. In other work with *B. ovatus*, mutants unable to utilize guar gum (galactomannan) or lacking α-galactosidase III were deficient in colonization of germ-free mice (Valentine et al., 1991). The authors interpreted these results as suggesting that the loss of even a minor substrate for *B. ovatus* was critical for its ability to compete. Overall, there is a paucity of good information on in vivo substrate utilization and this hinders sound interpretation of these mutant studies.

Protease Activity Nitrogen turnover in the colon is an efficient process with approximately similar amounts of nitrogen entering the tract from the ileum as are being excreted. Most of the nitrogen input (70–80%) is in the form of protein and peptides derived from the host or diet. By comparison, excreted nitrogen is more than 50% bacterial protein (Stephen and Cummings, 1980; Stephen, 1987; Chacko and Cummings, 1988). Thus there is a high degree of protein turnover in the colon, indicating that this must be a highly proteolytic environment. Although many of the species in the colon such as the clostridia are more proteolytic than the *Bacteroides* spp., the sheer numbers of the later species ensure them of a dominant role in protein degradation. Studies on colonic protein digestion are limited, but one study using an azocasein hydrolysis assay localized the majority of proteolytic activity to a particulate fraction containing either bacterial cells or activity tightly bound to particles (Macfarlane et al., 1986). The dominant proteolytic organisms in these samples were *Bacteroides* spp., which were present at about three orders of magnitude greater than other proteolytic genera such as *Propionibacterium*, *Clostridium* and *Enterococcus*. The protease activity of these organisms was cell bound and not measurably secreted into the medium. These results were verified using pure cultures of *B. fragilis* and *B. vulgatus*, which also had high levels of cell-bound protease activity. Similar studies with *B. thetaiotaomicron* revealed much

lower levels of protease activity (Macfarlane et al., 1986). In more detailed studies of a *B. fragilis* strain, it was found that three major proteases (34, 52 and 73 kDa) were produced and that these were largely membrane-associated, although one was released in the periplasm (Gibson and Macfarlane, 1988a). Inhibitor studies suggested that these proteases were a mixture of a serine, a cysteine, and a metallo-protease. The proteases had a limited substrate range with activity on casein, trypsin, chymotrypsin, azocasein but very little (or no) activity on collagen, azocoll, gelatin, bovine serum albumin, and elastin. The protease activity was constitutively expressed under a variety of growth conditions and throughout the growth cycle, but during stationary phase, the cells did release some activity into the supernatant (Gibson and Macfarlane, 1988b). Protease activity was optimal at alkaline pH, suggesting that it would be well suited for the descending colon where pH rises as a result of proteolytic activity of the gut microflora (Gibson and Macfarlane, 1988a). Nitrogen cycling in the large intestine goes beyond protein turnover, and the *Bacteroides* spp. have a variety of enzymes such as glutamate decarboxylase or deaminases that contribute to these functions, but there is little in the way of direct assessment of these activities in different bacterial groups.

Bile Acid Metabolism Another important metabolic activity of the colon is the transformation of bile acids and steroids. The primary bile acids, cholic and chenodeoxycholic acid conjugated on their carboxyl groups with either glycine or taurine, are synthesized in the liver from cholesterol and secreted into the small intestine. The enterohepatic circulatory system returns most of these compounds to the liver, but a sizable portion escapes to the colon, where they are immediately deconjugated (>80%), leaving the free bile acid present in the feces (Larson, 1988). The bile acid hydrolases (BAHs) are responsible for this activity, and they are produced by a number of intestinal anaerobes including *Bacteroides* spp. The enzyme activity has been found in *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus* and some strains of *B. distasonis* and *B. vulgatus* (Stellwag and Hylemon, 1976; Kawamoto et al., 1989). When BAH is found in these strains, it is always accompanied by a second transformation activity, 7-α-hydroxysteroid dehydrogenase, suggesting there may be some coordinate regulation of these two enzymes (Stellwag and Hylemon, 1976). While most reports indicate that the BAHs are constitutive, one report found that in *B. fragilis*, where BAH is periplasmic, the enzyme was produced in a growth-rate-dependent manner with significant induction during the stationary phase of growth (Hylemon and Stellwag, 1976). The enzymes (subunit mol wt of 32–36 kDa) do not seem to be highly oxygen sensitive and generally have a pH optimum in the acid range. Both taurine and glycine conjugates of the primary bile acids are rapidly hydrolyzed, but some of the conjugates of secondary bile acids were not hydrolyzed (Stellwag and Hylemon, 1976; Masuda, 1981; Larson, 1988).

As mentioned, several of the *Bacteroides* spp. have 7-α-hydroxysteroid dehydrogenase, which is required (in part) for epimerization of the 7-hydroxyl group. Several types of these enzymes differ with respect to their pyridine nucleotide specificity, but interestingly *B. thetaiotaomicron* and some strains of *B. fragilis* possess both NAD- and NADP-linked enzymes (MacDonald et al., 1975). In *B. fragilis*, enzyme activity was located in the cell membrane and cytoplasmic fractions, and in one report, it was concluded that the enzyme was expressed throughout the growth cycle (Hylemon and Sherrod, 1975; Hylemon and Stellwag, 1976). In contrast, both the NAD- and NADP-linked activities of *B. thetaiotaomicron* were coordinately induced in stationary phase. None of the *Bacteroides* spp. are known to possess the 7-β-hydroxysteroid dehydrogenase activity, so they are not responsible for the complete epimerization of the molecule. At the present time, the role of these transformations in the physiology of the *Bacteroides* is not clear. It has been suggested that the enzymes may play some role in energy metabolism, but no data support this idea.

Transformations of Toxic and Mutagenic Compounds The production of toxic compounds and fecal mutagens has been a topic of considerable interest due to their implied potential role in colon cancer. It has been known for some time that the intestinal microflora have the ability to transform substrates with possible toxicological consequences and that a mutagenic potential is associated with the microflora. The *Bacteroides* exhibit a wide array of activities that can participate in these transformations, and owing to their high numbers in the colon, it is likely they play a significant role. One class of hydrolytic enzymes that is important for many transformations are the glycosidases. The diet provides a number of plant glycosides, which when hydrolyzed release aglycones, some of which are toxic or mutagenic. In one recent report, it was shown that in samples obtained from colon contents, most of the glucosidase activities were associated with the bacterial fraction and that this activity increased in the distal colon (McBain and Macfarlane, 1998). Most

probable number estimates indicated that there were approximately 10^{11} bacteria per g of contents with glucosidase activity. Screening of strains representing the colonic microflora indicated that *B. fragilis*, *B. ovatus* and *B. vulgatus* displayed some of the highest glucosidase activities (McBain and Macfarlane, 1998). This is not surprising since as discussed above (see Metabolism), the *Bacteroides* spp. possess high levels of glycosidase activity with broad substrate specificities.

Glucuronides are formed in the liver where they are important for the detoxification of xenobiotic compounds. These xenobiotic conjugates are excreted in the bile and end up in the colon where levels of β-glucuronidase of microbial origin are high (Hawksworth et al., 1971; Larson, 1988). The hydrolysis of the glucuronides can result in the production of toxic compounds in the gut, from which they can be reabsorbed rather than excreted in the feces. Glucuronidase activity has been found throughout the colon but apparently is highest in the proximal colon (McBain and Macfarlane, 1998). When normal flora isolates are examined for β-glucuronidase activity, the *Bacteroides* spp. are well represented, although not all species or strains of a given species express this activity. Strains of *B. fragilis* and *B. vulgatus* were among the most active when using *p*-nitrophenyl-D-glucuronide as the substrate (Berg et al., 1980; McBain and Macfarlane, 1998), but examination of a *B. vulgatus* strain isolated from a Crohns disease patient revealed only small amounts of β-glucuronidase (Ruseler-van Embden et al., 1989). In another study ninety-seven β-glucuronidase-positive strains were isolated from feces, and the majority of these were *Bacteroides* spp., but the highest activities were found in *Peptostreptococcus* spp. and *Clostridium* spp. (Nanno et al., 1986). Further, it also was shown that the β-glucuronidase activity of the *Bacteroides* isolates could in fact release mutagenic metabolites from benzo(a)pyrene conjugates.

The *Bacteroides* are host to a variety of other enzymatic activities that might contribute to the toxic or mutagenic potential of feces including azo-reductases and nitro-reductases, but little data are available on the distribution of these enzymes (Chung et al., 1978; Kinouchi et al., 1982). However, several *Bacteroides* spp. have been directly implicated in production of fecal mutagens in the fecapentaene class. These are highly potent, 12 or 14 carbon glyceryl ether lipids with a conjugated pentaene chain, and they are strongly mutagenic in the Ames test (Gupta et al., 1983; Hirai et al., 1985). The actual precursor(s) of these compounds is not known, and not all people produce high levels of the precursor, but it is produced endogenously, requires anaerobic conditions, requires pH >6, and requires the presence of *Bacteroides* spp. (van Tassel et al., 1982; Hirai et al., 1985). In a survey of organisms capable of producing this activity, only *Bacteroides* spp. were active, and cells needed to be in stationary phase of the growth cycle (van Tassel et al., 1982). All of the *B. thetaiotaomicron* strains and 70% of *B. fragilis* were capable of producing the fecal mutagen, and several isolates of *B. ovatus* and *B. uniformis* also had the activity. Thus, there is no doubt that these mutagens are actively produced in the colon, but it is less clear whether they are clinically significant or linked to colon cancer.

Role of *Bacteroides* spp. in Development of the Intestinal Tract

It has been known for some time that when compared to conventional animals, the gastrointestinal tract of germ-free animals is markedly altered in its morphology and function (reviewed in Abrams, 1983). The cecum of germ-free mice is greatly enlarged and can account for 1/4 of the body weight. In contrast, the intestine of germ-free animals is smaller with about 15–30% less surface area and fewer villi, which are smaller in size (Gordon and Bruckner-Kardoss, 1961; Wells and Balish, 1980), and many histological differences including a poorly developed lamina propria and smaller Peyers patches that lack the characteristic germinal centers. Taken together, all of these changes result in a somewhat altered function of the tract, but association of the gastrointestinal tract with its indigenous microflora can reverse each of these changes (Abrams, 1983; Falk et al., 1998). In this regard, the analysis of intestinal epithelial sections from ex-germ-free rats monoassociated with *B. fragilis* revealed histological characteristics similar to those of conventional animals, including a thick lamina propria and normal villi (Wells and Balish, 1980).

Owing to their prominence in the normal flora, *Bacteroides* spp. are likely to be important for the normal development of the tract, but until recently, there was very little progress in deciphering the role of these or any organisms. A new model system in which genetically engineered *B. thetaiotaomicron* are used to colonize germ-free mice has been developed to elucidate specific interactions between the microbe and its host (Bry et al., 1996). Using this system, the normal pattern of production of fucosylated glycoconjugates on the surface of the intestinal epithelium was shown to be regulated at the level of transcription by *B. thetaiotaomicron* strains able to utilize fucose as a carbon source but not

by mutants disrupted in the fucose pathway (Hooper et al., 1999). It now appears that the microbe uses an inducer (FucR) to regulate its fucose utilization in response to the presence of the fucosylated glycoconjugates, and it also controls the synthesis of these via a FucR-regulated factor that modulates the synthesis of an α-1,2-fucosyltransferase in the intestinal cells. This coordinated expression program clearly benefits the microbe, but its importance to the host is less clear. The use of this model system was recently expanded in a study where transcription in intestinal epithelial cells was analyzed by DNA microarrays in response to colonization with *B. thetaiotaomicron* (Hooper et al., 2001). The results showed that a variety of mRNAs such as colipase, glutathione S-transferase, adenosine deaminase, angiogenin-3, lactase-phlorizin hydrolase, metallothionein I, and others were modulated by colonization. It was possible to determine that some of these genes were directly influenced by *B. thetaiotaomicron*, whereas others required different components of the microflora. Another study that examined the expression of matrilysin I, a matrix metalloprotease, concluded that induction of activity required colonization with *B. thetaiotaomicron* and that a soluble factor, not adherence to cells, was responsible for the appearance of matrilysin activity (Lopez-Boado et al., 2000). Overall, these new data support the idea that interactions between the gut flora and the gut are going to be exceedingly complex. Using these defined, genetically manipulable systems, it should be possible to learn more about the biochemical signals used to communicate between the host and microbe, allowing them to establish this exceptionally stable symbiosis.

Virulence and Pathogenesis

Association with Infection

The high frequency of *Bacteroides* spp. isolated from human infections underscores their pathogenic potential. These organisms account for approximately two thirds of all anaerobes isolated from clinical specimens. *Bacteroides fragilis* is a minor component of the species present in the human gut (generally <1% of the flora), but it accounts for about 50% of all anaerobes isolated from cases of intra-abdominal infections, infections of the female genital tract, deep wounds, and bacteremia (Finegold and George, 1989). The isolation of different *Bacteroides* spp. varies somewhat, but because antibiotic susceptibility patterns of the different species are heterogeneous, it is important to identify the species (Brook, 1989). In this regard, *B. fragilis* accounted for 63% of all *B. fragilis* group isolates, *B. thetaiotaomicron* for 14%, *B. vulgatus* and *B. ovatus* for 7% each, *B. distasonis* for 6%, and *B. uniformis* for 2%. The prevalence of *B. fragilis* in clinical specimens compared to the other members of the *Bacteroides* also was reviewed by Goldstein and Citron (1988). *Bacteroides fragilis* was the predominant species accounting for 45–69% of all *Bacteroides* isolated. *Bacteroides thetaiotaomicron* was the second most frequent isolate (8–20%), and the other species were the least frequently isolated (0–10%). Some recent data summarizing the distribution of *Bacteroides* spp. by site of infection are provided in Table 4. The virulence of *B. fragilis* is highlighted by its high frequency of recovery from blood cultures compared to other species of the genus. However, many factors must be considered when describing the pathogenic potential of an organism. For example, in cases of bacteremia, *B. fragilis* was isolated from more cases, though the mortality rate following *B. thetaiotaomicron* was higher (38%) compared to *B. fragilis* (24%; Brook, 1989). A controlled study to determine the true attributable mortality rate and mortality risk ratio found an excess of 19% attributable to *Bacteroides* spp. bacteremia. In addition, the presence of *Bacteroides* spp. bacteremia was independently associated with a nearly fivefold increase in relative risk of death (Redondo et al., 1995).

Bacteroides generally cause opportunistic infections that can occur any time the integrity of the mucosal wall of the intestine is compromised. Common predisposing conditions are gastrointestinal surgery, perforated or gangrenous appendicitis, perforated ulcer, diverticulitis, trauma and inflammatory bowel disease (Sheehan and Harding, 1989). Following inoculation of the site, a typical infection occurs in two phases. First is initiation, where the organisms need to adhere to a surface, resist clearing mechanisms and other host defenses, and withstand the increased oxygen tensions. Next, an abscess is formed where the organisms must be able to persist in an environment that is populated by numerous host cells and in which resources become limited owing to the metabolism of a large bacterial population. During the course of these two phases, *Bacteroides* spp. need to express a variety of different virulence factors. These will include cell surface structures such as capsular polysaccharide and lipopolysaccharide, adherence mechanisms, production of proteases, hydrolytic enzymes, neuraminidase, ability to scavenge iron from the host, production of enterotoxin in certain strains, and the ability to avoid host defense mechanisms. The specific *Bacteroides* virulence factors will be discussed next in the section Virulence Mechanisms; how-

Table 4. Distribution of the *Bacteroides fragilis* group species isolated from clinical specimens.

Sites of infection	Number of isolates	Percentage of total						References
		B. fragilis	*B. thetaiotaomicron*	*B. ovatus*	*B. vulgatus*	*B. distasonis*	*B. uniformis*	
Blood	165	78	14	4	3	2	0	Brook, 1988
	163	71	17	4	5	2	0	Cuchural et al., 1988
Abdomen	236	59	14	9	8	9	1	Brook, 1988
	408	57	19	12	5	6	0	Cuchural et al., 1988
Abscess or wound	506	66	15	7	4	5	2	Brook, 1988
	112	66	16	7	8	3	0	Cuchural et al., 1988
Pelvic	183	56	7	5	20	10	2	Brook, 1988
	69	61	20	6	12	1	0	Cuchural et al., 1988
Bile	22	41	18	18	9	9	5	Brook, 1988
	15	80	7	6	6	0	0	Cuchural et al., 1988
Bone	3	100	0	0	0	0	0	Brook, 1988
	22	67	19	14	0	0	0	Cuchural et al., 1988
Skin and soft tissue	280	64	19	10	2	3	0	Cuchural et al., 1988
Sinuses	4	50	25	0	25	0	0	Brook, 1988
Tumors	16	75	19	6	0	0	0	Brook, 1988
Perinatal	40	37	13	3	33	13	0	Cuchural et al., 1988
Lower respiratory tract	10	70	0	0	20	10	0	Cuchural et al., 1988
Drainage	28	77	7	9	7	0	0	Cuchural et al., 1988

ever, the reader should bear in mind that many of these factors reflect work done with *B. fragilis*, since this is the most pathogenic species and the majority of work on pathogenesis has focused on this organism.

Virulence Mechanisms

Capsular Polysaccharides The capsular polysaccharide complex (CPC) is a major factor associated with virulence and pathogenicity. For example, encapsulated strains were demonstrated to be more virulent than nonencapsulated strains by their higher rate of induction of bacteremia and greater enhancement of growth compared to other bacteria in mixed infections (Brook, 1989). An abscess is the hallmark of a *Bacteroides* infection, and formation of an abscess is a pathological response of the immune system to the presence of the *B. fragilis* CPC. *Bacteroides fragilis* is the only bacterium that has been shown to induce abscess formation when present as the sole infecting organism (Onderdonk et al., 1977). Using an experimental abscess model, it has been shown that implantation of cell-free CPC into the peritoneal cavities of rats induced formation of intra-abdominal abscesses. Most bacterial polysaccharides stimulate an antibody-mediated response, but *B. fragilis* capsule stimulates a T-cell mediated response (Shapiro et al., 1986).

CPC is involved in the abscess formation in several ways. The first is adherence, which is presumed to increase resistance to peritoneal clearance. The ability of *B. fragilis* and other *Bacteroides* to adhere to rat peritoneal mesothelium was shown to be due to the presence of capsular polysaccharide, while nonencapsulated species were less adherent (Onderdonk et al., 1978). In experimental intra-abdominal infections, the adherence of *B. fragilis* group species to peritoneal mesothelium was an advantage allowing it to outcompete other intestinal species following fecal contamination (Edmiston et al., 1990). However, immunization with capsular polysaccharide failed to reduce adherence (Onderdonk et al., 1977). In contrast, purified capsular polysaccharide alone inhibited *B. fragilis* adherence to rat peritoneal mesothelium (Onderdonk et al., 1978). As can be seen by these sometimes conflicting reports, much is still

not understood about the role of capsule, but clearly it is a major virulence factor that contributes to all phases of the infections. The second role of the *B. fragilis* CPC is its direct effect on immune system cells which results in secretion of cytokines. In vitro murine resident peritoneal cells and human peritoneal blood leukocytes respond to *B. fragilis* CPC by increased production of tumor necrosis factor alpha (TNF-α) and interleukin-8 (IL-8), respectively. This leads to recruitment of polymorphonuclear leukocytes (PMNs) and significant upregulation of intercellular adhesion molecule-1 (ICAM-1) on the mesothelial cells in the location of the adherent bacteria. This leads to accumulation of PMNs and initiation of abscess formation (Gibson et al., 1996; Gibson et al., 1998). Finally, CPC can aid in phagocytosis inhibition (Simon et al., 1982).

Immunochemical studies revealed that the surface polysaccharides of *B. fragilis* populations are antigenically heterogeneous. The antigenic composition varies within a bacterial population apparently by switching the expression of epitopes (Lutton et al., 1991; Patrick et al., 1999). This antigenic variation is presumed to be relevant to the virulence of *B. fragilis* (Lutton et al., 1991). The nature of the *B. fragilis* capsular polysaccharide complex has been elucidated and is composed of distinct polysaccharide structures containing a zwitterionic motif of repeated units of positively and negatively charged groups (Tzianabos et al., 1993). It was demonstrated that the presence of these charged groups was essential to induce intra-abdominal abscess in a rat model (Tzianabos et al., 1993). Using a combination of mutational and genome analysis (Krinos et al., 2001), it was shown that *B. fragilis* produces eight distinct polysaccharides designated PSA, PSB, PSC, PSD, PSE, PSF, PSG, and PSH. These eight capsule loci utilize an unprecedented amount of bacterial genome with about 133 kb dedicated to the synthesis of capsular polysaccharides. This, in fact, comprises about 2.5% of the whole *B. fragilis* genome. Each of these capsule loci is regulated independently by an ON-OFF phase variation mechanism, which results in the expression of different combinations of the eight polysaccharides within the same population, yielding mixed phenotypes (Krinos et al., 2001). This regulation involves the inversion of each individual transcription promoter regulatory region, resulting in an ON-OFF switch for each locus. The complex phase-variation control and the exorbitant number of capsule polysaccharides demonstrate the importance of polysaccharide surface diversity with implications for the physiology, ecology and virulence of this microorganism (Krinos et al., 2001).

Studies have demonstrated that PSA seems to have the essential composition for intra-abdominal abscess formation in animals, since a PSA mutant had a drastic reduction in abscess-inducing capability compared to parent strain (Coyne et al., 2001). In this regard, PSA is conserved in all serotypes tested, and this is consistent with the ability to induce abscess. Purified PSB alone is able to induce an abscess in vivo, but disruption of the PSB or PSC operons did not abolish abscess formation compared to wildtype strain (Comstock et al., 1999; Coyne et al., 2000).

Adherence The ability of a microorganism to adhere to host tissues is an important initial step in the colonization and multiplication in a host. The adhesive properties of *B. fragilis* are considered to be an important component of its virulence mechanism. It has been shown that adherence to a host cell is mediated by surface structures such as the capsular polysaccharide or pilus (fimbriae). *Bacteroides fragilis* isolates from abscesses and blood have a strong hemagglutination activity compared to strains isolated from nonclinical strains (Guzman et al., 1997). Some studies have shown that the increased ability of *B. fragilis* to adhere to laminin in vitro might be an advantage when compared to other potentially infecting species, and this could be part of the reason why *B. fragilis* is so successful as a pathogen (Eiring et al., 1995).

Pili- or fimbriae-like structures have been associated with *B. fragilis* adherence to epithelial cells and with red blood cell hemagglutination (Pruzzo et al., 1989; Brook and Myhal, 1991); however, other studies have demonstrated that adherence to red blood cells can be mediated by either capsule or pili-like structures (Guzman et al., 1997). Perhaps both structures act together to potentiate hemagglutination activity, since elimination of either pili or capsule reduces but does not abolish adherence (Guzman et al., 1997). Adherence of piliated and encapsulated strains of *B. fragilis* and *B. ovatus* to intestinal cells and mucus was shown to be at least five times greater than adherence of their nonpiliated or nonencapsulated counterparts (Brook and Myhal, 1991). Nevertheless, reports conflict regarding the role of fimbriae in *B. fragilis*. The studies of Oyston and Handley (1991) have shown no correlation between fimbriae and hemagglutination, even though fimbriae were actually isolated from the *B. fragilis* strains used in this study (van Doorn et al., 1992).

Another factor that is still poorly understood is the production of soluble lectin-like adhesins by strains of *Bacteroides* spp. (Rogemond and Guinet, 1986). A 70-kDa lectin with affinity for

∀-D-glucosamine and D-galactosamine was purified and shown to promote formation of micro-aggregates between *B. fragilis* and *Enterococcus faecalis* or *E. coli* (Rogemond and Guinet, 1986). This may have implications in the pathogenesis of synergistic polymicrobial infections allowing adhesion to host cells and aggregation to each other. These organisms are frequently found in mixed infections, and aggregation has been demonstrated between *B. fragilis* and *E. coli* (Reid et al., 1990).

Lipopolysaccharide and Other Surface Structures The *B. fragilis* lipopolysaccharide (LPS or endotoxin) is approximately 100–1000-fold less toxic than the potent LPS from *E. coli* and *Salmonella typhimurium* (Lindberg et al., 1990). Nevertheless, studies have suggested that LPS may be considered as a virulence factor (Zalesnik et al., 1986; Poxton and Edmund, 1995), since it is able to provoke abscess formation in experimental animal models. However, the dose of LPS required is about 5 times higher than the dose required for purified capsular polysaccharide, suggesting the possibility of some minor contamination of the LPS with CPC (Zalesnik et al., 1986). *Bacteroides fragilis* LPS induces secretion of the proinflammatory cytokine TNF-α through a CD_{14}-independent pathway (Gangloff et al., 1999), but the amount of *B. fragilis* LPS required to stimulate half-maximal TNF-α secretion is at least 1,500-fold higher than the amount of LPS from an enteric organism needed to reach the same levels of TNF-α expression (Gangloff et al., 1999). The weak endotoxic activity of *B. fragilis* is attributed to differences in its structural and chemical composition compared to typical Enterobacteriaceae LPS. *Bacteroides fragilis* LPS lacks classical O-antigen structures, has reduced acyl chains (5 instead of 6), has only one phosphorylated 2-keto-3-deoxyoctulosonic acid (KDO) moiety, and has shortened carbohydrate chains (Lindberg et al., 1990; Gangloff et al., 1999).

The release of outer-membrane vesicles (OMV) from *B. fragilis* also is presumed to play a role in the pathogenesis of *B. fragilis*. The OMV are released from the surface of whole cells of *B. fragilis* and carry a variety of hydrolyzing enzymes such as alkaline and acid phosphatases, esterase lipase, phosphohydrolyase, glucosaminidase, β-glucuronidase and α- and β-galactosidase (Patrick et al., 1996). These OMV are able to agglutinate horse and human erythrocytes, and this property may be used to deliver enzymes directly to host cells with the objective of increasing tissue damage. In addition, the *B. fragilis* OM contains an unidentified component with pro-inflammatory activity. It promotes neutrophil adherence to endothelium in vitro by inducing the expression of E-selectin on human endothelial cells by a soluble CD_{14}-dependent mechanism (Sato et al., 1998).

Neuraminidase The role of neuraminidase in the attachment of *B. fragilis* to host cells is controversial. Some studies have shown that hemagglutination and adherence to colon WiDr cells were not mediated by neuraminidase activity (Namavar et al., 1994). In contrast, other studies have shown that *B. fragilis* adheres to mammalian epithelial cells but not to PMNs by the action of an adhesin in a neuraminidase dose-dependent attachment (Guzman et al., 1990). Though this matter needs further clarification, the role of neuraminidase (NanH) in *B. fragilis* pathogenicity in vivo was investigated with *nanH* mutants. The findings have shown that the *nanH* mutant had a severe growth defect compared to the parent strain in a rat pouch model and in a tissue culture monolayer (Chinese hamster ovary [CHO] cells) model (Godoy et al., 1993). In the rat pouch experiments, >90% of the *nanH* mutants reverted to $nanH^+$ genotype within 48 h postinfection as glucose concentrations fell within the pouch during bacterial growth. This selective pressure resulting in the $nanH^+$ strains indicates that neuraminidase may play an important role in acquisition of carbon sources for bacterial cell growth in vivo (Godoy et al., 1993). Thus there may be two functions for neuraminidase during the course of infection, one for adherence and one for energy metabolism.

Iron Acquisition from Host Tissues The ability of pathogenic bacteria to scavenge iron in vivo from host tissues is an important virulence mechanism. Iron in mammalian tissues is tightly bound to proteins such as hemoglobin, transferrin or ferritin. Consequently, many aerobic bacteria and pathogens, in particular, will not grow in the presence of these proteins unless they possess specific iron-acquisition mechanisms. Although these mechanisms have been extensively investigated in aerobic bacteria, iron uptake systems in *B. fragilis* have been largely overlooked. Much of the iron requirement may be obtained from hemin; however, elemental iron is also required for growth even in the presence of hemin (Rocha et al., 1991). Apparently *B. fragilis* does not take up iron through the production of siderophores (Otto et al., 1988), but it can utilize iron from iron-bound transferrin, heme, hemoglobin and haptoglobin-hemoglobin complex (Verweij-van Vught et al., 1988; Otto et al., 1994). Furthermore, *B. fragilis* is able to overcome hemopexin-mediated heme limitation

by degradation of hemopexin, a heme-binding plasma protein, to make the protein-bound heme available for uptake (Rocha et al., 2001). This ability may enable *B. fragilis* to overcome the inhibitory effect of serum, which is commonly seen with many of the other *Bacteroides* species and can be overcome by the addition of hemin or protoporphyrin IX plus iron (Verweij-van Vught et al., 1986b). *Bacteroides fragilis* is better adapted to survive in low iron conditions than the less virulent species of the genus, and its enhanced virulence may be related to its ability to grow in serum with low free iron. The mechanisms of iron acquisition have not yet been worked out, but under iron-limited conditions, *B. fragilis* expresses four iron-repressible outer-membrane proteins (IROMP) of 89, 49, 44 and 23.5 kDa (Otto et al., 1988). It has been demonstrated that the 44-kDa IROMP is involved in the heme acquisition and is expressed in vivo (Otto et al., 1991). Other IROMP may be involved with other aspects of iron and/or heme uptake.

Enterotoxin Enterotoxigenic strains of *B. fragilis* (ETBF) are associated with diarrhea in humans and animals (Border et al., 1985; Myers and Shoop, 1987; Myers et al., 1989). Epidemiological studies have demonstrated that the presence of ETBF is significantly higher in adults and children with diarrhea (but with no other known etiological agents) when compared to the numbers of ETBF isolated from healthy controls (Kato et al., 1996; Zhang et al., 1999). The purified *B. fragilis* toxin (BFT) is a 20,000-Da zinc-containing metalloprotease capable of hydrolyzing gelatin, actin, tropomyosin and fibrinogen (Moncrief et al., 1995). Several studies have demonstrated that BFT induces physiological and morphological changes in intestinal epithelial cells both in vitro and in vivo leading to diarrhea and inflammation of the mucosa (Myers et al., 1989; Obiso et al., 1995; Saidi et al., 1997; Sanfilippo et al., 1998; Riegler et al., 1999). Three isoforms of the toxin gene have been described: *bft-1*, *bft-2* and *bft-3* (Chung et al., 1999a). Studies on the frequency of distribution of the genes have shown that the isoform *bft-1* is most common among adults with or without diarrhea, while *bft-1* and *bft-2* are equally distributed among children (D'Abusco et al., 2000). Interestingly, ETBF strains produce only one toxin, even though more than one *bft* gene may be present in the chromosome. As described in the section Genome Structure, molecular analysis has shown that a *bft* gene and a second metalloprotease gene are contained in a 6-kb DNA fragment, denoted as a pathogenicity island, which is present in all ETBF strains (Moncrief et al., 1998; Franco et al., 1999). In some cases, duplication of the whole pathogenicity island has been documented; however, this does not seem to affect expression of the *bft* genes.

The proposed mechanism of action for BFT is unique for a bacterial enterotoxin. BFT specifically cleaves the extracellular domain of the zonula adherens protein E-cadherin, altering cellular morphology and physiology and leading to increased intestinal permeability (Wu et al., 1998). However, the intestinal epithelial cells affected by BFT also seem to influence the modulation of the intestinal mucosal inflammatory response and tissue repair by inducing expression of the cytokines IL-8 and transforming growth factor beta (TGF-β), respectively (Sanfilippo et al., 2000; Kim et al., 2001).

The role of BFT is not clear, but studies comparing the frequencies of enterotoxigenic and nonenterotoxigenic strains isolated from various nonintestinal infection sites have found enterotoxigenic strains at higher frequencies in bacteremia (Kato et al., 1996; Chung et al., 1999a). It has been postulated that BFT facilitates the release of *B. fragilis* from the abscesses or other sites of infection, allowing them to disseminate into the bloodstream (Kato et al., 1996).

Proteases and Hydrolytic Enzymes The production of extracellular enzymes by pathogenic anaerobic bacteria is important for tissue damage during the course of an infection to supply nutrients for growth (Duerden, 1994). These enzymes also may contribute to the metabolic interdependency of *B. fragilis* and facultative species in polymicrobial infections (Duerden, 1994). *Bacteroides* spp. produce several enzymes such as hyaluronidase, heparinase, chondroitin sulfatase, phosphorylase, DNase, and fibrinogen-degrading protease (Rudek and Haque, 1976; Stefen and Hentges, 1981). Apparently these may contribute to the establishment and development of the infectious process, but very little experimental evidence exists to support their significance in *Bacteroides* spp. infections.

Inhibition of Neutrophil Function It is well established that in vitro anaerobic bacteria inhibit the phagocytic killing function of neutrophils. The phagocytic uptake and killing of several aerobic bacteria such as *E. coli* and *Proteus* sp. is diminished by the ability of *Bacteroides* species to compete for opsonins (Ingham et al., 1981; Jones and Gemmell, 1982; Namavar et al., 1983). Both heat-stable and heat-labile opsonins are depleted from serum incubated with *Bacteroides* species. This results in impairment of opsonization of the aerobic bacteria (Tofte et al.,

1980). Another mechanism of PMN inhibition may be mediated by the production of fermentation end products. It has been shown that short chain fatty acids inhibit PMN chemotaxis and activation of the respiratory burst function (Botta et al., 1985; Rotstein et al., 1985). Several studies have shown that the major product of *Bacteroides* fermentative metabolism, succinate, inhibits neutrophil migration and killing activity at acid pH but not neutral pH (Rotstein et al., 1985; Rotstein and Kao, 1988a). At an extracellular pH of 5.5, succinic acid is protonated, and in this form, it is able to cross the neutrophil plasma membrane and consequently lower the intracellular pH causing impairment of enzyme activity, leading to neutrophil dysfunction (Rotstein and Kao, 1988a; Rotstein et al., 1988b). More recently, Stehle et al. (2001) have shown that succinic acid at high concentrations (30 mM) significantly accelerates PMN apoptosis in vitro.

Synergy in Polymicrobial Infections

Intra-abdominal infections usually occur when gastrointestinal- or genitourinary-tract microorganisms enter into the peritoneal space as the result of a breach in the mucosal barrier (Johnson et al., 1997). The majority of intra-abdominal infections are polymicrobial associations containing facultative and anaerobic organisms, mainly *Bacteroides fragilis* and *E. coli*, as the predominant pathogens (Lorber and Swenson, 1975; Brook and Frazier, 2000). The importance of *Bacteroides* spp., particularly *B. fragilis* and *B. thetaiotaomicron*, in polymicrobial infections of abdominal, pelvic, skin and soft tissue areas was shown in a report from Brook (1989). The specific virulence factors that are involved in the pathogenesis of these mixed infections are dependent on the combination of species present at the site and the interactions of the different virulence factors of these species (Duerden, 1994). Some of these interactions have been described and are discussed next.

Bacterial synergy between anaerobic and aerobic bacteria has been demonstrated experimentally in animal models. The results have shown that this synergistic association increases mortality and abscess formation (Onderdonk et al., 1976; Dunn et al., 1984; Verweij-van Vught et al., 1986a; Rotstein and Kao, 1988a). Although the mechanisms involved in the interactions are not completely understood, several factors are known to play significant roles in the overall outcome. These factors include

1) The ability of *E. coli* to reduce intraperitoneal pO_2 and pH, creating favorable redox conditions that allow anaerobic growth and promote abscess formation (Sawyer et al., 1991). Extracellular acid pH and low oxygen tension have also been shown to alter leukocyte-killing mechanisms (Park et al., 1992). Moreover, low pH potentiates the inhibition of neutrophil functions by *B. fragilis* metabolic by-product succinate (Rotstein et al., 1988b)

2) *Bacteroides fragilis* inhibits phagocytosis and killing of *E. coli* by PMN. *Bacteroides fragilis* efficiently competes for opsonins and reduces aerobic bacteria opsonization and uptake by PMNs (Tofte et al., 1980; Jones and Gemmell, 1982)

3) Production of hemolysin by *E. coli* alters the host's ability to eliminate *E. coli* itself and other bacteria such as *B. fragilis* in experimental peritonitis (May et al., 2000)

4) Nutritional interactions in which the *E. coli* hemolysin and hemoglobin protease were shown to increase synergy of abscess formation in experimental models possibly by facilitating *B. fragilis* access to essential nutrients such as heme-iron from hemoglobin (May et al., 2000; Otto et al., 2002), and

5) The ability of *Bacteroides* species to induce local fibrin deposition impairs clearance of the bacteria in polymicrobial infections (Rotstein, 1993).

Antibiotic Susceptibility and Resistance

Susceptibility Profiles

The *Bacteroides* spp. are highly drug resistant owing to a combination of inherent resistance traits and acquired resistance determinants. For example, inherent resistance to aminoglycosides is nearly universal among anaerobic bacteria because of the inability to transport these drugs into the cell (Bryan et al., 1979) and because resistance to tetracycline, a front-line drug in the 1960s, has now reached >80% of isolates, due primarily to the dissemination of the *tetQ* gene (Salyers et al., 1995b; Shoemaker et al., 2001). Antimicrobial therapy plays an important role in the treatment of infections caused by *Bacteroides* spp.; therefore, susceptibility patterns have been watched closely to monitor the emergence of resistant strains. Summarized in Table 5 are the results from several antibiotic-resistance surveys of recent isolates. Over the years, metronidazole and chloramphenicol have remained effective antibiotics against the *Bacteroides*, with the finding of only an occasional chloramphenicol resistant strain and absence of metronidazole resistance in the United States (although occasional resistance has been noted on the European and Asian continents; Sebald et al., 1990). In contrast, *Bacteroides* spp. are resistant to most

Table 5. Summary of several susceptibility studies for *Bacteroides* spp. isolated between 1995 and 1999.

						MIC_{90}/strains (% resistant)[a]							
Organism	Pene	Cfxime	Cfox	Im	Mer	Pip	Pip + taz	Amp + sul	Cipro	Trov	Clin	Met	Cm
B. fragilis group													
Am1	>32,100	nd	16/8	.25/.2	.5/.2	nd	4/0	16/11	32/91	2/7	>16/29	1/0	nd
Am2	nd	128/18	32/5	1/0	1/0	128/17	8/.2	8/0.8	nd	4/7.3	256/16	nd	nd
Sp1	nd	32/7	64/13	1/0.5	1/0.5	256/23	16/0.5	8/0.5	nd	1/1.8	>256/34	1/0	4/0
Sp2	nd	128/11	32/8	0.5/0.9	nd	128/14	16/0.7	8/1	nd	nd	>256/26	1/0	4/0.1
SA	nd	nd	32/32	1/0	4/0	>128/39	nd	Nd	128/94	2/10	>128/29	2/0	4/0
						Strains (% resistant)							
B. fragilis													
Am1	100	nd	8	0.2	1	nd	0	11	90	7	29	0	nd
Am2	nd	20	5	0.2	0.2	21	0	1	nd	6	14	nd	nd
Sp1	nd	0.8	9	0.8	0.8	14	0.8	0.8	nd	1.5	33	0	0
Sp2	nd	7	5	1		8	1	2	nd	nd	22	0	0
B. thetaiotaomomicron													
Am1	100	nd	10	0	0	nd	0	10	93	1	23	0	nd
Am2	nd	21	5	0	0	20	0	2	nd	4	20	nd	nd
Sp1	nd	18	28	0	0	44	0	0	nd	0	36	0	0
Sp2	nd	14	10	0	nd	22	0	0	nd	nd	35	0	0
B. uniformis													
Am1	100	nd	29	0	5	nd	0	14	86	9	24	0	nd
Sp1	nd	12	0	0	0	23	0	0	nd	0	39	0	0
Sp2	nd	10	16	0	nd	13	0	0	nd	nd	46	0	0
B. vulgatus													
Am1	100	nd	9	0	3	nd	0	12	94	24	30	0	nd
Am2	nd	5	2	0	0	17	0	0	nd	14	27	nd	nd
Sp2	nd	7	0	0	nd	19	0	0	nd	nd	28	0	0
B. ovatus													
Am1	0	nd	5	0	0	nd	0	0	100	7	39	0	nd
Am2	nd	24	14	0	0	26	0	1	nd	6	21	nd	nd
Sp2	nd	24	13	0	nd	28	0	0	nd	nd	19	0	2
B. distasonis													
Am1	100	nd	11	0	0	nd	0	33	93	4	41	0	nd
Am2	nd	17	14	0	0	21	0	2	nd	1	11	nd	nd
Sp2	nd	27	24	0	nd	34	0	2	nd	nd	22	0	0

Abbreviations: MIC_{90}, minimal inhibitory concentation for 90% of strains tested; nd, no data; Pen, penicillin; Cfxime, ceftizoxime; Cfox, cefoxitin; Im, imipenem; Mer, meropenem; Pip, piperacillin; Pip + taz, piperacillin + tazobactam; Amp + sul, ampicillin + sulbactam; Cipro, ciprofloxacin; Trov, trovafloxacin; Clin, clindamycin; Met, metronidazole; and Cm, chloramphenicol.

[a]The MIC_{90} values are given in μg per ml and the % resistance refers to breakpoints of resistant strains recommended by the National Committee for Clinical Laboratory Standards (NCCLS). For the "*B. fragilis* group," values for the MIC_{90} and the % resistant strains are provided. The values given for the individual species are for % resistant strains only.

Data were from the following studies: Am1 (Aldridge et al., 2001), Am2 (Snydman et al., 1999), Spa (Betriu et al., 1999a), Sp2 (Betriu et al., 1999b), and SA (Lubbe et al., 1999).

cephalosporins and penicillins owing to the production of a variety of β-lactamases (Edwards, 1997). One exception is cefoxitin, which has shown good activity on all *Bacteroides* spp., although resistance rates up to 16% have been observed (Cuchural and Tally, 1986; Cornick et al., 1990). The carbapenems and the β-lactam + inhibitor combinations have retained outstanding activity and are the best of the β-lactam group (Table 5), but β-lactamases that destroy these drugs have been described. For the most part, the quinolone family of antibiotics has poor activity on *Bacteroides* spp. except for trovafloxacin, but significant resistance to this newer quinolone has been observed. Although the lincosamide, clindamycin, has been a very effective drug, resistance rates have risen dramatically reducing the utility of this drug in many hospitals.

Except for its resistance to imipenem, *B. fragilis* seems to be somewhat more antibiotic sensitive than other species or the genus as a whole. *Bacteroides thetaiotaomicron* and *B. ovatus* species generally are the most resistant and display the highest mean resistance rates for all antibiotics, but especially the β-lactam drugs (Snydman et al., 1999; Aldridge et al., 2001). On the other hand, high-level resistance

to carbapenems has been restricted primarily to *B. fragilis* isolates, which contain the *cfiA* β-lactamase gene and will be discussed in the section Mechanisms of Resistance. Resistance rates to clindamycin and trovafloxacin are highest among *B. vulgatus* strains (Snydman et al., 1999). No other trends have been noted, although one study suggested that with the exception of its resistance to clindamycin, the antibiotic resistance of *Bacteroides* spp. has decreased slightly (Snydman et al., 1999). It remains to be seen whether this is a sustainable trend.

Mechanisms of Resistance

Resistance to all major classes of antibiotics has been observed in the *Bacteroides* spp., and the physiological mechanisms of resistance cover the full array of mechanisms from target site alteration to antibiotic destruction. Clindamycin resistance and other MLS resistance are generally mediated by genes closely related to *ermF*, but recently two other MLS genes, *ermG* and *ermB*, have been found in *Bacteroides* spp. (Shoemaker et al., 2001; Table 6). These genes encode methyltransferase enzymes that methylate specific adenine residues on the 23S rRNA to inhibit binding of the drug to the ribosome target. This mechanism provides cross-resistance to most all macrolide, lincosamide, and streptogramin B type drugs. The *ermF* genes were first found on transmissible plasmids, and on these plasmids, they were present within three related compound transposons (Odelson et al., 1987; Smith et al., 1998; Table 6). The transposons each contained copies of IS*4351* in direct orientation that were adjacent to the *ermF* gene and responsible for its transcription. Tn*4351* and Tn*4400* were nearly identical, but Tn*4551* was twice the size and encoded a different set of genes downstream of *ermF*. The *ermF* genes are not closely related to any other *erm* genes and in fact have at most only 35% identity at the amino acid level. The *ermF* genes have been mainly found in different *Bacteroides* spp. and seem to be the major resistance gene in this genus. Recently, the *ermG* gene was found on a CTn, and in contrast to *ermF*, it was nearly identical to an MLS gene from *Bacillus sphaericus*, suggesting that it was a recent acquisition by the *Bacteroides*. Between 0 and 21% of recent MLS isolates (since 1996) had *ermG* (Cooper et al., 1996; Shoemaker et al., 2001). The *ermB* gene originally isolated from *Clostridium perfringens* also has been found in recent *Bacteroides* spp. isolates, indicating that the spread of MLS resistance continues to be a very dynamic process.

The most important form of β-lactam resistance continues to be the production of β-lactamase enzymes. Since 1955, the *Bacteroides* have been known for their production of penicillin inactivating enzymes, β-lactamases, and now nearly 100% of isolates tested have some measurable β-lactamase activity (Edwards, 1997; Rasmussen et al., 1997). By far the largest majority of these enzymes fall into the Bush class 2e, which are serine active-site cephalosporinases that are inhibited by clavulanate (Rasmussen et al., 1997). Each of the *Bacteroides* species has a specific or indigenous β-lactamase of this class. In the two cases where the β-lactamase genes have been sequenced (*B. fragilis cepA* and *B. uniformis cblA*), it was found that they are in the Ambler molecular Class A (Rogers et al., 1993; Smith et al., 1994; Mastrantonio et al., 1996). None of these enzymes can degrade cefoxitin, and the strains are sensitive to carbapenems as well. Although most species produce low levels of β-lactamase activity, in some cases the enzyme levels are upregulated and this results in resistance to all classes of β-lactam except for carbapenems, cephamycins, and inhibitor combinations (Rogers et al., 1994). For the *cepA* gene of *B. fragilis*, this activation resulted from the insertion of an IS element into the –35 region of the *cepA* promoter and resulted in high levels of *cepA* transcription. None of these species-specific β-lactamases are known to be transmissible. One unique class 2e β-lactamase was encoded by the *cfxA* gene and was found to be responsible for widespread, transmissible, cefoxitin resistance in a variety of *Bacteroides* spp. including *B. fragilis*, *B. vulgatus*, *B. uniformis* and *B. ovatus* (Parker and Smith, 1993). The CfxA β-lactamase slowly degraded cefoxitin but was otherwise typical of the Bush class 2e. Several other class 2e type enzymes have been described for the genus, but these have not yet been analyzed at the molecular level (Bush et al., 1995).

The other significant class of β-lactamases in the *Bacteroides*, the metallo-β-lactamases (which are in the Bush class 3), is responsible for imipenem resistance (Bush et al., 1995; Edwards, 1997). These are significant enzymes capable of hydrolyzing nearly all β-lactams including carbapenems and cephamycins, and they are not inhibited by clavulanate or sulbactam. DNA sequence analysis has shown that the *cfiA* gene or closely related homologs encode this potent β-lactamase, but it is only found in about 2% of *B. fragilis* clinical isolates, and in more than 60% of these strains, the gene is not expressed (Rasmussen et al., 1990; Thompson and Malamy, 1990; Podglajen et al., 1992; Podglajen et al., 2001). Strains that are imipenem resistant contain *cfiA*, which is transcriptionally activated by any one of several IS elements and is able to produce significant levels of the enzyme (Rasmussen and Kovacs, 1991; Podglajen et al., 1992; Podglajen et al., 1994; Edwards and Read,

Table 6. Antibiotic resistance determinants in *Bacteroides* spp.

R-determinant	Size (bp)	GenBank accession number	Function or activity	IS-activation	Transposon/ plasmid	Transmissible[a]
MLS						
ermF[b](*ermFS*,[c] *ermFU*)[d]	1,002	M14730	Methyltransferase	IS*4351*	Tn*4351*/pBF4	Yes
				IS*4351*	Tn*4400*/pBFTM10	Yes
				IS*4351*	Tn*4551*/pBI136	Yes
				ND	CTn5030	Yes
				ND	CTn*DOT*	Yes
ermG[e]	735	L42817	Methyltransferase	ND	CTn*7853*	Yes
ermB[f]	738	NR	Methyltransferase	ND	Unk	Unk
β-lactam						
cepA[g]	903	U05888	Bush class 2e β-lactamase	IS*1224*	ND	ND
cblA[h]	891	L08472	Bush class 2e β-lactamase	ND	ND	ND
cfxA[i]	966	U75371	Bush class 3 metallo-β-lactamase	ND	Tn*4555*	Yes
cfiA (*ccrA*)[j]	750	M34831		IS*1186*	ND	ND
				IS*1187*	ND	ND
				IS*1188*	ND	ND
				IS*942*	ND	ND
				IS*4351*	ND	ND
Tetracycline						
tetQ[k]	1,926	X58717	Elongation factor EF-G homolog	ND	CTn*DOT*	Yes
tetX[l]	1,167	M37699	NADP oxidoreductase	ND	Tn*4351*/pBF4	Yes
				ND	Tn*4400*/pBFTM10	Yes
				ND	CTn*DOT*	Yes
5-Nitroimidazole						
nimA[m]	531	X71444	5-Nitroimidazole reductase	IS*1168* (IS*1186*)	ND/pIP417	Yes
nimB[m]	495	X71443	5-Nitroimidazole reductase	IS*1168* (IS*1186*)	ND	Yes
nimC[n]	492	X76948	5-Nitroimidazole reductase	IS*1170* (IS*942*)	ND/pIP419	Yes
nimD[n]	493	X76949	5-Nitroimidazole reductase	IS*1168* (IS*1186*)	ND/pIP421	Yes
Quinolone						
gyrA[o]	2,538	AB017712	DNA gyrase	ND	ND	ND
bexA[p]	1,332	AB067769	MATE efflux pump	ND	ND	ND
Lincosamide						
linA[q]	513	AF251288	*O*-nucleotidyltransferase	ND	NBU2	Yes
Aminoglycoside						
aadS[r]	864	M72415	Adenyltransferase	ND	Tn4551/pBI136	Yes
				ND	CTn*DOT*	Yes

Abbreviations: MLS, macrolide-lincosamide-streptogramin; IS, insertion sequence; NR, not reported; ND, not detected; Unk, studies not done; and MATE, multidrug and toxic compound extrusion.

[a]Either self-transmissible or mobilizable by conjugation.

[b]Rasmussen et al. (1986).

[c]Smith (1987).

[d]Halulam et al. (1991).

[e]Cooper et al. (1996).

[f]Shoemaker et al. (2001).

[g]Rogers et al. (1993).

[h]Smith et al. (1994).

[i]Parker and Smith (1993).

[j]Rasmussen et al. (1990) and Thompson and Malamy (1990).

[k]Nikolich et al. (1992).

[l]Speer et al. (1991).

[m]Haggoud et al. (1994).

[n]Trinh et al. (1995).

[o]Onodera and Sato (1999).

[p]Miyamae et al. (2001).

[q]Wang et al. (2000).

[r]Smith et al. (1992).

2000; Table 6). Interestingly, *cfiA* seems to be restricted to a subgroup of the *B. fragilis* strains referred to as "*B. fragilis* DNA homology group II," whereas *cepA* is found in the more prevalent homology group I strains represented by the type strain ATCC 25285 (Podglajen et al., 1995). In association with this finding, it was shown that the IS elements known to activate *cfiA* also appear to be primarily associated with homology group II strains. These findings in part explain why imipenem resistance has been restricted to a small group of *B. fragilis* strains, but it remains to be seen whether these genes will become associated with transmissible elements and hence more widespread.

Two other mechanisms also may contribute to β-lactam resistance: reduced permeability of the drugs and reduced affinity of the penicillin-binding proteins (PBPs) for the drugs (Piddock and Wise, 1987; Cuchural et al., 1988; Edwards, 1997). Permeation studies of cephalosporins through the outer membrane of *B. fragilis* showed that values were about 1/10 of those seen for *E. coli* (Yotsuji et al., 1988a). Further studies yielded similar results and supported the general idea that hydrophilicity of the drugs was less a factor in permeation in *B. fragilis* than in enteric bacteria (Yotsuji et al., 1988a). The *Bacteroides* spp. appear to have at least three high mol wt PBPs (58,000–90,000 Da) and several smaller sized PBPs as estimated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE; Piddock and Wise, 1986; Piddock and Wise, 1987; Yotsuji et al., 1988b; Wexler and Halebian, 1990). The high mol wt PBPs have been correlated with similar proteins in *E. coli*, and each *Bacteroides* spp. has its own distinctive pattern, although even within species there were differences. These differences may account for some cases of β-lactam resistance. In two studies, cefoxitin resistance of *B. fragilis* and *B. thetaiotaomicron* strains appeared to involve decreased affinity of the PBPs for the drug and a similar study of a cephalothin-resistant *B. fragilis* strain showed that both reduced permeation and PBP affinity were responsible for resistance (Piddock and Wise, 1986; Yotsuji et al., 1988b; Wexler and Halebian, 1990).

Thus far, only two tetracycline-resistance genes, *tetQ* and *tetX*, have been found in the *Bacteroides* spp. (Table 6). The *tetX* gene encodes a novel NADP-linked oxidoreductase that inactivates tetracycline; however, this enzymatic activity requires aerobic conditions and does not seem to be active under anaerobic conditions (Guiney et al., 1984b; Speer et al., 1991). This gene has been found on a number of transposons that contain an *erm* gene but otherwise does not appear widespread. The *tetQ* gene on the other hand is responsible for the >80% tetracycline-resistance rates now seen in the genus and was found in 100% of tetracycline-resistant *Bacteroides* spp. (Nikolich et al., 1992; Shoemaker et al., 2001). This dissemination is in large part due to its presence on virtually all CTns. It is interesting to note that *tetQ* has not been found associated with plasmids in *Bacteroides* spp., but in the closely related *Prevotella ruminicola*, this gene was located on a plasmid (Flint et al., 1988). The available evidence suggests that the spread of *tetQ* is recent on the basis of the >96% nucleotide sequence identity among 33 *tetQ* genes isolated from strains representing all of the *Bacteroides* spp. (Shoemaker et al., 2001). The TetQ protein is in the TetM family of tetracycline-resistance determinants whose mode of action is ribosome protection. It appears that TetM is related to elongation factor G (EF-G), and in the presence of GTP, TetM reduces the amount of tetracycline bound to ribosomes (Burdett, 1991; Burdett, 1996; Trieber et al., 1998).

Resistance to metronidazole and other 5-nitroimidazoles has been attributed to two major mechanisms. Until the 1980s, resistant strains were infrequently isolated, often from patients undergoing extended treatment regimens (Ingham et al., 1978). Analyses of this class of resistant strain indicated that resistance was mediated by a global change in metabolism that resulted in a decreased reducing environment in the cell thus preventing activation of the drugs. This was thought to be accomplished by a reduction in pyruvate:ferredoxin oxidoreductase activity and a subsequent increase in lactate dehydrogenase, which balances the fermentation products (Narikawa et al., 1991). More recently, transmissible resistance to as much as 64 μg/ml of metronidazole has been observed in several European countries. This resistance phenotype has been associated with four *nim* genes that share between 67 and 90% sequence similarity at the protein level but do not match anything else in the public databases (Reysset et al., 1992; Trinh et al., 1995; Trinh and Reysset, 1996a; Trinh et al., 1996b). The genes have been found on a variety of different small mobilizable plasmids, as well as on what appears to be a mobilizable transposon. In nearly every case these genes are silent unless activated by IS elements, which in many cases are the same IS elements known to activate the *cfiA* genes (Haggoud et al., 1994; Trinh et al., 1995; Table 6). The mechanism of metronidazole resistance appears to rely on the ability of the Nim protein to reduce the drugs to their amine form, which precludes the reductive formation of the toxic nitroso-radical anions that form the basis of killing by the 5-nitroimidazoles (Carlier et al., 1997). Thus it is thought that the *nim* genes encode a 5-nitroimidazole reductase.

The intrinsic resistance of *Bacteroides* spp. to most quinolone and fluoroquinolone drugs is striking, but in addition there are reports that even the use of effective fluoroquinolones such as trovafloxacin or sparfloxacin can be compromised by the rapid emergence of resistant strains following 24 h of exposure to the drugs (Peterson et al., 1999). Also, *B. fragilis* isolates from Japan showed a significant increase in all fluoroquinolone minimal inhibitory concentrations (MICs) from 1983 to 1987 (Kato et al., 1988). It is likely that resistance to these drugs arises as a combination of active efflux and mutations in the *gyrA* gene that encodes a subunit of DNA gyrase that is the antibiotic target. Studies on the accumulation of norfloxacin in *B. fragilis* revealed the presence of an active efflux pump that was sensitive to inhibition by the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone (Miyamae et al., 1998; Ricci and Piddock, 2000). Further, the rate of efflux was dramatically increased by a single-step spontaneous mutation that resulted in simultaneous increases in the MICs for several fluoroquinolones, ethidium bromide, and puromycin (Miyamae et al., 1998). These data suggested the presence of a multidrug efflux pump, and in *B. thetaiotaomicron*, the gene for an efflux pump (designated *bexA*) was cloned and found to be in the multidrug and toxic compound extrusion (MATE) family. The presence of *bexA* was shown to impart increased resistance to fluoroquinolones and ethidium bromide (Miyamae et al., 2001). Comparison of the BexA protein to the *B. fragilis* genome sequence revealed the presence of at least three similar proteins (Miyamae et al., 2001).

Mutation of the DNA gyrase in the quinolone-resistance determining region (QRDR) also was shown to be responsible for the spontaneous resistance to levofloxacin of a *B. fragilis* mutant (Onodera and Sato, 1999). In a more extensive study, about half of the naturally occurring clinafloxacin-resistant strains (including *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus*, *B. uniformis* and *B. vulgatus*) exhibited *gyrA* mutations in the QRDR (Oh et al., 2001). These data suggest that while GyrA mutations are important for quinolone resistance, other mechanisms such as active efflux may also play a role.

As mentioned briefly above, aminoglycoside antibiotics are not effective against *Bacteroides* spp. There is an exception to this observation, and that is trospectomycin, an amino-cyclitol similar to spectinomycin that is very active against all *Bacteroides* spp. with MIC_{90} (MIC for 90% of strains tested) of ~8 μg/ml (Jacobus and Tally, 1988; Chisari et al., 1995). Studies on the mechanism of aminoglycoside resistance have shown that the ribosomes of *B. fragilis* are in fact sensitive to streptomycin and gentamycin, but the drugs do not accumulate in the cells (Bryan et al., 1979). This suggests that the lack of aminoglycoside transport is responsible for inherent resistance. It is interesting to note that one aminoglycoside-resistance gene, *aadS*, has been found in *Bacteroides* spp. (Smith et al., 1992b). This *aadS* gene encodes a streptomycin-dependent adenyltransferase and has been found on Tn*4551* and CTn*DOT*. The gene is actually expressed in *B. fragilis*, resulting in MIC values of >10 mg/ml of streptomycin, but its significance in *Bacteroides* antibiotic resistance really is not clear.

The Role of *Bacteroides* spp. in Interspecies Resistance Transfer

The extraordinary high numbers of the *Bacteroides* in the intestinal tract allows these organisms to interact physically with a variety of the 500 or so indigenous species, as well as with the constant parade of transients passing through the tract. Contact with such diverse species provides the opportunity for genetic exchange in both directions, so *Bacteroides* spp. could be an ideal reservoir or perhaps more accurately a clearing-house of antibiotic-resistance genes. The CTns help propel this gene transfer, and their widespread dissemination may in part explain the proliferation of mobilizable plasmids and transposons, which are in >70% of all *Bacteroides* spp. (Callihan et al., 1983; Smith et al., 1998; Wang et al., 2000). The mobilizable transposons in particular have a potentially wide host range and could at the least provide a mechanism to introduce new antibiotic-resistance genes into a variety of organisms.

A significant amount of data indicates that the *Bacteroides* can transfer antibiotic-resistance determinants to diverse species. First, resistance genes (*tetQ*) are transferred directly between the *Bacteroides* spp. and Gram-negative organisms in the oral cavity such as *Prevotella* (seven species) and *Porphyromonas gingivalis* (Leng et al., 1997; Roberts, 1998; Chung et al., 1999b). More recently, a penicillin-resistant isolate of the oral microbe *Prevotella intermedia* was found to have a β-lactamase gene >98% identical to the *cfxA* gene of *B. vulgatus*, and sequences homologous to Tn*4555* transposition genes also were found (Madinier et al., 2001). A significant number of tetracycline-resistant *Prevotella* in the rumen of cattle also have been shown to harbor *tetQ* (Nikolich et al., 1994). In fact, *tetQ* has now turned up in a number of different Gram-positive and Gram-negative anaerobes from a variety of human sources, and direct transfer of the gene from diverse donors to *Enterococcus*

faecalis recipients has been demonstrated (Leng et al., 1997; Chung et al., 1999b). This shows not only that broad, heterologous expression of these genes can be achieved but also that these genes can cross very broad species barriers. Other data sets reveal complex patterns of *erm* gene transfer. As mentioned above, an identical *ermG* gene (three nucleotides differ) was found in *Bacillus* and on a *Bacteroides* CTn (Cooper et al., 1996). Also, new data on the distribution of *ermF* have shown that this resistance determinant has entered into *Treponema denticola* (Roberts et al., 1996) and *Neisseria* species including *N. gonorrhoeae* (Roberts et al., 1999).

Isolation and Identification

The majority of anaerobes isolated from clinical specimens are Gram-negative anaerobic bacilli, mainly *Bacteroides*, *Fusobacterium*, *Prevotella* and *Porphyromonas*. It is recommended that anaerobes isolated from sterile sites and from serious infections should always be completely identified to species level (Citron and Appelbaum, 1993). This recommendation is particularly relevant to the *Bacteroides* group because of their virulence and resistance to many antimicrobial agents.

The procedures recommended for appropriate processing of clinical specimen collection, transport, and anaerobic growth techniques follow the same procedures for general anaerobic bacteriology described elsewhere (Summanen et al., 1993).

Methods of Isolation, Examination, and Identification

Direct Examination Direct examination of Gram-stained smears of clinical material is a simple method that provides useful information on bacterial morphology, which for the most part is similar for aerobic organisms and anaerobes. The typical *Bacteroides* cellular morphology is the presence of pleomorphic, pale, Gram-negative forms with round-ends, occurring singly or in pairs, with vacuoles (Holdeman et al., 1977).

The methodology of fluorescent polyvalent antibody conjugates for rapid identification of the *Bacteroides* group is commercially available and gives reliable results within a few hours (Wills et al., 1982). The immunological detection of *B. fragilis* and *Bacteroides* spp. from clinical specimens by fluorescence microscopy is in the range of 81 to 97% when compared to culture results (Slack et al., 1981; Viljanen et al., 1988; Patrick et al., 1995). Antibodies prepared against the common polysaccharide antigen of *B. fragilis* and related species are more suitable for the immunodetection of *B. fragilis* from clinical samples compared to monoclonal antibodies specific for the variable surface polysaccharide (Patrick et al., 1995). Though fluorescent antibody procedures for the identification of *Bacteroides* spp. have been useful in reference laboratories, they are not routinely used in the clinical microbiology laboratory.

Direct gas-liquid chromatography analysis of clinical specimens for the detection of short-chain fatty acid end products of anaerobic bacteria metabolism is a presumptive indicator of the presence of anaerobes in the infectious site (Gorbach et al., 1976). The presence of succinic acid was suggested to be a good indicator for the presence of *Bacteroides* in the infected material, but this has not been consistently reported (Gorbach et al., 1976; Watt et al., 1982).

Preliminary Identification Initial processing procedures for examination and inoculation of the specimen require an appropriate combination of enriched, nonselective, selective and differential media. The differential isolation and identification of the species belonging to Gram-negative anaerobic bacilli groups other then *Bacteroides* are dealt with in detail by Summanen et al. (1993) and Jousimies-Somer et al. (1999).

The nonselective recovery of *Bacteroides* can be carried out on various basal media, which include Brucella agar, brain-heart infusion agar, Columbia agar and Wilkins-Chalgren agar supplemented with 5–10% horse or sheep blood and hemin (5 μg/ml). Primary selective isolation of *Bacteroides* from human origin can be accomplished on either kanamycin-vancomycin laked blood Brucella (KVLB) agar (Summanen et al., 1993) or bile-containing media such as *Bacteroides* bile esculin (BBE) agar (Livingston et al., 1978). KVLB agar inhibits most facultative anaerobic bacteria but permits selective isolation mainly of *Prevotella* spp. and *Bacteroides* spp., while BBE is selective for *Bacteroides* spp.

The use of selective media allows rapid presumptive identification of *Bacteroides* spp. from clinical and nonclinical sources. Specimens inoculated on plates containing BBE can be examined following 18–24 h anaerobic incubation. *Bacteroides* spp. will typically form colonies >1 mm diameter and will be surrounded by a brown-black zone due to esculin hydrolysis. *Bacteroides vulgatus* is an exception owing to its esculin-negative phenotype (Summanen et al., 1993). As a general rule, any colony picked off BBE agar plates for further characterization must be subcultured in an enriched medium with

no antibiotic and incubated in an aerobic incubator to determine whether the isolate is an obligate anaerobe or a facultative anaerobe. Enterobacteriaceae resistant to gentamycin are able to form colonies on BBE agar. Other anaerobic Gram-negative rods species such as *Fusobacterium mortiferum* and *Bilophila* spp. also can form colonies on BBE. The former can be differentiated from *Bacteroides* by its sensitivity to kanamycin and colistin and resistance to rifamycin. The latter is nitrate positive (Summanen et al., 1993; Jousimies-Somer et al., 1999).

Definitive Identification The standard conventional methods for the identification and differentiation of the *Bacteroides* group species are largely based on the schemes proposed by Holdeman et al. (Holdeman et al., 1977; Holdeman et al., 1986) and involve the utilization of pre-reduced anaerobically sterilized media for the determination of sugar fermentation, biochemical tests and the detection of fermentative end products by gas/liquid chromatography (Table 7). A simplified and rapid scheme for biochemical and fermentative identification of bile resistant *Bacteroides* allows speciation of the group within 24 h after obtaining a pure culture (Citron et al., 1990). Another simplified identification scheme based on conventional methodology and that provides convenient identification at a reasonable cost is the use of Presumpto plates (Whaley et al., 1995). Generally a set of three quadrant plates containing four conventional media to generate 21 tests is used to separate most of the clinically relevant anaerobes with rapid recognition of five *Bacteroides* species.

Several rapid identification systems for anaerobic bacteria also are available and include the API 20A, Minitek, An-Ident, RapID-ANAII, and API ZYM systems. Most of these systems involve the use of several chromogenic substrates for the detection of constitutive enzymes. Generally, the investigator is able to obtain adequate identification of *Bacteroides* species, but additional tests are often required since some species in this genus are not easily distinguished by these phenotypic tests.

Whole-cell carbohydrate analysis by capillary gas chromatography provides rapid and accurate identification of *Bacteroides* spp. (Engelhard and Mutters, 1991). Identification of the strains can be performed within 4–6 hours after primary isolation, and the time-consuming conventional anaerobic identification procedures can be avoided.

Molecular Approaches

Nucleic Acid Probes A variety of identification schemes based on restriction endonuclease digestion, DNA or RNA hybridization, PCR or a combination of these technologies have been used for the identification of *Bacteroides* spp. Most uses of these approaches have been in research laboratories, but some of these technologies may be adapted to the clinical laboratories in the near future. The simplest hybridization approach is to take whole-chromosome DNA samples, transfer these to a hybridization membrane, and then probe the membranes with labeled chromosomal DNA from the type strains of the species (Morotomi et al., 1988) or species-specific DNA fragments (Kuritza et al., 1986b). Using this procedure, accurate identification was obtained for *B. fragilis* in experimental blood cultures, and quantification was obtained of *B. thetaiotaomicron*, *B. uniformis*, *B. distasonis*, *B. ovatus*, *Bacteroides* group 3452A and *B. vulgatus* from fecal samples. Groves and Clark (1987) utilized specific radiolabeled RNA probes, obtained from randomly cloned chromosomal DNA from the *B. fragilis* type strain, to detect chromosomal DNA from clinical isolates of *B. fragilis* blotted on nitrocellulose membrane. These are highly accurate tests, but the use of radiolabeled probe has limited their use in the clinical laboratory.

Restriction Endonuclease Analysis Restriction endonuclease analysis (REA) of the bacterial chromosome also has been used in determining the genetic relationship of microorganisms. This approach offers the advantage of being highly reproducible and accurate. To generate a fingerprint of the chromosome, total genomic DNA is digested with an appropriate restriction endonuclease and electrophoresed on an agarose gel. The resulting patterns are species specific owing to RFLP. Often the RFLP patterns are simplified by coupling them to hybridization techniques that use specific DNA probes. For example, a ribotype, shown to have taxonomic and epidemiological values, can be obtained by using labeled ribosomal operons for 16S rRNA or 23S rRNA (Tang et al., 1997). Using this approach, distinct patterns were observed for all of the *Bacteroides* spp. and each could be easily differentiated (Smith and Callihan, 1992a). In another study, *B. fragilis* isolated from blood and feces was differentiated on the basis of REA and ribotype profiles of genomic DNA digested with *Bam*HI and *Eco*RI, respectively (Kleivdal and Hofstad, 1995). This analysis of REA and rRNA gene profiles of *B. fragilis* revealed some genetic heterogeneity among the isolates and concluded that no particular ribotype profile could be associated with systemic infection. Ribotype profiles of *B. fragilis* may be useful for phylogenetic and epidemiological studies, but

Table 7. Biochemical reactions of the *Bacteroides* group.

Species	Growth in 20% bile	Esculin hydrolysis	Indole	Catalase	Fermentation of									*a*-Fucosidase	Products from PYG
					Sucrose	Trehalose	Arabinose	Cellobiose	Salicin	Xylan	Melezitose	Rhamnose	Glycogen		
B. caccae	+	+	−	−	+	+	+	+	−	−	+	+	−	+	SAp(iv)
B. distasonis	+	+	−	+	+	+	−	+	+	−	+	+/−	−	−	SApy(ivibl)
B. eggerthii	+	+	+	−	−	−	+	−	−	+	−	+	+	−	SAp(ivibl)
B. fragilis	+	+	−	+	+	−	−	+	−	−	−	−	+	+	SApy(ibivl)
B. merdae	+	+	−	−	+	+	−	+/−	+	−	+	+	−	−	SAp(ibiv)
B. ovatus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	SApy(ibivl)
B. stercoris	+	+	+	−	+	−	−	−	−	+/−	−	+	+/−	+/−	SAp(ibiv)
B. thetaiotaomicron	+	+	+	+	+	+	+	+	−	−	+/−	+	+	+	SApy(ivibl)
B. uniformis	+	+	+	−	+	−	+	+	+	+/−	−	−	+	+	Sapl(ivib)
B. vulgatus	+	−	−	−	+	−	+	−	−	−	−	+	+	+	SAp(ivibl)

Symbols: for biochemical tests, +, positive reaction; −, negative reaction; and +/−, variable reaction. For sugar fermentations, +, pH 5.5 or below; and −, pH 5.7 or above.
Abbreviations: PYG, peptone yeast glucose; A, acetate; IB, isobutyrate; IV, isovalerate; L, lactate; P, propionate; Y, phenyl acetate; and S, succinate. Fermentation products in parentheses are those only found in occasional strains.
[a]Fermentation products from PYG: upper case letters >1 meq of acid/100 ml of broth and lower case letters <1 meq of acid/100 ml.
From Johnson and Ault (1978), Holdeman et al. (1984), and Jousimies-Somer et al. (1999).

the complex patterns and heterogeneity make it difficult for routine taxonomic studies (Eribe and Olsen, 2000).

Comparison of the complex REA of chromosomal DNA profiles is difficult because of the hundreds of fragments generated by conventional methods. To address this, differentiation of *Bacteroides* has been obtained by pulse field gel electrophoresis (PFGE), which has the ability to separate the large DNA fragments typically generated with rare-cutting restriction endonucleases (Shaheduzzaman et al., 1997; Tang et al., 1997). In one study, *Bacteroides* spp. were identified on the basis of total genome size and the number and molecular size of restriction fragments generated with the rare-cutting restriction enzyme I-*Ceu*I. Restriction with I-*Ceu*I provided distinct species-specific RFLP profiles, and these profiles have a significant correlation with the classification based on 16S rRNA (Shaheduzzaman et al., 1997). PFGE is a simple and powerful technique for studying bacterial genome organization and may be useful for further studies on the phylogeny of the genus.

Polymerase Chain Reaction The use of PCR methodology has proven to be useful in the identification of *B. fragilis* and *B. thetaiotaomicron* directly from clinical specimens. PCR amplification of the neuraminidase, *nanH*, and glutamine synthase genes allowed direct detection of *B. fragilis* from clinical specimens containing other aerobic and anaerobic bacteria (Yamashita et al., 1994; Jotwani et al., 1995). It was shown that the presence of a specific PCR amplified *nanH* product completely correlated with positive cultures for *B. fragilis*. *Bacteroides thetaiotaomicron* also could be identified in clinical specimens by PCR amplification of a specific 721-bp chromosomal DNA sequence (Teng et al., 2000).

Restriction endonuclease analysis of PCR-amplified DNA also has been applied to *Bacteroides* species identification. Analysis of the RFLP pattern generated from PCR-amplified 16S rDNA digested with *Hpa*II and *Taq*I produced profiles that allowed identification of all *Bacteroides* species type strains and unequivocal identification of 93% of the *Bacteroides* clinical strains tested, compared to conventional methods (Stubbs et al., 2000). The application of 16S rDNA PCR-RFLP to species identification offers a rapid and accurate approach for identification of *Bacteroides* to the species level. A similar strategy was used by Kuwahara et al. (2001) to examine the 16S and 23S rRNA internal transcribed spacer region among *Bacteroides* spp. Amplification products of the spacer region digested with *MspI* produced distinctive species-specific RFLP patterns, and it was possible to differentiate 90 strains to the species level, including *B. fragilis*, *B. distasonis*, *B. ovatus*, *B. eggerthii*, *B. thetaiotaomicron*, *B. uniformis* and *B. vulgatus*.

PCR methodology has been used not only for the detection of *B. fragilis* in clinical specimens but also for epidemiological studies. For example, a nested PCR amplification of the *bft* gene was developed to trace enterotoxigenic strains of *B. fragilis* in human and animal feces (Shetab et al., 1998). Moreover, detection of *B. fragilis* enterotoxigenic strains by PCR was instrumental in a study on the geographical distribution of ETBF, which led to the isolation and detection of a new *bft*-isoform (*bft*-3) in Korea (Chung et al., 1999a). Comparison of PCR fingerprint profiles of clinical isolates of *B. fragilis* and *B. thetaiotaomicron* showed that all *B. fragilis* strains isolated from Germany belong to the DNA homology group I, and all *B. thetaiotaomicron* isolates are genetically homologous to the type strain. In contrast, *B. fragilis* and *B. thetaiotaomicron* strains isolated from the United States showed a higher degree of genetic heterogeneity (Carlos et al., 1997).

DNA homology studies in 1978 suggested the likelihood of two genetically distinct *B. fragilis* groups since they shared only 69% identical sequences (Johnson, 1978a). These two *B. fragilis* DNA homology groups are phenotypically homogeneous and their metabolic features do not offer enough information to differentiate these groups by conventional identification tests (Johnson and Ault, 1978b). In an example of the utility of molecular typing approaches, these genetically distinct groups have been separated by means of ribotyping, 16S rRNA sequence analysis, PCR amplification of the β-lactamase genes *cepA* and *cfiA*, RFLP, and multi-locus enzyme electrophoresis profiles (Podglajen et al., 1995; Ruimy et al., 1996; Gutacker et al., 2000). Presence of the *cepA* gene in DNA homology group I (*cfiA* is absent) and the presence of *cfiA* in the group II strains (*cepA* is absent) provide a PCR strategy by which the two groups can be easily distinguished. Cluster analysis based on multi-locus electrophoretic profiles of 15 genetic loci separated DNA homology group I into 81 distinct electrophoretic profile types associated with the presence of *cepA* gene, whereas 9 electrophoretic profiles were detected for strains belonging to group II carrying the *cfiA* gene (Gutacker et al., 2000). The differentiation of *B. fragilis* strains belonging to genetic group I or group II is useful for taxonomic and epidemiological studies, but there is no association between these groups and any clinical manifestations such as sepsis, abscesses, or diarrhea, geographical origin, or human or animal origin.

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Literature Cited

Abrahams, G. L., and V. R. Abratt. 1998. The NADH-dependent glutamate dehydrogenase enzyme of Bacteroides fragilis Bf1 is induced by peptides in the growth medium. Microbiol. 144:1659–1667.

Abrahams, G. L., K. D. Iles, and V. R. Abratt. 2001. The Bacteroides fragilis NAD-specific glutamate dehydrogenase enzyme is cell surface-associated and regulated by peptides at the protein level. Anaerobe 7:135–142.

Abrams, G. D. 1983. Impact of the intestinal microflora on intestinal structure and function. *In:* D. J. Hentges (Ed.) Human Intestinal Microflora in Health and Disease. Academic Press. New York, NY. 292–310.

Abratt, V. R., H. Zappe, and D. R. Woods. 1993. A reporter gene vector to investigate the regulation of glutamine synthetase in Bacteroides fragilis Bf1. J. Gen. Microbiol. 139:59–65.

Aldridge, K., D. Ashcraft, K. Cambre, C. L. Peirson, S. Jenkins, and J. E. Rosenblatt. 2001. Multicenter survey of the changing in vitro antimicrobial susceptibilities of clinical isolates of Bacteroides fragilis group, Prevotella, Fusobacterium, Porphyromonas, and Peptostreptococcus species. Antimicrob. Agents Chemother. 45:1238–1243.

Al-Jalili, T. A. R., and H. N. Shah. 1988. Protoheme, a dispensable growth factor for Bacteroides fragilis grown by batch and continuous culture in a basal medium. Curr. Microbiol. 17:13–18.

Allison, M. J., I. M. Robinson, and A. L. Baetz. 1979. Synthesis of a-ketoglutarate by reductive carboxylation of succinate in Veillonella, Selenomonas, and Bacteroides species. J. Bacteriol. 140:980–986.

Allison, M. J., A. L. Baetz, and J. Wiegel. 1984. Alternative pathways for biosynthesis of leucine and other amino acids in Bacteroides ruminicola and Bacteroides fragilis. Appl. Environ. Microbiol. 48:1111–1117.

Allsop, K., and D. J. Stickler. 1984. The enumeration of Bacteroides fragilis group organisms from sewage and natural waters. J. Appl. Bacteriol. 56:15–24.

Allsop, K., and D. J. Stickler. 1985. An assessment of Bacteroides fragilis group organisms as indicators of human fecal pollution. J. Appl. Bacteriol. 58:95–99.

Azeddoug, H., G. Reysset, and M. Sebald. 1992. Characterization of restriction endonuclease BfrBI from Bacteroides fragilis strains BE3 and AIP 10006. FEMS Microbiol. Lett. 74:133–136.

Baggio, L., and M. Morrision. 1996. The NAD(P)H-utilizing glutamate dehydrogenase of Bacteroides thetaiotaomicron belongs to enzyme family I, and its activity is affected by trans-acting gene(s) positioned downstream of gdhA. J. Bacteriol. 178:7212–7220.

Baughn, A. D., and M. H. Malamy. 2002. A mitochondrial-like aconitase in the bacterium Bacteroides fragilis:implications for the evolution of the mitochondrial Krebs cycle. Proc. Natl. Acad. Sci. USA 99(7):4662–4667.

Bedzyk, L. A., N. B.Shoemaker, K. E.Young, and A. A. Salyers. 1992. Insertion and excision of Bacteroides conjugative chromosomal elements. J. Bacteriol. 174:166–172.

Berg, J. O., L. Lindqvist, and C. E. Nord. 1980. Purification of glycohydrolases from Bacteroides fragilis. Appl. Environ. Microbiol. 40:40–47.

Bernhardt, H., and M. Knoke. 1989. Recent studies on the microbial ecology of the upper gastrointestinal tract. Infection 17:259–263.

Betriu, C., M. Gomez, M. L. Palau, A. Sanchez, and J. J. Picazo. 1999a. Activities of new antimicrobial agents against Bacteroides fragilis group: Comparison with the activities of 14 other agents. Antimicrob. Agents Chemother. 43:2320–2322.

Betriu, C., A. Sanchez, M. Gomez, M. L. Palau, and J. J. Picazo. 1999b. In-vitro susceptibilities of species of the Bacteroides fragilis group to newer b-lactam agents. J. Antimicrob. Chemother. 43:133–136.

Bonheyo, G., D. Graham, N. B. Shoemaker, and A. A. Salyers. 2001a. Transfer region of a Bacteroides conjugative transposon, CTnDOT. Plasmid 45:41–51.

Bonheyo, G. T., B. D. Hund, N. B. Shoemaker, and A. A. Salyers. 2001b. Transfer region of a Bacteroides conjugative transposon contains regulatory as well as structural genes. Plasmid 46:202–209.

Booth, S. J., R. L. van Tassel, J. L. Johnson, and T. D. Wilkens. 1979. Bacteriophages of Bacteroides. Rev. Infect. Dis. 1:325–334.

Border, M., B. D. Firehammer, D. S. Shoop, and L. L. Myers. 1985. Isolation of Bacteroides fragilis from the feces of diarrhetic calves and lambs. J. Clin. Microbiol. 21:472–473.

Bornside, G. H., W. E. Donovan, and M. B. Myers. 1976. Intracolonic tensions of oxygen and carbon dioxide in germfree, conventional, and gnotobiotic rats. Proc. Soc. Exp. Biol. Med. 151:437–441.

Botta, G. A., C. Eftimiadi, M. Tonetti, T. J. M. van Steenbergen, and J. De Graaff. 1985. Influence of volatile fatty acids on human granulocyte chemotaxis. FEMS Microbiol. Lett. 27:68–72.

Brook, I. 1989. Pathogenicity of Bacteroides fragilis group. Ann. Clin. Lab. Sci. 19:360–376.

Brook, I., and M. L. Myhal. 1991. Adherence of Bacteroides fragilis group species. Infect. Immun. 59:742–744.

Brook, I., and E. H. Frazier. 2000. Aerobic and anaerobic microbiology in intra-abdominal infections associated with diverticulitis. J. Med. Microbiol. 49:827–830.

Bry, L., P. G. Falk, T. Midtvedt, and J. I. Gordon. 1996. A model of host-microbial interactions in an open mammalian ecosystem. Science 273:1380–1383.

Bryan, L. E., S. K. Kowland, and H. M. van den Elzen. 1979. Mechanisms of aminoglycoside antibiotic resistance in anaerobic bacteria: Clostridium perfringens and Bacteroides fragilis. Antimicrob. Agents Chemother. 15:7–13.

Burdett, V. 1991. Purification and characterization of Tet(M), a protein that renders ribosomes resistant to tetracycline. J. Biol. Chem. 266:2872–2877.

Burdett, V. 1996. Tet(M)-promoted release of tetracycline from ribosomes is GTP dependent. J. Bacteriol. 178:3246–3251.

Burt, S. J., and D. R. Woods. 1977. Transfection of the anaerobe Bacteroides thetaiotaomicron with phage DNA. J. Gen. Microbiol. 103:181–187.

Bush, K., G. A. Jacoby, and A. A. Medeiros. 1995. A functional classification scheme for b-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. 39:1211–1233.

Caldwell, D. R., and C. Arcand. 1974. Inorganic and metal-organic growth requirements of the genus Bacteroides. J. Bacteriol. 120:322–333.

Callihan, D. R., F. E. Young, and V. L. Clark. 1983. Identification of three homology classes of small, cryptic plasmids in intestinal Bacteroides species. Plasmid 9:17–30.

Carlier, J. P., N. Sellier, M. N. Rager, and G. Reysset. 1997. Metabolism of a 5-nitroimidazole in susceptible and resistant isogenic strains of Bacteroides fragilis. Antimicrob. Agents Chemother. 41:1495–1499.

Carlos, M. C., S. H. Gerardo, D. M. Citron, E. J. Goldstein, G. Schonian, and A. C. Rodloff. 1997. Use of polymerase chain reaction fingerprint to compare clinical isolates of Bacteroides fragilis and Bacteroides thetaiotaomicron from Germany and the United States. Clin. Infect. Dis. 25 (Suppl. 2):S295–S298.

Carlsson, J., J. Wrethen, and G. Beckman. 1977. Superoxide dismutase in Bacteroides fragilis and related Bacteroides species. J. Clin. Microbiol. 6:280–284.

Caspari, D., and J. M. Macy. 1983. The role of carbon dioxide in glucose metabolism of Bacteroides fragilis. Arch. Microbiol. 135:16–24.

Chacko, H., and J. H. Cummings. 1988. Nitrogen losses from the human small bowel: Obligatory losses and the effect of physical form of food. Gut 29:809–815.

Chen, M., and M. J. Wolin. 1981. Influence of heme and vitamin B12 on growth and fermentation of Bacteroides species. J. Bacteriol. 145:466–471.

Chen, Y., T. Kinouchi, K. Kataoka, S. Akimoto, and Y. Ohnishi. 1995. Purification and characterization of a fibrinogen-degrading protease in Bacteroides fragilis strain YCH46. Microbiol. Immunol. 39:967–977.

Cheng, Q., and A. A. Salyers. 1995a. Use of suppressor analysis to find genes involved in the colonization deficiency of a Bacteroides thetaiotaomicron mutant unable to grow on the host-derived mucopolysaccharides chondroitin sulfate and heparin. Appl. Environ. Microbiol. 61:734–740.

Cheng, Q., M. C. Yu, A. R. Reeves, and A. A. Salyers. 1995b. Identification and characterization of a Bacteroides gene, csuF, which encodes an outer membrane protein that is essential for growth on chondroitin sulfate. J. Bacteriol. 177:3721–3727.

Cheng, Q., B. J. Paszkiet, N. B. Shoemaker, J. F. Gardner, and A. A. Salyers. 2000. Integration and excision of a Bacteroides conjugative transposon, CTnDOT. J. Bacteriol. 182:4035–4043.

Chisari, G., A. M. Lo Bue, and G. Nicoletti. 1995. The in vitro activity of trospectomycin against anaerobic bacteria. J. Chemother. 7:515–518.

Cho, K. H., and A. A. Salyers. 2001a. Biochemical analysis of interactions between outer membrane proteins that contribute to starch utilization by Bacteroides thetaiotaomicron. J. Bacteriol. 183:7224–7230.

Cho, K. H., D. Cho, G.-R. Wang, and A. A. Salyers. 2001b. New regulatory gene that contributes to control of Bacteroides thetaiotaomicron starch utilization genes. J. Bacteriol. 183:7198–7205.

Chung, K.-T., G. E. Fulk, and M. Egan. 1978. Reduction of azo dyes by intestinal anaerobes. Appl. Environ. Microbiol. 35:558–562.

Chung, G. T., A. A. Franco, S. G. Wu, G. E. Rhie, H. B. Cheng, H. Oh, and C. L. Sears. 1999a. Identification of a third metalloprotease toxin gene in extraintestinal isolates of Bacteroides fragilis. Infect. Immun. 67:4945–4949.

Chung, W. O., K. Young, Z. Leng, and M. C. Roberts. 1999b. Mobile elements carrying ermF and tetQ genes in Gram-positive and Gram-negative bacteria. J. Antimicrob. Chemother. 44:329–335.

Citron, D. M., E. J. Baron, S. M. Finegold, and E. J. Goldstein. 1990. Short prereduced anaerobically sterilized (PRAS) biochemical scheme for identification of clinical isolates of bile-resistant Bacteroides species. J. Clin. Microbiol. 28:2220–2223.

Citron, D. M., and P. C. Appelbaum. 1993. How far should a clinical laboratory go in identifying anaerobic isolates, and who should pay? Clin. Infect. Dis. 16 (Suppl. 4):S435–S438.

Comstock, L. E., M. J. Coyne, A. O. Tzianabos, A. Pantosti, A. B. Onderdonk, and D. L. Kasper. 1999. Analysis of a capsular polysaccharide biosynthesis locus of Bacteroides fragilis. Infect. Immun. 67:3525–3532.

Cooper, S. W., E. G. Szymczak, N. V. Jacobus, and F. P. Tally. 1984. Differentiation of Bacteroides ovatus and Bacteroides thetaiotaomicron by means of bacteriophage. J. Clin. Microbiol. 20:1122–1125.

Cooper, A. J., N. B. Shoemaker, and A. A. Salyers. 1996. The erythromycin resistance gene from the Bacteroides conjugal transposon Tcr Emr 7853 is nearly identical to ermG from Bacillus sphaericus. Antimicrob. Agents Chemother. 40:506–508.

Cooperstock, M. S., and A. J. Zeed. 1983. Intestinal flora of infants. *In:* D. J. Hentges (Ed.) Human Intestinal Microflora in Health and Disease. Academic Press. New York, NY. 79–101.

Cornax, R., R. A. Morinigo, I. G. Paez, M. A. Munoz, and J. J. Borrego. 1990. Application of direct plaque assay for detection and enumeration of bacteriophages of Bacteroides fragilis from contaminated water samples. Appl. Environ. Microbiol. 56:3170–3173.

Cornick, N. A., G. J. Cuchural, D. R. Snydman, N. V. Jacobus, P. Iannini, G. Hill, T. Cleary, J. P. O'Keefe, C. Pierson, and S. M. Finegold. 1990. The antimicrobial susceptibility patterns of the Bacteroides fragilis group in the United States, 1987. J. Antimicrob. Chemother. 25:1011–1019.

Coyne, M. J., W. Kalka-Moll, A. O. Tzianabos, D. L. Kasper, and L. E. Comstock. 2000. Bacteroides fragilis NCTC9343 produces at least three distinct capsular polysaccharides: cloning, characterization, and reassignment of polysaccharide B and C biosynthesis loci. Infect. Immun. 68:6176–6181.

Coyne, M. J., A. O. Tzianabos, B. C. Mallory, V. J. Carey, and D. L. Kasper. 2001. Polysaccharide biosynthesis locus required for virulence of Bacteroides fragilis. Infect. Immun. 69:4342–4350.

Cuchural, G. J., and F. P. Tally. 1986. Bacteroides fragilis: Current susceptibilities, mechanisms of drug resistance, and principles of antimicrobial therapy. Drug Intell. Clin. Pharm. 20:567–573.

Cuchural Jr., G. J., S. Hurlbut, M. H. Malamy, and F. P. Tally. 1988. Permeability to b-lactams in Bacteroides fragilis. J. Antimicrob. Chemother. 22:785–790.

D'Abusco, A. S., M. D. Grosso, S. Censini, A. Covacci, and A. Pantosti. 2000. The alleles of the bft gene are distributed differently among enterotoxigenic Bacteroides

fragilis strains from human sources and can be present in double copies. J. Clin. Microbiol. 38:607–612.

Degnan, B. A., and G. T. Macfarlane. 1995. Carbohydrate utilization patterns and substrate preferences in Bacteroides thetaiotaomicron. Anaerobe 1:33.

Degnan, B. A., S. Macfarlane, and G. T. Macfarlane. 1997a. Utilization of starch and synthesis of a combined amylase/alpha-glucosidase by the human colonic anaerobe Bacteroides ovatus. J. Appl. Microbiol. 83:359–366.

Degnan, B. A., S. Macfarlane, M. E. Quigley, and G. T. Macfarlane. 1997b. Starch utilization by Bacteroides ovatus isolated from the human large intestine. Curr. Microbiol. 34:290–296.

D'elia, J. N., and A. A. Salyers. 1996a. Contribution of a neopullulanase, a pullulanase, and an a-glucosidase to growth of Bacteroides thetaiotaomicron on starch. J. Bacteriol. 178:7173–7179.

D'elia, J. N., and A. A. Salyers. 1996b. Effect of regulatory protein levels on utilization of starch by Bacteroides thetaiotaomicron. J. Bacteriol. 178:7180–7186.

Duerden, B. I. 1980. The isolation and identification of Bacteroides spp,.from normal human fecal flora. J. Med. Microbiol. 13:69–78.

Duerden, B. I. 1994. Virulence factors in anaerobes. Clin. Infect. Dis. 18 (Suppl. 4):S253–S259.

Dunn, D. L., O. D. Rotstein, and R. L. Simmons. 1984. Synergistic intraperitoneal infection caused by Escherichia coli and Bacteroides fragilis within fibrin clots. Arch. Surg. 119:139–144.

Edmiston Jr., C. E., M. P. Goheen, S. Kornhall, F. E. Jones, and R. R. Condon. 1990. Fecal peritonitis: Adherence to serosal mesothelium and resistance to peritoneal lavage. World J. Surg. 14:176–183.

Edwards, R. 1997. Resistance to beta-lactam antibiotics in Bacteroides spp. [review, 101 refs]. J. Med. Microbiol. 46:979–986.

Edwards, R., and P. N. Read. 2000. Expression of the carbapenemase gene (cfiA) in Bacteroides fragilis. J. Antimicrob. Chemother. 46:1009–1012.

Eiring, P., B. Manncke, K. Gerbracht, and H. Werner. 1995. Bacteroides fragilis adheres to laminin significantly stronger than Bacteroides thetaiotaomicron and other species of the genus. Zentralbl Bakteriol. 282(3):279–86.

Engelhard, E., and R. Mutters. 1991. Rapid differentiation of the species of the genus Bacteroides sensu stricto by capillary gas chromatography of cellular carbohydrates. J. Appl. Bacteriol. 70:216–220.

Eribe, R. E., and I. Olsen. 2000. Strain differentiation in Bacteroides fragilis by ribotyping and computer-assisted gel analysis. APMIS 108:429–438.

Falk, P. G., L. V. Hooper, T. Midtvedt, and J. I. Gordon. 1998. Creating and maintaining the gastrointestinal ecosystem: What we know and need to know from gnotobiology [review, 214 refs]. Microbiol. Molec. Biol. Rev. 62:1157–1170.

Fallingborg, J. 1999. Intraluminal pH of the human gastrointestinal tract. Danish Med. Bull. 46:183–196.

Finegold, S. M., H. R. Attebery, and V. L. Sutter. 1974. Effect of diet on human fecal flora: comparison of Japanese and American diets. Am. J. Clin. Nutr. 27:1456–1469.

Finegold, S. M., V. L. Sutter, and G. E. Mathisen. 1983. Normal indigenous intestinal flora. *In:* D. J. Hentges (Ed.) Human Intestinal Microflora in Health and Disease. Academic Press. New York, NY. 3–31.

Finegold, S. M., and W. L. George. 1989. Anaerobic Infections in Humans. Academic Press. San Diego, CA.

Flint, H. J., A. M. Thomson, and J. Bisset. 1988. Plasmid associated transfer of tetracycline resistance in Bacteroides ruminicola. Appl. Environ. Microbiol. 54:855–860.

Franco, A. A., R. K. Cheng, G. T. Chung, S. G. Wu, H. B. Oh, and C. L. Sears. 1999. Molecular evolution of the pathogenicity island of enterotoxigenic Bacteroides fragilis strains. J. Bacteriol. 181:6623–6633.

Fuller, M. D., and D. R. Caldwell. 1982. Tetrapyrrole utilization of Bacteroides fragilis. Can. J. Microbiol. 28:1304–1310.

Gangloff, S. C., N. Hijiya, A. Haziot, and S. M. Goyert. 1999. Lipopolysaccharide structure influence the macrophage response via CD14-independent and CD14-dependent pathways. Clin. Infect. Dis. 28:491–496.

Garrity, G. M. (Ed.). 2001. Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY.

Gibson, S. A., and G. T. Macfarlane. 1988a. Characterization of proteases formed by Bacteroides fragilis. J. Gen. Microbiol. 134:2231–2240.

Gibson, S. A., and G. T. Macfarlane. 1988b. Studies on the proteolytic activity of Bacteroides fragilis. J. Gen. Microbiol. 134:19–27.

Gibson 3rd, F. C., A. O. Tzianabos, and A. B. Onderdonk. 1996. The capsular polysaccharide complex of Bacteroides fragilis induces cytokine production from human and murine phagocytic cells. Infect. Immun. 64:1065–1069.

Gibson 3rd, F. C., A. B. Onderdonk, D. L. Kasper, and A. O. Tzianabos. 1998. Cellular mechanism of intraabdominal abscess formation by Bacteroides fragilis. J. Immunol. 160:5000–5006.

Glass, T. L., and P. B. Hylemon. 1980. Characterization of the pyridine nucleotide-nonspecific glutamate dehydrogenase from Bacteroides thetaiotaomicron. J. Bacteriol. 141:1320–1330.

Godoy, V. G., M. M. Dallas, T. A. Russo, and M. H. Malamy. 1993. A role for Bacteroides fragilis neuraminidase in bacterial growth in two model systems. Infect. Immun. 61:4415–4426.

Goldstein, E. J., and D. M. Cirton. 1988. Annual incidence, epidemiology, and comparative in vitro susceptibilities to cefoxitin, cefotetan, cefmetazole, and ceftizoxime of recent community-acquired isolates of the Bacteroides fragilis group. J. Clin. Microbiol. 26:2361–2366.

Gorbach, S. L., J. W. Mayhew, J. G. Bartlett, H. Thadepalli, and A. B. Onderdonk. 1976. Rapid diagnosis of anaerobic infections by direct gas-liquid chromatography of clinical specimens. J. Clin. Invest. 57:478–484.

Gordon, H. A., and E. Bruckner-Kardoss. 1961. Effect of normal microbial flora on intestinal surface area. Am. J. Physiol. 201:175–178.

Gregory, E. M., W. E. C. Moore, and L. V. Holdeman. 1978. Superoxide dismutase in anaerobes: Survey. Appl. Environ. Microbiol. 35:988–991.

Gregory, E. M., and C. H. Dapper. 1983. Isolation of iron-containing superoxide dismutase from Bacteroides fragilis: Reconstitution as a Mn-containing enzyme. Arch. Biochem. Biophys. 220:293–300.

Groves, D. J., and V. L. Clark. 1987. Preparation of ribonucleic acid probes specific for Bacteroides fragilis. Diagn. Microbiol. Infect. Dis. 7:273–278.

Guiney, D. G., P. Hasegawa, and C. Davis. 1984a. Expression in Escherichia coli of cryptic tetracycline resistance genes from Bacteroides R plasmids. Plasmid 11:248–252.

Guiney, D. G., P. Hasegawa, and C. E. Davis. 1984b. Plasmid transfer from Escherichia coli to Bacteroides fragilis: Differential expression of antibiotic resistance phenotypes. Proc. Natl. Acad. Sci. USA 81:7203–7206.

Gupta, I., J. Baptista, W. R. Bruce, C. T. Che, R. Furrer, J. S. Gingerich, A. A. Grey, L. Marai, P. Yates, and J. J. Krepinsky. 1983. Structures of fecapentaenes, the mutagens of bacterial origin isolated from human feces. Biochemistry 22:241–245.

Gutacker, M., C. Valsangiacomo, and J.-C. Piffaretti. 2000. Identification of two genetic groups in Bacteroides fragilis by multilocus enzyme electrophoresis: Distribution of antibiotic resistance (cfiA, cepA) and enterotoxin (bft) encoding genes. Microbiol. 146:1241–1254.

Guthrie, E. P., N. Shoemaker, and A. A. Salyers. 1985. Cloning and expression in Escherichia coli of a gene coding for chondroitin lyase from Bacteroides thetaiotaomicron. J. Bacteriol. 164:510–515.

Guzman, C. A., M. Plate, and C. Pruzzo. 1990. Role of neuraminidase-dependent adherence in Bacteroides fragilis attachment to human epithelial cells. FEMS Microbiol. Lett. 59:187–192.

Guzman, C. A., F. Biavasco, and C. Pruzzo. 1997. News and Notes: adhesiveness of Bacteroides fragilis strains isolated from feces of health donors, abscesses, and blood. Curr. Microbiol. 34:332–334.

Haggoud, A., G. Reysset, H. Azeddoug, and M. Sebald. 1994. Nucleotide sequence analysis of two 5-nitroimidazole resistance determinants from Bacteroides strains and of a new insertion sequence upstream of the two genes. Antimicrob. Agents Chemother. 38:1047–1051.

Halulam, M. C., S. Manning, and F. L. Macrina. 1991. Nucleotide sequence of ermFU, a macrolide-lincosamide-streptogramin (MLS) resistance gene encoding an RNA methylase from the conjugal element of Bacteroides fragilis V503. Nucleic Acids Res. 19:3453.

Harris, M. A., and C. A. Reddy. 1977. Hydrogenase activity and the hydrogen-fumarate electron transport system in Bacteroides fragilis. J. Bacteriol. 131:922–928.

Hawksworth, G., B. S. Drasar, and M. J. Hill. 1971. Intestinal bacteria and the hydrolysis of glycosidic bonds. J. Med. Microbiol. 4:451–459.

Hill, R. T., J. R. Parker, H. J. K. Goodman, D. T. Jones, and D. R. Woods. 1989. Molecular analysis of a novel glutamine synthetase of the anaerobe Bacteroides fragilis. J. Gen. Microbiol. 135:3271–3279.

Hirai, N., D. G. Kingston, R. L. Van Tassell, and T. D. Wilkins. 1985. Isolation and structure elucidation of fecapentaenes-12, potent mutagens from human feces. J. Nat. Prod. 48:622–630.

Hold, G. L., P. G. Quie, V. J. Russell, E. Furrie, and H. J. Flint. 2002. Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. FEMS Microbiol. Ecol. 39:33–39.

Holdeman, L. V., I. J. Good, and W. E. C. Moore. 1976. Human fecal flora: Variation in bacterial composition within individuals and a possible effect of emotional stress. Appl. Environ. Microbiol. 31:359–375.

Holdeman, L. V., E. P. Cato, and W. E. C. Moore. 1977. Anaerobe Laboratory Manual. Virginia Polytechnic Institute and State University. Blacksburg, VA.

Holdeman, L. V., R. W. Kelley, and W. E. C. Moore. 1986. Family I Bacteroidaceae and genus I Bacteroides. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. 1st ed. Williams and Wilkins. Baltimore, MD.

Hooper, L. V., J. Xu, P. G. Falk, T. Midtvedt, and J. I. Gordon. 1999. A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. Proc. Natl. Acad. Sci. USA 96:9833–9838.

Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J. I. Gordon. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science 291:881–884.

Hwa, V., and A. A. Salyers. 1992a. Analysis of two chondroitin sulfate utilization mutants of Bacteroides thetaiotaomicron that differ in their abilities to compete with the wild type in the gastrointestinal tracts of germ-free mice. Appl. Environ. Microbiol. 58:869–876.

Hwa, V., and A. A. Salyers. 1992b. Evidence for differential regulation of genes in the chondroitin sulfate utilization pathway of Bacteroides thetaiotaomicron. J. Bacteriol. 174:342–344.

Hylemon, P. B., and P. V. Phibbs. 1974. Evidence against the presence of cyclic AMP and related enzymes in selected strains of Bacteroides fragilis. Biochem. Biophys. Res. Commun. 60:88–95.

Hylemon, P. B., and J. A. Sherrod. 1975. Multiple forms of 7-a-hydroxysteroid dehydrogenase in selected strains of Bacteroides fragilis. J. Bacteriol. 122:418–424.

Hylemon, P. B., and E. J. Stellwag. 1976. Bile acid biotransformation rates of selected Gram-positive and Gram-negative intestinal anaerobic bacteria. Biochem. Biophys. Res. Commun. 69:1088–1094.

Hylemon, P. B., J. L. Young, R. F. Roadcap, and P. V. Phibbs. 1977. Uptake and incorporation of glucose and mannose by whole cells of Bacteroides thetaiotaomicron. Appl. Environ. Microbiol. 34:488–494.

Ingham, H. R., S. Eaton, C. W. Venables, and P. C. Adams. 1978. Bacteroides fragilis resistant to metronidazole after long-term therapy. Lancet 1:214.

Ingham, H. R., P. R. Sisson, R. L. Middleton, H. K. Narang, A. A. Codd, and J. B. Selkon. 1981. Phagocytosis and killing of bacteria in aerobic and anaerobic conditions. J. Med. Microbiol. 14:391–399.

Jacobus, N. V., and F. P. Tally. 1988. Activity of trospectomycin against Bacteroides fragilis and other Bacteroides species. Antimicrob. Agents Chemother. 32:584–586.

Johnson, J. L. 1978a. Taxonomy of the bacteroides. I: Deoxyribonucleic acid homologies among Bacteroides fragilis and other saccharolytic Bacteroides species. Int. J. Syst. Bacteriol. 28:245–256.

Johnson, J. L., and D. A. Ault. 1978b. Taxonomy of the Bacteroides:II. Correlation of phenotypic characteristics with deoxyribonucleic acid homology groupings for Bacteroides fragilis and other saccharolytic Bacteroides species. Int. J. Syst. Bacteriol. 28:257–268.

Johnson, C. C., J. Baldessarre, and M. E. Levison. 1997. Peritonitis: Update on pathophysiology, clinical manifestations, and management. Clin. Infect. Dis. 24:1035–1047.

Jones, G. R., and C. G. Gemmell. 1982. Impairment by Bacteroides species of opsonization and phagocytosis of enterobacteria. J. Med. Microbiol. 15:351–361.

Jotwani, R., N. Kato, H. Kato, K. Watanabe, and K. Ueno. 1995. Detection of Bacteroides fragilis in clinical specimens by polymerase chain reaction amplification of the neuraminidase gene. Curr. Microbiol. 31:215–219.

Jousimies-Somer, H. R., P. H. Summanen, and S. M. Finegold. 1999. Bacteroides, Porphyromonas, Prevotella, Fusobacterium, and other anaerobic Gram-negative rods and cocci. *In:* P. R. Murray, E. J. Baron,

M. A. Pfaller, F. C. Tenover, and R. H. Yolken (Eds.) Manual of Clinical Microbiology. ASM Press. Washington, DC. 690–711.

Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules. *In:* H. N. Munro (Ed.) Mammalian Protein Metabolism. Academic Press. New York, NY. 21–132.

Kato, N., M. Miyauchi, Y. Muto, K. Watanabe, and K. Ueno. 1988. Emergence of fluoroquinolone resistance in Bacteroides fragilis accompanied by resistance to b-lactam antibiotics. Antimicrob. Agents Chemother. 32:1438.

Kato, N., H. Kato, K. Watanabe, and K. Ueno. 1996. Association of enterotoxigenic Bacteroides fragilis with bacteremia. Clin. Infect. Dis. 23 (Suppl. 1):S83–S86.

Kawamoto, K., I. Horibe, and K. Uchida. 1989. Purification and characterization of a new hydrolase for conjugated bile acids, chenodeoxycholytaurine hydrolase from Bacteroides vulgatus. J. Biochem. 106:1049–1053.

Keller, R., and N. Traub. 1974. The characterization of Bacteroides fragilis bacteriophage recovered from animal sera: observations on the nature of bacteroides phage carrier cultures. J. Gen. Virol. 24:179–189.

Kim, J. M., Y. K. Oh, Y. J. Kim, H. B. Oh, and Y. J. Cho. 2001. Polarized secretion of CXC chemokines by human intestinal epithelial cells in response to Bacteroides fragilis enterotoxin: NF-kB plays a major role in the regulation of IL-8 expression. Clin. Exp. Immunol. 123:421–427.

Kinouchi, T., Y. Manabe, K. Wakisaka, and Y. Ohnishi. 1982. Biotransformation of 1-nitropyrene in intestinal anaerobic bacteria. Microbiol. Immunol. 26:993–1005.

Kleivdal, H., and T. Hofstad. 1995. Chromosomal restriction endonuclease analysis and ribotyping of Bacteroides fragilis. APMIS 103:180–184.

Kory, M. M., and S. J. Booth. 1986. Characterization of Bacteroides fragilis bacteriophages and comparison to their DNAs. Curr. Microbiol. 14:199–203.

Kotarski, S. F., J. Linz, D. M. Braun, and A. A. Salyers. 1985. Analysis of outer membrane proteins which are associated with growth of Bacteroides thetaiotaomicron on chondroitin sulfate. J. Bacteriol. 163:1080–1086.

Kreader, C. A. 1995. Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution. Appl. Environ. Microbiol. 61:1171–1179.

Kreader, C. A. 1998. Persistence of PCR-detectable Bacteroides distasonis from human feces in river water. Appl. Environ. Microbiol. 64:4103–4105.

Krinos, C. M., M. J. Coyne, K. G. Weinacht, A. O. Tzianabos, D. L. Kasper, and L. E. Comstock. 2001. Extensive surface diversity of a commensal microorganism by multiple DNA inversions. Nature 414:555–558.

Kuritza, A. P., and A. A. Salyers. 1983. Digestion of proteoglycan by Bacteroides thetaiotaomicron. J. Bacteriol. 153:1186.

Kuritza, A. P., C. E. Getty, P. Shaughnessy, R. Hesse, and A. A. Salyers. 1986a. DNA probes for identification of clinically important Bacteroides species. J. Clin. Microbiol. 23:343–349.

Kuritza, A. P., P. Shaughnessy, and A. A. Salyers. 1986b. Enumeration of polysaccharide-degrading Bacteroides species in human feces by using species-specific DNA probes. Appl. Environ. Microbiol. 51:385–390.

Kuwahara, T., I. Norimatsu, H. Nakayama, S. Akimoto, K. Kataoka, H. Arimochi, and Y. Ohnishi. 2001. Genetic variation in 16S-23S rDNA internal transcribed spacer regions and the possible use of this genetic variation for molecular diagnosis of Bacteroides species. Microbiol. Immunol. 45:191–199.

Kuwahara, T., M. R. Sarker, H. Ugai, S. Akimoto, S. M. Shaheduzzaman, H. Nakayama, T. Miki, and Y. Ohnishi. 2002. Physical and genetic map of the Bacteroides fragilis YCH46 chromosome. FEMS Microbiol. Lett. 207:193–197.

Larson, G. L. 1988. Deconjugation of biliary metabolites by microflora, β-glucuronidases, sulphatases and cysteine conjugate b-lyases and their subsequent enterohepatic circulation. *In:* I. R. Rowland (Ed.) Role of Gut Flora in Toxicity and Cancer. Academic Press. London, UK. 79–108.

Leng, Z., D. E. Riley, R. E. Berger, J. N. Krieger, and M. C. Roberts. 1997. Distribution and mobility of the tetracycline resistance determinant tetQ. J. Antimicrob. Chemother. 40:551–559.

Lesser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller. 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microbiol. 68:673–690.

Li, L. Y., N. B. Shoemaker, and A. A. Salyers. 1995. Location and characteristics of the transfer region of a Bacteroides conjugative transposon and regulation of transfer genes. J. Bacteriol. 177:4992–4999.

Lindberg, A. A., A. Weintraub, U. Zahringer, and R. T. Rietschel. 1990. Structure-activity relationships in lipopolysaccharides of Bacteroides fragilis. Rev. Infect. Dis. 12 (Suppl. 2):S133–S141.

Lindner, J. G., J. H. Marcelis, N. M. de Vos, and J. A. Hoogkamp-Korstanje. 1979. Intracellular polysaccharide of Bacteroides fragilis. J. Gen. Microbiol. 111:93–99.

Lipeski, L., E. P. Guthrie, M. O'Brien, S. F. Kotarski, and A. A. Salyers. 1986. Comparison of proteins involved in chondroitin sulfate utilization by three colonic Bacteroides species. Appl. Environ. Microbiol. 51:978–984.

Livingston, S. J., S. D. Kominos, and R. D. Yee. 1978. New medium for selection and presumptive identification of the Bacteroides fragilis group. J. Clin. Microbiol. 7:448–453.

Long, S. S., and R. M. Swenson. 1977. Development of anaerobic fecal flora in healthy newborn infants. J. Pediatr. 91:298–301.

Lopez-Boado, Y. S., C. L. Wilson, L. V. Hooper, J. I. Gordon, and S. J. Hultgren. 2000. Bacterial exposure induces and activates matrilysin in mucosal epithelial cells. J. Cell Biol. 148:1305–1315.

Lorber, B., and R. M. Swenson. 1975. The bacteriology of intra-abdominal infections. Surg. Clin. North Am. 55:1349–1354.

Lubbe, M. M., P. L. Botha, and L. J. Chalkley. 1999. Comparative activity of eighteen antimicrobial agents against anaerobic bacteria isolated in South Africa. Eur. J. Clin. Microbiol. Infect. Dis. 18:46–54.

Lutton, D. A., S. Patrick, A. D. Crockard, L. D. Stewart, M. J. Larkin, E. Dermott, and T. A. McNeil. 1991. Flow cytometry analysis of within-strain variation in polysaccharide expression by Bacteroides fragilis by use of murine monoclonal antibodies. J. Med. Microbiol. 35:229–237.

MacDonald, I. A., C. N. Williams, D. E. Mahony, and W. M. Christie. 1975. NAD- and NADP-dependent 7-a-hydroxysteroid dehydrogenases from Bacteroides fragilis. Biochim. Biophys. Acta 384:12–24.

Macfarlane, G. T., J. H. Cummings, and C. Allison. 1986. Protein degradation by human intestinal bacteria. J. Gen. Microbiol. 132:1647–1656.

Macfarlane, G. T., S. Hay, S. Macfarlane, and G. R. Gibson. 1990. Effect of different carbohydrates on growth, polysaccharidase, and glycosidase production by Bacteroides ovatus in batch and continuous culture. J. Appl. Bacteriol. 68:179–187.

Macfarlane, G. T., and J. H. Cummings. 1991a. The colonic flora, fermentation, and large bowel digestive function. *In:* S. F. Phillips, J. H. Pemberton, and R. G. Shorter (Eds.) The Large Intestine: Physiology, Pathophysiology, and Disease. Raven Press. New York, NY. 51–92.

Macfarlane, G. T., and G. R. Gibson. 1991b. Co-utilization of polymerized carbon sources by Bacteroides ovatus grown in a two-stage continuous culture system. Appl. Environ. Microbiol. 57:1–6.

Macy, J. M., I. Probst, and G. Gottschalk. 1975. Evidence for cytochrome involvement in fumarate reduction and adenosine 5′-triphosphate synthesis by Bacteroides fragilis grown in the presence of hemin. J. Bacteriol. 123:436–442.

Macy, J. M., L. G. Ljungdahl, and G. Gottschalk. 1978. Pathway of succinate and propionate formation in Bacteroides fragilis. J. Bacteriol. 134:84–91.

Macy, J. M. 1984. The biology of gastrointestinal Bacteroides. Ann. Rev. Microbiol. 33:561–594.

Madinier, I., T. Fosse, J. Giudicelli, and R. Labia. 2001. Cloning and biochemical characterization of a class A beta-lactamase from Prevotella intermedia. Antimicrob. Agents Chemother. 45:2386–2389.

Marteau, P., P. Pochart, J. Dore, C. Bera-Maillet, A. Bernalier, and G. Corthier. 2001. Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl. Environ. Microbiol. 67:4939–4942.

Mastrantonio, P., R. Cardines, and P. Spigaglia. 1996. Oligonucleotide probes for detection of cephalosporinases among Bacteroides strains. Antimicrob. Agents Chemother. 40:1014–1016.

Masuda, N. 1981. Deconjugation of bile salts by Bacteroides and Clostridium. Microbiol. Immunol. 25:1–11.

May, A. K., T. G. Gleason, R. G. Sawyer, and T. L. Pruett. 2000. Contribution of Escherichia coli alpha-hemolysin to bacterial virulence and to intraperitoneal alterations in peritonitis. Infect. Immun. 68:176–183.

McBain, A. J., and G. T. Macfarlane. 1998. Ecological and physiological studies on large intestinal bacteria in relation to production of hydrolytic and reductive enzymes involved in formation of genotoxic metabolites. J. Med. Microbiol. 47:407–416.

McCarthy, R. E., S. F. Kotarski, and A. A. Salyers. 1985. Location and characteristics of enzymes involved in the breakdown of polygalacturonic acid by Bacteroides thetaiotaomicron. J. Bacteriol. 161:493–499.

McCarthy, R. E., M. Pajeau, and A. A. Salyers. 1988. Role of starch as a substrate for Bacteroides vulgatus growing in the human colon. Appl. Environ. Microbiol. 54:1916.

Mevissen-Verhage, E. A. E., J. H. Marcelis, M. N. De Vos, W. Amerongen, and J. Verhoef. 1987. Bifidobacterium, Bacteroides, and Clostridium spp. in fecal samples from breast-fed and bottle-fed infants with and without iron supplement. J. Clin. Microbiol. 25:285–289.

Mikamo, H., K. Kawazoe, Y. Sato, and T. Tamaya. 1999. Elastase activity of anaerobes isolated from amniotic fluid with preterm premature rupture of membranes. Am. J. Obstet. Gynecol. 180:378–380.

Miyamae, S., H. Nikaido, Y. Tanaka, and F. Yoshimura. 1998. Active efflux of norfloxacin by Bacteroides fragilis. Antimicrob. Agents Chemother. 42:2121.

Miyamae, S., O. Ueda, F. Yoshimura, J. Hwang, Y. Tanaka, and H. Nikaido. 2001. A MATE family multidrug efflux transporter pumps out fluoroquinolones in Bacteroides thetaiotaomicron. Antimicrob. Agents Chemother. 45:3341–3346.

Miyamoto, Y. and K. Itoh. 2000. Bacteroides acidifaciens sp. nov., isolated from the caecum of mice. Int. J. Syst. Evol. Microbiol. 50:145–148.

Moncrief, J. S., R. Obiso, L. A. Barroso, J. J. Kling, R. L. Wright, R. L. van Tassel, D. M. Lyerly, and T. D. Wilkens. 1995. The enterotoxin of Bacteroides fragilis is a metalloprotease. Infect. Immun. 63:181.

Moncrief, J. S., A. J. Duncan, R. L. Wright, L. A. Barroso, and T. D. Wilkens. 1998. Molecular characterization of the fragilysin pathogenicity islet of enterotoxigenic Bacteroides fragilis. Infect. Immun. 66:1735–1739.

Moore, W. E. C., and L. V. Holdeman. 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. 27:961–979.

Moore, W. E. C., L. V. Moore, E. P. Cato, T. D. Wilkins, and E. T. Kornegay. 1987. Effect of high-fiber and high-oil diets on the fecal flora of swine. Appl. Environ. Microbiol. 53:1638–1644.

Moore, W. E., and L. H. Moore. 1995. Intestinal floras of populations that have a high risk of colon cancer. Appl. Environ. Microbiol. 61:3202–3207.

Morgan, R. M., and F. M. Macrina. 1997. bctA: A novel pBF4 gene necessary for conjugal transfer in Bacteroides spp. Microbiology 143:2155–2165.

Morotomi, M., T. Ohno, and M. Mutai. 1988. Rapid and correct identification of intestinal Bacteroides spp. with chromosomal DNA probes by whole-cell dot blot hybridization. Appl. Environ. Microbiol. 54:1158–1164.

Morris, J. G. 1991. Characteristics of anaerobic metabolism. *In:* B. I. Duerden and B. S. Drasar (Eds.) Anaerobes in Human Disease. John Wiley. New York, NY. 16–37.

Murakami, M., O. Ozawa, T. Kanematsu, and Y. Yamada. 1990. A new restriction endonuclease from Bacteroides distasonis (BdiI). Agric. Biol. Chem. 54:275–277.

Murakami, M., O. Ozawa, T. Kanematsu, and Y. Yamada. 1991. Characterization of restriction endonuclease BdiSI, an isoschizomer of SfeI from Bacteroides distasonis strain S-7. Agric. Biol. Chem. 55:261–263.

Myers, L. L., D. S. Shoop, B. D. Firehammer, and M. M. Border. 1985. Association of enterotoxigenic Bacteroides fragilis with diarrheal disease in calves. J. Infect. Dis. 152:1344–1347.

Myers, L. L., and D. S. Shoop. 1987. Association of enterotoxigenic Bacteroides fragilis with diarrheal disease in young pigs. Am. J. Vet. Res. 48:774–775.

Myers, L. L., D. S. Shoop, J. E. Collins, and W. C. Bradbury. 1989. Diarrheal disease caused by enterotoxigenic Bacteroides fragilis in infant rabbits. J. Clin. Microbiol. 27:2025–2030.

Namavar, F., A. M. J. J. Verweij-van Vught, M. Bal, T. J. van Steenbergen, J. De Graaff, and D. M. MacLaren. 1983. Effect of anaerobic bacteria on killing of Proteus mirabilis by human polymorphonuclear leukocytes. Infect. Immun. 40:930–935.

Namavar, F., M. W. Van der Bijl, B. J. Appelmelk, J. De Graaff, and D. M. MacLaren. 1994. The role of neuraminidase in haemagglutination and adherence to colon WiDr cells by Bacteroides fragilis. J. Med. Microbiol. 40:393–396.

Nanno, M., M. Morotomi, H. Takayama, T. Kuroshima, R. Tanaka, and M. Mutai. 1986. Mutagenic activation of biliary metabolites of benzo(a)pyrene by b-glucuronidase-positive bacteria in human feces. J. Med. Microbiol. 22:351–355.

Narikawa, S., T. Suzuki, M. Yamamoto, and M. Nakamura. 1991. Lactate dehydrogenase activity as a cause of metronidazole resistance in Bacteroides fragilis NCTC 11295. J. Antimicrob. Chemother. 28:47–53.

Nikolich, M. P., N. B. Shoemaker, and A. A. Salyers. 1992. A Bacteroides tetracycline resistance gene represents a new class of ribosome protection tetracycline resistance. Antimicrob. Agents Chemother. 36:1005–1012.

Nikolich, M. P., G. Hong, N. B. Shoemaker, and A. A. Salyers. 1994. Evidence for natural horizontal transfer of tetQ between bacteria that normally colonize humans and bacteria that normally colonize livestock. Appl. Environ. Microbiol. 60:3255–3260.

Obiso, R., D. M. Lyerly, R. L. van Tassel, and T. D. Wilkens. 1995. Proteolytic activity of the Bacteroides fragilis enterotoxin causes fluid secretion and intestinal damage in vivo. Infect. Immun. 63:3820–3826.

Odelson, D. A., J. L. Rasmussen, C. J. Smith, and F. L. Macrina. 1987. Extrachromosomal systems and gene transmission in anaerobic bacteria. Plasmid 17:87–109.

Oh, H., N. El Amin, T. Davies, P. C. Appelbaum, and C. Edlund. 2001. gyrA mutations associated with quinolone resistance in Bacteroides fragilis group strains. Antimicrob. Agents Chemother. 45:1977–1981.

Ohkuma, M., S. Noda, Y. Hongoh, and T. Kudo. 2002. Diverse bacteria related to the bacteroides subgroup of the CFB phylum within the gut symbiotic communities of various termites. Biosci. Biotechnol. Biochem. 66:78–84.

Olsen, I. and H. N. Shah. 2001. Review and outcome of the meetings held in Manchester, UK, June 2000 by the international committee on the systematics of Prokaryotes Subcommittee on the Taxonomy of Gram-negative Anaerobic Rods. Anaerobe 7:329–331.

Onderdonk, A. B., J. G. Bartlett, T. J. Louie, N. Sullivan-Seigler, and S. L. Gorbach. 1976. Microbial synergy in experimental intra-abdominal abscess. Infect. Immun. 13:22–26.

Onderdonk, A. B., D. L. Kasper, R. L. Cisneros, and J. G. Bartlett. 1977. The capsular polysaccharide of Bacteroides fragilis as a virulent factor: Comparison of the pathogenic potential of encapsulated and unencapsulated strains. J. Infect. Dis. 136:82–89.

Onderdonk, A. B., N. Moon, D. L. Kasper, and J. G. Bartlett. 1978. Adherence of Bacteroides fragilis in vivo. Infect. Immun. 19:1083–1087.

Onodera, Y., and K. Sato. 1999. Molecular cloning of the gyrA and gyrB genes of Bacteroides fragilis encoding DNA gyrase. Antimicrob. Agents Chemother. 43:2423–2429.

Otto, B. R., A. M. Verweij-van Vught, J. van Doorn, and D. M. MacLaren. 1988. Outer membrane proteins of Bacteroides fragilis and Bacteroides vulgatus in relation to iron uptake and virulence. Microb. Pathog. 4:279–287.

Otto, B. R., M. Sparrius, A. M. J. J. Verweij-van Vught, and D. M. MacLaren. 1990. Iron regulated outer membrane protein of Bacteroides fragilis involved in heme uptake. Infect. Immun. 58:3954–3958.

Otto, B. R., W. R. Verweij, M. Sparrius, A. M. Verweij-van Vught, C. E. Nord, and D. M. MacLaren. 1991. Human immune response to an iron-repressible outer membrane protein of Bacteroides fragilis. Infect. Immun. 59:2999–3003.

Otto, B. R., M. Sparrius, D. J. Wors, F. K. de Graaf, and D. M. MacLaren. 1994. Utilization of haem from the haptoglobin-haemoglobin complex by Bacteroides fragilis. Microb. Pathog. 17:137–147.

Otto, B. R., S. J. van Dooren, C. M. Dozois, J. Luirink, and B. Oudega. 2002. Escherichia coli hemoglobin protease autotransporter contributes to synergistic abscess formation and heme-dependent growth of Bacteroides fragilis. Infect. Immun. 70:5–10.

Oyston, P. C., and P. S. Handley. 1991. Surface components of Bacteroides fragilis involved in adhesion and haemagglutination. J. Med. Microbiol. 34:51–55.

Pan, N., and J. A. Imlay. 2001. How does oxygen inhibit central metabolism in the obligate anaerobe Bacteroides thetaiotaomicron. Molec. Microbiol. 39:1562–1571.

Park, M. K., R. A. M. Myers, and L. Marzella. 1992. Oxygen tension and infections: modulation of microbial growth, activity of antimicrobial agents, and immunologic responses. Clin. Infect. Dis. 14:720–740.

Parker, A. C., and C. J. Smith. 1993. Genetic and biochemical analysis of a novel ambler class A b-lactamase responsible for cefoxitin resistance in Bacteroides species. Antimicrob. Agents Chemother. 37:1028–1036.

Paster, B. J., F. E. Dewhirst, I. Olsen, and G. J. Fraser. 1994. Phylogeny of Bacteroides, Prevotella, and Porphyromonas spp. and related bacteria. J. Bacteriol. 176:725–732.

Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabudhe, and F. E. Dewhirst. 2001. Bacterial diversity in human subgingival plaque. J. Bacteriol. 183:3770–3783.

Patrick, S., L. D. Stewart, N. Damani, K. G. Wilson, D. A. Lutton, M. J. Larkin, I. Poxton, and R. Brown. 1995. Immunological detection of Bacteroides fragilis in clinical samples. J. Med. Microbiol. 43:99–109.

Patrick, S., J. P. McKenna, S. O'Hagan, and E. Dermott. 1996. A comparison of the haemagglutinating and enzymic activities of Bacteroides fragilis whole cells and outer membrane vesicles. Microb. Pathog. 20:191–202.

Patrick, S., D. Gilpin, and L. Stevenson. 1999. Detection of intrastrain antigenic variation of Bacteroides fragilis surface polysaccharides by monoclonal antibody labeling. Infect. Immun. 67:4346–4351.

Pennington, C. D., and E. M. Gregory. 1986. Isolation and reconstitution of iron- and manganese-containing superoxide dismutases from Bacteroides thetaiotaomicron. J. Bacteriol. 166:528–532.

Peterson, M. L., L. D. Hovde, D. H. Wright, A. D. Hoang, J. K. Raddatz, P. J. Boysen, and C. Rotschafer. 1999. Fluoroquinolone resistance in Bacteroides fragilis following sparfloxacin exposure. Antimicrob. Agents Chemother. 43:2251–2255.

Piddock, L. J., and R. Wise. 1986. Properties of the penicillin-binding proteins of four species of the genus Bacteroides. Antimicrob. Agents Chemother. 29:825–832.

Piddock, L. J., and R. Wise. 1987. Cefoxitin resistance in Bacteroides species: evidence indicating two mechanisms causing decreased susceptibility. J. Antimicrob. Chemother. 19:170.

Podglajen, I., J. Breuil, F. Bordon, L. Gutmann, and E. Collatz. 1992. A silent carbapenemase gene in strains of Bacteroides fragilis can be expressed after a one-step mutation. FEMS Microbiol. Lett. 91:21–30.

Podglajen, I., J. Breuil, and E. Collatz. 1994. Insertion of a novel DNA sequence, IS1186, upstream of the silent carbapenemase gene cfiA, promotes expression of carbapenem resistance in clinical isolates of Bacteroides fragilis. Molec. Microbiol. 12:105–114.

Podglajen, I., J. Breuil, I. Casin, and E. Collatz. 1995. Genotypic identification of two groups within the species Bacteroides fragilis by ribotyping and by analysis of PCR- generated fragment patterns and insertion sequence content. J. Bacteriol. 177:5270–5275.

Podglajen, I., J. Breuil, C. Monsempes, and E. Collatz. 2001. Multiple mobile promoter regions for the rare carbapenem resistance gene of Bacteroides fragilis. J. Bacteriol. 183:3531–3535.

Poxton, I., and D. M. Edmund. 1995. Biological activity of Bacteroides lipopolysaccharide-reappraisal. Clin. Infect. Dis. 20 (Suppl. 2):S149–S153.

Privitera, G., A. Dublanchet, and M. Sebald. 1979a. Transfer of multiple antibiotic resistance between subspecies of Bacteroides fragilis. J. Infect. Dis. 139:97–101.

Privitera, G., M. Sebald, and F. Fayolle. 1979b. Common regulatory mechanism of expression and conjugative ability of a tetracycline resistance plasmid in Bacteroides fragilis. Nature 278:657–659.

Pruzzo, C., C. A. Guzman, and B. Dainelli. 1989. Incidence of hemaggultination activity among pathogenic and nonpathogenic Bacteroides fragilis strains and the role of capsule and pili in HA and adherence. FEMS Microbiol. Lett. 50:113–118.

Rasmussen, J. L., D. A. Odelson, and F. L. Macrina. 1986. Complete nucleotide sequence and transcription of ermF, a macrolide-lincosamide-streptogramin B resistance determinant from Bacteroides fragilis. J. Bacteriol. 168:523–533.

Rasmussen, B. A., Y. Gluzman, and F. P. Tally. 1990. Cloning and sequencing of the class B beta-lactamase gene (ccrA) from Bacteroides fragilis TAL3636. Antimicrob. Agents Chemother. 34:1590–1592.

Rasmussen, B. A., and E. Kovacs. 1991. Identification and DNA sequence of a new Bacteroides fragilis insertion sequence-like element. Plasmid 25:141–144.

Rasmussen, B. A., K. Bush, and F. P. Tally. 1997. Antimicrobial resistance in anaerobes [review, 123 refs]. Clin. Infect. Dis. 24 (Suppl. 1):S110–S120.

Redondo, M. C., M. D. Arbo, J. Grindlinger, and D. R. Snydman. 1995. Attributable mortality of bacteremia associated with the Bacteroides fragilis group. Clin. Infect. Dis. 20:1492–1496.

Reeves, A. R., J. N. D'elia, J. Frias, and A. A. Salyers. 1996. A Bacteroides thetaiotaomicron outer membrane protein that is essential for utilization of maltooligosaccharides and starch. J. Bacteriol. 178:823–830.

Reeves, A. R., G.-R. Wang, and A. A. Salyers. 1997. Characterization of four outer membrane proteins that play a role in utilization of starch by Bacteroides thetaiotaomicron. J. Bacteriol. 179:643–649.

Reid, G., A. W. Bruce, M. Llano, J. A. McGroarty, and M. Blake. 1990. Bacterial aggregation in sepsis. Curr. Microbiol. 20:185–190.

Reinecke, S. N., and R. M. Morgan. 1991. BfaI, a new MaeI isoschizomer from Bacteroides fragilis, recognizes the sequence 5′CTAG 3′. Nucleic Acids Res. 19:1152–1152.

Reyes, J. C., and F. J. Florencio. 1994. A new type of glutamine synthetase in cyanobacteria: the protein encoded by the glnN gene supports nitrogen assimilation in Synechocystis sp. strain PCC 6803. J. Bacteriol. 176:1260–1267.

Reysset, G., A. Haggoud, W. J. Su, and M. Sebald. 1992. Genetic and molecular analysis of pIP417 and pIP419: Bacteroides plasmids encoding 5-nitroimidazole resistance. Plasmid 27:181–190.

Ricci, V., and L. J. Piddock. 2000. Accumulation of norfloxacin by Bacteroides fragilis. Antimicrob. Agents Chemother. 44:2361–2366.

Riegler, M., M. Lotz, C. Sears, C. Pothoulakis, I. Castagliuolo, C. C. Wang, R. Sedivy, T. Sogukoglu, E. Cosentini, G. Bischof, W. Feil, B. Teleky, G. Hamilton, J. T. LaMont, and E. Wenzl. 1999. Bacteroides fragilis toxin 2 damages human colonic mucosa in vitro. Gut 44:504–510.

Roberton, A. M., and P. G. Glucina. 1982. Fructose 6-phosphate phosphorylation in Bacteroides species. J. Bacteriol. 150:1056–1060.

Roberts, M. C., W. O. Chung, and D. Roe. 1996. Characterization of tetracycline and erythromycin resistance determinants in Treponema denticola. Antimicrob. Agents Chemother. 40:1690–1694.

Roberts, M. C. 1998. Antibiotic resistance in oral/respiratory bacteria. Crit. Rev. Oral Biol. Med. 9:522–540.

Roberts, M. C., W. O. Chung, D. Roe, M. Xia, C. Marquez, G. Borthagaray, W. L. Whittington, and K. K. Holmes. 1999. Erythromycin resistant Neissereia gonorrhoeae and oral commensal Neisseria spp. carry known rRNA methylase genes. Antimicrob. Agents Chemother. 43:1367–1372.

Robinson, I. M., M. J. Allison, and J. A. Bucklin. 1981. Characterization of the cecal bacteria of normal pigs. Appl. Environ. Microbiol. 41:959–955.

Rocha, E. R., M. de Uzeda, and J. H. Brock. 1991. Effect of ferric and ferrous iron chelators on growth of Bacteroides fragilis under anaerobic conditions. FEMS Microbiol. Lett. 68:45–50.

Rocha, E. R., and C. J. Smith. 1995. Biochemical and genetic analyses of a catalase from the anaerobic bacterium Bacteroides fragilis. J. Bacteriol. 177:3111–3119.

Rocha, E. R., T. Selby, J. P. Coleman, and C. J. Smith. 1996. Oxidative stress response in an anaerobe, Bacteroides fragilis: A role for catalase in protection against hydrogen peroxide. J. Bacteriol. 178:6895–6903.

Rocha, E. R., and C. J. Smith. 1999. Role of the alkyl hydroperoxide reductase (ahpCF) gene in oxidative stress defense of the obligate Anaerobe bacteroides fragilis. J. Bacteriol. 181:5701–5710.

Rocha, E. R., G. Owens Jr., and C. J. Smith. 2000. The redox-sensitive transcriptional activator OxyR regulates the peroxide response regulon in the obligate anaerobe Bacteroides fragilis. J. Bacteriol. 182:5059–5069.

Rocha, E. R., A. Smith, C. J. Smith, and J. H. Brock. 2001. Growth inhibition of Bacteroides fragilis by hemopexin: Proteolytic degradation of hemopexin to overcome heme limitation. FEMS Microbiol. Lett. 199:73–78.

Rogemond, V., and R. M. F. Guinet. 1986. Lectin-like adhesins in the Bacteroides fragilis group. Infect. Immun. 53:99–102.

Rogers, M. B., A. C. Parker, and C. J. Smith. 1993. Cloning and characterization of the endogenous cephalosporinase gene, cepA, from Bacteroides fragilis reveals a new subgroup of Ambler class A b-lactamases. Antimicrob. Agents Chemother. 37:2391–2400.

Rogers, M. B., T. K. Bennett, C. M. Payne, and C. J. Smith. 1994. Insertional activation of cepA leads to high-level b-lactamase expression in Bacteroides fragilis clinical isolates. J. Bacteriol. 176:4376–4384.

Rolfe, R. D., D. J. Hentges, J. T. Barrett, and B. J. Campbell. 1977. Oxygen tolerance of human intestinal anaerobes. Am. J. Clin. Nutr. 30:1762–1769.

Rotstein, O. D., T. L. Pruett, and R. L. Simmons. 1985. Mechanisms of microbial synergy in polymicrobial surgical infections [review, 136 refs]. Rev. Infect. Dis. 7:151–170.

Rotstein, O. D., and J. Kao. 1988a. The spectrum of Escherichia coli—Bacteroides fragilis pathogenic synergy in an intraabdominal infection model. Can. J. Microbiol. 34:352–357.

Rotstein, O. D., P. E. Nasmith, and S. Grinstein. 1988b. pH-dependent impairment of the neutrophil respiratory burst by the Bacteroides byproduct succinate. Clin. Invest. Med. 11:259–265.

Rotstein, O. D. 1993. Interactions between leukocytes and anaerobic bacteria in polymicrobial surgical infections [review, 46 refs]. Clin. Infect. Dis. 16 (Suppl. 4):S190–S194.

Rudek, W., and R.-U. Haque. 1976. Extracellular enzymes of the genus Bacteroides. J. Clin. Microbiol. 4:458–460.

Ruimy, R., I. Podglajen, J. Breuil, R. Christen, and E. Collatz. 1996. A recent fixation of cfiA genes in a monophyletic cluster of Bacteroides fragilis is correlated with the presence of multiple insertion elements. J. Bacteriol. 178:1914–1918.

Ruseler-van Embden, J. G., R. van der Helm, and L. M. van Lieshout. 1989. Degradation of intestinal glycoproteins by Bacteroides vulgatus. FEMS Microbiol. Lett. 49:37–41.

Saidi, R. F., K. Jaeger, M. H. Montrose, S. G. Wu, and C. Sears. 1997. Bacteroides fragilis toxin rearranges the actin cytoskeleton of HT29/C1 cells without direct proteolysis of actin or decrease in F-actin content. Cell Motil. Cytoskel. 37:159–165.

Saito, H., I. Yamamoto, and M. Ishimoto. 1988. Reversible inactivation of glutamate dehydrogenase in Bacteroides fragilis: Purification and characterization of high activity- and low activity-enzymes. J. Gen. App. Microbiol. 34:377–385.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molec. Biol. Evol. 4:406–425.

Salyers, A. A., J. K. Palmer, and T. D. Wilkins. 1977a. Laminarinase (b-glucanase) activity in Bacteroides from the human colon. Appl. Environ. Microbiol. 33:1118–1124.

Salyers, A. A., J. R. Vercellotti, S. E. West, and T. D. Wilkins. 1977b. Fermentation of mucin and plant polysaccharides by strains of Bacteroides from the human colon. Appl. Environ. Microbiol. 33:319–322.

Salyers, A. A., and M. O'Brien. 1980. Cellular location of enzymes involved in chondroitin sulfate breakdown by Bacteroides thetaiotaomicron. J. Bacteriol. 143:772–780.

Salyers, A. A., M. O'Brien, and S. F. Kotarski. 1982. Utilization of chondroitin sulfate by Bacteroides thetaiotaomicron growing in carbohydrate-limited continuous culture. J. Bacteriol. 150:1008–1015.

Salyers, A. A., and J. A. Z. Leedle. 1983. Carbohydrate metabolism in the human colon. *In:* D. J. Hentges (Ed.) Human Intestinal Microflora in Health and Disease. Academic Press. New York, NY. 129–146.

Salyers, A. A. 1984. Bacteroides of the human lower intestinal tract. Ann. Rev. Microbiol. 38:293–313.

Salyers, A. A., M. Pajeau, and R. E. McCarthy. 1988. Importance of mucopolysaccharides as substrates for Bacteroides thetaiotaomicron growing in the intestinal tracts of exgermfree mice. Appl. Environ. Microbiol. 54:1979–1976.

Salyers, A. A., and M. Pajeau. 1989. Competitiveness of different polysaccharide utilization mutants of Bacteroides thetaiotaomicron in the intestinal tracts of germfree mice. Appl. Environ. Microbiol. 55:2572–2578.

Salyers, A. A., N. B. Shoemaker, and L. Y. Li. 1995a. In the driver's seat: The Bacteroides conjugative transposons and the elements they mobilize [review, 22 refs]. J. Bacteriol. 177:5727–5731.

Salyers, A. A., N. B. Shoemaker, A. M. Stevens, and L. Y. Li. 1995b. Conjugative transposons: An unusual and diverse set of integrated gene transfer elements. Microbiol. Rev. 59:579–590.

Salyers, A. A., G. Bonheyo, and N. Shoemaker. 2000. Starting a new genetic system: Lessons from Bacteroides. Methods 20:35–46.

Sanfilippo, L., T. J. Baldwin, M. G. Menozzi, S. P. Borriello, and Y. R. Mahida. 1998. Heterogeneity in responses by primary adult human colonic epithelial cells to purified enterotoxin of Bacteroides fragilis. Gut 43:651–655.

Sanfilippo, L., C. K. Li, R. Seth, T. J. Balwin, M. G. Menozzi, and Y. R. Mahida. 2000. Bacteroides fragilis enterotoxin induces the expression of IL-8 and transforming growth factor-beta (TGF-beta) by human colonic epithelial cells. Clin. Exp. Immunol. 119:456–463.

Sarker, M. R., S. Akimoto, S. Skimoto, T. Ono, T. Kinouchi, and Y. Ohnishi. 1995a. Molecular cloning of the leuB gene from Bacteroides fragilis by functional complementation in Escherichia coli. Microbiol. Immunol. 39:19–25.

Sarker, M. R., S. Akimoto, H. Ugai, T. Kuwahara, and Y. Ohnishi. 1995b. Nucleotide sequence of the gene encoding b-isopropylmalate dehydrogenase (leuB) from Bacteroides fragilis. Microbiol. Immunol. 39:525–529.

Sato, T. T., J. C. Kovacich, R. M. Boyle, T. L. Haddix, A. Weintraub, and T. H. Pohlman. 1998. CD14-dependent activation of human endothelial cells by Bacteroides fragilis outer membrane. J. Surg. Res. 74:103–111.

Sawyer, R. G., M. D. Spengler, R. B. Adams, and T. L. Pruett. 1991. The peritoneal environment during infection: The effect of monomicrobial and polymicrobial bacteria on pO_2 and pH. Ann. Surg. 213:253–260.

Scholle, R. R., H. E. Steffen, H. J. K. Goodman, and D. R. Woods. 1990. Expression and regulation of a Bacteroides fragilis sucrose utilization system cloned in Escherichia coli. Appl. Environ. Microbiol. 56:1944–1948.

Sebald, M., G. Reysset, and J. Breuil. 1990. What's new in 5-nitro-imidazole resistance in the Bacteroides fragilis group? *In:* S. P. Borriello (Ed.) Clinical and Molecular Aspects of Anaerobes. Wrightson Biomedical Publishing. Petersfield, UK. 217–225.

Sghir, A., G. Gramet, A. Suau, V. Rochet, P. Pochart, and J. Dore. 2000. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl. Environ. Microbiol. 66:2263–2266.

Shah, H. N., and R. A. D. Williams. 1982. Dehydrogenase patterns in the taxonomy of Bacteroides. J. Gen. Microbiol. 128:2955–2965.

Shah, H. N., K. M. Elhag, T. A. R. Al-Jalili, and A. R. Mundegar. 1987. Glucose-6-phosphate dehydrogenase

and malate dehydrogenase enzyme electrophoretic patterns amongst strains of Bacteroides fragilis. J. Gen. Microbiol. 133:1975–1981.

Shah, H. N., and M. D. Collins. 1989. Proposal to restrict the genus Bacteroides (Castellani and Chalmers) to Bacteroides fragilis and closely related species. Int. J. Syst. Bacteriol. 39:85–87.

Shaheduzzaman, S. M., S. Akimoto, T. Kuwahara, T. Kinouchi, and Y. Ohnishi. 1997. Genome analysis of Bacteroides by pulsed-field gel electrophoresis: Chromosome sizes and restriction patterns. DNA Res. 4:19–25.

Shapiro, M. E., D. L. Kasper, D. F. Zalesnik, S. Spriggs, A. B. Onderdonk, and R. W. Finberg. 1986. Cellular control of abscesses formation: Role of T-cells in the regulation of abscesses formed in response to Bacteroides fragilis. J. Immunol. 137:314–346.

Sheehan, G., and G. Harding. 1989. Intraperitoneal infections. *In:* S. M. Finegold and W. L. George (Eds.)Anaerobic Infections in Humans. Academic Press. San Diego, CA. 349–384.

Shetab, R., S. H. Cohen, T. Prindiville, Y. J. Tang, M. Cantrell, D. Rahmani, and J. Silva Jr. 1998. Detection of Bacteroides fragilis enterotoxin gene by PCR. J. Clin. Microbiol. 36:1729–1732.

Shipman, J. A., J. E. Berleman, and A. A. Salyers. 2000. Characterization of four outer membrane proteins involved in binding starch to the cell surface of Bacteroides thetaiotaomicron. J. Bacteriol. 182:5365–5372.

Shoemaker, N. B., C. Getty, E. P. Guthrie, and A. A. Salyers. 1986. Regions in Bacteroides plasmids pBFTM10 and pB8-51 that allow Escherichia coli-Bacteroides shuttle vectors to be mobilized by IncP plasmids and by a conjugative Bacteroides tetracycline resistance element. J. Bacteriol. 166:959–965.

Shoemaker, N. B., H. Vlamakis, K. Hayes, and A. A. Salyers. 2001. Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon. Appl. Environ. Microbiol. 67:561–568.

Simon, G. L., M. S. Klempner, D. L. Kasper, and S. L. Gorbach. 1982. Alterations in opsonophagocytic killing by neutrophils of Bacteroides fragilis associated with animal and laboratory passage: Effect of capsular polysaccharide. J. Infect. Dis. 145:72–77.

Slack, M. P. E., D. T. Griffiths, and H. H. Johnston. 1981. The fluoretec system for rapid diagnosis of bacteroides infections by direct immunofluorescence of clinical specimens. J. Clin. Pathol. 34:1381–1384.

Smalley, D., E. R. Rocha, and C. J. Smith. 2002. Aerobic-type ribonucleotide reductase in the anaerobe Bacteroides fragilis. J. Bacteriol. 184:895–903.

Smith, C. J., and M. P. Bryant. 1979. Introduction to the metabolic activities of intestinal bacteria. Am. J. Clin. Nutr. 32:149–157.

Smith, C. J. 1985. Polyethylene glycol-facilitated transformation of Bacteroides fragilis with plasmid DNA. J. Bacteriol. 164:466–469.

Smith, C. J. 1987. Nucleotide sequence analysis of Tn4551: Use of ermFS operon fusions to detect promoter activity in Bacteroides fragilis. J. Bacteriol. 169:4589–4596.

Smith, C. J., A. Parker, and M. B. Rogers. 1990. Plasmid transformation of Bacteroides spp. by electroporation. Plasmid 24:100–109.

Smith, C. J., and D. R. Callihan. 1992a. Analysis of rRNA restriction fragment length polymorphisms from Bacteroides spp. and Bacteroides fragilis isolates associated with diarrhea in humans and animals. J. Clin. Microbiol. 30:806–812.

Smith, C. J., C. Owen, and L. Kirby. 1992b. Activation of a cryptic streptomycin-resistance gene in the Bacteroides erm transposon, Tn4551. Molec. Microbiol. 6:2287–2297.

Smith, C. J., and A. C. Parker. 1993. Identification of a circular intermediate in the transfer and transposition of Tn4555, a mobilizable transposon from Bacteroides spp. J. Bacteriol. 175:2682–2691.

Smith, C. J., T. K. Bennett, and A. C. Parker. 1994. Molecular and genetic analysis of the Bacteroides uniformis cephalosporinase gene, cblA, encoding the species-specific beta-lactamase. Antimicrob. Agents Chemother. 38:1711–1715.

Smith, C. J., G. D. Tribble, and D. P. Bayley. 1998. Genetic elements of Bacteroides species: a moving story [review, 81 refs]. Plasmid 40:12–29.

Snydman, D. R., N. V. Jacobus, L. A. McDermott, S. Supran, G. J. Cuchural Jr., S. Finegold, L. Harrell, D. W. Hecht, P. Iannini, S. Jenkins, C. Pierson, J. Rihs, and S. L. Gorbach. 1999. Multicenter study of in vitro susceptibility of the Bacteroides fragilis group, 1995 to 1996, with comparison of resistance trends from 1990 to 1996. Antimicrob. Agents Chemother. 43:2417–2422.

Soki, J., I. Szoke, and E. Nagy. 1999. Characterisation of a 5.5-kb cryptic plasmid present in different isolates of Bacteroides spp. originating from Hungary. J. Med. Microbiol. 48:25–31.

Southern, J. A., J. R. Parker, and D. R. Woods. 1986. Expression and purification of glutamine synthetase cloned from Bacteroides fragilis. J. Gen. Microbiol. 132:2827–2835.

Speer, B. S., L. Bedzyk, and A. A. Salyers. 1991. Evidence that a novel tetracycline resistance gene found on two Bacteroides transposons encodes an NADP-requiring oxidoreductase. J. Bacteriol. 173:176–183.

Sperry, J. F., M. D. Appleman, and T. D. Wilkins. 1977. Requirement of heme for growth of Bacteroides fragilis. Appl. Environ. Microbiol. 34:386–390.

Stefen, E. K., and D. J. Hentges. 1981. Hydrolytic enzymes of anaerobic bacteria isolated from human infections. J. Clin. Microbiol. 14:153–156.

Stehle, H. W., B. Leblebicilglu, and J. D. Waters. 2001. Short-chain carboxylic acids produced by Gram-negative anaerobic bacteria can accelerate or delay polymorphonuclear leukocyte apoptosis in vitro. J. Periodontol. 72:1059–1063.

Stellwag, E. J., and P. B. Hylemon. 1976. Purification and characterization of bile salt hydrolase from Bacteroides fragilis subsp. fragilis. Biochim. Biophys. Acta 452:165–176.

Stephen, A. M., and J. H. Cummings. 1980. The microbial contribution to human fecal mass. J. Med. Microbiol. 13:45–56.

Stephen, A. M. 1987. Dietary fibre and colonic nitrogen metabolism. Scand. J. Gastroenterol. 129 (Suppl.):110–115.

Stevens, A. M., J. M. Sanders, N. B. Shoemaker, and A. A. Salyers. 1992. Genes involved in production of plasmid-like forms by a Bacteroides conjugal chromosomal element share amino acid homology with two-component regulatory systems. J. Bacteriol. 174:2935–2942.

Stevens, A. M., N. B. Shoemaker, L. Y. Li, and A. A. Salyers. 1993. Tetracycline regulation of genes on Bacteroides conjugative transposons. J. Bacteriol. 175:6134–6141.

Stiffler, P. W., R. Keller, and N. Traub. 1974. Isolation and characterization of several cryptic plasmids from clinical isolates of Bacteroides fragilis. J. Infect. Dis. 130:544–547.

Storz, G., and J. A. Imlay. 1999. Oxidative stress. Curr. Opin. Microbiol. 2:188–194.

Stubbs, S. L., J. S. Brazier, P. R. Talbot, and B. I. Duerden. 2000. PCR-restriction fragment length polymorphism analysis for identification of Bacteroides spp. and characterization of nitroimidazole resistance genes. J. Clin. Microbiol. 38:3209–3213.

Suau, A., R. Bonnet, M. Sutren, and J. J. Godon. 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. 65:4799–4807.

Summanen, P. H., E. J. Baron, D. M. Citron, C. Strong, H. M. Wexler, and S. M. Finegold. 1993. Wadsworth Anaerobic Bacteriology Manual. Star Publishing. Belmont, CA.

Takeuchi, T., Y. Nakaya, N. Kato, K. Watanabe, and K. Morimoto. 1999. Induction of oxidative DNA damage in anaerobes. FEBS Lett. 450:178–180.

Tally, F. P., P. R. Stewart, V. L. Sutter, and J. E. Rosenblatt. 1975. Oxygen tolerance of fresh clinical anaerobic bacteria. J. Clin. Microbiol. 1:161–164.

Tang, T. W., G. W. Procop, and D. H. Persing. 1997. Molecular diagnostics of infectious diseases. Clin. Chem. 43:2021–2038.

Tang, Y. P., M. M. Dallas, and M. H. Malamy. 1999. Characterization of the Batl (Bacteroides aerotolerance) operon in Bacteroides fragilis: Isolation of a B. fragilis mutant with reduced aerotolerance and impaired growth in in vivo model systems. Molec. Microbiol. 32:139–149.

Tang, Y. P., and M. H. Malamy. 2000. Isolation of Bacteroides fragilis mutants with in vivo growth defects by using Tn4400′, a modified Tn4400 transposition system, and a new screening method. Infect. Immun. 68:415–419.

Tannock, G. W. 1977. Characteristics of Bacteroides isolates from the cecum of conventional mice. Appl. Environ. Microbiol. 33:745–750.

Tartera, C., and J. Jofre. 1987. Bacteriophages active against Bacteroides fragilis in sewage polluted waters. Appl. Environ. Microbiol. 53:1632–1637.

Tartera, C., F. Lucena, and J. Jofre. 1989. Human origin of Bacteroides fragilis bacteriophages present in the environment. Appl. Environ. Microbiol. 55:2969–2701.

Teng, L. J., P. R. Hsueh, J. C. Tsai, F. L. Chiang, C. Y. Chen, S. W. Ho, and K. T. Luh. 2000. PCR assay for species-specific identification of Bacteroides thetaiotaomicron. J. Clin. Microbiol. 38:1672–1675.

Thompson, J. S., and M. H. Malamy. 1990. Sequencing the gene for an imipenem-cefoxitin-hydrolyzing enzyme (CfiA) from Bacteroides fragilis TAL2480 reveals strong similarity between CfiA and Bacillus cereus beta-lactamase II. J. Bacteriol. 172:2584–2593.

Tierny, Y., M. Bechet, J. C. Joncquiert, H. C. Dubourguier, and J. B. Guillaume. 1994. Molecular cloning and expression in Escherichia coli of genes encoding pectate lyase and pectin methylesterase activities from Bacteroides thetaiotaomicron. J. Appl. Bacteriol. 76:592–602.

Tofte, R. W., P. K. Peterson, D. Schmeling, J. Bracke, Y. Kim, and P. G. Quie. 1980. Opsonization of four Bacteroides species: role of the classical complement pathway and immunoglobulin. Infect. Immun. 27:784–792.

Tribble, G. D., A. C. Parker, and C. J. Smith. 1999. Genetic structure and transcriptional analysis of a mobilizable, antibiotic resistance transposon from Bacteroides. Plasmid 42:1–12.

Trieber, C. A., N. Burkhardt, K. H. Nierhaus, and D. E. Taylor. 1998. Ribosomal protection from tetracycline mediated by Tet(O): Tet(O) interaction with ribosomes is GTP-dependent. Biol. Chem. 379:847–855.

Trinh, S., A. Haggoud, G. Reysset, and M. Sebald. 1995. Plasmids pIP419 and pIP421 from Bacteroides: 5-nitroimidazole resistance genes and their upstream insertion sequence elements. Microbiol. 141:927–935.

Trinh, S., and G. Reysset. 1996a. Detection by PCR of the nim genes encoding 5-nitroimidazole resistance in Bacteroides spp. J. Clin. Microbiol. 34:2078–2084.

Trinh, S., A. Haggoud, and G. Reysset. 1996b. Conjugal transfer of the 5-nitroimidazole resistance plasmid pIP417 from Bacteroides vulgatus BV-17: characterization and nucleotide sequence analysis of the mobilization region. J. Bacteriol. 178:6671–6676.

Tzianabos, A. O., A. B. Onderdonk, B. Rosner, R. L. Cisneros, and D. L. Kasper. 1993. Structural features of polysaccharides that induce intra-abdominal abscesses. Science 262:416–419.

Valentine, P. C., F. C. Gherardini, and A. A. Salyers. 1991. The Bacteroides ovatus chromosomal locus which contains an alpha-galactosidase gene may be important for colonization of the gastrointestinal tract. Appl. Environ. Microbiol. 57:1615–1623.

Valentine, P. C., and A. A. Salyers. 1992. Use of inducible disaccharidases to assess the importance of different carbohydrate sources for Bacteroides ovatus growing in the intestinal tracts of germfree mice. Appl. Environ. Microbiol. 58:2698–2700.

Van de Peer, Y., and R. De Wachter. 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Applic. Biosci. 10:569–570.

van Doorn, J., B. Oudega, and D. M. MacLaren. 1992. Characterization and detection of the 40 kDa fimbrial subunit of Bacteroides fragilis BE1. Microb. Pathog. 13:75–79.

van Tassel, R. L., D. K. MacDonald, and T. D. Wilkins. 1982. Production of a fecal mutagen by Bacteroides spp. Infect. Immun. 37:975–980.

Varel, V. H., and M. P. Bryant. 1974. Nutritional features of Bacteroides fragilis sp. fragilis. Appl. Microbiol. 28:251–257.

Verweij-van Vught, A. M., F. Namavar, W. A. Vel, M. Sparrius, and D. M. MacLaren. 1986a. Pathogenic synergy between Escherichia coli and Bacteroides fragilis or B. vulgatus in experimental infections: A non-specific phenomenon. J. Med. Microbiol. 21:43–47.

Verweij-van Vught, A. M., M. Sparrius, F. Namavar, W. A. Vel, and D. M. MacLaren. 1986b. Nutritional requirements of Bacteroides fragilis and Bacteroides vulgatus with reference to iron and virulence. FEMS Microbiol. Lett. 36:51.

Verweij-van Vught, A. M., B. R. Otto, F. Namavar, M. Sparrius, and D. M. MacLaren. 1988. Ability of bacteroides species to obtain iron from iron salts, haem-compounds and transferrin. FEMS Microbiol. Lett. 49:223–228.

Viljanen, M. K., L. Linko, and O. P. Lehtonen. 1988. Detection of Bacteroides fragilis, Bacteroides thetaiotaomicron and Bacteroides ovatus in clinical specimens by immunofluorescence with monoclonal antibody to B.

fragilis lipopolysaccharide. J. Clin. Microbiol. 26:448–452.

Wallace, B. L., J. E. Bradley, and M. Rogolsky. 1981. Plasmid analyses of clinical isolates of Bacteroides fragilis and other Bacteroides species. J. Clin. Microbiol. 383:388.

Wang, R.-F., W.-W. Cao, and C. E. Cerniglia. 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Environ. Microbiol. 62:1242–1247.

Wang, J., N. B. Shoemaker, G. R. Wang, and A. A. Salyers. 2000. Characterization of a Bacteroides mobilizable transposon, NBU2, which carries a functional lincomycin resistance gene. J. Bacteriol. 182:3559–3571.

Watt, B., P. A. Geddes, O. A. Greenan, S. K. Napier, and A. Mitchel. 1982. Gas-liquid chromatography in the diagnosis of anaerobic infections: A three year experience. J. Clin. Pathol. 35:709–714.

Weaver, J., T. R. Whitehead, M. A. Cotta, P. C. Valentine, and A. A. Salyers. 1992. Genetic analysis of a locus on the Bacteroides ovatus chromosome which contains xylan utilization genes. Appl. Environ. Microbiol. 58:2764–2770.

Welch, R. A., K. R. Jones, and F. L. Macrina. 1979. Transferable lincosamide-macrolide resistance in Bacteroides. Plasmid 2:261–268.

Wells, C. L., and E. Balish. 1980. Gastrointestinal ecology and histology of rats monoassociated with anaerobic bacteria. Appl. Environ. Microbiol. 39:265–267.

Wexler, H. M., and S. Halebian. 1990. Alterations to the penicillin binding proteins in the Bacteroides fragilis group: A mechanism for non-b-lactamase mediated cefoxitin resistance. J. Antimicrob. Chemother. 26:7–20.

Whaley, D. N., L. S. Wiggs, P. H. Miller, P. U. Srivastava, and J. M. Miller. 1995. Use of presumpto plates to identify anaerobic bacteria. J. Clin. Microbiol. 33:1196–1202.

Whitehead, T. R., and R. B. Hespell. 1990. The genes for three xylan-degrading activities from Bacteroides ovatus are clustered in a 3.8 kilobase region. J. Appl. Bacteriol. 172:2408–2412.

Whitehead, T. R. 1995. Nucleotide sequences of xylan-inducible xylanase and xylosidase/arabinoisidase genes from Bacteroides ovatus V975. Biochim. Biophys. Acta 1244:239–241.

Whittle, G., B. D. Hund, N. B. Shoemaker, and A. A. Salyers. 2001. Characterization of the 13-kilobase ermF region of the Bacteroides conjugative transposon CTnDOT. Appl. Environ. Microbiol. 67:3488–3495.

Wiegel, J. 1981. a-Isopropylmalate synthase as a marker for the leucine biosynthetic pathway in several clostridia and in Bacteroides fragilis. Arch. Microbiol. 130:385–390.

Wilkens, T. D., D. L. Wagner, B. J. Veltri Jr., and E. M. Gregory. 1978. Factors affecting production of catalase by Bacteroides. J. Clin. Microbiol. 8:553–557.

Wills, A. E., A. Taylor, A. Pantosti, I. Phillips, and S. Tabaqchali. 1982. Comparison of antisera in the fluorescent antibody test for detection of Bacteroides spp. in clinical specimens. J. Clin. Pathol. 35:304–308.

Wood, J., K. P. Scott, G. Avgustin, C. J. Newbold, and H. J. Flint. 1998. Estimation of the relative abundance of different Bacteroides and Prevotella ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. Appl. Environ. Microbiol. 64:3683–3689.

Wu, S. G., K. C. Lim, J. Huang, R. F. Saidi, and C. Sears. 1998. Bacteroides fragilis enterotoxin cleaves the zonula adherens protein, E-cadherin. Proc. Natl. Acad. Sci. USA 95:14979–14984.

Yamamoto, I., A. Abe, H. Saito, H., M. and Ishimoto. 1984. The pathway of ammonia assimilation in Bacteroides fragilis. J. Gen. App. Microbiol. 30:499–508.

Yamamoto, I., A. Abe, and M. Ishimoto. 1987a. Properties of glutamate dehydrogenase purified from Bacteroides fragilis. J. Biochem. 101:1391–1397.

Yamamoto, I., H. Saito, and M. Ishimoto. 1987b. Regulation and synthesis and reversible inactivation in vivo of dual coenzyme-specific glutamate dehydrogenase in Bacteroides fragilis. J. Gen. Microbiol. 133:2773–2780.

Yamashita, Y., S. Kohno, H. Koga, K. Tomono, and M. Kaku. 1994. Detection of Bacteroides fragilis in clinical specimens by PCR. J. Clin. Microbiol. 32:679–683.

Yotsuji, A., J. Mitsuyama, R. Hori, T. Yasuda, I. Saikawa, M. Inoue, and S. Mitsuhashi. 1988a. Mechanism of action of cephalosporins and resistance caused by decreased affinity for penicillin binding proteins in Bacteroides fragilis. Antimicrob. Agents Chemother. 32:1848–1853.

Yotsuji, A., J. Mitsuyama, R. Hori, T. Yasuda, I. Saikawa, M. Inoue, and S. Mitsuhashi. 1988b. Outer membrane permeation of Bacteroides fragilis by cephalosporins. Antimicrob. Agents Chemother. 32:1097–1099.

Zalesnik, D. F., Z. L. Zhang, A. B. Onderdonk, and D. L. Kasper. 1986. Effect of subinhibitory doses of clindamycin on the virulence of Bacteroides fragilis: role of lipopolysaccharide. J. Infect. Dis. 154:40–46.

Zhang, G., B. Svenungsson, A. Karnell, and A. Weintraub. 1999. Prevalence of enterotoxigenic Bacteroides fragilis in adult patients with diarrhea and healthy controls. Clin. Infect. Dis. 29:590–594.

Zhu, X., T. Zhong, Y. Pandya, and R. D. Joerger. 2002. 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Appl. Environ. Microbiol. 68:124–137.

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CHAPTER 6.2

The Genus *Porphyromonas*

FRANK C. GIBSON AND CAROLINE ATTARDO GENCO

Introduction

Members of the genus *Porphyromonas* are characterized by the production of porphyrin pigments. The genus *Porphyromonas* was recognized as a separate taxon on the basis of ribosomal RNA homology and 16S rRNA data (Olsen et al., 1999). The most extensively characterized species of the genus is *Porphyromonas gingivalis* (a Gram-negative anaerobe), which has been long considered an important pathogen associated with human periodontal disease. Periodontal diseases comprise a group of inflammatory diseases of the gingiva and supporting structures of the periodontium. They (the most common of the destructive oral inflammatory diseases) are characterized by the bacterially initiated conversion of a healthy gingival region to one that is inflamed (gingivitis) and by the destruction of the supporting structures of the teeth (periodontitis). While a large number of different bacterial species exist in the periodontal environment, it is now recognized that the large number of bacteria (bacterial load) per se does not result in the biological progression from health to periodontal disease. Rather the establishment and growth of a very few of the more than 300 proposed different bacterial species resident in the subgingival niche are apparently periodontopathic (Holt et al., 2000), and these include *Fusobacterium nucleatum*, *Bacteroides forsythus*, *Prevotella intermedia*, *Treponema denticola* and *P. gingivalis*. *Porphyromonas gingivalis* is essentially absent during periodontal health, but during disease progression to periodontitis, can reach a very significant percentage of the pathogenic microbiota. Eradication of *P. gingivalis* from the subgingival microbial population correlates with resolution of the disease (Moritz et al., 1998).

Description of the Genus

The twelve species of the genus *Porphyromonas* have been isolated from the oral cavities of humans, dogs, cats, and nonhuman primates. More than likely, members of the genus are also associated with a large number of other warm-blooded animals. These organisms were originally classified in the genus Bacteroides, which is phylogenetically closely related (Fig. 1). Members of the genus are obligatorily anaerobic, nonsporeforming, nonmotile rods (0.5–0.8 by 1.0–3.5 μm; Table 1). They have also been described as cocco-bacilli depending on their stage of growth when they are examined. Characteristic of the genus is the production of large amounts of cell-associated protoheme. The major fermentation end products of growth on complex carbohydrates (except in the case of asaccharolytic *P. gingivalis*) and proteins are n-butyrate, propionate and acetate. These end-products account for much of the malodor associated with oral infections. Small amounts of isovalerate, isobutyrate, succinate and phenylacetate are also produced. While several of the strains (i.e., *P. gingivalis* and *P. macacae*) possess significant proteolytic activity, the other strains are relatively nonproteolytic.

Porphyromonas gingivalis is a nonmotile, asaccharolytic, obligatory anaerobic coccobacillus exhibiting smooth, raised colonies. When grown on a blood agar surface, the colonies are initially white to cream colored. With time (4–8 days), these colonies darken from their edge towards the center to a deep red to black color, which correlates with the concentration of protoheme. The species produce a large number of enzymes, which are active against a broad spectrum of host proteins. *Porphyromonas gingivalis*-derived proteinases can cleave a number of host defense molecules, including proteinase inhibitors, immunoglobulins, iron-containing proteins, bactericidal proteins, extracellular matrix proteins, and proteins intimately involved in phagocytic functions, such as complement fixation (Holt et al., 2000). Recent studies using molecular and immunological approaches have revealed that most of the *P. gingivalis* enzymatic activity is due to the production of cysteine proteinases (see section on Gingipains in this Chapter). Metabolically, the ability of *P. gingivalis* to secrete these cysteine proteinases in a host provides it with

distinct advantages for its survival and growth, including the ability to use host proteins for its growth and metabolism. Since these proteinases cleave both synthetic and host proteins after arginine and lysine residues, their growth is significantly enhanced in the presence of protein hydrolysates (trypticase, proteinase peptone, and yeast extract). *Porphyromonas gingivalis* produces a variety of virulence determinants, which include capsules, fimbriae, lipopolysaccharides, outer membrane proteins, and proteinases. Although these virulence molecules have been extensively described in vitro, their function within the confines of a host remain to be definitively determined.

Significance

Periodontal diseases represent a group of chronic inflammatory diseases of the gingiva and supporting periodontal tissues. Next to dental caries, periodontal disease is one of the most common oral diseases of mankind, and like caries, is initiated by bacterial infection. Although the effect of periodontal health (loss of dentition) on general human health has previously been regarded as a quality of life issue, recent epidemiological data, case studies, and initial scientific observations have begun to challenge this presumption. These studies suggest that periodontal disease may represent a significant risk factor for serious diseases such as cardiovascular and cerebrovascular disease (Beck et al., 1998b; Morrison et al., 1999), as well as potentially being an important cause of pre-term birth in pregnant women (Dasanayake et al., 2001; see section on Systemic Complications of Periodontal Disease in this Chapter). Should future basic research studies confirm these epidemiological associations and indeed demonstrate the causative or exacerbative relationship of periodontal disease and these more severe human diseases, additional studies will be necessary to provide a mechanistic understanding of the processes governing this association. This should ultimately lead to the development of novel prophylactic strategies to prevent the establishment of periodontal disease, such strategies lessening the present dependence on therapeutic strategies to control progression of disease following clinical recognition.

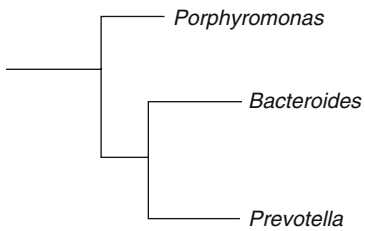

Fig. 1. Phylogenetic relationship of the genus *Porphyromonas* to other members of the family Bacteroideaceae.

Habitats

The oral cavity is a unique anatomical site in the body. It is composed of multiple epithelial and mucosal surfaces, as well as calcified hard tissues. These tissues are constantly bathed by the saliva, a broth of glandular secretions in which variable levels of microorganisms and food particles are suspended (Fig. 2). The gingival sulcus, a 0.5 mm deep crevice (in healthy subjects) between the tooth and the gum, is the portal of entry and

Table 1. The genus *Porphyromonas*.

Classification	Site	Comments
P. asaccharolytica	Periodontal pocket	Type strain for the genus
P. cangingivalis	Subgingival plaque	Periodontal disease in dogs
P. canoris	Subgingival plaque	Periodontal disease in dogs
P. cansulci	Subgingival plaque	Periodontal disease in dogs
P. catoniae	Gingival crevice	Healthy and diseased human gingiva
P. circumdentaria	Gingival margins of mouth	Diseased sites in cats
P. crevioricanis	Gingival crevicular fluids	From beagles
P. endodontalis	Acute periapical	Human abscess
P. gingivalis	Gingival sulcus	Generalized aggressive periodontitis
	Tongue	Periodontal abscesses
	Cheek	Periapical abscesses
	Saliva	
P. gingivicanis	Gingival crevicular fluids	From beagles
P. gulae	Gingival sulcus	From various animal hosts
P. levii	Foot rot lesion	Steer
P. macacae	Periodontal pocket	Nonhuman primate
P. salivosa	Periodontal pocket	Isolated form cat

From Love et al., 1992; Love et al., 1994; M. D. Collins et al., 1994; Hirasawa and Takada, 1994; Paster et al., 1994; Willems and Collins, 1995; Fournier et al., 2001.

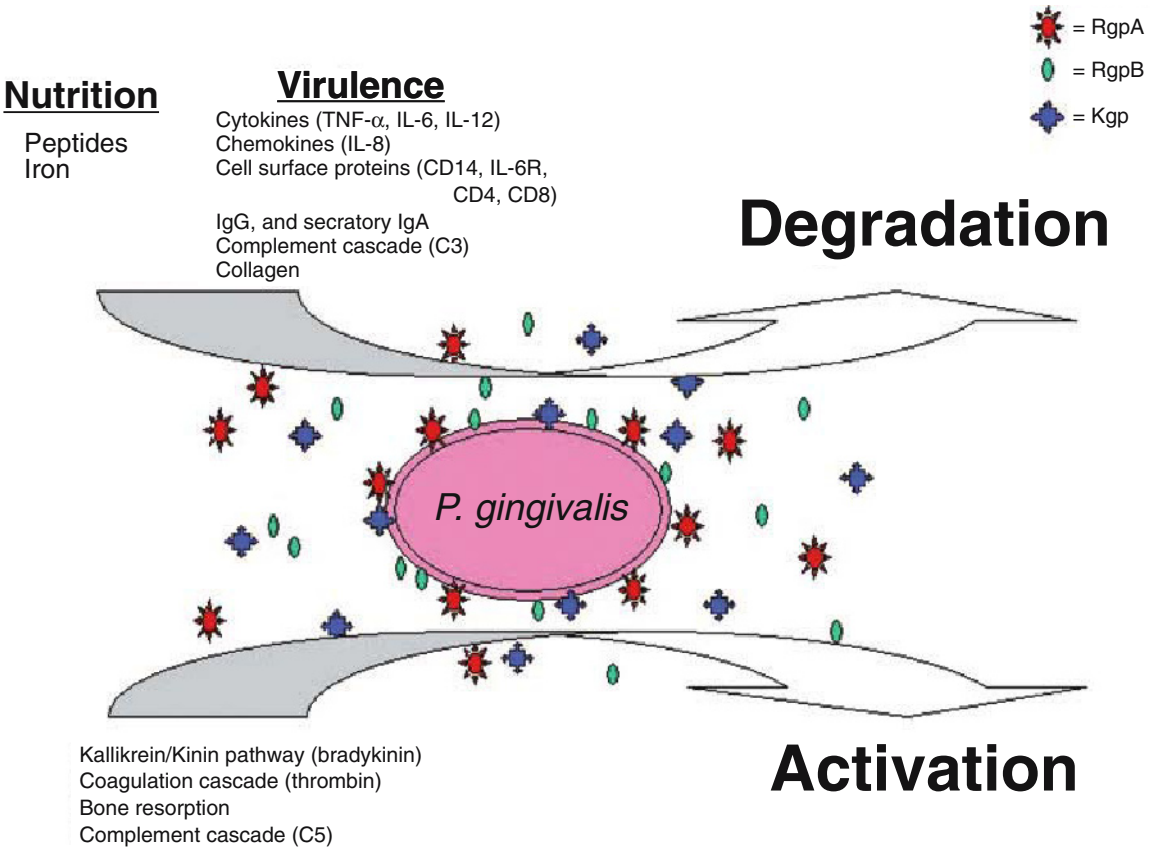

Fig. 2. Differences between a healthy periodontium and a site with generalized aggressive periodontal disease. Healthy site (on left). Site with generalized aggressive periodontal disease (on right). In a healthy site, the subgingival microflora is comprised predominantly of Gram-positive bacteria, although low numbers of Gram-negative bacteria such as *P. gingivalis* may be recovered from periodontally healthy people. There is an intact tooth attachment apparatus with numerous attachment fibrils, few inflammatory cells, and normal levels of alveolar bone. The shift to generalized aggressive periodontal disease results in a change in the composition of the subgingival microflora from predominantly Gram-positive bacteria to predominantly Gram-negative bacteria and the isolation of *P. gingivalis*. There is a subsequent host inflammatory response to the elevated levels of *P. gingivalis* in the gingival sulcus that is characterized by an increase in neutrophils (acute lesion) and mononuclear cells (chronic lesion) at the infected site. Additionally there is loss of the tooth attachment apparatus including loss of attachment fibrils with subsequent erosion of alveolar bone. In severe cases of periodontal destruction, exfoliation of teeth can occur. Emerging data support that *P. gingivalis* invades the tissues subjacent to the gingival sulcus and bacteremia occurs, and this may be related in part to the epidemiological associations of periodontal disease with accelerated cardiovascular disease and preterm-births.

niche for a unique bacterial ecosystem composed of highly adapted microorganisms, most notably *P. gingivalis*. *Porphyromonas gingivalis* appears to colonize solely subgingival sites of the oral cavity but has also been recovered from the tongue, tonsils, saliva, and supragingival dental plaque samples (Socransky and Haffajee, 1992). *Porphyromonas gingivalis* constitutes less than 5% of the cultivable subgingival flora of individuals with gingivitis, but its numbers can increase dramatically in advanced periodontal disease. Since *P. gingivalis* is recovered almost exclusively from disease sites, its primary source of infection remains unknown. Likewise the precise mechanism by which *P. gingivalis* enters the human oral cavity and becomes established within the oral mucosa is not well defined. Evidence indicates that *P. gingivalis* is largely an opportunistic pathogen. Although studies support the transmission of *P. gingivalis* by intimate contact, they do not indicate that transmission results in disease. In addition, data do not exclude the possibility that pathogenic strains have virulence factors that are not present in colonizing healthy individuals. Transient changes in the host immune response and the indigenous microflora may be important cues to *P. gingivalis* to produce a set of factors that are required for pathogenesis (Forng et al., 2000). In addition, the ability of *P. gingivalis* to colonize periodontal sites appears to correlate with the ability of the organism to obtain nutrients for growth and with the anaer-

obic microenvironment within this site (see section on Hemin Acquisition in this Chapter).

Porphyromonas gingivalis encounters a series of obstacles, which are aimed at exclusion of the organism, during colonization of the gingival pocket and include antimicrobial effects of saliva, competition for attachment sites by the antecedent microbiota of the dental plaque, the physical barriers of the gingival environment, as well as the host immune response to this organism. Previous work has demonstrated that *P. gingivalis* can bind to other bacteria as well as saliva-coated hydroxyapatite particles via fimbriae and thus gain a foothold in the oral cavity and the gingival sulcus. Additionally, fimbriae are important in promoting *P. gingivalis* attachment to, and subsequent invasion of, gingival epithelial cells (see section on Fimbriae in this Chapter).

Isolation and Identification

The isolation and identification of the bacterial pathogens associated with periodontal disease have been complicated by the large number of bacterial species (more than 300) present in the oral environment (Holt et al., 2000). Despite this complexity, only a few are clinically associated with this disease, and *P. gingivalis* is the predominate organism associated with generalized aggressive periodontal disease. The genus *Porphyromonas* is a member of the family Bacteroideaceae. Members of this family are obligatory anaerobic, nonmotile, nonsporeforming and Gram negative. The porphyromonads can be distinguished from other members of the family Bacteroideaceae by black-pigmented colonies that develop from cream-colored colonies during growth on blood agar plates, a consequence of storing iron on the surface of these bacteria in the form of protoheme (Shah et al., 1979). Colonies are shiny, convex with smooth edges that appear wet, and slightly mucoid. Proteins constitute the primary source of metabolic energy for members of the genus *Porphyromonas*. *Porphyromonas gingivalis* is an aerotolerant, asaccharolytic member of the genus. Saccharolytic members (including *P. levii* and *P. macacae*) weakly ferment glucose, lactose and/or mannose. The major long chain fatty acid produced by the genus *Porphyromonas* is *iso*-$C_{15:0}$, and the G+C content is 46–54 mol% (Olsen et al., 1999). Isolation of *Porphyromonas* from dental plaque was previously routinely performed on vancomycin-kanamycin blood agar plates. More recently, a selective medium containing colistin, bacitracin and nalidixic acid has been developed for isolation of *P. gingivalis* (Hunt et al., 1986).

Clinical Diagnosis of Periodontal Disease

Following bacterial colonization of the host, the first phase of periodontal disease is characterized by gingivitis (i.e., the conversion of healthy gingival tissue to inflamed tissue). Progression from gingivitis to periodontal disease is determined by several factors including erosion of the supporting attachment apparatus of the teeth and loss of osseous tissue. Assessment of periodontal disease is primarily defined radiographically and by assessment of the degree of tooth attachment loss using a periodontal probe. In severe cases of periodontal disease early loss of dentition can occur. Recently, a reclassification of the various forms of periodontal diseases of humans has been performed on the basis of sets of defined clinical definitions (Armitage, 1999). Previous classification schemes of periodontal disease were inadequate and consisted of classifications such as early onset (juvenile) periodontitis (EOP) including localized juvenile periodontitis (LJP; etiological agent, *Actinobacillus actinomycetemcomitans*) or adult periodontal diseases including rapidly progressive periodontal disease (RPP; etiological agent, *P. gingivalis*); however, it was recognized that the temporal aspects of these conditions were usually not known to the clinician. The new classification scheme consists of the major headings: I. Chronic Periodontitis; II. Aggressive Periodontitis; III. Periodontitis as a Manifestation of Systemic Diseases; IV. Necrotizing Periodontal Disease; and V. Periodontitis Associated with Endodontic Lesions. Thus, previous classification of LJP is now localized aggressive periodontitis, and the classification of RPP is either generalized aggressive periodontitis or chronic periodontitis (Armitage, 1999). This new classification scheme will provide defined criteria on which to base detailed experimental studies into the precise etiology, pathogenesis and treatment regimens for periodontal disease.

Localized and generalized forms of both chronic and aggressive periodontal disease exist. Localized forms typically involve <30% of the sites, while generalized periodontal disease involves 30% of the sites. A severity index based on the observed amount of attachment loss (slight [1–2 mm], moderate [3–4 mm], or severe [5 mm]) can be applied in each case. Periodontal lesions of patients with generalized aggressive periodontal disease present with a predominately neutrophilic cellular infiltrate in the early stages of disease. Interestingly, the cellular composition of a chronic lesion is predominantly monocyte-rich and consists primarily of B cells, plasma cells and T cells. Commonly, a flow-rate

increase is observed in the gingival crevicular fluid (a plasma-derived fluid).

Patients with HIV commonly present with oral infections, and certain lesions such as oral candidiosis or hairy leukoplakia play a part in the diagnosis and staging of HIV infection (Greenspan and Greenspan, 1996). Additionally, severe periodontal infections such as necrotizing ulcerative periodontitis though rare in healthy people are observed in 6–10% of people with significant HIV-related infection. Although the specific immune defect during HIV infection that mediates the acceleration of periodontal disease is not known, it is speculated that the deficiency in T helper cell-mediated immunity plays a significant role in the establishment of necrotizing ulcerative periodontitis (Greenspan and Greenspan, 1996). The specific impact of HIV infection on more aggressive forms of *P. gingivalis*-mediated periodontal disease is yet to be determined.

Because the immune response is important in controlling infectious disease, understanding the impact of immunosuppression on periodontal disease is important. Organ transplant recipients receive chemical immunosuppressive drugs such as cyclosporin or nifedipine to prevent tissue rejection. A common side effect of cyclosporin therapy is gingival enlargement (in up to 30% of patients). However, the effect of cyclosporin-mediated immunosuppression on accelerated periodontal disease is contentious, as both acceleration and protection from periodontal breakdown have been reported (Thomason et al., 1994). Similarly, cancer therapy may exacerbate periodontal infections. High levels of radiation adversely affect the periodontium by involving blood vessels to the periodontium and the periodontal ligament, thus limiting vascular flow and affecting bone repair. Furthermore, irradiated teeth suffer from greater attachment loss than do non-irradiated teeth. Additionally patients with certain types of leukemia that receive chemotherapy and present with neutrophil dysfunction or neutropenia commonly present with a highly attenuated inflammatory response and limited soft tissue healing (Epstein and Stevenson-Moore, 2001).

Treatment of Periodontal Disease

Gingivitis and periodontal disease are the two most common forms of inflammatory diseases of the periodontium. Gingivitis is a reversible disease that commonly can be treated by improving the patient's daily plaque control, as well as regularly scheduled professional cleaning. For patients with periodontal disease, the treatment regimen is significantly different, and commonly the therapy often must be tailored to each individual case on the basis of pattern of attachment loss, local anatomical differences, and type of periodontal disease (Research, Science and Therapy Committee of the American Academy of Periodontology, 2001). Therapy for patients with chronic periodontal disease is aimed at reducing the factors responsible for eliciting the breakdown of the periodontium. This includes scaling (removal of the buildup of dental calculus in the periodontal pocket) and root planing (smoothing of the root surface to promote epithelial reattachment and healing); antimicrobial chemotherapy and surgery to correct the defects and promote tooth attachment and bone regeneration. Scaling and root planing, combined with personal plaque reduction measures, have been shown to be effective for the control of periodontal disease, particularly by shifting the composition of the subgingival microflora from one consisting of Gram-negative anaerobic organisms such as *P. gingivalis* to one more closely associated with periodontal health (Magnusson et al., 1984), reducing clinical measures of inflammation, as well as promoting clinical attachment and decreasing probing depths. However, not all sites infected with *P. gingivalis* respond to scaling and root planing, particularly deep periodontal pockets. In these cases, additional modes of therapy should be used. Other therapies that show promise include the use of growth factors, matrix proteins, and stimulators of bone morphogenesis. The primary goal of all surgical therapy is to significantly decrease the microbial burden and provide an environment to restore periodontal attachment and regeneration of lost tissue. The primary risk factor that complicates the potential benefits of surgical therapy is tobacco use (Haber et al., 1993). Other factors can also complicate the intervention process and include host factors such as diabetes.

Commonly, antimicrobial chemotherapy is incorporated into the clinical treatment regimen of periodontal disease and includes both local and/or systemic therapy. Those antibiotics most commonly used are highly effective against Gram-negative anaerobic organisms (*P. gingivalis*, *B. forsythus* and *P. intermedia*) and include metronidozole and tetracycline. More recently, nonsteroidal, anti-inflammatory drugs such as flurbiprofen, ibuprofen and naproxen have shown benefits in reducing loss of alveolar bone, the osseous tissue supporting the tooth. In addition, subantimicrobial doses of doxycycline regimen have also been shown to be effective in treatment of periodontal disease. In 2000, the United States Food and Drug Administration (FDA) approved the systemic collagenase inhibitor therapy consisting of 20 mg of doxycycline hyclate as an adjunct to scaling and root planing;

however, only limited improvement in periodontal status has been reported (Research, Science and Therapy Committee of the American Academy of Periodontology, 2001). Local delivery of antibiotics has also been shown to be beneficial for periodontal therapy, as the antibiotic is delivered directly to the diseased site at bactericidal levels. The FDA has approved several local delivery systems including ethylene vinyl acetate fiber with tetracycline, a gelatin chip with chlorohexidine, a polymer with minocycline, and a bioabsorbable gel containing doxycycline hyclate (Research, Science and Therapy Committee of the American Academy of Periodontology, 2001).

Porphyromonas gingivalis Virulence Factors

Fimbriae

With only one or two exceptions, all of the *P. gingivalis* strains so far examined contain fimbriae arranged in a peritrichous fashion over the surface of the cell (Handley and Tipler, 1986; Fig. 3). These structures have been isolated, purified and examined both chemically and structurally (Sojar et al., 1991; Yoshimura et al., 1993; Lee et al., 1995). The gene encoding the *P. gingivalis* fimbriae (*fimA*) is resident on the chromosome as a single copy, and all *P. gingivalis* strains so far examined contain the gene (Dickinson et al., 1988; Takahashi et al., 1992; Fujiwara et al., 1993; Sharma et al., 1993; Washington et al., 1993). The *P. gingivalis* fimbriae are not related to fimbriae produced by other black-pigmented Bacteroides species or to most of the other Gram-negative prokaryotes. Different *P. gingivalis* strains produce fimbriae that are heterogeneous with respect to antigenicity, molecular weight of the fimbrillin subunit, N′-terminal sequence of the fimbrillin protein, and DNA sequence (Yoshimura et al., 1984; Yoshimura et al., 1985; Lee et al., 1991; Ogawa et al., 1991; Loos and Dyer, 1992; Fujiwara et al., 1993). Amino acid sequence analysis of the *P. gingivalis* fimbrillins has placed them into four to six major groups (Hamada et al., 1994). Considerable heterogeneity in *fimA* exists amongst *P. gingivalis* strains. Since only one *fimA* gene copy is found on the chromosome, the variation in the fimbrillin gene is most likely due to mutational events and/or genetic exchange between strains and not due to antigenic variation of the fimbriae within the strain. To date, no fimbrillin phase variation has been identified within a *P. gingivalis* strain. Recently a two-component regulatory system has been described in *P. gingivalis*, which appears to be involved in regulation of fimbrillin expression (Hayashi et al., 2000).

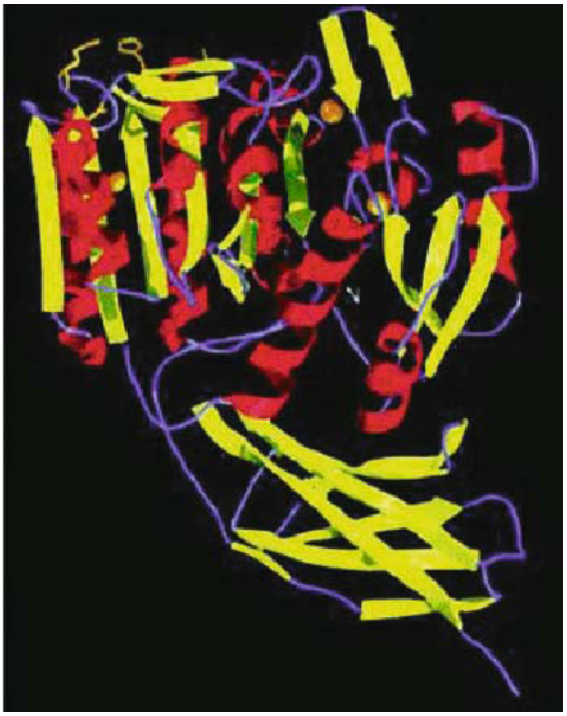

Fig. 3. *Porphyromonas gingivalis* virulence factors. This Gram-negative pathogen possesses a broad armamentarium of virulence factors that likely play a significant role in the establishment of periodontal disease. Although not precisely known, it is well established that the development of periodontal disease in response to *P. gingivalis* infection is the result of a complex interplay between the host defenses and the virulence factors expressed by this organism.

There is strong evidence that *P. gingivalis* fimbriae are responsible for binding the bacterium to host tissues. Purified native fimbriae, recombinant fimbriae, synthetic peptides based on the coding region of *fimA*, and anti-fimbriae sera are effective in preventing the attachment of *P. gingivalis* cells to epithelial, fibroblast, and endothelial cell culture systems (Lee et al., 1992; Sharma et al., 1993; Njoroge et al., 1997; Nassar et al., 2002). *Porphyromonas gingivalis fim*A knockout mutants also exhibit a nonadherent and noninvasive phenotype in epithelial, fibroblast, and endothelial cell culture systems (Weinberg et al., 1997). Ogawa et al. (1997) investigated the contribution of various regions of the fimbriae binding to a human gingival fibroblast cell line. Peptides containing amino acids 1–20, 69–80, and 171–181 strongly inhibited binding of purified fimbriae. All of the inhibitory peptides contained the amino acid sequence XLTX. The addition of *P. gingivalis* fimbrillin-specific peptides to the N-terminal domain of fimbrillin to human umbilical vein endothelial cells (HUVEC) also stimulates

interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1 in these cells (Khlgatian et al., 2002), as well as the cell adhesion molecules intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and P- and E-selectins (Khlgatian et al., 2002). It has recently been reported that *P. gingivalis* fimbriae promote adhesion to gingival epithelial cells through interaction with β1 integrins (Yilmaz et al., 2002).

The intact fimbriae, fimbrillin subunits, or synthetic fimbrial peptides of *P. gingivalis* are also capable of eliciting several important host-associated biological responses, which could result in harmful effects in vivo. These include the production of fibroblast-derived thymocyte-activating factor from human gingival fibroblasts (Hirose, 1990), IL-1, neutrophil chemotactic factor KC, and tumor necrosis factor (TNF)-α from mouse peritoneal macrophages (Hanazawa et al., 1991; Hanazawa et al., 1992; Murakami et al., 1993), and IL-6, IL-8 and TNF-α in human peripheral blood monocytes (Ogawa et al., 1991; Hamada et al., 1994). The fimbriae also appear to function as adjuvants (Ogawa et al., 1991; Hamada et al., 1994), as well as being capable of stimulating mitogenic and polyclonal B-cell activation in mouse splenocytes (Ogawa et al., 1991). The fimbriae are also highly immunogenic, eliciting both an antibody and cell mediated response in serum and saliva (Ogawa et al., 1992; Kusumoto et al., 1993; Hamada et al., 1994; Isogai et al., 1994). Animal experiments have also been conducted, which strongly implicate a role for fimbriae in tissue destruction (Evans et al., 1992a; Malek et al., 1994). Immunization of gnotobiotic rats with *P. gingivalis* fimbriae resulted in the protection of the animals from periodontal damage when challenged with live *P. gingivalis* (Evans et al., 1992a). Infection of gnotobiotic rats or mice with a fimA knock-out strain of *P. gingivalis* resulted in a markedly reduced bone loss in comparison to animals infected with the wildtype strain (Malek et al., 1994).

Minor Fimbriae

Electron microscopic examination of a *P. gingivalis* fimA mutant revealed that while the long, thin, classical fimbriae were absent, the surface of these cells was sparsely covered with short fimbriae-like structures, which were unreactive with anti-fimbrial antibody (Hamada et al., 1996). Hamada et al. (1996) isolated and characterized these "minor fimbriae" from the mutant strain, and they were found to have a higher estimated molecular weight than the major fimbrillin, 67 kDa and 41 kDa, respectively. Amino acid compositional analysis of the two fimbriae showed that they contained similar amino acids and were rich in hydrophobic amino acids. The two fimbriae were also antigenically distinct from one another. Antiserum against the 67-kDa fimbriae recognized the short (0.1–0.5 μm) fimbriae found on the mutant but did not react with the long (0.5–1.0 μm) fimbriae in the wildtype strain. While the wildtype fimbriae were involved in adherence to both epithelial cells and fibroblasts, as well as initiating hemagglutination reactions, the minor fimbriae did not possess any hemagglutinating activity. The minor fimbrial protein induces IL-1, IL-6 and TNF expression in macrophages (Hamada et al., 2002). Recent work in a rat animal model also supports a role for the *P. gingivalis* minor fimbriae in bone loss (Umemoto and Hamada, 2003).

Gingipains

The *P. gingivalis* cysteine proteinases (gingipains) have been recognized as major virulence factors of this organism owing to their ability to degrade and inactivate a number of host structural and defense proteins. The *P. gingivalis* gingipains cleave synthetic and natural substrates after arginine and lysine residues and have been designated "gingipain R" and "gingipain K," respectively. The genes encoding the arginine-(*rgpA* and *rgpB*) and the lysine-(*kgp*) specific gingipains have been well characterized (Potempa et al., 1995a; Potempa et al., 1995b). The translated portion of both the *rgpA* and *kgp* genes is composed of four functional regions: the signal peptide, the NH_2-terminal prosequence, the mature proteinase domain, and the COOH-terminal hemagglutinin domain (Okamoto et al., 1996). The COOH-terminal hemagglutinin domain has been demonstrated to bind fibrinogen, fibronectin, hemoglobin, and a number of additional substrates. The *rgpB* gene, which encodes the RgpB protein, is missing the COOH-terminal hemagglutinin domain (Potempa et al., 1995a; Genco et al., 1999; Fig. 4). In some strains of *P. gingivalis*, RgpA, RgpB and Kgp remain attached to the outer membrane, while in others these proteins are found extracellularly. The gingipains are also found associated with outer membrane blebs (Fig. 4). The crystal structure of RgpB has recently been reported (Fig. 5).

RgpA and RgpB are very potent vascular permeability enhancement (VPE) factors of *P. gingivalis*, inducing this activity through plasma prekallikrein activation and subsequent bradykinin release. By itself, Kgp is not able to induce VPE in human plasma, but working in concert with RgpA and RgpB, the pair can induce VPE by cleaving bradykinin directly from high molecular weight kininogen (Fig. 6). Also, RgpA is a

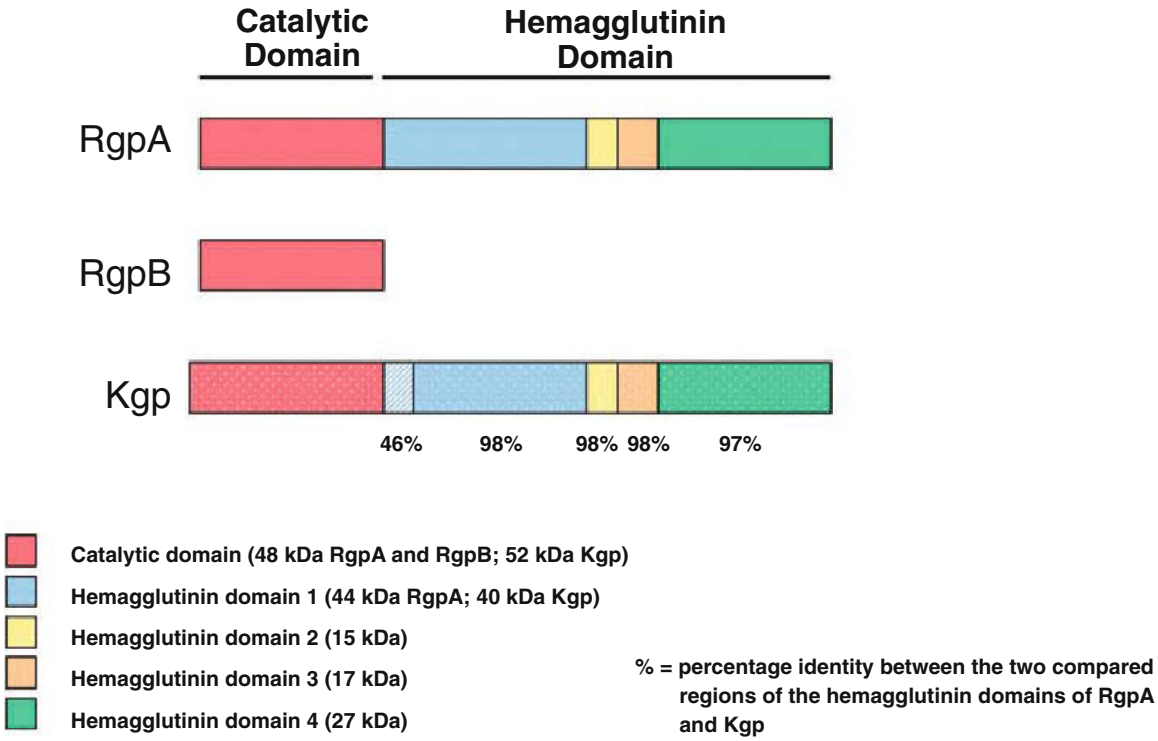

Fig. 4. Structure of the gingipains. The division of gingipains into two groups (arginine-specific [RgpA and RgpB] cysteine-proteinases and lysine-specific [Kgp]) is based on amino acid substrate specificity. RgpA and RgpB are encoded by two different genes, *rgpA* and *rgpB*. Structurally RgpA possesses a catalytic domain (48 kDa) and a hemagglutinin domain comprised of four subdomains (44, 15, 17, and 27 kDa), while RgpB possesses the catalytic domain and a small nonfunctional portion of the hemagglutinin domain. Kgp structurally resembles RgpA, as Kgp possesses both a catalytic (52 kDa) and hemagglutinin domain with four subdomains (40, 15, 17, and 27 kDa). The percentage values define the percent identity of the two compared regions of the hemagglutinin domains of RgpA and Kgp.

very efficient enzyme in terms of the generation of a potent chemotactic factor, C5a, through direct cleavage of C5, which likely contributes to the significant leukocyte infiltration at *P. gingivalis*-induced periodontitis lesions. At the same time, the enzyme degrades C3 and in this way eliminates the creation of C3-derived opsonins, thus rendering *P. gingivalis* resistant to phagocytosis (Cutler et al., 1993). The massive accumulation of neutrophils in the inflamed periodontal tissue importantly contributes to the very high levels of active granular proteinases (elastase, cathepsin G, gelatinase, and collagenase) in gingival crevicular fluid that may be responsible for connective tissue destruction. In such a highly proteolytic environment, subgingival plaque bacteria would clearly thrive owing to the presence of high concentrations of peptides and amino acids (Fig. 6), thus further aggravating tissue destruction. In addition, RgpA and RgpB have been reported to degrade host matrix proteins, which in turn may lead to increased exposure of the cryptic ligands that can result in enhanced fimbria-mediated binding of *P. gingivalis* to periodontal tissues. Recent studies indicating that RgpA can activate factor X suggest that this gingipain could be responsible for the production of thrombin (Imamura et al., 1997).

Among the plasma proteins, fibrinogen seems to be the major target for Kgp. In vitro, this enzyme degrades the fibrinogen A α-chain within minutes, thus rendering it nonclottable. The nonrestricted Kgp activity in periodontal pockets has been postulated to contribute to a bleeding tendency, especially since it also destroys the procoagulant portion of high molecular weight kininogen (Imamura et al., 1995). The ability of the hemagglutinin domains of *P. gingivalis* RgpA and Kgp to hemagglutinate erythrocytes has also been well documented (Potempa et al., 2000). The proteinase-hemagglutinin complexes may thus be important in the uptake of essential growth factors, via hemagglutination, hemolysis of erythrocytes, and subsequent degradation of hemoglobin (Potempa et al., 2000).

Several reports have further defined the role of the *P. gingivalis* gingipains in virulence using various animal models (Genco et al., 1999). Inactivation of *P. gingivalis* cysteine proteinases

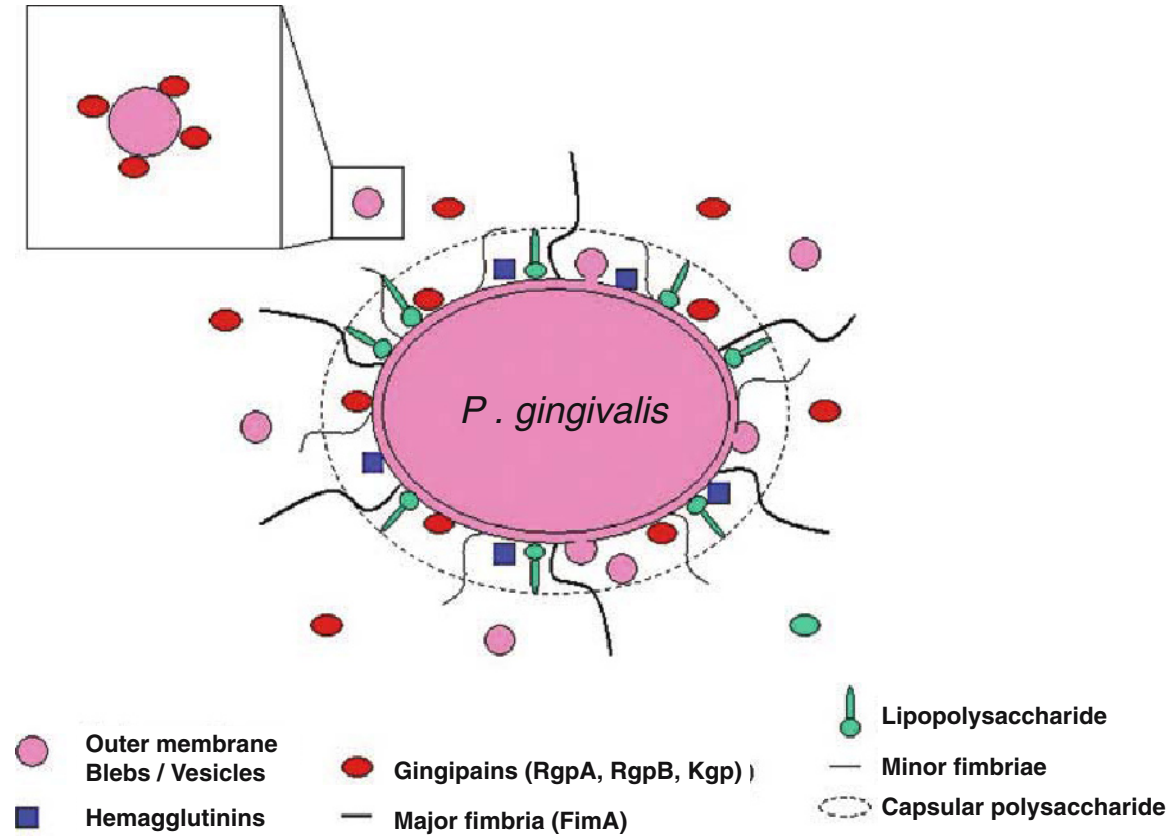

Fig. 5. The X-ray crystallographic structure of RgpB. The catalytic domain of RgpB is subdivided into two subdomains comprised of 4- and 6-strand β-sheets sandwiched between single β-helices. The crystal structures of RgpA and Kgp are currently not known. From Eichinger et al. (1999).

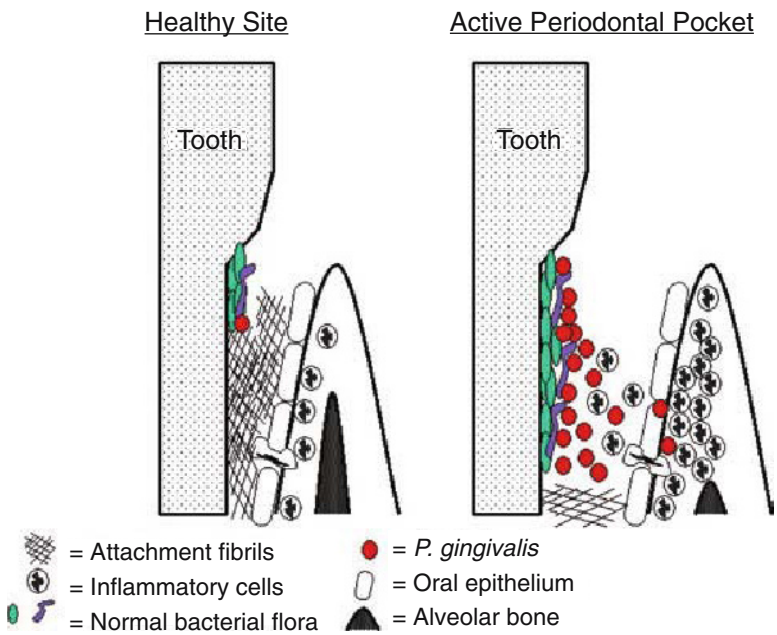

Fig. 6. Known functional roles of the gingipains of *P. gingivalis*. Gingipains are found in either cell bound or secreted forms and have been shown to possess both degradative and immune activating activities, which likely play a significant role in the establishment of periodontal disease.

including the gingipains prior to infection in mice resulted in a decrease in *P. gingivalis* virulence in a mouse chamber model (Genco et al., 1999). *Porphyromonas gingivalis rgpA rgpB* mutants have been shown to exhibit a marked reduction in virulence in a mouse abscess model (Nakayama et al., 1995). The gingipains may contribute to the virulence potential of *P. gingivalis* in a multifactorial manner by influencing adherence to host tissues, degrading host proteins, and disturbing host defenses (Potempa et al., 2000). Further, it is known that RgpA and RgpB may act as processing proteinases responsible for self-maturation, as well as for maturation of Kgp, fimbrillin, and a 75-kDa outer membrane protein. Since fimbrillin, RgpA, RgpB, and Kgp have been documented in the virulence potential of *P. gingivalis*, gingipains may play a central role in the pathogenesis of periodontal disease via the production of pathophysiologically significant bacterial proteins.

Hemagglutinins

Porphyromonas gingivalis produces several distinct hemagglutinins, which are cell surface associated proteins that aid in binding of the bacterium to host cells. Multiple hemagglutinin genes have been identified from *P. gingivalis* (Holt et al., 2000). As discussed above, polypeptides corresponding to the C-terminal domains of the gingipains possess hemagglutnin activity (Potempa et al., 2000). Three additional hemagglutinins have been described. Han et al. (1996) cloned and sequenced the *hagA* gene, which was found to be 7,887 bp in length, encoding a protein of 2,628 amino acids with a molecular mass of 283.3 kDa. Within its open reading frame, four large, contiguous, direct repeats (varying from 1,318 to 1,368 bp) were identified. The repeat unit (HArep), which was assumed to contain the hemagglutinin domain, was also present in other recently reported protease and hemagglutinin genes in *P. gingivalis*. The presence of multiple repeat units may provide a means for *hagA* to undergo rearrangements (duplications or deletions of a repeat unit) and thus antigenic variation. In addition to HagA, HagB and HagC have been characterized and demonstrated to hemagglutinate red blood cells (Lepine et al., 1996b); however, the exact role of these hemagglutinins in pathogenesis has not been defined.

Hemin Acquisition Nutrients are limited in the subgingival crevice and successful colonization by *P. gingivalis* is dependent on the ability of this organism to capture essential nutrients for growth. Perhaps the most obvious of these nutrient requirements is the *P. gingivalis* requirement for hemin (Cutler et al., 1995). Although hemin is a major component of gingival crevice fluid, host iron-chelating proteins such as haptoglobin, albumin, and hemopexin make hemin unavailable for microorganisms (Genco and Dixon, 2001). A *P. gingivalis* outer membrane receptor (HmuR), which is required for both hemoglobin and hemin utilization in *P. gingivalis*, has been described and well characterized (Simpson et al., 2000). Inactivation of *hmuR* resulted in a diminished ability of *P. gingivalis* to bind hemoglobin and to grow with hemoglobin or hemin as sole iron source. Recombinant HmuR has also been demonstrated to bind hemin and hemoglobin (Olczak et al., 2001). Thus, HmuR appears to serve as a major outer membrane hemoglobin receptor in *P. gingivalis*.

The lysine specific cysteine protease (Kgp) has also been demonstrated to specifically bind hemoglobin; however, it is not clear which part of the Kgp complex participates in hemoglobin binding (Sroka et al., 2001). Kgp can also cleave hemoglobin (Nakayama et al., 1995; Abe et al., 1998; DeCarlo et al., 1999; Lewis et al., 1999; Simpson et al., 2000; Sroka et al., 2001). *Porphyromonas gingivalis kgp* mutants grow poorly with hemin or hemoglobin as sole iron sources, further supporting a role for Kgp in hemin and hemoglobin utilization (Genco, 1995a; Simpson et al., 1999). Furthermore, a *P. gingivalis hmuR kgp* mutant displayed a nonpigmented colony phenotype, bound less hemoglobin as compared to the parent strain, and did not grow with hemin or hemoglobin as sole iron source. Although Kgp can be found associated with the *P. gingivalis* outer membrane, it is premature to classify Kgp as a receptor. The amino acid sequence of Kgp has no similarity to TonB-dependent outer membrane proteins. Rather Kgp may function as a soluble hemoglobin binding protein (hemophore), which captures hemoglobin and delivers it to a second outer-membrane-associated receptor, possibly the hemoglobin receptor HmuR. Indeed, we have demonstrated that HmuR interacts with Kgp. This may represent the most efficient system of heme/hemoglobin delivery in *P. gingivalis*. Kgp has been proposed to function as a hemophore that facilitates hemoglobin utilization via HmuR. While HmuR may be capable of permitting growth of *P. gingivalis* with hemoglobin, an interaction with Kgp may increase the overall efficiency of the system.

In addition to the hemoglobin receptor, HmuR, it has been reported that *P. gingivalis* possesses three additional putative TonB-dependent hemin receptors (HemR, Tla and IhtA). It is possible that HemR, Tla and IhtA could function to bind hemin directly; however, conclusive evidence for the role of these proteins

in hemin binding has not been reported. A *P. gingivalis tla* mutant was demonstrated to grow with high levels of hemin; however, growth was decreased with low levels of hemin (Aduse-Opoku et al., 1997). These results indicate that Tla is involved in hemin transport; however, it is not known whether Tla functions in heme capture or in heme binding via a receptor-like mechanism. Definitive roles for HemR and IhtA proteins in hemin transport have not been delineated since Karunakaran et al. (1997) and Dashper et al. (2000) were unable to construct *P. gingivalis* mutants in these proteins.

Lipopolysaccharide (LPS) In comparison to the classical LPS of *Salmonella typhimurium* and *Escherichia coli*, *P. gingivalis* LPS was significantly less endotoxic when assayed by the Limulus amoebocyte lysate assay, Schwartzman reaction, lethality in galactosamine-sensitized mice, and pyrogenicity assay (Nair et al., 1983; Fujiwara et al., 1990; Takada et al., 1990). Endotoxic activity is confined to lipid A, while significant immunobiological activity is contained within the O-antigen (Takada et al., 1990). Studies by Ogawa et al. (1994b) and Tanamoto et al. (1997a) demonstrating that *P. gingivalis* LPS is a poor activator of IL-1 and TNF-α, as compared to enterobacterial LPS, are in contrast to other studies which demonstrated that *P. gingivalis* LPS stimulates equivalent or higher amounts of IL-1/IL-1β (Hanazawa et al., 1985; Koga et al., 1985; Lindemann et al., 1988; Yamazaki et al., 1992; Agarwal et al., 1995) and TNF-α (Lindemann et al., 1988; Agarwal et al., 1995) from human and murine cells. Bramanti et al. (1989) and Sismey-Durrant and Hopps (1991) also reported that *P. gingivalis* LPS was able to stimulate the production of prostaglandin E_2 (PGE_2) from mouse macrophages and rat and human gingival fibroblasts. Similar results reported by Tamura et al. (Tamura et al., 1992; Tamura et al., 1992) have also demonstrated IL-1 and IL-8 stimulation by *P. gingivalis* LPS in human gingival fibroblast cells. While there are reports that *P. gingivalis* LPS is capable of inducing IL-6 in human gingival fibroblasts (Bartold and Haynes, 1991), this induction is thought to be the result of an indirect effect of the LPS stimulating the production of IL-1β (Yamazaki et al., 1992). *Porphyromonas gingivalis* LPS does not stimulate the expression of E-selectin in human umbilical cord endothelial cells, nor does it stimulate neutrophil adhesion to these cells. Employing an in vivo mouse model, Reife et al. (1995) demonstrated that purified *P. gingivalis* LPS mediated a variety of inflammatory responses; however, it did not induce an inflammatory cellular infiltrate, the production of P- or E-selectin, or the production of monocyte chemoattractant protein (MCP)-1 or fibroblast-induced cytokine. The differences in the immunopharmacologic activity of *P. gingivalis* LPS are thought to be due to differences in the chemical structure of *P. gingivalis* LPS and that of the enterobacterial molecule (i.e., specifically, the absence of the 4′-phosphate group and the location and nature of the fatty acids on the *P. gingivalis* lipid A; Takada, 1992; Ogawa, 1994a; Tanamoto et al., 1997b).

Shapira et al. (1994) determined that *P. gingivalis* LPS stimulates cytokine secretion in monocytes by binding to CD14 after the LPS has interacted with soluble serum factors. In contrast to that reported for the enteric LPS, *P. gingivalis* LPS was not able to activate nonmyeloid cells and was, for the most part, significantly less effective than the enteric LPS in activating myeloid cells (Ogawa, 1994a; Darveau et al., 1995; Cunningham et al., 1996; Tanamoto et al., 1997b). It appears that this inability of *P. gingivalis* LPS to stimulate myeloid cells is due to its approximately 100-fold lower ability to bind LPS binding protein (LBP; Cunningham et al., 1996) compared to that observed for the *E. coli* LPS. The mechanism by which soluble CD14 binds to LPS-LBP complex is different in *P. gingivalis* compared to *E. coli* (Shapira et al., 1994). Since *P. gingivalis* LPS-LBP complex is capable of binding to soluble CD14 (Cunningham et al., 1996), some other factor(s) must be responsible for the inability of these complexes to stimulate nonmyeloid cells when they come in contact with LPS from *P. gingivalis*. The low biological activity of *P. gingivalis*, especially its very low endotoxicity, may reflect the organism's ability to colonize and grow in sterile tissue undetected by the host (Darveau et al., 1995).

Capsule

Many bacteria produce capsular polysaccharides, and the importance of capsular polysaccharides in infectious diseases has been studied in detail (Lindberg, 1999). Epidemiological and case report data indicate that, of the infections caused by anaerobic bacteria, the majority of these isolates are encapsulated (Kasper et al., 1977; Onderdonk et al., 1977; Brook and Walker, 1983; Brook, 1994; Califano et al., 1999) and include *Bacteroides* sp., *Prevotella* sp., *Fusobacterium* sp. and *Porphyromonas* sp. (Brook, 1994). The sugar composition of *P. gingivalis* capsular polysaccharide has been shown to consist of glucose, glucosamine, galactosamine and galactosaminuronic acid residues, while the LPS saccharide chains are comprised primarily of rhamnose, glucose and galactose (Schifferle et al., 1989). Bramanti et al. (1989) reported that

the polysaccharide component of *P. gingivalis* LPS was rhamnose, glucose, galactose, mannose, glucosamine and galactosamine. More recent chemical analysis of *P. gingivalis* surface polysaccharides by Farquharson et al. (2000) reported the saccharide structure of *P. gingivalis* capsular polysaccharide consisted of mannuronic acid, glucuronic acid, galactose, and 2-acetamido-2-deoxy-D-glucose. To date, six capsular serotypes of *P. gingivalis* have been described (Laine et al., 1996); however, a significant number of nontypeable *P. gingivalis* capsule serotypes appear to exist (Laine et al., 1997). Epidemiological studies are beginning to define the distribution of these serotypes associated with human periodontal disease (Laine et al., 1997; Califano et al., 1999).

Porphyromonas gingivalis capsular polysaccharide appears to promote phagocytosis of this organism by polymorphonuclear neutrophils (PMNs), as a polysaccharide mutant was more resistant to opsonophagocytic uptake by PMNs (Genco et al., 1995b). Van Winkelhoff et al. (1993) reported that encapsulated *P. gingivalis* causes serious forms of infection using a murine model. Laine and van Winkelhoff (1998) confirmed this observation and demonstrated that mice challenged with encapsulated *P. gingivalis* developed more severe infections than mice challenged with unencapsulated strains. Lithium diiodosalicylate extracts of *P. gingivalis* possess vaccine potential; however, this extract is complex and likely contains several antigens including capsular polysaccharide (Chen et al., 1990). A conjugate vaccine consisting of *P. gingivalis* capsular polysaccharide and *P. gingivalis* fimbriae was shown to prevent *P. gingivalis* infection using a murine subcutaneous challenge model (Choi et al., 1998). Additionally emerging animal model data support that purified *P. gingivalis* CPS (capsular polysaccharide) may represent a novel vaccine candidate as mice immunized with *P. gingivalis* CPS developed elevated serum levels of IgM and IgG that reacted with *P. gingivalis* whole organisms, and these animals were protected from *P. gingivalis*-elicited oral bone loss (Gonzalez, 2003).

Genetic Variability and Genomics

Porphyromonas gingivalis can modulate gene expression through genomic rearrangement and transposition, a factor that could contribute to this organism's ability to evade host detection and become established in the oral cavity. As discussed above, the structures of the *rgpA* and *rgpB* genes are very similar except that the *rgpB* gene does not possess most of the hemagglutinin domain present in the C-terminal region of the *rgpA* gene. It has been postulated that the two *rgp* genes were generated through the duplication of an ancestor *rgp* gene, insertion of the hemagglutinin domain region into one copy of the two resulting *rgp* genes (or deletion of the region from one *rgp*) and homologous recombination between the proteinase domain regions of the two *rgp* genes. It is appealing to speculate that this type of rearrangement may be responsible for the genetic diversity of this family of genes. Interestingly, the presence of nonreciprocal recombination in *P. gingivalis* was experimentally proven using suicide/integration plasmid systems.

Comparison of the nucleotide sequences of *rgpA* and *kgp* indicates that a majority of the C-terminal sequences of these genes are identical. It has been suggested that recombinational rearrangement (such as transposition or gene conversion) may have occurred in this nucleotide region between *kgp* and *rgpA*. Gene conversion type recombination has been observed in *P. gingivalis* (Nakayama et al., 1995; Okamoto et al., 1996), and thus it is reasonable to postulate that recombination between *P. gingivalis rgpA* and *kgp* could occur by such a mechanism. There are numerous examples in which gene conversion results in antigenic variation of surface proteins in a variety of bacterial pathogens (Seifert and So, 1988). The ability to undergo antigenic variation protects pathogens from immunological challenge by their hosts.

In addition to gene conversion, transposition may be a mechanism for recombinational rearrangements in *P. gingivalis* (Barkocy-Gallagher et al., 1996; Simpson et al., 1999). It is well established that genes proximal to an insertion element can be transposed or deleted (Ajioka, 1989; Galas, 1989). Interestingly, the *P. gingivalis* endogenous insertion sequence (IS) element IS*1126* was found 3′ to the *kgp* gene, and it was suggested that this IS element could serve as one end of a composite transposon (Barkocy-Gallagher et al., 1996). Recombination within the locus encoding Kgp could thus have occurred via a transposition event. In support of this contention, Simpson et al. (1999) found that IS*1126* is capable of transposition in *P. gingivalis*. Analysis of a *P. gingivalis* strain in which IS*1126* was mobilized indicated that IS*1126* had inserted directly upstream of the *kgp* gene. The demonstration of the mobilization of the second endogenous *P. gingivalis* insertion element, PGIS*2*, in the *P. gingivalis* chromosome has also been reported (Wang et al., 1997). Thus, the ability of IS elements to be mobilized within *P. gingivalis*, together with the finding that these elements are commonly found flanking the *kgp* locus, indicates that the variability in this locus may be due to genomic rearrangements facilitated by trans-

positional events. Further support of an active recombination system in *P. gingivalis* comes from the isolation of the *P. gingivalis recA* homolog (Fletcher et al., 1997).

The *P. gingivalis* genome has been completely sequenced and has revealed the presence of numerous IS elements and transposons in this organism (The Institute for Genomic Research website). Recently a DNA array based on the complete genome sequence has been generated and is available to examine gene expression. It is anticipated that with the complete sequence in hand, additional bacterial virulence factors will be defined and the regulation of these factors in response to specific environmental cues will be examined.

Regulation

During the transition from health to disease, a number of environmental changes are apparent in the oral ecological niche occupied by *P. gingivalis*. The gingival sulcus and periodontal pockets do not represent favorable environments for oral microbial flora to proliferate and colonize, and only a limited number of highly evolved species can colonize this area (Socransky and Haffajee, 1992). This is assumed to result from the pH, temperature, and oxygen availability, which limit the growth of a number of bacterial species. In addition, these environmental changes serve as important cues for this organism to regulate gene expression. The mean temperature of the gingival sulcus is 35°C, with a consistent range of 30–38°C (Socransky and Haffajee, 1991). The mean temperature is slightly higher (36.8°C) in inflamed periodontal pockets (Kung et al., 1990; Fedi and Killoy, 1992). Like other pathogenic microorganisms, *P. gingivalis* mounts a heat shock response when exposed to elevated temperature (an important homeostatic mechanism; Lu and McBride, 1994; Vayssier et al., 1994). GroEL (HSP60 family) and DnaK (HSP70 family) homologs have been described in *P. gingivalis* (Lu and McBride, 1994; Vayssier et al., 1994). In addition to reporting the response to temperature changes, Vayssier et al. (1994) have also reported that an increase in oxygen tension and acidic pH induce GroEL. Amano et al. (1994) have reported on a heat shock inducible 55-kDa protein, which may be related to HSP60. Further studies are needed to clarify the exact types and numbers of heat-shock proteins synthesized by *P. gingivalis* in response to an upward shift in temperature and to determine the function of these proteins and their involvement in pathogenicity.

Increased expression of *P. gingivalis* fimbrillin and superoxide dismutase (SOD) in response to a shift in growth temperature from 37 to 39°C has been reported (Amano et al., 1994). In contrast, in a separate study, *P. gingivalis* was observed to exhibit an increase in *fimA* promoter activity when the growth temperature was downshifted from 37 to 34°C (Xie et al., 1997). Although the precise biological function of temperature-regulated fimbrillin expression and SOD activity is not clear, it was suggested that downregulation of fimbrillin expression may help *P. gingivalis* evade the host immune system and that elevated SOD activity may enable *P. gingivalis* to survive in environments containing high levels of neutrophil-generated superoxide (Amano et al., 1994).

The pH range within gingival pockets is reported to be 7.0–8.5 (Cimasoni, 1983). The pH in the periodontal pocket increases accordingly as the periodontal pockets deepen and the host inflammatory response is induced (Bickel and Cimasoni, 1985). The optimal pH for growth of *P. gingivalis* is within the range 7.5–8.0 (Marsh et al., 1994); however, *P. gingivalis* has often been recovered from disease sites with elevated pH. The ability of *P. gingivalis* to grow at elevated pH may confer a great advantage to this bacterium over other species and may explain its association with disease. One factor contributing to the growth stability of *P. gingivalis* in this pH range may be the expression of the *sod* gene. Lynch and Kuramitsu (1999) have reported that in addition to oxidative stress and temperature, *sod* expression is also influenced by pH. Compared with acidic conditions (pH 6.0), *sod* expression increases significantly at neutral (pH 7.0) or slightly alkaline (pH 8.0) conditions (Lynch and Kuramitsu, 1999). Trypsin-like and chymotrypsin-like activity have also been observed to increase as pH increases, until maximal activity is reached at pH 8.0, suggesting that during initial stages of infection, *P. gingivalis* responds to the environmental pH changes by producing enzymes with maximal host tissue damage potential (McDermid et al., 1988). It has now become evident that the majority of trypsin-like activity produced by *P. gingivalis* is due to the production of the arginine- and lysine-specific cysteine proteinases (see section on Gingipains in this Chapter). It has been demonstrated that the optimal activity of these enzymes is achieved at a pH of 8.0. The increased activity of the *P. gingivalis* gingipains may serve to inactivate the host immune system components such as complement and immunoglobulins.

The hemin concentration in the periodontal pocket can vary significantly depending on the degree of inflammation and bleeding at this site. The regulation of *P. gingivalis* gene products involved in hemin-binding and transport is controlled by the level of hemin and iron (Carman

et al., 1990; Grenier, 1991; Smalley et al., 1991; Cutler et al., 1995). Transcription of the *hmuR* and *kgp* genes is negatively regulated by iron, and the *hmuR* and *kgp* promoters contain binding sequences for ferric uptake regulator protein (Fur; Forng et al., 2000; C. A. Genco, unpublished data). In several Gram-negative pathogens, Fur functions as a general global regulator and controls the expression of not only genes required for iron transport but also genes required for virulence. The recent report of a *P. gingivalis fur* homolog (C.A. Genco, unpublished data), together with the identification of several iron regulated receptors, suggests that the global negative regulator Fur may be operative in *P. gingivalis*.

In addition, both the induction and repression of expression of specific virulence factors in response to hemin have been reported (Cutler et al., 1995). Limitation of hemin during *P. gingivalis* growth affects hemolytic, trypsin-like protease and hemagglutination activities of *P. gingivalis* whole cell and extracellular vesicles. The expression of *P. gingivalis* proteinases can vary in response to hemin, as well as to specific growth phase (Carman et al., 1990). Trypsin-like protease activity was higher in hemin-excess *P. gingivalis* cultures, while collagenolytic activity was higher in hemin-restricted *P. gingivalis* cultures (Carman et al., 1990). An increase in extracellular vesicle production by hemin-limited *P. gingivalis* cultures has also been reported (McKee et al., 1986), and vesicles isolated from hemin-limited *P. gingivalis* cultures have also been shown to possess higher trypsin-like protease and hemagglutination activities (Smalley et al., 1991). Although hemin-restricted cultures have a lower overall level of trypsin-like activity and a reduction in total culture biomass, the activity present in extracellular vesicles from hemin-restricted cultures is greater than that of hemin-excess cultures (Smalley et al., 1991). Recent studies have demonstrated that expression of the *P. gingivalis* hemagglutinin genes *hagB*, *hagC*, and *hagD* is regulated by hemin (Lepine and Progulske-Fox, 1996a; Han et al., 1998). Modifications of the antigenicity of *P. gingivalis* LPS have also been reported in response to hemin limitation (Cutler et al., 1996). Hemin limitation and growth in the presence of 1% serum or saliva have also been reported to reduce transcription of the *fimA* gene.

Growth of *P. gingivalis* under hemin-restricted conditions results in increased virulence as assessed in a mouse model (Bramanti and Holt, 1993), and this increase may be explained by the increased expression of *P. gingivalis* outer membrane components and their associated enzymatic activities, which directly contribute to the overall pathogenic potential when this organism enters a hemin-limited environment. *Porphyromonas gingivalis* may adapt to changes in this environment by turning off the expression of some virulence factors and turning on the expression of others. The balance of virulence factor expression may be controlled through a number of yet to be identified regulatory proteins acting together or alone.

Recent studies have also begun to define the role of density-dependent gene regulation in *P. gingivalis*. This organism is frequently found within a mixed-species biofilm that exists in the gingival crevice. Additionally *P. gingivalis* can invade, replicate and persist in high density within gingival epithelial cells. In many Gram-negative and Gram-positive bacteria, the density dependent regulation of gene expression is controlled through quorum sensing. Quorum sensing involves the synthesis and detection of extracellular signaling molecules termed "autoinducers." In *Vibrio harveyi*, the *luxS* gene is required for type 2 autoinducer production. Recently a functional *luxS*-dependent signaling pathway was identified in *P. gingivalis*. The regulation of this system was demonstrated to be in response to cell density and osmolarity. In addition, differential display PCR showed that the inactivation of the *P. gingivalis luxS* resulted in the upregulation of HemR and RgpA (Choi et al., 2001).

During the early onset of periodontal disease, polymorphonuclear leukocyte infiltration within the subepithelial connective tissue and the loss of epithelial cell attachment to the tooth enamel are observed. Subsequently, T-cells and macrophages are recruited into the area underlying the pocket epithelial cells. This array of antagonistic cells is accompanied by the release of biologically active cytokines such as IL-1, IL-6, and TNF-α (Cutler et al., 1995; Darveau et al., 1998). To date little is known about the expression of specific bacterial components in response to the host inflammatory response; however, the induction of a host inflammatory response in response to bacterial infection clearly changes the local environment in which *P. gingivalis* resides. Although there is no hard evidence yet that the host response influences gene expression in *P. gingivalis*, this concept warrants further investigation. With the development of new methodologies, bacterial genes that are regulated by the host response should be easily identified.

Innate Host Immune Response

The innate immune response during an acute infection can be broadly broken down in to four stages: 1) recognition and processing of the

foreign particle, 2) antigen presentation, 3) immune cell recruitment with cell activation, and 4) directed killing of the foreign material with subsequent resolution of the inflammatory response. In the case of chronic diseases such as periodontal disease, it is not understood why the host is unable to fully resolve the infection. Mounting evidence supports that both host immunological defects and bacterial virulence strategies play important roles in the chronic nature of periodontal disease. As the topic of innate immunity has been studied in detail for over 100 years, in this section, we will concentrate primarily on the innate host response to *P. gingivalis*.

During periodontal health, the tissues adjacent to and underneath the gingival epithelium commonly possess a modest accumulation of neutrophils that are believed to be important in clearing any transient bacteria that gain access to these tissues. However, in patients in the acute or active stage of periodontal disease, the periodontium presents with a neutrophilic cellular infiltrate that switches to a predominating monocytic cellular infiltrate in chronic lesions. Understanding the complex cellular interactions that occur during periodontal disease is critical to defining a mechanistic approach to determine what bacterial factors might be responsible for this response. Gingival crevicular fluid obtained from periodontal disease sites has high levels of IL-1β, IL-8, and IL-10 and the chemokine RANTES (regulated upon activation, normal T cell expressed and secreted; Gamonal et al., 2000), IL-6, transforming growth factor, PGE_2, IL-2, TNF-α, and interferon (IFN)-γ (Salvi et al., 1998). The mechanisms by which *P. gingivalis* stimulates cytokine and chemokine production are not well known, but recent in vitro studies have been performed with *P. gingivalis* as well as with purified antigens from this organism. Numerous studies have been performed using various experimental criteria, and as a result it has become obvious that the host cell type, the number of bacteria, and the amount of antigen being tested are critical to the reported observations.

Innate Response of Relevant Host Cells In Vitro

ORAL EPITHELIAL CELLS. Oral epithelium, and more specifically gingival epithelium, represents one of the initial host barriers to *P. gingivalis* when this organism is present in the gingival sulcus. Several studies have begun to characterize the host response of oral epithelial cells to *P. gingivalis*. Challenge of oral epithelial cells with *P. gingivalis* elicits a TNF-α and IL-1β response. Additional studies have demonstrated that these cells also express cell adhesion molecules on their surface in response to *P. gingivalis* infection and include ICAM-1 and VCAM-1 (Wang et al., 1999). Interestingly, work by Darveau et al. (1998) has demonstrated that gingival epithelial cells challenged with *P. gingivalis* LPS fail to produce IL-8; furthermore, *P. gingivalis* LPS stimulation functions as a potent inhibitor of subsequent *E. coli* LPS stimulation of IL-8. This mechanism has been coined "localized chemokine paralysis." This mechanism, along with the ability of *P. gingivalis* gingipains to digest cytokines and chemokines (see section on Gingipains in this Chapter), is an attractive mechanism by which *P. gingivalis* may be able to circumvent the host response.

POLYMORPHONUCLEAR NEUTROPHILS. Anatomically, healthy people possess a layer of neutrophils that reside immediately below the epithelium of the gingival sulcus. It has been suggested that these cells perform immune surveillance and quickly clear any low level contamination of the subepithelial tissues. During the switch from periodontal health to periodontal disease, these infected sites typically present with an influx of PMNs. Recent studies have begun to focus on the response of PMNs to *P. gingivalis* infection, as well as investigating the potential of *P. gingivalis* to modulate the response of these cells during the infectious process. In vitro, PMNs challenged with live *P. gingivalis* produce the proinflammatory cytokines TNF-α, IL-1β and IL-6. Interestingly these cells also express IL-10 (a cytokine commonly associated with regulation of the host immune response). More recent studies have concentrated on the host responses to *P. gingivalis* fimbriae, LPS and gingipains. PMNs produce IL-8 and MCP-1 in response to live *P. gingivalis* infection. Interestingly, *P. gingivalis* viability is not necessary for stimulating chemokine production from PMNs as viable and heat-killed *P. gingivalis* elicits similar levels of these chemokines. Recent studies have focused on the PMN response to defined *P. gingivalis* antigens including LPS, fimbriae and gingipains. *Porphyromonas gingivalis* LPS upregulates complement receptor 3 on the PMN surface, as well as stimulates IL-1β, TNF-α and IL-8. Purified fimbrillin monomer (43-kDa protein), as well as a 24-mer-peptide sequence from the fimbrillin protein, possesses potent IL-8 stimulating activities. The effect of gingipains on PMN function is not known directly; however, recent studies using defined gingipain mutants of *P. gingivalis* show that gingipains downregulate the transcription of genes responsible for chemokine production (Nassar et al., 2002).

MONOCYTES. Monocytes represent the primary cell population present in chronic

periodontal lesions. Despite this observation, relatively few studies have focused on the interaction of monocytes and *P. gingivalis*; however, it is well known that monocytes commonly serve as primary antigen presenting cells. Of the studies that have been performed, the majority of work has focused on the inflammatory response of monocytes to *P. gingivalis* or defined antigens of this organism such as LPS. Studies have focused on the use of both human monocytic cells, as well as cells of murine origin, and despite some differences, the trend is that *P. gingivalis* stimulation of monocytes leads to the expression of TNF-α, IL-1β, MCP-1, IL-6, IL-8, nitric oxide, as well as PGE_2; however, *P. gingivalis* strain-dependent activation of monocytes has been reported. The relationship between tobacco use and periodontal disease is well documented. It has been reported that nicotine affects the host by upregulating inflammatory mediator expression of monocytes in response to *P. gingivalis* LPS. The result of this upregulation is elevated IL-1β production, a molecule whose activity is associated with bone remodeling and is commonly detected in crevicular fluids of patients with periodontal disease.

It is now well established that Gram-negative enteric LPS signals host cell activation through the Toll-like receptor (TLR)-4. TLRs are a family of pattern recognition receptors that function as cognate receptors, and currently there are 10 identified TLRs. Recent data suggested that *P. gingivalis* LPS stimulates human monocytes to produce cytokines; this process involves CD14 and TLR-2, and the p38 mitogen-activated protein (MAP) kinase activation is involved in this pathway. As discussed above, *P. gingivalis* LPS is not a potent endotoxin, and this is likely due to the unique fatty acid composition of the lipid A of *P. gingivalis*.

A limited number of studies have been performed to assess the effect of other defined antigens of *P. gingivalis* with monocytes. It has been suggested that fimbriae can stimulate monocyte differentiation, TNF-α and the chemokine KC (keratinocyte-derived cytokine) production. After the initial interaction of *P. gingivalis* fimbriae with macrophages, neutrophil chemotactic factor and protein kinase C are rapidly induced (Hanazawa et al., 1992; Murakami et al., 1994). Associated with this interaction is the formation of a 68-kDa phosphorylated protein within the confines of the macrophage, the induction being mediated by prior induction of protein kinase C. Fimbriae have also been suggested to inhibit caspase-3, an important molecule in cell apoptosis. In these studies, it was demonstrated that fimbria-specific antibody was able to neutralize this activity. As decreased apoptosis appears to be linked to chronic infections, these results suggest that *P. gingivalis* prevents monocyte cell apoptosis and thus facilitates the chronic nature of this disease.

DENDRITIC CELLS. A relatively new area is the role that dendritic cells may play in *P. gingivalis*-mediated periodontal disease. Recent studies by Cutler et al. (1999) suggest that dendritic cells and T-cells aggregate at sites of periodontal damage. Using in vitro techniques, it has been shown that these dendritic cells produce a variety of molecules in response to *P. gingivalis* LPS including accessory molecules involved in antigen presentation, the inflammatory mediators IL-1β and PGE_2, as well as the immunoregulatory cytokines IL-10 and IL-12. It is suggested that the high levels of IL-10 detected in dendritic cell supernatant fluids may downregulate the local immune response in a way that facilitates the chronic nature of *P. gingivalis*-mediated periodontal disease.

T CELL RESPONSES. The primary response of T cells is to coordinate the host effector response that includes mobilization of both innate and adaptive arms of a host response to rid the host of the infectious agent. Using specialized receptors, T cells recognize foreign antigens presented to these cells by antigen-presenting cells such as monocytes, and subsequently these cells undergo clonal expansion and secrete a plethora of cytokines that are responsible for dictating the host response. Little is known about the T cell-specific responses that occur during *P. gingivalis*-mediated periodontal disease. The numbers of T cells present in periodontal lesions extracted from patients with adult periodontal disease are elevated as compared to those of normal subjects (Gemmell and Seymour, 1998); however, the cytokine responses to *P. gingivalis* are not very different. The use of T cell clones established from periodontal disease patients has demonstrated that these cells produce chemokines (MCP-1, MIP-1α and RANTES) and cytokines (IFN-γ, IL-4 and IL-10) in response to *P. gingivalis* challenge, supporting the importance of IFN-γ and a Th1-like response in the progression of periodontal disease (Gemmell et al., 1999). CD_4^+ T cell specific responses are critical to the progression of oral bone loss in response to *P. gingivalis* oral challenge, and mice deficient in IFN-γ and IL-6 are also resistant to *P. gingivalis*-mediated oral bone loss (Baker et al., 1999; Baker et al., 2001). Recently, attempts have been made to better understand the predominating T cell response of patients with periodontal disease due to *P. gingivalis*, in an attempt to define whether Th1 or Th2 responses predominate. On the basis of antibody data, it appears that a *P. gingivalis*-specific Th1 response predominates in humans during periodontal disease as compared with

normal control subjects. What is not known, however, is why this mechanism is not protective. It is intriguing to speculate that by modifying the host T cell-mediated immune response, human periodontal disease can be limited. Future studies will better define the role of T cell-mediated host responses to *P. gingivalis*-elicited periodontal disease.

VASCULAR ENDOTHELIUM. The emergence of epidemiological data suggesting a connection between *P. gingivalis*-mediated periodontal disease and systemic diseases such as cardiovascular and cerebrovascular disease (see section on Systemic Complications of Periodontal Disease in this Chapter) has prompted investigators to study the interaction of *P. gingivalis* with vascular endothelium. Seminal studies by Deshpande et al. (1998a) demonstrated that *P. gingivalis* is able to actively invade endothelium, and these studies have been confirmed by Dorn et al. (1999). HUVECs or human aortic endothelial cells (HAECs) challenged with heat-killed *P. gingivalis* produce IL-8, while challenge of these cells with live *P. gingivalis* fails to produce this response. Although these cells do not secrete IL-8, live *P. gingivalis* challenge does stimulate IL-8 gene transcription. The precise mechanism by which *P. gingivalis* "uncouples" IL-8 production is unknown; however, through use of genetically defined *P. gingivalis* strains, it appears that gingipains are important in modifying this response at the transcriptional level, as well as by degrading secreted IL-8 (Nassar et al., 2002). *Porphyromonas gingivalis* LPS and fimbria are able to stimulate chemoattractants such as IL-8 and MCP-1 from endothelial cells challenged with *P. gingivalis*. Additionally, emerging data demonstrate that endothelial cells infected with *P. gingivalis* produce an array of cell adhesion molecules. The importance of cell adhesion molecules is the localization of immune cells present in the circulation to a site of infection. HUVECs infected with *P. gingivalis* produce P- and E-selectins, as well as ICAM-1 and VCAM-1, as compared with resting endothelial cells (Khlgatian et al., 2002). In the event that a clear experimental connection between periodontal disease and cardiovascular disease is established, it will be important to understand the cellular response of vascular cells to *P. gingivalis* in more detail.

COMPLEX CELL CULTURE SYSTEMS. Recently it has become clear that the result of using single cells to determine the host response to *P. gingivalis* challenge may be misleading and that the use of complex cell culture systems infected with more than one *P. gingivalis* cell could be revealing. Infection of oral epithelial cells with *P. gingivalis* results in inhibition of trans-epithelial migration of PMNs. *Porphyromonas gingivalis* infection of endothelial cells also appears to downregulate the cells in such a manner as to make them unresponsive to *E. coli* LPS challenge. This localized chemokine paralysis appears to play an important role in the host response to *P. gingivalis* infection by limiting inflammatory cell migration and in part explains why *P. gingivalis* is not efficiently cleared during infection. *Porphyromonas gingivalis* or *P. gingivalis* fimbriae leads to a synergistic chemokine response in endothelial/PMN or endothelial/macrophage cultures that is not observed when either cell type is infected in monoculture. The elevated IL-8 and MCP-1 responses may likely govern the activation and migration of PMNs and monocytes to this site; however, precise assays to define this aspect of inflammation are still needed.

COMPLEMENT. As an important molecule of the innate immune response, complement has opsonic properties, functions as a chemoattractant for localizing inflammatory cells at the site of infection, and mediates killing activity. Control of complement activity is therefore an important part of the regulatory mechanism of the host innate response. Complement deposition occurs by two distinct pathways, either in the presence of specific antibody (classical pathway) or in direct response to microbial products (alternate or properdin pathway). The classical pathway of complement activation is important in the clearance of *P. gingivalis*; however, as is discussed in the section dealing with adaptive host immunity (see the section Adaptive Immune Response in this Chapter), patients with *P. gingivalis*-mediated periodontal disease produce antibodies with weak complement activating properties.

PATTERN RECOGNITION MOLECULES AND TOLL-LIKE RECEPTORS. One important area that is emerging in innate immunity is the role of pattern recognition molecules in modulating the response of a cell to a specific microbial structure. Currently the mechanism by which *P. gingivalis* activates cells is under intensive study to determine which pattern recognition receptors are engaged, what signal transduction events are activated following receptor-ligand binding, and what subsets of genes are activated as a result of this binding. Much of the early work in this area focused on the ligand-ligand interactions of *E. coli* LPS, as this form of endotoxin is highly associated with endotoxic shock and vascular collapse. A synthesis of this data indicates that enteric LPS interacts with TLR-4 via a CD14-dependent process (Heumann and Roger, 2002), and NFκB is translocated prior to initiation of gene transcription, which results in a TNF-α-mediated proinflammatory response that dominates the host

endotoxic response; however, the data for *P. gingivalis* infection and the interaction of specific virulence factors with pattern recognition molecules are not as clear. It is well accepted that *P. gingivalis* LPS binds to CD1; however, the specific binding site that is engaged by *P. gingivalis* LPS is different than the site used by *E. coli* LPS. Toll-like receptor (TLR 2 rather than TLR-4) is engaged by *P. gingivalis* LPS on vascular endothelium; however, it appears that TLR-4 is engaged by *P. gingivalis* LPS on monocytes. Recent data support involvement of both TLR-2 and TLR-4 in the context of live *P. gingivalis* infection, as macrophages from C3H/HeJ mice (which have a point-mutated TLR-4 rendering it nonfunctional; Poltorak et al., 1998) respond to *P. gingivalis* challenge albeit to a lesser extent than do wildtype murine macrophages (C. A. Genco, unpublished data). Because pattern recognition affects the host response as well as the complexity of the interaction of *P. gingivalis* with cells of various lineages, additional work in this area is required and should increase understanding of *P. gingivalis* surface structures important in stimulating a host response and lead to new treatments (based on circumventing these processes) for *P. gingivalis*-mediated periodontal disease.

Adaptive Immune Response

The production of specific antibody to a bacterial pathogen is a common host response that in most cases is highly efficient in promoting clearance of the invading organism. These antibodies assist in clearance primarily by 1) opsonizing the organism, thus allowing neutrophil Fc receptor-mediated recognition and killing, and 2) facilitating complement deposition. Individuals colonized with *P. gingivalis* possess elevated levels of serum antibody specific for this organism and its individual components (Kinane et al., 2000). Currently there is no antibody response that can be used clinically to demonstrate the presence of periodontal disease or for differential diagnosis. Initial studies by Moulton et al. (1981) demonstrated that the serum of patients with adult periodontal disease contain elevated levels of *P. gingivalis*-specific IgG, IgM and IgA. Following this report, a majority of the subsequent studies from the United States or other countries support that levels of *P. gingivalis*-specific IgG are elevated in the serum of patients with generalized aggressive periodontal disease; however, this is controversial, as other reports suggest no differences or highly variable results between patients and controls. Yet despite this potent antibody response, the host is unable to adequately control *P. gingivalis*-mediated periodontal disease. The reasons for this lack of control are not known; however, several important possibly concurrent processes involving host factors (such as antibody avidity, antibody subtype, and host Fc receptor profiles) and bacterial factors (such as degradation of antibody by gingipains and the *P. gingivalis* serotype present in the oral cavity) could provide an explanation.

On the basis of several studies, it appears that the high levels of IgG produced in response to *P. gingivalis* infection are primarily of low avidity. The reason for the low avidity of these antibodies is not known. However, IgG antibodies of low avidity do not bind efficiently to the bacterium and would not be expected to efficiently opsonize *P. gingivalis*. Assessments of the IgG subclass during generalized aggressive periodontal disease indicate that a range of isotypes is produced by patients with *P. gingivalis* periodontitis (Kinane et al., 2000). Studies by Whitney et al. (1992) reported high levels of IgG with reactivities increasing in the order $IgG_2 > IgG_3 > IgG_1 > IgG_4$ in patients with generalized aggressive periodontitis; however, most of the IgG_2 appears to be directed toward the LPS and capsular polysaccharide of *P. gingivalis* (Ogawa et al., 1990; Califano et al., 1999). Interestingly, the preponderance of IgG_2 produced in response to *P. gingivalis* challenge may, in fact, facilitate the chronic nature of generalized aggressive periodontal disease as IgG_2 possesses weak complement fixation activity and is a poor opsonin as compared with the other IgG isotypes.

More recent studies have begun to assess the levels of IgG produced to defined *P. gingivalis* antigens during periodontal disease. Patients with generalized aggressive periodontal disease possess high levels of IgG (predominantly IgG_2) directed to a gingipain complex consisting of RgpA-Kgp. Additional studies indicate that patients produce high levels of IgG to the individual gingipains RgpA, RgpB and Kgp as compared with control subjects, and the RgpA- and RgpB-specific (but not Kgp-specific) IgG can facilitate opsonophagocytosis of *P. gingivalis* by naïve human neutrophils. The IgG isotype response in the serum of periodontitis patients to *P. gingivalis* fimbriae is predominately IgG_3, while other antigens appear to elicit IgG_1-specific and IgG_4-specific responses.

In addition, it has been demonstrated that gingipains, specifically RgpA, possess the ability to degrade IgG (Holt et al., 2000). This strategy would certainly create an environment "locally cleared" of antibody by both *P. gingivalis* secreted and membrane-bound gingipains. These degraded antibodies by not recognizing *P. gingivalis*, mediating opsonophagocytosis, or mediating complement fixation would create a local

environment favorable to *P. gingivalis* survival within the host.

Systemic Complications of Periodontal Disease

Diabetes Diabetes affects more than 13 million people in the United States and approximately 600,000 new cases are diagnosed each year. Diabetes has been identified as a significant risk factor for periodontal disease and can aggravate the severity of periodontal infections (Matthews, 2002; Teng et al., 2002). It has been shown that even in children, insulin-dependent diabetes is associated with an increase in periodontal disease. Factors that increase the severity of inflammatory diseases, such as impaired neutrophil function, excessive inflammatory response, collagen turnover defects, impaired wound healing, and periodontal flora changes, have been associated with diabetes pathogenesis. Patients with insulin-dependent diabetes express high levels of proinflammatory mediators compared with matched non-diabetic, normal individuals. Both PGE_2 and IL-1β have been found in significantly higher levels in the gingival crevicular fluid of diabetic patients as compared with non-diabetic control subjects when matched for periodontal disease severity. Similar results have been observed for TNF-α expression. Recent studies also indicate that periodontal disease can adversely impact glycemic control in diabetes. Periodontal treatment that decreases the bacterial challenge and the resulting inflammatory periodontal destruction can improve glycemia in some diabetic patients. Overall, the evidence supports the view that the relationship between diabetes and periodontal disease is bidirectional (Soskolne and Klinger, 2001). Although information about the molecular pathogenesis of periodontal infections in diabetes is just becoming available, the molecular and cellular aspects of host-parasite interactions are poorly understood and warrant definitive investigation.

Cardiovascular Disease Several reports have described an association between periodontal disease and coronary artery disease. These include case control studies, which demonstrated significant associations after correction for cholesterol, smoking, hypertension, social class, and body mass index (Beck and Offenbacher, 1998a; Beck et al., 1998b; Beck et al., 2001). The biological consequences of oral infection with *P. gingivalis* and the risk for acute myocardial infarction and other vascular phenomena are supported by the following: 1) *P. gingivalis* can induce aggregation of platelets (Pham et al., 2002) and trigger cytokines, which play a role in atheroma formation through bacterial products such as LPS and fimbriae. 2) *P. gingivalis* can cause local inflammation (which leads to ulceration of the gingivae) and local vascular changes (which increase the incidence and the severity of transient bacteremias when the gingivae are traumatized). Procedures such as dental extraction, periodontal surgery, tooth scaling, and even tooth brushing can lead to the presence of oral bacteria in circulating blood. 3) Injured or activated endothelial cells, resulting from *P. gingivalis*-induced alterations in the endothelial cell, may show a variety of atherogenic properties including increased procoagulant activity, secretion of vasoactive and inflammatory mediators, and expression of adhesion molecules. 4) *P. gingivalis* may be released into the bloodstream, which could have direct or indirect effects. Direct effects would include the aggregation of platelets triggered by *P. gingivalis* surface antigens, gingipains, and fimbriae, leading to thrombus formation. Indirect effects may be mediated via LPS and other bacterial components that could trigger cytokine production by macrophages in situ in atheromatous plaque. This could lead to accumulation of lipid and foam cell formation and trigger endothelial cells to form IL-1β, which increases thrombus adherence. The consequences of these direct and indirect effects may include bacterial induced thrombi, which attach to atheromatous plaques in the heart vessel wall, leading to ischemic heart disease and the risk for myocardial infarction. Chronic and multiple transient bacteremias could also trigger macrophages and endothelial cells to blood vessels and increase formation of atheromatous plaques to which thrombi could then attach, precipitating acute myocardial infarction.

The gingival sulcus is believed to be the most likely area of entry of oral bacteria into the bloodstream and the integrity of the basement membrane of the oral mucosa and particularly the gingival sulcus is paramount in the protection of patients at risk for cardiovascular disease. The ability of *P. gingivalis* to invade and replicate within endothelial cells (Deshpande et al., 1998b) suggests that this organism possesses mechanisms for survival within vascular endothelial cells. Injured or activated endothelial cells (during infection) may show various atherogenic properties including increased procoagulant activity, secretion of vasoactive and inflammatory mediators, and expression of adhesion molecules. E-selectin, ICAM-1, and VCAM-1 have been detected in atherosclerotic areas of human arteries, and inflammatory cells are abundant at these sites. Invasion of vascular and heart endothelial cells by *P. gingivalis* after entry into the bloodstream may contribute to the pathology of cardiovascular disease.

PRE-TERM BIRTH Recent epidemiological studies have implicated periodontal disease as a risk factor for pre-term birth (Offenbacher et al., 1998). Human studies in patients with periodontal disease and animal studies have suggested that infection with *P. gingivalis* may be an important component in the underlying association in linking periodontal disease to pre-term birth. Several plausible hypotheses support a link between maternal periodontal disease and pre-term birth. In particular, maternal periodontal infection may influence pre-term delivery through mechanisms involving inflammatory mediators. Using a subcutaneous chamber mouse model, it was demonstrated that *P. gingivalis* infection was associated with hepatic and uterine translocation, and this dissemination correlated with fetuses that exhibited growth restriction (Collins et al., 1994). Likewise, *P. gingivalis* challenge was demonstrated to induce maternal elevated proinflammatory cytokine responses (TNF) and decreased anti-inflammatory cytokine response (IL-10). These systemic changes have been associated with fetal growth restriction. Translocation of *P. gingivalis* or specific bacterial components into the placenta may induce placental immune responses that impair placental function, thereby mediating fetal growth restriction. Clearly additional studies in well-defined animal models will further define the mechanisms associated with *P. gingivalis*-induced inflammatory responses in the context of pre-term birth.

Animal Models

A variety of animal models (such as rodents, dogs, sheep and nonhuman primates) have proven useful for expanding our understanding of *P. gingivalis*-elicited periodontal disease. No single animal model effectively mimics all aspects of periodontal disease. Thus a variety of models are needed to provide a complete picture of the pathogenesis of *P. gingivalis*-elicited periodontal disease. A recent review article discusses many of the studies performed in animals to address various aspects of *P. gingivalis* infection and host response, as well as discusses the benefits and limitations of each of these animal model systems (Genco et al., 1998).

Initial studies to address the association of dental plaque and periodontal disease pathogenesis were performed in dogs (Genco et al., 1998). These studies demonstrated a link between the presence of plaque and subsequent development of periodontal disease. Unfortunately, *P. gingivalis* does not appear to play a role in the development of periodontitis in dogs. A seminal study by Holt et al. (1988) demonstrated that the primate *Macaca fascicularis* orally challenged with *P. gingivalis* developed periodontitis, thus establishing a direct link between *P. gingivalis* infection and the development of periodontal disease. Rodents, owing to their small size and cost, have proven very useful in defining various aspects of periodontitis. A murine abscess model has been used by many investigators to study *P. gingivalis* disease pathogenesis. In this model, localized subcutaneous injection of *P. gingivalis* leads to an initial focal lesion with the development of secondary spreading lesions. This abscess model has also demonstrated that attenuation of *P. gingivalis* virulence directly impacts disease progression, as gingipain double knockout mutant ($RgpA^-$ and $RgpB^-$) exhibited less abscess formation than did wildtype *P. gingivalis* (Nakayama et al., 1995). A murine subcutaneous chamber model has proven particularly useful for studying the temporal interaction between *P. gingivalis* and the host immune system, with particular emphasis in studying PMN/*P. gingivalis* interactions (Genco et al., 1998). Additionally both mice and rats have proven effective for studying the impact of *P. gingivalis* oral challenge and subsequent development of oral bone loss (Baker et al., 1994; Malek et al., 1994; Gibson and Genco, 2001).

In addition to animal studies of *P. gingivalis* infection's effect on induction of disease, other studies have looked at mechanisms for preventing *P. gingivalis*-mediated periodontal disease. Although conflicting reports exist, it is established that patients with generalized aggressive periodontitis develop specific antibody to *P. gingivalis*; however, this antibody does not appear to provide protection. By contrast, several studies have demonstrated that immunization of rodents and primates with either *P. gingivalis* whole organism preparations or specific antigens purified from this organism protects against periodontitis (Evans et al., 1992b; Moritz et al., 1998; Gibson and Genco, 2001; Rajapakse et al., 2002). Additionally, strategies specifically designed to assess control of the innate immune response as a way to block *P. gingivalis* from causing periodontal disease are yielding interesting data. Recently, the importance of the proinflammatory cytokines TNF-α and IL-1β in the bone loss process has been demonstrated by the observation that nonhuman primates given TNF-α and IL-1β receptor agonists are protected from accelerated inflammatory cell response to *P. gingivalis* oral challenge using a ligature based model. Novel vaccine candidates for use in the prevention of periodontal disease will undoubtedly be found.

Emerging epidemiological data support that periodontal disease represents an important risk factor for cardiovascular disease and pre-term

child delivery (Beck et al., 2001; Dasanayake et al., 2001; Genco et al., 2002). Moreover, periodontal disease is also more prevalent in diabetics (Zachariasen, 1991). Owing to the clinical connection between systemic disease and periodontal disease, animal models to study the impact of *P. gingivalis* infection on cardiovascular disease, diabetes, and pre-term birth will continue to be useful (Genco et al., 1998).

Conclusions and Future Directions

It is apparent that we have entered an exciting era of research as it relates to the interactions of *P. gingivalis* and the host. The complete repertoire of genes in *P. gingivalis* is available as a result of the DNA sequencing of the genome of this organism. Knowledge of the entire genomic sequence will allow us to identify additional virulence genes and to determine the components of the system(s) that regulate them. As seen with other microorganisms, the mechanisms of *P. gingivalis* virulence gene regulation in response to environmental cues may be extremely complex. Understanding the mechanism of virulence gene regulation in response to the local environment of the host will provide crucial information in the development of effective treatments targeted at eradication of these periodontal disease pathogens. The recent studies, which document that periodontal infection may represent a risk factor for other systemic conditions (Beck and Offenbacher, 1998a; Offenbacher et al., 1998), further support efforts into understanding the unique interactions between *P. gingivalis* and the response to environmental cues of the host. A number of different animal models have been used to evaluate the complex interactions between *P. gingivalis* and the host and have been an important research tool to study the pathogenesis of *P. gingivalis*-mediated periodontal diseases. Numerous investigations have elucidated important aspects of the complex interactions of *P. gingivalis* with the intact host through the use of various animal models. These animal models have enabled investigators to more clearly define the role of specific *P. gingivalis* factors in virulence and periodontal disease pathogenesis. Likewise, we have learned a great deal about the role of the host response in disease pathology; however, it is clear that there is much more to be gained from further studies in animal models. A concentrated effort is required to more completely characterize the pathogenesis of periodontal breakdown in rodent and primate models, since these models seem to hold the greatest promise in pathogenesis studies. Without baseline information, development and testing of potential therapies designed to disrupt the pathogenesis of disease will be significantly delayed. A concerted effort is also required to evaluate the role of periodontal infections on systemic diseases, including coronary artery disease, diabetes, adverse pregnancy outcomes, and other infections, such as endocarditis, respiratory infections, and HIV infection in relevant animal models.

Literature Cited

Abe, N., T. Kadowaki, K. Okamoto, K. Nakayama, M. Ohishi, and K. Yamamoto. 1998. Biochemical and functional properties of lysine-specific cysteine proteinase (Lys-gingipain) as a virulence factor of Porphyromonas gingivalis in periodontal disease. J. Biochem. (Tokyo) 123(2):305–312.

Aduse-Opoku, J., J. M. Slaney, M. Rangarajan, J. Muir, K. A. Young, and M. A. Curtis. 1997. The Tla protein of Porphyromonas gingivalis W50: A homolog of the RI protease precursor (PrpRI) is an outer membrane receptor required for growth on low levels of hemin. J. Bacteriol. 179(15):4778–4788.

Agarwal, S., N. P. Piesco, L. P. Johns, and A. E. Riccelli. 1995. Differential expression of IL-1 beta, TNF-alpha, IL-6, and IL-8 in human monocytes in response to lipopolysaccharides from different microbes. J. Dent. Res. 74(4):1057–1065.

Ajioka, J., and D. L. Hartl. 1989. Population dynamics of transposable elements. *In:* D. E. Berg and M. M. Howe. Mobile DNA. ASM Press. Washington, DC. 939–958.

Amano, A., A. Sharma, H. T. Sojar, H. K. Kuramitsu, and R. J. Genco. 1994. Effects of temperature stress on expression of fimbriae and superoxide dismutase by Porphyromonas gingivalis. Infect. Immun. 62(10):4682–4685.

Armitage, G. C. 1999. Development of a classification system for periodontal disease and conditions. Ann. Periodontol. 4(1):1–6.

Baker, P. J., R. T. Evans, and D. C. Roopenian. 1994. Oral infection with Porphyromonas gingivalis and induced alveolar bone loss in immunocompetent and severe combined immunodeficient mice. Arch. Oral Biol. 39(12):1035–1040.

Baker, P. J., M. Dixon, R. T. Evans, L. Dufour, E. Johnson, and D. C. Roopenian. 1999. CD4(+) T cells and the proinflammatory cytokines gamma interferon and interleukin-6 contribute to alveolar bone loss in mice. Infect. Immun. 67(6):2804–2809.

Baker, P. J., J. Garneau, L. Howe, and D. C. Roopenian. 2001. T-cell contributions to alveolar bone loss in response to oral infection with Porphyromonas gingivalis. Acta Odontol. Scand. 59(4):222–225.

Barkocy-Gallagher, G. A., N. Han, J. M. Patti, J. Whitlock, A. Progulske-Fox, and M. S. Lantz. 1996. Analysis of the prtP gene encoding porphypain, a cysteine proteinase of Porphyromonas gingivalis. J. Bacteriol. 178(10):2734–2741.

Bartold, P. M., and D. R. Haynes. 1991. Interleukin-6 production by human gingival fibroblasts. J. Periodont. Res. 26(4):339–345.

Beck, J. D., and S. Offenbacher. 1998a. Oral health and systemic disease: Periodontitis and cardiovascular disease. J. Dent. Educ. 62(10):859–870.

Beck, J. D., S. Offenbacher, R. Williams, P. Gibbs, and R. Garcia. 1998b. Periodontitis: A risk factor for coronary heart disease? Ann. Periodontol. 3(1):127–141.

Beck, J. D., J. R. Elter, G. Heiss, D. Couper, S. M. Mauriello, and S. Offenbacher. 2001. Relationship of periodontal disease to carotid artery intima-media wall thickness: The atherosclerosis risk in communities (ARIC) study. Arterioscler. Thromb. Vasc. Biol. 21(11):1816–1822.

Bickel, M., and G. Cimasoni. 1985. The pH of human crevicular fluid measured by a new microanalytical technique. J. Periodont. Res. 20(1):35–40.

Bramanti, T. E., G. G. Wong, S. T. Weintraub, and S. C. Holt. 1989. Chemical characterization and biologic properties of lipopolysaccharide from Bacteroides gingivalis strains W50, W83, and ATCC 33277. Oral Microbiol. Immunol. 4(4):183–192.

Bramanti, T. E., and S. C. Holt. 1993. Hemin uptake in Porphyromonas gingivalis: Omp26 is a hemin-binding surface protein. J. Bacteriol. 175(22):7413–7420.

Brook, I., and R. I. Walker. 1983. Infectivity of organisms recovered from polymicrobial abscesses. Infect. Immun. 42(3):986–989.

Brook, I. 1994. The role of encapsulated anaerobic bacteria in synergistic infections. FEMS Microbiol. Rev. 13(1):65–74.

Califano, J. V., R. E. Schifferle, J. C. Gunsolley, A. M. Best, H. A. Schenkein, and J. G. Tew. 1999. Antibody reactive with Porphyromonas gingivalis serotypes K1-6 in adult and generalized early-onset periodontitis. J. Periodontol. 70(7):730–735.

Carman, R. J., M. D. Ramakrishnan, and F. H. Harper. 1990. Hemin levels in culture medium of Porphyromonas (Bacteroides) gingivalis regulate both hemin binding and trypsinlike protease production. Infect. Immun. 58(12):4016–4019.

Chen, P. B., L. B. Davern, R. Schifferle, and J. J. Zambon. 1990. Protective immunization against experimental Bacteroides (Porphyromonas) gingivalis infection. Infect. Immun. 58(10):3394–3400.

Choi, J. I., R. E. Schifferle, F. Yoshimura, and B. W. Kim. 1998. Capsular polysaccharide-fimbrial protein conjugate vaccine protects against Porphyromonas gingivalis infection in SCID mice reconstituted with human peripheral blood lymphocytes. Infect. Immun. 66(1):391–393.

Choi, J., S. W. Chung, and S. J. Kim. 2001. Establishment of Porphyromonas gingivalis-specific T-cell lines from atherosclerosis patients. Oral Microbiol. Immunol. 16(5):316–318.

Cimasoni, G. 1983. Crevicular fluid updated. Monogr. Oral Sci. 12(III–VII):1–152.

Collins, J. G., H. W. Windley 3rd, R. R. Arnold, and S. Offenbacher. 1994. Effects of a Porphyromonas gingivalis infection on inflammatory mediator response and pregnancy outcome in hamsters. Infect. Immun. 62(10):4356–4361.

Collins, M. D., D. N. Love, J. Karjalainen, A. Kanervo, B. Forsblom, A. Willems, S. Stubbs, E. Sarkiala, G. D. Bailey, D. I. Wigney et al. 1994b. Phylogenetic analysis of members of the genus Porphyromonas and description of Porphyromonas cangingivalis sp. nov. and Porphyromonas cansulci sp. nov. Int. J. Syst. Bacteriol. 44(4):674–679.

Cunningham, M. D., C. Seachord, K. Ratcliffe, B. Bainbridge, A. Aruffo, and R. P. Darveau. 1996. Helicobacter pylori and Porphyromonas gingivalis lipopolysaccharides are poorly transferred to recombinant soluble CD14. Infect. Immun. 64(9):3601–3608.

Cutler, C. W., R. R. Arnold, and H. A. Schenkein. 1993. Inhibition of C3 and IgG proteolysis enhances phagocytosis of Porphyromonas gingivalis. J. Immunol. 151(12):7016–7029.

Cutler, C. W., J. R. Kalmar, and C. A. Genco. 1995. Pathogenic strategies of the oral anaerobe, Porphyromonas gingivalis. Trends Microbiol 3(2):45–51.

Cutler, C. W., P. I. Eke, C. A. Genco, T. E. Van Dyke, and R. R. Arnold. 1996. Hemin-induced modifications of the antigenicity and hemin-binding capacity of Porphyromonas gingivalis lipopolysaccharide. Infect. Immun. 64(6):2282–2287.

Cutler, C. W., R. Jotwani, K. A. Palucka, J. Davoust, D. Bell, and J. Banchereau. 1999. Evidence and a novel hypothesis for the role of dendritic cells and Porphyromonas gingivalis in adult periodontitis. J. Periodont. Res. 34(7):406–412.

Darveau, R. P., M. D. Cunningham, T. Bailey, C. Seachord, K. Ratcliffe, B. Bainbridge, M. Dietsch, R. C. Page, and A. Aruffo. 1995. Ability of bacteria associated with chronic inflammatory disease to stimulate E-selectin expression and promote neutrophil adhesion. Infect. Immun. 63(4):1311–1317.

Darveau, R. P., C. M. Belton, R. A. Reife, and R. J. Lamont. 1998. Local chemokine paralysis, a novel pathogenic mechanism for Porphyromonas gingivalis. Infect. Immun. 66(4):1660–1665.

Dasanayake, A. P., D. Boyd, P. N. Madianos, S. Offenbacher, and E. Hills. 2001. The association between Porphyromonas gingivalis-specific maternal serum IgG and low birth weight. J. Periodontol. 72(11):1491–1497.

Dashper, S. G., A. Hendtlass, N. Slakeski, C. Jackson, K. J. Cross, L. Brownfield, R. Hamilton, I. Barr, and E. C. Reynolds. 2000. Characterization of a novel outer membrane hemin-binding protein of Porphyromonas gingivalis. J. Bacteriol. 182(22):6456–6462.

DeCarlo, A. A., M. Paramaesvaran, P. L. Yun, C. Collyer, and N. Hunter. 1999. Porphyrin-mediated binding to hemoglobin by the HA2 domain of cysteine proteinases (gingipains) and hemagglutinins from the periodontal pathogen Porphyromonas gingivalis. J. Bacteriol. 181(12):3784–3791.

Deshpande, R. G., M. B. Khan, and C. A. Genco. 1998a. Invasion of aortic and heart endothelial cells by Porphyromonas gingivalis. Infect. Immun. 66(11):5337–5343.

Deshpande, R. G., M. Khan, and C. A. Genco. 1998b. Invasion strategies of the oral pathogen porphyromonas gingivalis: Implications for cardiovascular disease. Invasion Metastasis 18(2):57–69.

Dickinson, D. P., M. A. Kubiniec, F. Yoshimura, and R. J. Genco. 1988. Molecular cloning and sequencing of the gene encoding the fimbrial subunit protein of Bacteroides gingivalis. J. Bacteriol. 170(4):1658–1665.

Dorn, B. R., W. A. Dunn Jr., and A. Progulske-Fox. 1999. Invasion of human coronary artery cells by periodontal pathogens. Infect. Immun. 67(11):5792–5798.

Eichinger, A., Beisel, H.G., Jacob, U., Huber, R. Medrano, F.J., Banbula, A., Potempa, J., Travis, J., and Bode, W. 1999. Crystal structure of gingipain R: an Arg-specific bacterial cysteine proteinase with a caspase-like fold. EMBO J. 18:5453–5462.

Epstein, J. B., and P. Stevenson-Moore. 2001. Periodontal disease and periodontal management in patients with cancer. Oral Oncol. 37(8):613–619.

Evans, R. T., B. Klausen, and R. J. Genco. 1992a. Immunization with fimbrial protein and peptide protects against Porphyromonas gingivalis-induced periodontal tissue destruction. Adv. Exp. Med. Biol. 327:255–262.

Evans, R. T., B. Klausen, H. T. Sojar, G. S. Bedi, C. Sfintescu, N. S. Ramamurthy, L. M. Golub, and R. J. Genco. 1992b. Immunization with Porphyromonas (Bacteroides) gingivalis fimbriae protects against periodontal destruction. Infect. Immun. 60(7):2926–2935.

Farquharson, S. I., G. R. Germaine, and G. R. Gray. 2000. Isolation and characterization of the cell-surface polysaccharides of Porphyromonas gingivalis ATCC 53978. Oral Microbiol. Immunol. 15(3):151–157.

Fedi Jr., P. F., and W. J. Killoy. 1992. Temperature differences at periodontal sites in health and disease. J. Periodontol. 63(1):24–27.

Fletcher, H. M., R. M. Morgan, and F. L. Macrina. 1997. Nucleotide sequence of the Porphyromonas gingivalis W83 recA homolog and construction of a recA-deficient mutant. Infect. Immun. 65(11):4592–4597.

Forng, R. Y., C. Champagne, W. Simpson, and C. A. Genco. 2000. Environmental cues and gene expression in Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans. Oral Dis. 6(6):351–365.

Fournier, D., C. Mouton, P. Lapierre, T. Kato, K. Okuda, and C. Menard. 2001. Porphyromonas gulae sp. nov., an anaerobic, gram-negative coccobacillus from the gingival sulcus of various animal hosts. Int. J. Syst. Evol. Microbiol. 51(Pt. 3):1179–1189.

Fujiwara, T., T. Ogawa, S. Sobue, and S. Hamada. 1990. Chemical, immunobiological and antigenic characterizations of lipopolysaccharides from Bacteroides gingivalis strains. J. Gen. Microbiol. 136 (Pt. 2):319–326.

Fujiwara, T., S. Morishima, I. Takahashi, and S. Hamada. 1993. Molecular cloning and sequencing of the fimbrilin gene of Porphyromonas gingivalis strains and characterization of recombinant proteins. Biochem. Biophys. Res. Commun. 197(1):241–247.

Galas, D., and M. Chandler. 1989. Bacterial insertion sequences. *In:* D. E. Berg and M. M. Howe. Mobile DNA. ASM Press. Washington, DC. 109–162.

Gamonal, J., A. Acevedo, A. Bascones, O. Jorge, and A. Silva. 2000. Levels of interleukin-1 beta, -8, and -10 and RANTES in gingival crevicular fluid and cell populations in adult periodontitis patients and the effect of periodontal treatment. J. Periodontol. 71(10):1535–1545.

Gemmell, E., and G. J. Seymour. 1998. Cytokine profiles of cells extracted from humans with periodontal diseases. J. Dent. Res. 77(1):16–26.

Gemmell, E., D. A. Grieco, M. P. Cullinan, B. Westerman, and G. J. Seymour. 1999. The proportion of interleukin-4, interferon-gamma and interleukin-10-positive cells in Porphyromonas gingivalis--specific T-cell lines established from P. gingivalis-positive subjects. Oral Microbiol. Immunol. 14(5):267–274.

Genco, C. A. 1995a. Regulation of hemin and iron transport in Porphyromonas gingivalis. Adv. Dent. Res. 9(1):41–47.

Genco, C. A., R. E. Schifferle, T. Njoroge, R. Y. Forng, and C. W. Cutler. 1995b. Resistance of a Tn4351-generated polysaccharide mutant of Porphyromonas gingivalis to polymorphonuclear leukocyte killing. Infect. Immun. 63(2):393–401.

Genco, C. A., T. Van Dyke, and S. Amar. 1998. Animal models for Porphyromonas gingivalis-mediated periodontal disease. Trends Microbiol 6(11):444–449.

Genco, C. A., J. Potempa, J. Mikolajczyk-Pawlinska, and J. Travis. 1999. Role of gingipains R in the pathogenesis of Porphyromonas gingivalis-mediated periodontal disease. Clin. Infect. Dis. 28(3):456–465.

Genco, C. A., and D. W. Dixon. 2001. Emerging strategies in microbial haem capture. Molec. Microbiol. 39(1):1–11.

Genco, R., S. Offenbacher, and J. Beck. 2002. Periodontal disease and cardiovascular disease: Epidemiology and possible mechanisms. J. Am. Dent. Assoc. 133(Suppl.): 14S–22S.

Gibson 3rd, F. C., and C. A. Genco. 2001. Prevention of Porphyromonas gingivalis-induced oral bone loss following immunization with gingipain R1. Infect. Immun. 69(12):7959–7963.

Gonzalez, D., A. O. Tzianabos, C. A. Genco, and F. C. Gibson. 2003. Immunization with Porphyromonas gingivalis capsular polysaccharide prevents P. gingivalis-elicited oral bone loss in a murine model. Infect. Immun. 71:2283–2287.

Greenspan, D., and J. S. Greenspan. 1996. HIV-related oral disease. Lancet 348(9029):729–733.

Grenier, D. 1991. Hemin-binding property of Porphyromonas gingivalis outer membranes. FEMS Microbiol. Lett. 61(1):45–49.

Haber, J., J. Wattles, M. Crowley, R. Mandell, K. Joshipura, and R. L. Kent. 1993. Evidence for cigarette smoking as a major risk factor for periodontitis. J. Periodontol. 64(1):16–23.

Hamada, S., T. Fujiwara, S. Morishima, I. Takahashi, I. Nakagawa, S. Kimura, and T. Ogawa. 1994. Molecular and immunological characterization of the fimbriae of Porphyromonas gingivalis. Microbiol. Immunol. 38(12): 921–930.

Hamada, N., H. T. Sojar, M. I. Cho, and R. J. Genco. 1996. Isolation and characterization of a minor fimbria from Porphyromonas gingivalis. Infect. Immun. 64(11):4788–4794.

Hamada, N., K. Watanabe, M. Arai, H. Hiramine, and T. Umemoto. 2002. Cytokine production induced by a 67-kDa fimbrial protein from Porphyromonas gingivalis. Oral Microbiol. Immunol. 17(3):197–200.

Han, N., J. Whitlock, and A. Progulske-Fox. 1996. The hemagglutinin gene A (hagA) of Porphyromonas gingivalis 381 contains four large, contiguous, direct repeats. Infect. Immun. 64(10):4000–4007.

Han, N., G. Lepine, J. Whitlock, L. Wojciechowski, and A. Progulske-Fox. 1998. The porphyromonas gingivalis prtP/kgp homologue exists as two open reading frames in strain 381. Oral Dis. 4(3):170–179.

Hanazawa, S., K. Nakada, Y. Ohmori, T. Miyoshi, S. Amano, and S. Kitano. 1985. Functional role of interleukin 1 in periodontal disease: Induction of interleukin 1 production by Bacteroides gingivalis lipopolysaccharide in peritoneal macrophages from C3H/HeN and C3H/HeJ mice. Infect. Immun. 50(1):262–270.

Hanazawa, S., Y. Murakami, K. Hirose, S. Amano, Y. Ohmori, H. Higuchi, and S. Kitano. 1991. Bacteroides (Porphyromonas) gingivalis fimbriae activate mouse peritoneal macrophages and induce gene expression and production of interleukin-1. Infect. Immun. 59(6):1972–1977.

Hanazawa, S., Y. Murakami, A. Takeshita, H. Kitami, K. Ohta, S. Amano, and S. Kitano. 1992. Porphyromonas gingivalis fimbriae induce expression of the neutrophil chemotactic factor KC gene of mouse peritoneal macrophages: Role of protein kinase C. Infect. Immun. 60(4):1544–1549.

Handley, P. S., and L. S. Tipler. 1986. An electron microscope survey of the surface structures and hydrophobicity of oral and non-oral species of the bacterial genus Bacteroides. Arch. Oral Biol. 31(5):325–335.

Hayashi, J., K. Nishikawa, R. Hirano, T. Noguchi, and F. Yoshimura. 2000. Identification of a two-component signal transduction system involved in fimbriation of Porphyromonas gingivalis. Microbiol. Immunol. 44(4):279–282.

Heumann, D., and T. Roger. 2002. Initial responses to endotoxins and Gram-negative bacteria. Clin. Chim. Acta 323(1–2):59–72.

Hirasawa, M., and K. Takada. 1994. Porphyromonas gingivicanis sp. nov. and Porphyromonas crevioricanis sp. nov., isolated from beagles. Int. J. Syst. Bacteriol. 44(4):637–640.

Hirose, K. 1990. Stimulatory effects of Bacteroides gingivalis fimbriae on production of fibroblasts-derived thymocyte-activating factor (FTAF) by human gingival fibroblasts [in Japanese]. Meikai Daigaku Shigaku Zasshi 19(1):127–136.

Holt, S. C., J. Ebersole, J. Felton, M. Brunsvold, and K. S. Kornman. 1988. Implantation of Bacteroides gingivalis in nonhuman primates initiates progression of periodontitis. Science 239(4835):55–57.

Holt, S. C., L. Kesavalu, S. Walker, and C. A. Genco. 2000. Virulence factors of Porphyromonas gingivalis. Periodontology 20:168–238.

Hunt, D. E., J. V. Jones, and V. R. Dowell Jr.. 1986. Selective medium for the isolation of Bacteroides gingivalis. J. Clin. Microbiol. 23(3):441–445.

Imamura, T., J. Potempa, R. N. Pike, and J. Travis. 1995. Dependence of vascular permeability enhancement on cysteine proteinases in vesicles of Porphyromonas gingivalis. Infect. Immun. 63(5):1999–2003.

Imamura, T., J. Potempa, S. Tanase, and J. Travis. 1997. Activation of blood coagulation factor X by arginine-specific cysteine proteinases (gingipain-Rs) from Porphyromonas gingivalis. J. Biol. Chem. 272(25):16062–16067.

Isogai, E., H. Isogai, S. Takagi, N. Ishii, N. Fujii, K. Kimura, M. Hayashi, and F. Yoshimura. 1994. Fimbria-specific immune response in various inbred mice inoculated with Porphyromonas gingivalis 381. Oral Microbiol. Immunol. 9(2):118–122.

Karunakaran, T., T. Madden, and H. Kuramitsu. 1997. Isolation and characterization of a hemin-regulated gene, hemR, from Porphyromonas gingivalis. J. Bacteriol. 179(6):1898–1908.

Kasper, D. L., M. E. Hayes, B. G. Reinap, F. O. Craft, A. B. Onderdonk, and B. F. Polk. 1977. Isolation and identification of encapsulated strains of Bacteroides fragilis. J. Infect. Dis. 136(1):75–81.

Khlgatian, M., H. Nassar, H. H. Chou, F. C. Gibson 3rd, and C. A. Genco. 2002. Fimbria-dependent activation of cell adhesion molecule expression in Porphyromonas gingivalis-infected endothelial cells. Infect. Immun. 70(1):257–267.

Kinane, D. F., J. Mooney, and J. L. Ebersole. 2000. Humoral immune response to Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in periodontal disease. Periodontology 20:289–340.

Koga, T., T. Nishihara, T. Fujiwara, T. Nisizawa, N. Okahashi, T. Noguchi, and S. Hamada. 1985. Biochemical and immunobiological properties of lipopolysaccharide (LPS) from Bacteroides gingivalis and comparison with LPS from Escherichia coli. Infect. Immun. 47(3):638–647.

Kung, R. T., B. Ochs, and J. M. Goodson. 1990. Temperature as a periodontal diagnostic. J. Clin. Periodontol. 17(8):557–563.

Kusumoto, Y., T. Ogawa, and S. Hamada. 1993. Generation of specific antibody-secreting cells in salivary glands of BALB/c mice following parenteral or oral immunization with Porphyromonas gingivalis fimbriae. Arch. Oral Biol. 38(5):361–367.

Laine, M. L., B. J. Appelmelk, and A. J. van Winkelhoff. 1996. Novel polysaccharide capsular serotypes in Porphyromonas gingivalis. J. Periodont. Res. 31(4):278–284.

Laine, M. L., B. J. Appelmelk, and A. J. van Winkelhoff. 1997. Prevalence and distribution of six capsular serotypes of Porphyromonas gingivalis in periodontitis patients. J. Dent. Res. 76(12):1840–1844.

Laine, M. L., and A. J. van Winkelhoff. 1998. Virulence of six capsular serotypes of Porphyromonas gingivalis in a mouse model. Oral Microbiol. Immunol. 13(5):322–325.

Lee, J. Y., H. T. Sojar, G. S. Bedi, and R. J. Genco. 1991. Porphyromonas (Bacteroides) gingivalis fimbrillin: Size, amino-terminal sequence, and antigenic heterogeneity. Infect. Immun. 59(1):383–389.

Lee, J. Y., H. T. Sojar, G. S. Bedi, and R. J. Genco. 1992. Synthetic peptides analogous to the fimbrillin sequence inhibit adherence of Porphyromonas gingivalis. Infect. Immun. 60(4):1662–1670.

Lee, J. Y., H. T. Sojar, A. Amano, and R. J. Genco. 1995. Purification of major fimbrial proteins of Porphyromonas gingivalis. Protein Expr. Purif. 6(4):496–500.

Lepine, G., and A. Progulske-Fox. 1996a. Duplication and differential expression of hemagglutinin genes in Porphyromonas gingivalis. Oral Microbiol. Immunol. 11(2): 65–78.

Lepine, G., R. P. Ellen, and A. Progulske-Fox. 1996b. Construction and preliminary characterization of three hemagglutinin mutants of Porphyromonas gingivalis. Infect. Immun. 64(4):1467–1472.

Lewis, J. P., J. A. Dawson, J. C. Hannis, D. Muddiman, and F. L. Macrina. 1999. Hemoglobinase activity of the lysine gingipain protease (Kgp) of Porphyromonas gingivalis W83. J. Bacteriol. 181(16):4905–4913.

Lindberg, A. A. 1999. Polyosides (encapsulated bacteria). C. R. Acad. Sci. III 322(11):925–932.

Lindemann, R. A., J. S. Economou, and H. Rothermel. 1988. Production of interleukin-1 and tumor necrosis factor by human peripheral monocytes activated by periodontal bacteria and extracted lipopolysaccharides. J. Dent. Res. 67(8):1131–1135.

Loos, B. G., and D. W. Dyer. 1992. Restriction fragment length polymorphism analysis of the fimbrillin locus, fimA, of Porphyromonas gingivalis. J. Dent. Res. 71(5): 1173–1181.

Love, D. N., G. D. Bailey, S. Collings, and D. A. Briscoe. 1992. Description of Porphyromonas circumdentaria sp. nov. and reassignment of Bacteroides salivosus (Love, Johnson, Jones, and Calverley 1987) as Porphyromonas (Shah and Collins 1988) salivosa comb. nov. Int. J. Syst. Bacteriol. 42(3):434–438.

Love, D. N., J. Karjalainen, A. Kanervo, B. Forsblom, E. Sarkiala, G. D. Bailey, D. I. Wigney, and H. Jousimies-Somer. 1994. Porphyromonas canoris sp. nov., an asaccharolytic, black-pigmented species from the gingival sulcus of dogs. Int. J. Syst. Bacteriol. 44(2):204–208.

Lu, B., and B. C. McBride. 1994. Stress response of Porphyromonas gingivalis. Oral Microbiol. Immunol. 9(3):166–173.

Lynch, M. C., and H. K. Kuramitsu. 1999. Role of superoxide dismutase activity in the physiology of Porphyromonas gingivalis. Infect. Immun. 67(7):3367–3375.

Magnusson, I., J. Lindhe, T. Yoneyama, and B. Liljenberg. 1984. Recolonization of a subgingival microbiota following scaling in deep pockets. J. Clin. Periodontol. 11(3):193–207.

Malek, R., J. G. Fisher, A. Caleca, M. Stinson, C. J. van Oss, J. Y. Lee, M. I.. Cho, R. J. Genco, R. T. Evans, and D. W. Dyer. 1994. Inactivation of the Porphyromonas gingivalis fimA gene blocks periodontal damage in gnotobiotic rats. J. Bacteriol. 176(4):1052–1059.

Marsh, P. D., A. S. McDermid, A. S. McKee, and A. Baskerville. 1994. The effect of growth rate and haemin on the virulence and proteolytic activity of Porphyromonas gingivalis W50. Microbiology 140 (Pt. 4):861–865.

Matthews, D. C. 2002. The relationship between diabetes and periodontal disease. J. Can. Dent. Assoc. 68(3):161–164.

McDermid, A. S., A. S. McKee, and P. D. Marsh. 1988. Effect of environmental pH on enzyme activity and growth of Bacteroides gingivalis W50. Infect. Immun. 56(5):1096–1100.

McKee, A. S., A. S. McDermid, A. Baskerville, A. B. Dowsett, D. C. Ellwood, and P. D. Marsh. 1986. Effect of hemin on the physiology and virulence of Bacteroides gingivalis W50. Infect. Immun. 52(2):349–355.

Moritz, A. J., D. Cappelli, M. S. Lantz, S. C. Holt, and J. L. Ebersole. 1998. Immunization with Porphyromonas gingivalis cysteine protease: Effects on experimental gingivitis and ligature-induced periodontitis in Macaca fascicularis. J. Periodontol. 69(6):686–697.

Morrison, H. I., L. F. Ellison, and G. W. Taylor. 1999. Periodontal disease and risk of fatal coronary heart and cerebrovascular diseases. J. Cardiovasc. Risk 6(1):7–11.

Mouton, C., P. G. Hammond, J. Slots, and R. J. Genco. 1981. Serum antibodies to oral Bacteroides asaccharolyticus (Bacteroides gingivalis): Relationship to age and periondontal disease. Infect. Immun. 31(1):182–192.

Murakami, Y., S. Hanazawa, K. Nishida, H. Iwasaka, and S. Kitano. 1993. N-acetyl-D-galactosamine inhibits TNF-alpha gene expression induced in mouse peritoneal macrophages by fimbriae of Porphyromonas (Bacteroides) gingivalis, an oral anaerobe. Biochem. Biophys. Res. Commun. 192(2):826–832.

Murakami, Y., S. Hanazawa, A. Watanabe, K. Naganuma, H. Iwasaka, K. Kawakami, and S. Kitano. 1994. Porphyromonas gingivalis fimbriae induce a 68-kilodalton phosphorylated protein in macrophages. Infect. Immun. 62(12):5242–5246.

Nair, B. C., W. R. Mayberry, R. Dziak, P. B. Chen, M. J. Levine, and E. Hausmann. 1983. Biological effects of a purified lipopolysaccharide from Bacteroides gingivalis. J. Periodont. Res. 18(1):40–49.

Nakayama, K., T. Kadowaki, K. Okamoto, and K. Yamamoto. 1995. Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of Porphyromonas gingivalis. Evidence for significant contribution of Arg-gingipain to virulence. J. Biol. Chem. 270(40):23619–23626.

Nassar, H., H. H. Chou, M. Khlgatian, F. C. Gibson 3rd, T. E. Van Dyke, and C. A. Genco. 2002. Role for fimbriae and lysine-specific cysteine proteinase gingipain K in expression of interleukin-8 and monocyte chemoattractant protein in Porphyromonas gingivalis-infected endothelial cells. Infect. Immun. 70(1):268–276.

Njoroge, T., R. J. Genco, H. T. Sojar, N. Hamada, and C. A. Genco. 1997. A role for fimbriae in Porphyromonas gingivalis invasion of oral epithelial cells. Infect. Immun. 65(5):1980–1984.

Offenbacher, S., H. L. Jared, P. G. O'Reilly, S. R. Wells, G. E. Salvi, H. P. Lawrence, S. S. Socransky, and J. D. Beck. 1998. Potential pathogenic mechanisms of periodontitis associated pregnancy complications. Ann. Periodontol. 3(1):233–350.

Ogawa, T., Y. Kusumoto, S. Hamada, J. R. McGhee, and H. Kiyono. 1990. Bacteroides gingivalis-specific serum IgG and IgA subclass antibodies in periodontal diseases. Clin. Exp. Immunol. 82(2):318–325.

Ogawa, T., Y. Kono, M. L. McGhee, J. R. McGhee, J. E. Roberts, S. Hamada, and H. Kiyono. 1991. Porphyromonas gingivalis-specific serum IgG and IgA antibodies originate from immunoglobulin-secreting cells in inflamed gingiva. Clin. Exp. Immunol. 83(2):237–244.

Ogawa, T., Y. Kusumoto, H. Kiyono, J. R. McGhee, and S. Hamada. 1992. Occurrence of antigen-specific B cells following oral or parenteral immunization with Porphyromonas gingivalis fimbriae. Int. Immunol. 4(9):1003–1010.

Ogawa, T. 1994a. Immunobiological properties of chemically defined lipid A from lipopolysaccharide of Porphyromonas (Bacteroides) gingivalis. Eur. J. Biochem. 219(3):737–742.

Ogawa, T., H. Uchida, and K. Amino. 1994b. Immunobiological activities of chemically defined lipid A from lipopolysaccharides of Porphyromonas gingivalis. Microbiology 140 (Pt. 5):1209–1216.

Ogawa, T., H. Ogo, and A. Kinoshita. 1997. Antagonistic effect of synthetic peptides corresponding to the binding regions within fimbrial subunit protein from Porphyromonas gingivalis to human gingival fibroblasts. Vaccine 15(2):230–236.

Okamoto, K., T. Kadowaki, K. Nakayama, and K. Yamamoto. 1996. Cloning and sequencing of the gene encoding a novel lysine-specific cysteine proteinase (Lys-gingipain) in Porphyromonas gingivalis: Structural relationship with the arginine-specific cysteine proteinase (Arg-gingipain). J. Biochem. (Tokyo) 120(2):398–406.

Olczak, T., D. W. Dixon, and C. A. Genco. 2001. Binding specificity of the Porphyromonas gingivalis heme and hemoglobin receptor HmuR, gingipain K, and gingipain R1 for heme, porphyrins, and metalloporphyrins. J. Bacteriol. 183(19):5599–5608.

Olsen, I., H. N. Shah, and S. E. Gharbia. 1999. Taxonomy and biochemical characteristics of Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. Periodontol. 2000 20:14–52.

Onderdonk, A. B., D. L. Kasper, R. L. Cisneros, and J. G. Bartlett. 1977. The capsular polysaccharide of Bacteroides fragilis as a virulence factor: Comparison of the pathogenic potential of encapsulated and unencapsulated strains. J. Infect. Dis. 136(1):82–89.

Paster, B. J., F. E. Dewhirst, I. Olsen, and G. J. Fraser. 1994. Phylogeny of Bacteroides, Prevotella, and Porphyromonas spp. and related bacteria. J. Bacteriol. 176(3):725–732.

Pham, K., D. Feik, B. F. Hammond, T. E. Rams, and E. J. Whitaker. 2002. Aggregation of human platelets by

gingipain-R from Porphyromonas gingivalis cells and membrane vesicles. Platelets 13(1):21–30.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. Science 282(5396):2085–2088.

Potempa, J., N. Pavloff, and J. Travis. 1995a. Porphyromonas gingivalis: A proteinase/gene accounting audit. Trends Microbiol. 3(11):430–434.

Potempa, J., R. Pike, and J. Travis. 1995b. The multiple forms of trypsin-like activity present in various strains of Porphyromonas gingivalis are due to the presence of either Arg-gingipain or Lys-gingipain. Infect. Immun. 63(4):1176–1182.

Potempa, J., A. Banbula, and J. Travis. 2000. Role of bacterial proteinases in matrix destruction and modulation of host responses. Periodontology 24:153–192.

Rajapakse, P. S., N. M. O'Brien-Simpson, N. Slakeski, B. Hoffmann, and E. C. Reynolds. 2002. Immunization with the RgpA-Kgp proteinase-adhesin complexes of Porphyromonas gingivalis protects against periodontal bone loss in the rat periodontitis model. Infect. Immun. 70(5):2480–2486.

Reife, R. A., R. A. Shapiro, B. A. Bamber, K. K. Berry, G. E. Mick, and R. P. Darveau. 1995. Porphyromonas gingivalis lipopolysaccharide is poorly recognized by molecular components of innate host defense in a mouse model of early inflammation. Infect. Immun. 63(12):4686–4694.

Research, Science and Therapy Committee of the American Academy of Periodontology. 2001. Treatment of plaque-induced gingivitis, chronic periodontitis, and other clinical conditions. J. Periodontol. 72(12):1790–1800.

Salvi, G. E., C. E. Brown, K. Fujihashi, H. Kiyono, F. W. Smith, J. D. Beck, and S. Offenbacher. 1998. Inflammatory mediators of the terminal dentition in adult and early onset periodontitis. J. Periodont. Res. 33(4):212–225.

Schifferle, R. E., M. S. Reddy, J. J. Zambon, R. J. Genco, and M. J. Levine. 1989. Characterization of a polysaccharide antigen from Bacteroides gingivalis. J. Immunol. 143(9):3035–3042.

Seifert, H. S., and M. So. 1988. Genetic mechanisms of bacterial antigenic variation. Microbiol. Rev. 52(3):327–336.

Shah, H. N., R. Bonnett, B. Mateen, and R. A. Williams. 1979. The porphyrin pigmentation of subspecies of Bacteroides melaninogenicus. Biochem. J. 180(1):45–50.

Shapira, L., S. Takashiba, S. Amar, and T. E. Van Dyke. 1994. Porphyromonas gingivalis lipopolysaccharide stimulation of human monocytes: Dependence on serum and CD14 receptor. Oral Microbiol. Immunol. 9(2):112–117.

Sharma, A., H. T. Sojar, J. Y. Lee, and R. J. Genco. 1993. Expression of a functional Porphyromonas gingivalis fimbrillin polypeptide in Escherichia coli: Purification, physicochemical and immunochemical characterization, and binding characteristics. Infect. Immun. 61(8):3570–3573.

Simpson, W., C. Y. Wang, J. Mikolajczyk-Pawlinska, J. Potempa, J. Travis, V. C. Bond, and C. A. Genco. 1999. Transposition of the endogenous insertion sequence element IS1126 modulates gingipain expression in Porphyromonas gingivalis. Infect. Immun. 67(10):5012–5020.

Simpson, W., T. Olczak, and C. A. Genco. 2000. Characterization and expression of HmuR, a TonB-dependent hemoglobin receptor of Porphyromonas gingivalis. J. Bacteriol. 182(20):5737–5748.

Sismey-Durrant, H. J., and R. M. Hopps. 1991. Effect of lipopolysaccharide from Porphyromonas gingivalis on prostaglandin E2 and interleukin-1-beta release from rat periosteal and human gingival fibroblasts in vitro. Oral Microbiol. Immunol. 6(6):378–380.

Smalley, J. W., A. J. Birss, A. S. McKee, and P. D. Marsh. 1991. Haemin-restriction influences haemin-binding, haemagglutination and protease activity of cells and extracellular membrane vesicles of Porphyromonas gingivalis W50. FEMS Microbiol. Lett. 69(1):63–67.

Socransky, S. S., and A. D. Haffajee. 1991. Microbial mechanisms in the pathogenesis of destructive periodontal diseases: A critical assessment. J. Periodont. Res. 26(3, Pt. 2):195–212.

Socransky, S. S., and A. D. Haffajee. 1992. The bacterial etiology of destructive periodontal disease: Current concepts. J. Periodontol. 63(4, Suppl):322–331.

Sojar, H. T., J. Y. Lee, G. S. Bedi, M. I. Cho, and R. J. Genco. 1991. Purification, characterization and immunolocalization of fimbrial protein from Porphyromonas (bacteroides) gingivalis. Biochem. Biophys. Res. Commun. 175(2):713–719.

Soskolne, W. A., and A. Klinger. 2001. The relationship between periodontal diseases and diabetes: An overview. Ann. Periodontol. 6(1):91–98.

Sroka, A., M. Sztukowska, J. Potempa, J. Travis, and C. A. Genco. 2001. Degradation of host heme proteins by lysine- and arginine-specific cysteine proteinases (gingipains) of Porphyromonas gingivalis. J. Bacteriol. 183(19):5609–5616.

Takada, H., H. Hirai, T. Fujiwara, T. Koga, T. Ogawa, and S. Hamada. 1990. Bacteroides lipopolysaccharides (LPS) induce anaphylactoid and lethal reactions in LPS-responsive and -nonresponsive mice primed with muramyl dipeptide. J. Infect. Dis. 162(2):428–434.

Takada, H., and S. Kotani. 1992. Structure-function relationships of lipid A. *In:* D. Morrison, and J. Ryan. Bacterial Endotoxic Lipopolysaccharides, Volume 1: Molecular Biochemistry and Cellular Biology. CRC Press. New York, NY. 107–134.

Takahashi, Y., F. Yoshimura, M. Kawanami, and H. Kato. 1992. Detection of fimbrilin gene (fimA) in Porphyromonas (Bacteroides) gingivalis by Southern blot analysis. J. Periodont. Res. 27(6):599–603.

Tamura, M., M. Tokuda, S. Nagaoka, and H. Takada. 1992. Lipopolysaccharides of Bacteroides intermedius (Prevotella intermedia) and Bacteroides (Porphyromonas) gingivalis induce interleukin-8 gene expression in human gingival fibroblast cultures. Infect. Immun. 60(11):4932–4937.

Tanamoto, K., S. Azumi, Y. Haishima, H. Kumada, and T. Umemoto. 1997a. Endotoxic properties of free lipid A from Porphyromonas gingivalis. Microbiology 143 (Pt. 1):63–71.

Tanamoto, K., S. Azumi, Y. Haishima, H. Kumada, and T. Umemoto. 1997b. The lipid A moiety of Porphyromonas gingivalis lipopolysaccharide specifically mediates the activation of C3H/HeJ mice. J. Immunol. 158(9):4430–4436.

Teng, Y. T., G. W. Taylor, F. Scannapieco, D. F. Kinane, M. Curtis, J. D. Beck, and S. Kogon. 2002. Periodontal health and systemic disorders. J. Can. Dent. Assoc. 68(3):188–192.

Thomason, J. M., R. A. Seymour, and J. Ellis. 1994. The periodontal problems and management of the renal transplant patient. Ren. Fail. 16(6):731–745.

Umemoto, T., and N. Hamada. 2003. Characterization of biologically active cell surface components of a periodontal pathogen: The roles of major and minor fimbriae of Porphyromonas gingivalis. J. Periodontol. 74(1):119–122.

Van Winkelhoff, A. J., B. J. Appelmelk, N. Kippuw, and J. de Graaff. 1993. K-antigens in Porphyromonas gingivalis are associated with virulence. Oral Microbiol. Immunol. 8(5):259–265.

Vayssier, C., D. Mayrand, and D. Grenier. 1994. Detection of stress proteins in Porphyromonas gingivalis and other oral bacteria by western immunoblotting analysis. FEMS Microbiol. Lett. 121(3):303–307.

Wang, C. Y., V. C. Bond, and C. A. Genco. 1997. Identification of a second endogenous Porphyromonas gingivalis insertion element. J. Bacteriol. 179(11):3808–3812.

Wang, P. L., M. Shinohara, N. Murakawa, M. Endo, S. Sakata, M. Okamura, and K. Ohura. 1999. Effect of cysteine protease of Porphyromonas gingivalis on adhesion molecules in gingival epithelial cells. Jpn. J. Pharmacol. 80(1):75–79.

Washington, O. R., M. Deslauriers, D. P. Stevens, L. K. Lyford, S. Haque, Y. Yan, and P. M. Flood. 1993. Generation and purification of recombinant fimbrillin from Porphyromonas (Bacteroides) gingivalis 381. Infect. Immun. 61(3):1040–1047.

Weinberg, A., C. M. Belton, Y. Park, and R. J. Lamont. 1997. Role of fimbriae in Porphyromonas gingivalis invasion of gingival epithelial cells. Infect. Immun. 65(1):313–316.

Whitney, C., J. Ant, B. Moncla, B. Johnson, R. C. Page, and D. Engel. 1992. Serum immunoglobulin G antibody to Porphyromonas gingivalis in rapidly progressive periodontitis: Titer, avidity, and subclass distribution. Infect. Immun. 60(6):2194–2200.

Willems, A., and M. D. Collins. 1995. Reclassification of Oribaculum catoniae (Moore and Moore 1994) as Porphyromonas catoniae comb. nov. and emendation of the genus Porphyromonas. Int. J. Syst. Bacteriol. 45(3):578–581.

Xie, H., S. Cai, and R. J. Lamont. 1997. Environmental regulation of fimbrial gene expression in Porphyromonas gingivalis. Infect. Immun. 65(6):2265–2271.

Yamazaki, K., F. Ikarashi, T. Aoyagi, K. Takahashi, T. Nakajima, K. Hara, and G. J. Seymour. 1992. Direct and indirect effects of Porphyromonas gingivalis lipopolysaccharide on interleukin-6 production by human gingival fibroblasts. Oral Microbiol. Immunol. 7(4):218–224.

Yilmaz, O., K. Watanabe, and R. J. Lamont. 2002. Involvement of integrins in fimbriae-mediated binding and invasion by Porphyromonas gingivalis. Cell Microbiol. 4(5):305–314.

Yoshimura, F., K. Takahashi, Y. Nodasaka, and T. Suzuki. 1984. Purification and characterization of a novel type of fimbriae from the oral anaerobe Bacteroides gingivalis. J. Bacteriol. 160(3):949–957.

Yoshimura, F., T. Takasawa, M. Yoneyama, T. Yamaguchi, H. Shiokawa, and T. Suzuki. 1985. Fimbriae from the oral anaerobe Bacteroides gingivalis: Physical, chemical, and immunological properties. J. Bacteriol. 163(2):730–734.

Yoshimura, F., Y. Takahashi, E. Hibi, T. Takasawa, H. Kato, and D. P. Dickinson. 1993. Proteins with molecular masses of 50 and 80 kilodaltons encoded by genes downstream from the fimbrilin gene (fimA) are components associated with fimbriae in the oral anaerobe Porphyromonas gingivalis. Infect. Immun. 61(12):5181–5189.

Zachariasen, R. D. 1991. Diabetes mellitus and periodontal disease. Compendium 12(5):324, 326–8, 330 passim.

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CHAPTER 6.3

An Introduction to the Family Flavobacteriaceae

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Introduction

An overview of the family Flavobacteriaceae (Reichenbach, 1992, emend. Bernardet et al., 1996, Bernardet et al., 2002) is appropriate because its genera have widely diverse ecological niches and physiological characteristics. Even within some genera, individual species may show contrasting characteristics. Indeed, although some genera were grouped long ago on the basis of common habitats and phenotypic traits (Reichenbach, 1989; The Order Cytophagales in second edition), others were subsequently attributed on the basis of molecular studies. However, the characteristics shared by most genera are presented in this introductory chapter so as to avoid repetition in subsequent chapters on member genera. The term "flavobacteria," although devoid of nomenclatural status, will be used in this chapter for convenience to represent all members of the family.

In previous editions of *Bergey's Manual of Systematic Bacteriology* and of *The Prokaryotes*, the organisms now included in the family Flavobacteriaceae were distributed among different and unrelated chapters, i.e., in *Bergey's Manual* in the chapters "Genus *Flavobacterium*" (Holmes et al., 1984a) and "Order I. *Cytophagales*" (Reichenbach, 1989), and in the second edition of *The Prokaryotes* in the chapters The Genera *Flavobacterium*, *Sphingobacterium* and *Weeksella* and The Order Cytophagales; (see the sections Phylogeny and Taxonomy in this Chapter). Conversely, these chapters also dealt with other organisms that were subsequently not included in the family (Bernardet et al., 1996). In spite of the considerable taxonomic modifications that occurred since their publication, these chapters (see also Reichenbach and Dworkin, 1981a; Reichenbach and Dworkin, 1981b) contain a huge amount of useful information that is still mostly valid; they deserve to be consulted by those interested in these organisms.

The very first suggestion of a family Flavobacteriaceae was in a PhD thesis study (Jooste, 1985). The family was then mentioned by Reichenbach (1989) in the first edition of *Bergey's Manual of Systematic Bacteriology*, although not formally described (Holmes, 1997). The family was validated (Reichenbach, 1992), its description published (Bernardet et al., 1996) and subsequently emended following the description of new genera (Bernardet et al., 2002). The family Flavobacteriaceae belongs to the phylum *Cytophaga-Flavobacterium-Bacteroides* (CFB; see the chapter on The Order Cytophagales in second edition), together with several other families and many isolated taxa (Bernardet et al., 2002; see the sections Phylogeny and Taxonomy in this Chapter). With the exception of the family Bacteroidaceae, this phylum falls within the competence of the Subcommittee on the Taxonomy of *Flavobacterium* and *Cytophaga*-like Bacteria of the International Committee on Systematics of Prokaryotes website. This subcommittee has issued minimal standards for the description of new taxa in the family (Bernardet et al., 2002).

The genus *Flavobacterium* (Bergey et al., 1923, emend. Bernardet et al., 1996) is the type genus of the family Flavobacteriaceae that currently also includes *Aequorivita* (Bowman and Nichols, 2002), *Arenibacter* (Ivanova et al., 2001; Nedashkovskaya et al., 2003c), *Bergeyella* (Vandamme et al., 1994a), *Capnocytophaga* (Leadbetter et al., 1979), *Cellulophaga* (Johansen et al., 1999), *Chryseobacterium* (Vandamme et al., 1994a), *Coenonia* (Vandamme et al., 1999), *Croceibacter* (Cho and Giovannoni, 2003), *Empedobacter* (Vandamme et al., 1994a), "*Fucobacter*" (Sakai et al., 2002), *Gelidibacter* (Bowman et al., 1997), *Mesonia* (Nedashkovskaya et al., 2003a), *Muricauda* (Bruns et al., 2001), *Myroides* (Vancanneyt et al., 1996), *Ornithobacterium* (Vandamme et al., 1994b), *Polaribacter* (Gosink et al., 1998), *Psychroflexus* (Bowman et al., 1998), *Psychroserpens* (Bowman et al., 1997), *Riemerella* (Segers et al., 1993), *Salegentibacter* (McCammon and Bowman, 2000), *Tenacibaculum* (Suzuki et al., 2001), *Ulvibacter* (Nedashkovskaya et al., 2003b),

Vitellibacter (Nedashkovskaya et al., 2003d), *Weeksella* (Holmes et al., 1986a) and *Zobellia* (Barbeyron et al., 2001). The family also comprises two generically misclassified organisms, [*Cytophaga*] *latercula* (Lewin, 1969) and [*Cytophaga*] *marinoflava* (Colwell et al., 1966).

In this edition, those genera that contain many species are treated in individual chapters, while those that share some particular physiological or ecological characteristics and those that are clinically significant in humans or animals are grouped together in particular chapters. The chapters are as follows: "The Genus *Flavobacterium*," by J.-F. Bernardet and J. Bowman; "The Genus *Chryseobacterium*," by J.-F. Bernardet, C. Hugo and B. Bruun; "Marine Members of the Family Flavobacteriaceae" (i.e., the genera *Aequorivita*, *Arenibacter*, *Cellulophaga*, *Croceibacter*, "*Fucobacter*," *Gelidibacter*, *Mesonia*, *Muricauda*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter* and *Zobellia*, as well as [*Cytophaga*] *latercula* and [*Cytophaga*] *marinoflava*), by J. Bowman; "Capnophilic Bird Pathogens in the Family Flavobacteriaceae: *Riemerella*, *Ornithobacterium*, and *Coenonia*," by P. Vandamme, M. Hafez, and K.-H. Hinz; "The Genus *Capnocytophaga*," by E. Leadbetter; "The Genera *Myroides* and *Empedobacter*," by C. Hugo and B. Bruun; and "The Genera *Bergeyella* and *Weeksella*," by C. Hugo and B. Bruun.

Phylogeny

The current view of phylogenetic relationships between the family Flavobacteriaceae and other taxa in the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum, and among taxa in the family, mostly results from extensive 16S rRNA/rDNA sequence analyses and DNA-rRNA hybridization experiments performed over nearly twenty years (e.g., Gherna and Woese, 1992; Nakagawa and Yamasato, 1993; Bernardet et al., 1996; Bowman et al., 1998; Suzuki et al., 2001; Nakagawa et al., 2002). Although considerable progress was made since then, the very first phylogenetic tree of this phylum, based on 16S rRNA oligonucleotide catalogs, already clearly distinguished a *Bacteroides* branch from another branch grouping organisms now attributed to the genera *Cellulophaga*, *Empedobacter*, *Flavobacterium* and *Sphingobacterium* (Paster et al., 1985). Procedures to determine the almost complete 16S rRNA sequence, as well as efficient methods of alignment, treeing algorithms, and statistical analyses, are now readily available, which has made it possible to resolve phylogenetic relationships much more accurately and reliably (Bernardet et al., 2002). However, since differences in 16S rRNA sequences of up to 5% have been found among strains of some species included in the family (Clayton et al., 1995; Triyanto and Wakabayashi, 1999), the sequences of additional strains should be obtained and compared. Several publications, including some dealing with members of the family, showed that the use of 16S rRNA sequence analysis to infer phylogenetic relationships should be restricted to the generic and suprageneric levels and that the resolution of the technique is not adapted to the delineation of new species (see Bernardet et al. [2002] and references therein).

Yamamoto and Harayama (1996) have shown that a phylogenetic analysis based on the DNA gyrase B subunit gene (*gyrB*) may have a greater degree of resolution than that based on the 16S rRNA sequence, because protein-encoding genes evolve faster than rRNA genes. When this technique was applied to many representatives of the CFB phylum, the resulting phylogenetic tree was almost equivalent to that resulting from 16S rRNA sequence analysis (Suzuki et al., 2001).

Phylogenetic relationships among prokaryotes were also deduced from signatures in different protein sequences. These signatures support a linear rather than tree-like relationship between the main eubacterial phyla, "suggesting that the major evolutionary changes within *Bacteria* have taken place in a directional manner" (Gupta, 2000). In this study, members of the CFB phylum were shown to belong to a "*Chlamydia-Cytophaga*-green sulfur bacteria group" that constitutes an intermediate evolutionary step between *Spirochetes* and ε- and δ-proteobacteria, a result in accordance with previous 16S rRNA data (Olsen et al., 1994). Hence, although their degree of resolution is different, the various molecules used for phylogenetic analyses do yield concordant data.

The position of the family Flavobacteriaceae in the CFB phylum is shown in Fig. 1. The phylogenetic relationships of the taxa classified in the family are shown in Fig. 2. Invalid taxa belonging to the family for which 16S rRNA sequence is available have been included in Fig. 2 for information; their names are given in quotation marks.

Taxonomy

Taxonomy of the family Flavobacteriaceae was reviewed in detail when minimal standards for

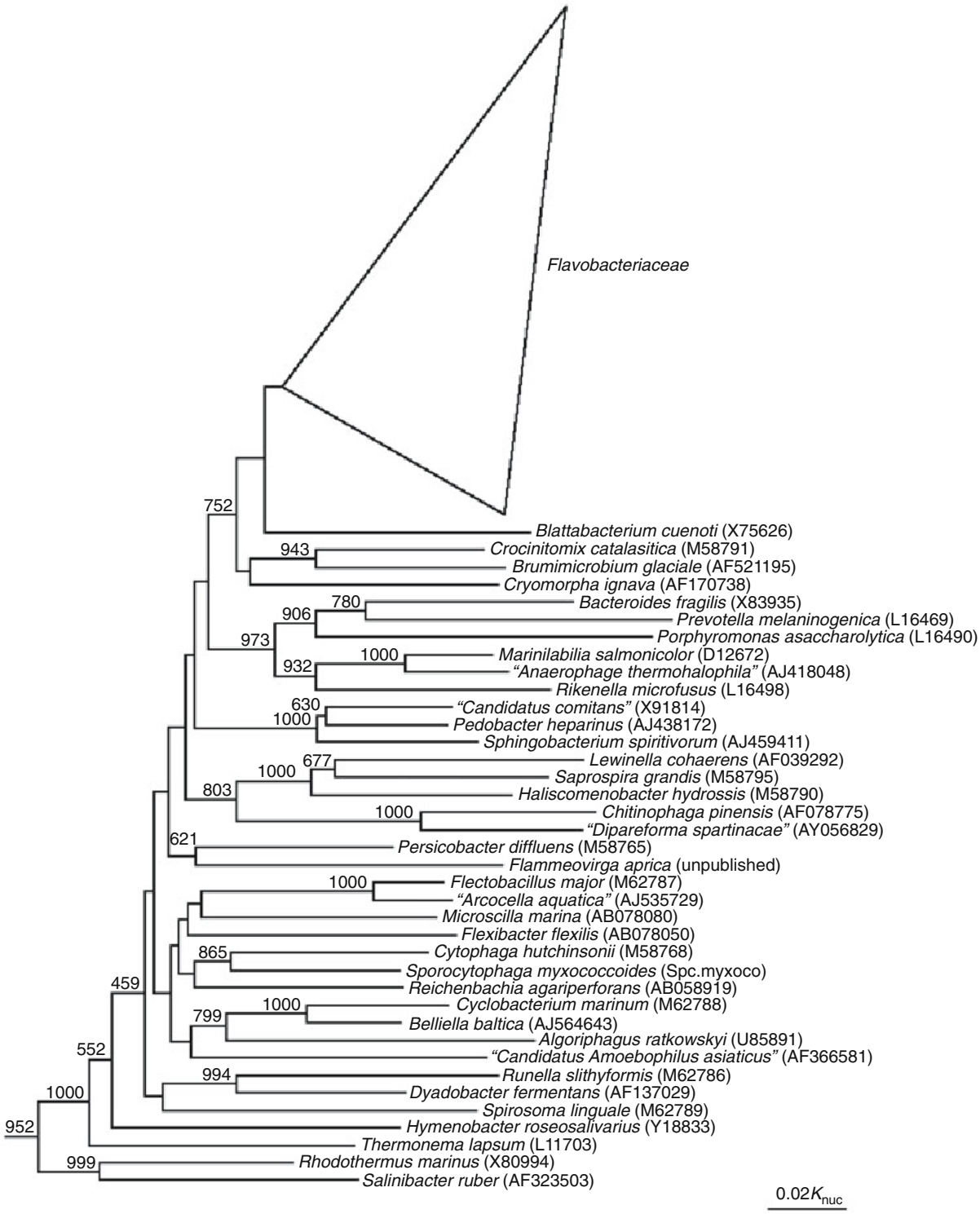

Fig. 1. Phylogenetic position of the family Flavobacteriaceae in the *Cytophaga-Flavobacterium-Bacteroides* phylum based on 16S rRNA sequence comparisons using the neighbor-joining method (Saitou and Nei, 1987). Sequences are taken from the DNA Data Bank of Japan (DDBJ) and GenBank nucleotide databases, apart from the sequence of *Sporocytophaga myxococcoides*, which comes from the Ribosomal Database Project (Maidak et al., 1999). All validly described genera in the phylum are represented by the sequence of the type strain of their type species. Accession numbers for the sequences are given in parentheses. Scale bar, 0.02 K_{nuc} (Kimura, 1980). The numbers on the branches represent the confidence limits estimated by a bootstrap analysis (Felsenstein, 1985) of 1000 replicates; confidence limits less than 50% are not shown. Sequences were aligned using the Clustal W ver. 1.8 software package (Thompson et al., 1994). The alignments were modified manually against the 16S rRNA secondary structure of *Escherichia coli* (Gutell et al., 1985). Positions at which the secondary structures varied in the strains (positions 66–104, 143–220, 447–487, 841–845, 991–1045, 1134–1140, and 1446–1456) and all sites which were not determined in any sequence were excluded from the analysis. *Agrobacterium tumefaciens*, *Bacillus subtilis*, and *Escherichia coli* were used as outgroups.

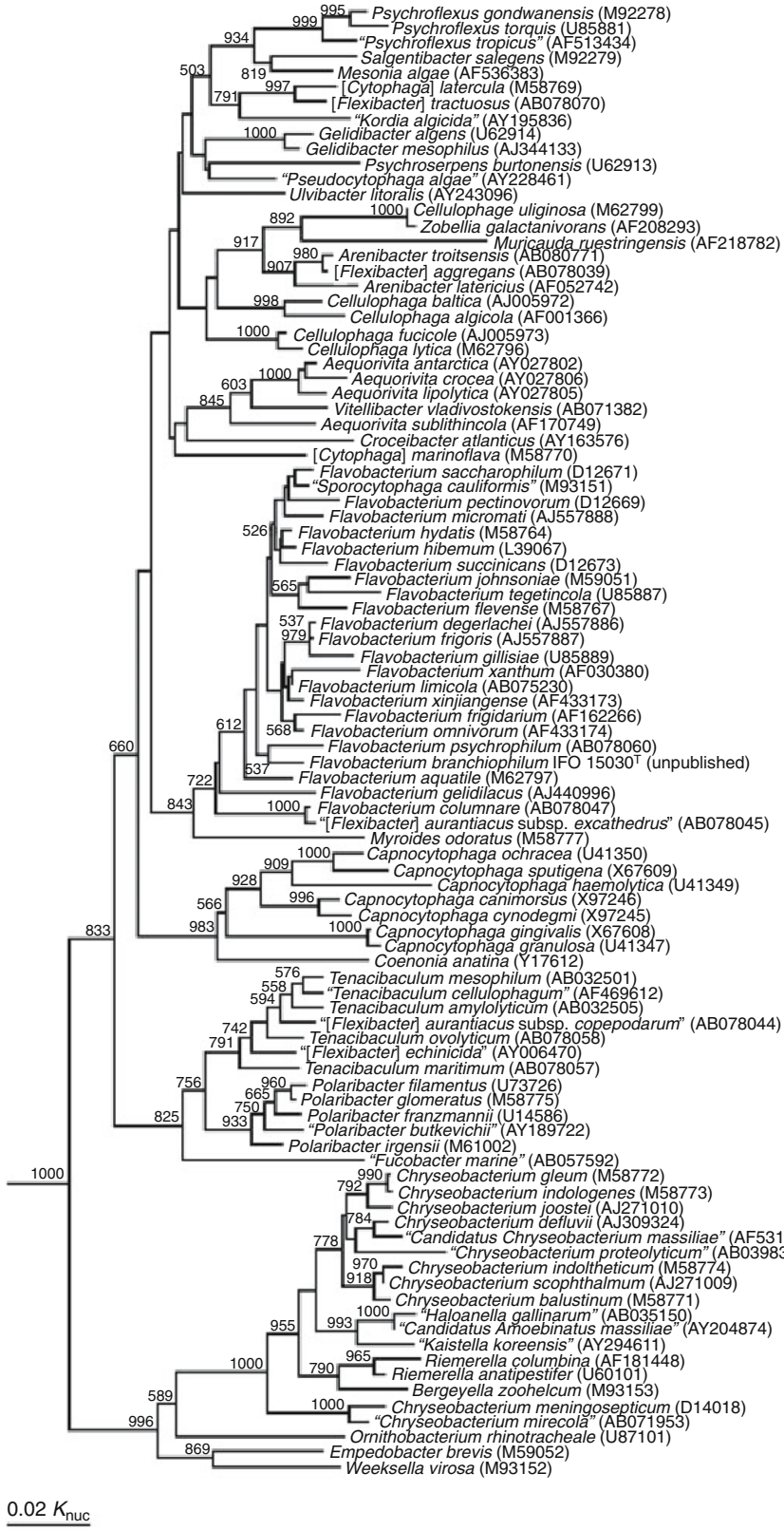

Fig. 2. Phylogenetic relationships among representatives of the family Flavobacteriaceae based on comparisons of 16S rRNA sequences. All species of Flavobacteriaceae are included except *Myroides odoratimimus*, for which no data are available. Invalid taxa for which 16S rRNA sequence is available have also been included for information; their names are in quotation marks. Other details are given in the legend to Fig. 1.

the description of new taxa in the family were proposed (Bernardet et al., 2002). At that time, the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum comprised the families Bacteroidaceae, Cytophagaceae, Flavobacteriaceae, Sphingobacteriaceae and Spirosomaceae, as well as a number of taxa unattributed to any family. A new family Cryomorphaceae was described since then for various polar organisms (Bowman et al., 2003). The taxonomic status of these families is rather different: while the families Bacteroidaceae (Paster et al., 1994), Cryomorphaceae (Bowman et al., 2003), Flavobacteriaceae (Bernardet et al., 1996) and Sphingobacteriaceae (Steyn et al., 1998) were defined on firm phenotypic and genomic grounds, taxa included in the families Cytophagaceae (Reichenbach, 1989; The Order Cytophagales in second edition) and Spirosomaceae (Raj and Maloy, 1990) were actually shown to be only distantly related (see Bernardet et al. [2002] and references therein), making their thorough emendation necessary. Indeed, these two families were deleted from the CFB phylum (renamed "Bacteroidetes") in the "Taxonomic Outlines of the Prokaryotic Genera" for the second edition of *Bergey's Manual of Systematic Bacteriology*, while several new families were proposed. For instance, a family Blattabacteriaceae was also created to accommodate the closest relatives of flavobacteria, the endosymbionts of insects (see the section Habitat in this Chapter).

The definition of higher taxa in the CFB phylum is even more confusing. Taxa currently belonging to the family Flavobacteriaceae were included in the order Cytophagales when it was described (Leadbetter, 1974), as was the family itself when it was created (Reichenbach, 1989). However, the delineation of the order and its connections with the CFB phylum was far from clear as shown by the variable taxa attributed to the order in the successive editions of *Bergey's Manual* and *The Prokaryotes* (Leadbetter, 1974; Reichenbach and Dworkin, 1981b; Reichenbach, 1989; see The Order Cytophagales in second edition). Moreover, the genus *Flavobacterium* was dealt with in separate chapters (Holmes et al., 1984a; see The Genera *Flavobacterium*, *Sphingobacterium* and *Weeksella* in the second edition) and its relationships with members of the Cytophagales were questioned (Reichenbach, 1989). Phylogenetic studies considerably improved the situation at the genus and family levels, but the structure and limits of the order remained unclear (Nakagawa and Yamasato, 1993; Nakagawa and Yamasato, 1996; Bernardet et al., 1996; Bernardet et al., 2002). The order Cytophagales was maintained (although its composition was not specified) as the type order of a new class Flavobacteria in the "bacterial megaclassification" proposed by Cavalier-Smith (2002), whereas the order disappeared from the above-mentioned "Taxonomic Outlines of the Prokaryotic Genera" in which three new orders (i.e., Bacteroidales, Flavobacteriales and Sphingobacteriales) were proposed, each being the sole order of three corresponding new classes (i.e., Bacteroidetes, Flavobacteria and Sphingobacteria). Interestingly, the families included in the class Flavobacteria (proposed in these two studies) are quite different: for instance, the family Bacteroidaceae may be included in the class (Cavalier-Smith, 2002) or not. These differing views demonstrate that much additional data are still necessary to homogenize the delineation and naming of higher taxa, and that investigators experienced in the taxonomy of this group should be consulted. Taxa even higher than classes were also proposed: the three classes defined for the *Bergey's Manual* comprise the phylum "*Bacteroides*", while the megaclassification includes the classes Flavobacteria and Chlorobea in a division *Sphingobacteria* (Cavalier-Smith, 2002). Although the relationships of the CFB phylum with the genus *Chlorobium* and other green sulfur bacteria were indeed demonstrated by 16S rRNA (e.g., Olsen et al., 1994; Paster et al., 1994) and protein (Gupta, 2000) data, the definition of the division based on the presence of sphingolipids is questionable, since only a few taxa included in the division contain sphingolipids in their cytoplasmic membrane (e.g., *Bacteroides*, *Prevotella*, *Porphyromonas*, *Sphingobacterium* and *Flectobacillus*) and since sphingolipids also exist in bacterial taxa that do not belong to the division and even in fungi (Olsen and Jantzen, 2001).

Table 1 (updated from Bernardet et al., 2002) lists currently recognized genera in the family Flavobacteriaceae as well as their type species, the different species, and their type strain, the source and G+C content of the type strains, the most relevant references (which are not necessarily the original descriptions), the list of collections in which the type strains were first deposited, and the names previously attributed to some species. Invalid taxa and the two generically misclassified species [*Cytophaga*] *latercula* and [*Cytophaga*] *marinoflava* are included for information. The current taxonomic status and standing in nomenclature of flavobacteria may be found at the List of Bacterial Names with Standing in Nomenclature (Euzéby, 1997). Taxonomy of each genus in the family

Table 1. Currently recognized genera and species classified in the family Flavobacteriaceae.[a]

Genera and species[b]	Type strain	G + C (mol%)	Source	Reference(s)
Genus *Aequorivita*				Bowman and Nichols, 2002
Aequorivita antarctica[T]	ACAM 640	39	Under-ice sea water, Prydz Bay, Antarctica	Bowman and Nichols, 2002
Aequorivita crocea	ACAM 642	34	Sea water, Mertz Polynya, Antarctica	Bowman and Nichols, 2002
Aequorivita lipolytica	ACAM 641	36	Sea water, Mertz Polynya, Antarctica	Bowman and Nichols, 2002
Aequorivita sublithincola	ACAM 643	37	Sublithic cyanobacterial community, Vestfold Hills, Antarctica	Bowman and Nichols, 2002
Genus *Arenibacter*				Ivanova et al., 2001
Arenibacter latericius[T]	KMM 426	38	Marine sediment, Indian Ocean, 1988	Ivanova et al., 2001
Arenibacter troitsensis	KMM 3674	40	Marine sediment, Sea of Japan, 2000	Nedashkovskaya et al., 2003c
Genus *Bergeyella*				Vandamme et al., 1994a
Bergeyella zoohelcum[T,c]	NCTC 11660	35	Human sputum, United States	Holmes et al., 1986b
Genus *Capnocytophaga*				Leadbetter et al., 1979; Holt and Kinder, 1989
Capnocytophaga canimorsus	ATCC 35979	37	Human blood after dog bite, United States	Brenner et al., 1989; Vandamme et al., 1996
Capnocytophaga cynodegmi	ATCC 49044	36	Dog mouth, United States, 1979	Brenner et al., 1989; Vandamme et al., 1996
Capnocytophaga gingivalis[AL]	ATCC 33624	40	Periodontitis in human, United States, 1978	Leadbetter et al., 1979; Vandamme et al., 1996
Capnocytophaga granulosa	JCM 8566	42	Human dental plaque, Japan	Yamamoto et al., 1994; Vandamme et al., 1996
Capnocytophaga haemolytica	JCM 8565	44	Human dental plaque, Japan	Yamamoto et al., 1994; Vandamme et al., 1996
Capnocytophaga ochracea[T,AL]	ATCC 27872	39	Human oral cavity	Leadbetter et al., 1979; Vandamme et al., 1996
Capnocytophaga sputigena[AL]	ATCC 33612	38	Periodontitis in human, United States, 1978	Leadbetter et al., 1979; Vandamme et al., 1996
Genus *Cellulophaga*				Johansen et al., 1999
Cellulophaga algicola	ACAM 630	37	Surface of marine alga, Antarctica	Bowman, 2000
Cellulophaga baltica	LMG 18535	33	Surface of marine alga, Svaneke, Denmark	Johansen et al., 1999
Cellulophaga fuciola	LMG 18536	32	Surface of marine alga, Hirsholm, Denmark	Johansen et al., 1999
Cellulophaga lytica[T,AL,d]	ATCC 23178	33	Beach mud, Limon, Costa Rica	Lewin, 1969; Reichenbach, 1989; Johansen et al., 1999
Cellulophaga uliginosa[e]	ATCC 14397	42	Marine sediment	ZoBell and Upham, 1944; Reichenbach, 1989; Bowman, 2000
Genus *Chryseobacterium*				Holmes et al., 1984a; Vandamme et al., 1944a
Chryseobacterium balustinum[AL,f]	NCTC 11212	33	Blood of freshwater fish, France, 1959	Holmes et al., 1984a
Chryseobacterium defluvii	DSM 14219	?	Activated fludge, Germany	Kämpfer et al., 2003
Chryseobacterium gleum[T,AL,g]	ATCC 35910	37	Human vaginal swab, United Kingdom, 1979	Holmes et al., 1984b
Chryseobacterium indologenes[AL,h]	NCTC 10796	38	Human trachea at autopsy, 1958	Yabuuchi et al., 1983
Chryseobacterium indoltheticum[AL,i]	ATCC 27950	34	Marine mud	Campbell and Williams, 1951
Chryseobacterium joostei	LMG 18212	37	Raw cow milk, South Africa, 1981	Hugo et al., 2003
Chryseobacterium meningosepticum[AL,j]	ATCC 13253	37	Human cerebrospinal fluid, USA, 1949	King, 1959; Holmes et al., 1984a
"Chryseobacterium proteolyticum"	NIBHT 17664	37	Soil, rice field, Tsukuba, Japan	Yamaguchi and Yokoe, 2000
Chryseobacterium scophthalmum[k]	CCM 4109	34	Gills of marine fish, United Kingdom, 1987	Mudarris et al., 1994

Genus *Coenonia*				Vandamme et al., 1999
Coenonia anatina[T]	LMG 14382	35	Peking duck, Germany, 1991	Vandamme et al., 1999
Genus *Croceibacter*				Cho and Giovannoni, 2003a
Croceibacter atlanticus[T]	HTCC 2559	35	Seawater, Sargasso Sea, Atlantic Ocean, 2001	Cho and Giovannoni, 2003a
Genus *Empedobacter*				Vandamme et al., 1994a
Empedobacter brevis[T,AL,l]	NCTC 11099	33	Human bronchial secretion, Switzerland, 1976	Holmes et al., 1978; Holmes et al., 1984a
Genus *Flavobacterium*				Bernardet et al., 1996
Flavobacterium aquatile[T,AL,m]	ATCC 11947	33	Deep well, United Kingdom	Holmes et al., 1984a; Bernardet et al., 1996
Flavobacterium branchiophilum[n]	ATCC 35035	34	Gills of salmon, Japan, 1977	Wakabayashi et al., 1989; Bernardet et al., 1996
Flavobacterium columnare[o]	NCIMB 2248	32	Kidney of salmon, United States, 1955	Bernardet and Grimont, 1989b; Bernardet et al., 1996
Flavobacterium degerlachei	LMG 21915	34	Microbial mats, Lake Ace, Vestfold Hills, Antarctica	Van Trappen et al., 2003b
Flavobacterium flevense[AL,p]	ATCC 27944	35	Freshwater lake, The Netherlands	van der Meulen et al., 1974; Bernardet et al., 1996
Flavobacterium frigidarium	ACTT 700810	35	Marine sediment, Adelaide Island, Antarctica	Humphry et al., 2001
Flavobacterium frigoris	LMG 21922	34	Microbial mats, Watts Lake, Vestfold Hills, Antarctica	Van Trappen et al., 2003a
Flavobacterium gelidilacus	LMG 21477	30	Microbial mats, freshwater and saline lakes, Antarctica	Van Trappen et al., 2003b; McCammon and Bowman, 2000
Flavobacterium gillisiae	ACAM 601	32	Sea ice, Prydz Bay, Antarctica	McCammon et al., 1998
Flavobacterium hibernum[q]	ACAM 376	34	Freshwater lake, Antarctica	Strohl and Tait, 1978
Flavobacterium hydatis[AL,r]	ATCC 29551	34	Gills of salmon, United States, 1974	Bernardet et al., 1996
Flavobacterium johnsoniae[AL,s]	ATCC 17061	35	Soil or mud, United Kingdom	Reichenbach, 1989; Bernardet et al., 1996
Flavobacterium limicola	JCM 11473	34	River sediment, Ibaraki Prefecture, Japan	Tamaki et al., 2003
Flavobacterium micromati	LMG 21919	33	Microbial mats, Grace Lake, Vestfold Hills, Antarctica	Van Trappen et al., 2003b
Flavobacterium omnivorum	AS 1.2747	35	Frozen soil, China No. 1 glacier, Xinjiang, China, 1999	Zhu et al., 2003
Flavobacterium pectinovorum[t]	NCIMB 9059	35	Soil, United Kingdom	Reichenbach, 1989; Bernardet et al., 1996
Flavobacterium psychrophilum[u]	NCIMB 1947	33	Kidney of salmon, United States	Bernardet and Grimont, 1989b; Bernardet et al., 1996
Flavobacterium saccharophilum[v]	NCIMB 2072	36	River Wey, United Kingdom, 1976	Agbo and Moss, 1979; Reichenbach, 1989; Bernardet et al., 1996
Flavobacterium succinicans[w]	DSM 4002	37	Fin of salmon, United States, 1954	Anderson and Ordal, 1961; Reichenbach, 1989; Bernardet et al., 1996
Flavobacterium tegetincola	ACAM 602	34	Cyanobacterial mat, marine salinity lake, Antarctica	McCammon and Bowman, 2000
Flavobacterium xinjiangense	AS 1.2749	34	Frozen soil, China No. 1 glacier, Xinjiang, China, 1999	Zhu et al., 2003
Flavobacterium xanthum[x]	IAM 12026	36	Soil, Showa station, Antarctica, 1967	Inoue and Komagata, 1976; Reichenbach, 1989; McCammon and Bowman, 2000

Table 1. *Continued*

Genera and species[b]	Type strain	G + C (mol%)	Source	Reference(s)
Genus *"Fucobacter"*				Sakai et al., 2002
"Fucobacter marina"[T]	FERM BP 5402	32	Water, Sea of Japan	Sakai et al., 2002
Genus *Gelidibacter*				Bowman et al., 1979a
Gelidibacter algens[T]	ACAM 536	36	Sea ice, Antarctica	Bowman et al., 1979a
Gelidibacter mesophilus	CECT 5103	40	Mediterranean water, Valencia, Spain	Macián et al., 2002
Genus *Mesonia*				Nedashkovskaya et al., 2003a
Mesonia algae[T]	KMM 3909	33	Green alga, Sea of Japan, 2000	Nedashkovskaya et al., 2003a
Genus *Muricauda*				Bruns et al., 2001
Muricauda ruestringensis[T]	DSM 13258	41	Marine sediment, Wadden Sea, Germany	Bruns et al., 2001
Genus *Myroides*				Vancanneyt et al., 1996
Myroides odoratus[T,AL,y]	ATCC 4651	36	Unknown	Holmes et al., 1977, 1984a; Vancanneyt et al., 1996
Myroides odoratimimus	NCTC 11180	32	Human wound, United Kingdom	Vancanneyt et al., 1996
Genus *Ornithobacterium*				Vandamme et al., 1994b
Ornithobacterium rhinotracheale[T]	CCUG 23171	38	Respiratory tract of turkey, United Kingdom	Vandamme et al., 1994b
Genus *Polaribacter*				Gosink et al., 1998
Polaribacter filamentus[T]	ATCC 700397	32	Surface sea water, Alaska, 1992	Gosink et al., 1998
Polaribacter franzmannii	ATCC 700399	32	Sea ice, Antarctica, 1992	Gosink et al., 1998
Polaribacter glomeratus[z]	ACAM 171	33	Marine salinity lake, Antarctica, 1984	McGuire et al., 1987; Gosink et al., 1998
Polaribacter irgensii[aa]	ATCC 700398	31	Sea water, Antarctica, 1986	Gosink et al., 1998
Genus *Psychroflexus*				Bowman et al., 1998
Psychroflexus gondwanensis[bb]	ACAM 44	39	Hypersaline lake, Antarctica, 1986	Dobson et al., 1993; Bowman et al., 1998
Psychroflexus torquis[T]	ACAM 623	33	Sea ice, Antarctica	Bowman et al., 1998
Genus *Psychroserpens*				Bowman et al., 1997a
Psychroserpens burtonensis[T]	ACAM 188	28	Marine salinity lake, Antarctica	Bowman et al., 1997a
Genus *Riemerella*				Segers et al., 1993a
Riemerella anatipestifer[T,AL,cc]	ATCC 11845	35	Duck blood, United States	Segers et al., 1993a
Riemerella columbina	LMG 11607	36	Pigeon palatine cleft, Germany, 1989	Vancanneyt et al., 1999
Genus *Salegentibacter*				McCammon and Bowman, 2000
Salegentibacter salegens[T,dd]	ACAM 48	37	Water, Organic Lake, Antarctica, 1986	Dobson et al., 1993; McCammon and Bowman, 2000
Genus *Tenacibaculum*				Suzuki et al., 2001
Tenacibaculum amylolyticum	IFO 16310	31	Marine alga, Palau, Philippines	Suzuki et al., 2001
Tenacibaculum maritimum[T,ee]	ATCC 43398	32	Diseased marine fish, Japan, 1977	Wakabayashi et al., 1986; Bernardet and Grimont 1989b; Suzuki et al., 2001
Tenacibaculum mesophilum	IFO 16307	32	Marine sponge, Numazu, Japan	Suzuki et al., 2001
Tenacibaculum ovolyticum[ff]	ATCC 51887	30	Marine fish egg, Norway, 1989	Hansen et al., 1992; Suzuki et al., 2001

Genus *Ulvibacter*				
Ulvibacter litoralis[T]	KMM 3912	37	Green alga, Sea of Japan, 2000	Nedashkovskaya et al., 2003b
Genus *Vitellibacter*				Nedashkovskaya et al., 2003d
Vitellibacter vladivostokensis[T,gg]	KMM 3516	41	Holothurian, Sea of Japan, 1997	Nedashkovskaya et al., 2003d
Genus *Weeksella*				Holmes et al., 1986a
Weeksella virosa[T]	NCTC 11634	37	Human urine, United States	Holmes et al., 1986a
Genus *Zobellia*				Barbeyron et al., 2001
Zobellia galactanivorans[T,hh]	DSM 12802	43	Red marine alga, Brittany, France	Barbeyron et al., 2001
Zobellia uliginosa[ii]	ATCC 14397	42	Marine sediment	ZoBell and Upham, 1944; Reichenbach, 1989; Barbeyron et al., 2001
Unaffiliated taxa				Bernardet et al., 1996; Hanzawa et al., 1995
[*Cytophaga*] *latercula*	TCCC 23177	32	Seawater aquarium outflow, La Jolla, United States	Lewin, 1969; Reichenbach, 1989
[*Cytophaga*] *marinoflava*	NCIMB 397	37	Seawater, United Kingdom	Colwell et al., 1966; Reichenbach, 1989

Abbreviations: [T], type species; AL, the species is cited on the Approved Lists of Bacterial Names (Skerman et al., 1980; Moore et al., 1985); ACAM, Australian Collection of Antarctic Microorganisms, University of Tasmania, Hobart, Tasmania, Australia; AS, culture collection of the Institute of Microbiology, Academia Sinica, Beijing, China; ATCC, American Type Culture Collection, Manassas, Va., USA; CCM, Czech Collection of Microorganisms, Brno, Czech Republic; CCUG, Culture Collection University of Göteborg, Göteborg, Sweden; CECT, Colección Española de Cultivos Tipo, Valencia, Spain; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; FERM, culture collection of the National Institute of Biosciences and Human Technology, Tsukuba, Japan; HTCC, culture collection of the Department of Microbiology, Oregon State University, Corvallis, Oregon; IAM, culture collection of the Institute of Applied Microbiology, University of Tokyo, Japan; IFO, culture collection of the Institute for Fermentation, Osaka, Japan; JCM, Japanese Collection of Microorganisms, Tokyo, Japan; KMM, Collection of Marine Micro-organisms, Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia; LMG, BCCM/LMG bacteria collection, Laboratorium voor Microbiologie, University of Gent, Gent, Belgium; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, London, England; NIBHT, culture collection of the National Institute of Bioscience and Human Technology, Tsukuba, Japan.

[a]Names in quotation marks have not been validly published. Previous names and corrected epithets are taken from Euzéby (1997). Accession number is that in the recognized culture collection in which the type strain was first deposited.

[b]Previous names of genera and species are as stated in footnotes c–ii.

[c][*Weesella*] *zoohelcum* Holmes et al. 1986b.

[d][*Cytophaga*] *lytica* Lewin 1969.

[e][*Flavobacterium*] *uliginosum* ZoBell and Upham 1944, Weeks 1974, *"Agarbacterium uliginosum"* Breed 1957a, [*Cytophaga*] *uliginosa* Reichenbach 1989 (this taxon has successively been reclassified in the genus *Cellulophaga* (Bowman, 2000) and in the genus *Zobellia* (Barbeyron et al., 2001); hence, it is provisionally listed within both genera in this table).

[f][*Flavobacterium*] *balustinum* Harrison 1929.

[g][*Flavobacterium*] *gleum* Holmes et al. 1984b.

Table 1. *Footnote Continued*

[h][*Flavobacterium*] *indologenes* Yabuuchi et al. 1983.
[i][*Flavobacterium*] *indoltheticum* Campbell and Williams 1951, *"Beneckea indolthetica"* Campbell 1957.
[j][*Flavobacterium*] *meningosepticum* King 1959.
[k]*"Cytophaga scophthalmis,"* name as listed in 1989 in the catalogue of strains of the Czech Collection of Microorganisms, [*Flavobacterium*] *scophthalmum* Mudarris et al. 1994.
[l]"[*Bacillus*] *brevis"* Lustig 1890, *"Bacterium brave"* Chester 1901, "[*Flavobacterium*] *brevis"* Bergey et al. 1923, *"Pseudobacterium brevis"* Krasil'nikov 1949, *"Empedobacter breve"* Prévot 1961, [*Flavobacterium*] *breve* Holmes and Owen 1982.
[m]"[*Bacillus*] *aquatilis"* Frankland and Franklnad 1889, *"Bacterium aquatilis"* Chester 1897, "[*Flavobacterium*] *aquatilis"* Bergey et al. 1923, "[*Chromobacterium*] *aquatilis"* Topley and Wilson 1929, "[*Empedobacter*] *aquatile"* Brison et al. 1960.
[n]*Flavobacterium branchiophila* Wakabayashi et al. 1989.
[o]"[*Bacillus*] *columnaris"* Davis 1922, "[*Chondrococcus*] *columnaris"* Ordal and Rucker 1944, [*Cytophaga*] *columnaris* Garnjobst 1945, Reichenbach 1989, [*Flexibacter*] *columnaris* Leadbetter 1974, Bernardet and Grimont 1989b.
[p][*Cytophaga*] *flevensis* van der Meulen et al. 1974, Reichenbach 1989.
[q]*"Flavobacterium xylanivorum,"* name as deposited in the 16S rRNA sequence databases and in the ATCC.
[r]*"Flavobacterium ameridies,"* name as deposited in the 16S rRNA sequence databases.
[s][*Cytophaga*] *aquatilis* Strohl and Tait 1978.
[t][*Cytophaga*] *johnsonae* Stanier 1947, Reichenbach 1989, "[*Cytophaga*] *johnsonii"* Stanier 1957.
[u]"*Flavobacterium pectinovorum"* Dorey 1959, "[*Empedobacter*] *pectinovorum"* Kaiser 1961, [*Cytophaga*] *pectinovora* Reichenbach 1989.
[v][*Cytophaga*] *psychrophila* Borg 1960, Reichenbach 1989, [*Flexibacter*] *psychrophilus* Bernardet and Grimont 1989.
[w][*Cytophaga*] *saccharophila* Agbo and Moss 1979.
[x][*Cytophaga*] *succinicans* Anderson and Ordal 1961, Reichenbach 1989, "[*Flexibacter*] *succinicans*" Leadbetter 1974.
[y]"[*Cytophaga*] *xantha"* Inoue and Komagata 1976.
[z]"[*Flavobacterium*] *odoratum"* Stutzer *in* Stutzer and Kwaschnina 1929.
[aa][*Flectobacillus*] *glomeratus* McGuire et al. 1987.
[bb]*"Antarcticum vesiculatum," "Vesiculatum antarctica,"* names as deposited in the 16S rRNA sequence databases.
[cc][*Flavobacterium*] *gondwanense* Dobson et al. 1993, *Psychroflexus gondwanense* Bowman et al. 1998; the original spelling of the specific epithet was corrected on validation (Bowman et al., 1999).
[dd]*"Pfeifferella anatipestifer"* Hendrickson and Hibert 1932, [*Moraxella*] *anatipestifer* Bruner and Fabricant 1954, [*Pasteurella*] *anatipestifer* Breed 1957b.
[ee][*Flavobacterium*] *salegens* Dobson et al. 1993.
[ff]"[*Flexibacter*] *marinus"* Hikida et al. 1979, [*Flexibacter*] *maritimus* Wakabayashi et al. 1986, [*Cytophaga*] *marina* Reichenbach 1989.
[gg][*Flexibacter*] *ovolyticus* Hansen et al. 1992.
[hh]*"Vladibactr vitellinus,"* name as deposited in the 16S rRNA sequence databases.
[ii]"[*Cytophaga*] *drobachiensis*" Potin et al. 1991, *Zobellia galactanovorans* Barbeyron et al. 2001; the original spelling of the specific epithet was corrected on notification (International Journal of Systematic and Evolutionary Microbiology, 2001).

is detailed in individual chapters as presented in the Introduction.

Habitat

The wide range of different habitats among (and sometimes within) the genera is one of the most intriguing characteristics of the family Flavobacteriaceae. Although the authors of the corresponding chapters in the previous editions of *Bergey's Manual of Systematic Bacteriology* and of *The Prokaryotes* (Holmes et al., 1984a; Reichenbach, 1989; see The Genera *Flavobacterium*, *Sphingobacterium* and *Weeksella*; The Order Cytophagales in this Volume) were actually referring to wider groups of organisms (see the Introduction), the habitats they listed are still valid for the current members of the family, as shown by a recent review (Jooste and Hugo, 1999). Taking into account the taxa described since then, the various habitats of flavobacteria may be as follows: soil; freshwater environments; marine environments; food and dairy products and their processing environments; specimens from humans; hospital equipment and devices; diseased dogs and cats; diseased amphibians and reptiles; diseased freshwater and marine fish; diseased mollusks, crustaceans, and sea urchins; diseased birds; eggs and digestive tract of insects; vacuoles or cytoplasm of amoebae; and diseased plants (see Table 1). All of these habitats are dealt with in detail in the chapters listed in the Introduction.

Another, very particular, habitat of flavobacteria was revealed by 16S rRNA analysis: some bacterial intracellular symbionts of insects are the closest relatives of bacteria in the family Flavobacteriaceae (Bernardet et al., 2002). First discovered in various cockroaches and termites, these uncultivable organisms were classified in the genus *Blattabacterium* (Dasch et al., 1984; Bandi et al., 1994). Related bacteria were subsequently detected in other roach species (Clark and Kambhampati, 2003) as well as in ladybird beetles; in the latter case, these intracellular bacteria were shown to kill male embryos they enter and to be maternally inherited (Hurst et al., 1997; Hurst et al., 1999). Other vertically transmitted bacteria in mites, ticks and wasps were attributed to the CFB group, although a close relationship with the flavobacteria was not demonstrated. These organisms also appear to be responsible for various reproductive anomalies in their hosts, such as parthenogenesis or feminization of haploid genetic males (Weeks et al., 2001; Zchori-Fein et al., 2001).

New insights on the distribution of flavobacteria in various environments have resulted from recent studies investigating the phylogenetic composition of bacterial communities. Some of the techniques used (such as phenotypic tests, fatty acid analysis, or sequencing of the 16S rRNA) required that bacteria be isolated first (Bowman et al., 1997; Jaspers et al., 2001; Nakagawa et al., 2001; Johansen and Binnerup, 2002; Van Trappen et al., 2002). Other studies used culture-independent identification methods such as fluorescent in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes, denaturing-gradient gel electrophoresis (DGGE), or sequencing of cloned 16S rRNA genes fragments (Manz et al., 1996; Hugenholtz et al., 1998; O'Sullivan et al., 2002). Most of these surveys considered the whole CFB group, whose members were shown to be much more abundant in most environments than previously estimated from cultured isolates (Eilers et al., 2000; Jaspers et al., 2001). Important populations of CFB were found in freshwater environments (Manz et al., 1999; Brümmer et al., 2000; Jaspers et al., 2001; Kirchman, 2002), marine environments (Cottrell and Kirchman, 2000; Eilers et al., 2000; Kirchman, 2002) especially in polar regions (Bowman et al., 1997; Ravenschlag et al., 2001), soils (Borneman et al., 1996; Johansen and Binnerup, 2002), and even special industrial environments (Whiteley and Bailey, 2000). Although important variations in the structure of bacterial communities may occur over time, the CFB usually constitute one of the major groups of heterotrophic bacteria (Kirchman, 2002). They frequently participate in biofilms (Burchard and Sorongon, 1998; Manz et al., 1999; Brümmer et al., 2000; O'Sullivan et al., 2002) and seem to play an important role in biogeochemical cycles, using their diverse enzymes to degrade a variety of complex organic substrates (Reichenbach, 1989; Kirchman, 2002; Johansen and Binnerup, 2002; see The Order Cytophagales in second edition; see also the section Identification in this Chapter). Other surveys of bacterial communities identified more specifically members of the family Flavobacteriaceae in various environments. Many organisms, sharing a high 16S rRNA sequence homology with described taxa in the family or representing new taxa, were found among the particle-attached bacteria in a river estuary (Crump et al., 1999), in various mangrove samples from the subtropical zone of Japan (Nakagawa et al., 2001), in bacterioplankton assemblages from the Arctic Ocean (Bano and Hollibaugh, 2002), in a glacier in China (Zhu et al., 2003), and in several Antarctic lakes (Van Trappen et al., 2002). Indeed, surveys performed in these freshwater or marine, tropical or polar environments have resulted in the description of many new taxa in the family. They are dealt with in chapters on their respective genera.

Identification

The following description of the family Flavobacteriaceae is from Bernardet et al. (2002):

Emended description of the family Flavobacteriaceae Reichenbach 1992: Cells are short to moderately long rods with parallel or slightly irregular sides and rounded or slightly tapered ends. They are usually 0.3 to 0.6 μm wide and 1 to 10 μm long though members of some species may form filamentous flexible cells (e.g., *Flavobacterium* and *Tenacibaculum*) or coiled and helical cells (*Polaribacter*, *Psychroflexus*, and *Psychroserpens* strains) under certain growth conditions; ring-shaped cells are not formed. Cells in old cultures may form spherical or coccoid bodies (e.g., *Flavobacterium*, *Gelidibacter*, *Psychroserpens*, and *Tenacibaculum*). Gram negative. Nonsporeforming. Gas vesicles are produced in members of some *Polaribacter* species. Flagella are usually absent; the only *Polaribacter irgensii* strain available is flagellated, but motility has not been observed in wet mounts. Nonmotile (*Bergeyella*, *Chryseobacterium*, *Coenonia*, *Empedobacter*, *Myroides*, *Ornithobacterium*, *Polaribacter*, *Psychroserpens*, *Riemerella*, *Salegentibacter* and *Weeksella* strains, and *Psychroflexus gondwanensis* strains) or motile by gliding (*Capnocytophaga*, *Cellulophaga*, *Gelidibacter*, *Flavobacterium*, *Tenacibaculum* and *Zobellia* strains, and *Psychroflexus torquis* strains).

Growth is aerobic (*Bergeyella*, *Cellulophaga*, *Chryseobacterium*, *Empedobacter*, *Flavobacterium*, *Gelidibacter*, *Myroides*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum*, *Weeksella*, and *Zobellia* strains) or microaerobic to anaerobic (*Capnocytophaga*, *Coenonia*, *Ornithobacterium*, and *Riemerella* strains). The optimum temperature is usually in the range from 25 to 35°C, but members of some species or genera are psychrophilic or psychrotrophic (*Flavobacterium psychrophilum* and the Antarctic *Flavobacterium* species, as well as *Gelidibacter*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, and *Salegentibacter* strains). Members of some taxa are halophilic to varying degrees (*Cellulophaga*, *Gelidibacter*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum* and *Zobellia* strains).

Colonies are nonpigmented (*Bergeyella*, *Coenonia*, *Ornithobacterium*, and *Weeksella* strains) or pigmented by carotenoid or flexirubin pigments or both (*Capnocytophaga*, *Cellulophaga*, *Chryseobacterium*, *Empedobacter*, *Flavobacterium*, *Gelidibacter*, *Myroides*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Riemerella*, *Salegentibacter*, *Tenacibaculum* and *Zobellia* strains).

Menaquinone 6 is either the only respiratory quinone or the major respiratory quinone. Chemoorganotrophic. Intracellular granules of poly-β-hydroxybutyrate are absent. Sphingophospholipids are absent. Homospermidine is the major polyamine though agmatine, cadaverine, and putrescine are frequently present as minor components. Crystalline cellulose (i.e., filter paper) is not decomposed. The DNA base composition ranges from 27 to 44 GC mol%.

Mostly saprophytic in terrestrial and aquatic habitats. Several members of the family are commonly isolated from diseased humans or animals, some species are considered true pathogens. The type genus is *Flavobacterium* Bergey, Harrison, Breed, Hammer, and Huntoon 1923, as emended in 1996 (Bernardet et al., 1996).

Other taxa included in the family Flavobacteriaceae are the genera *Bergeyella*, *Capnocytophaga*, *Cellulophaga*, *Chryseobacterium*, *Coenonia*, *Empedobacter*, *Gelidibacter*, *Myroides*, *Ornithobacterium*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Riemerella*, *Salegentibacter*, *Tenacibaculum*, *Weeksella* and *Zobellia*. Several species unaffiliated to any genus also belong to the family. Several intracellular symbionts of insects and intracellular parasites of amoebae are closely related to the family.

Since this emended description was published, several new species in the genera *Cellulophaga* (Bowman, 2000), *Chryseobacterium* (Hugo et al., 2003; Kämpfer et al., 2003), *Flavobacterium* (McCammon and Bowman, 2000; Humphry et al., 2001; Tamaki et al., 2003; Van Trappen et al., 2003a; Van Trappen et al., 2003b; Zhu et al., 2003), and *Gelidibacter* (Macián et al., 2002), as well as the new genera *Aequorivita* (Bowman and Nichols, 2002), *Arenibacter* (Ivanova et al., 2001; Nedashkovskaya et al., 2003c), *Croceibacter* (Cho and Giovannoni, 2003), *Mesonia* (Nedashkovskaya et al., 2003a), *Muricauda* (Bruns et al., 2001), *Ulvibacter* (Nedashkovskaya, 2003b) and *Vitellibacter* (Nedashkovskaya et al., 2003d), have been validly published or validated after publication (Cho and Giovannoni, 2003). Although not validly published, "*Chryseobacterium proteolyticum*" (Yamaguchi and Yokoe, 2000) and "*Fucobacter marina*" (Sakai et al., 2002) also belong to the family. New taxa, however, do not make another emendation of the family necessary, since their characteristics are in accordance with the current description. The only exception is *Muricauda ruestringensis*, which is facultatively anaerobic and may exhibit long cellular appendages (Bruns et al., 2001).

Table 2 lists the differentiating characteristics of taxa classified in the family, updated from Bernardet et al. (2002). Those taxa that have not been validly published, as well as the two generically misclassified species [*Cytophaga*] *latercula* and [*Cytophaga*] *marinoflava*, are included for information. Additional phenotypic characteristics that differentiate 1) the *Capnocytophaga* species, *Coenonia anatina*, *Ornithobacterium rhinotracheale* and *Riemerella anatipestifer* are described in Vandamme et al. (Vandamme et al., 1994b; Vandamme et al., 1999); 2) the genera *Aequorivita*, *Arenibacter*, *Cellulophaga*, *Croceibacter*, "*Fucobacter*," *Gelidibacter*, *Mesonia*, *Muricauda*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter*, and *Zobellia*, as well as [*Cytophaga*] *latercula* and [*Cytophaga*] *marinoflava*, are described in Bowman et al. (Bowman et al., 1997; Bowman et al., 1998), Gosink et al. (1998), Johansen et al. (1999), McCammon and Bowman (2000), Barbeyron et al. (2001), Bruns et al. (2001), Ivanova et al. (2001), Suzuki et al. (2001), Bowman and Nichols (2002), Macián et al. (2002), Sakai et al.

Table 2. Differentiating characteristics of taxa classified in the family Flavobacteriaceae.[a]

Characteristic	*Aequo-rivita* (4)	*Areni-bacter* (2)	*Bergeyella zoohelcum*	*Capno-cytophaga* (7)	*Cellulo-phaga* (5)	*Chryseo-bacterium* (9)	*Coenonia anatina*	*Crocei-bacter atlan-ticus*	[*Cyto-phaga*] *latercula*	[*Cyto-phaga*] *marinoflava*	*Empedobacter brevis*	*Flavobacterium* (22)	"*Fucobacter marina*"
Habitat	FL (te, me) or S	FL (me) or S	P or S	P or S	FL (me) or S	FL (fe, me, te), S or P	P	FL (me) or S	FL (me) or S	FL (me) or S	P or S	FL (fe, me, te), S or P	FL (me) or S
Cell morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods or filaments	Rods	Rods	Rods or filaments	Rods
Pigment production	+ C	+ C	–	+ F[b]	+ F and/or C	+ F[c]	–	+ C	+ C	+ not F	(+) F	+ F and/or C	+
Gliding motility	–	–	–	+	+	–	–	–	–/(+)	+	–	12[e]	–
Sea water requirement	v	+	–	–	v	–	–	+	+	–	–	–	+
Capnophilic metabolism	–	–	–	+	–	–	+	–	–	–	–	–	–
Growth at (°C)													
25	+	+	+	ND	+	+	(+)	+	+	+	+	18[e]	+
37	–	+	+	+	v	v	+	–	–	–	+[f]	–	ND
42	–	v	–[g]	ND	–	v	–	–	–	–	–	–	ND
Growth on													
MacConkey agar	ND	ND	–	–	ND	v[h]	–	ND	ND	ND	+	ND	ND
β-Hydroxy-butyrate	ND	ND	–	ND	ND	+[j]	ND	ND	ND	ND	+	ND	ND
Acid production from													
Glucose	–	v	–	+	v	+[k]	+	ND	ND	+	+[f]	v	+
Sucrose	–	ND	–	+[l]	v	*v*	–	ND	ND	+	–	v	–
Production of													
DNase	v	v	–	ND	v	+	ND	+	ND	ND	+	v	ND
Urease	v	v	+	v	v	v*	–	+	ND	ND	*v**	v	+
Oxidase	ND	+	+	–	v	+	+	–	+	+	+	18[e]	+
Catalase	+	+	+	v	+	+	+	+	–	+	+	+/(+)	+
Indole	ND	–	+	–	ND	+[k]	–	–	ND	–	+	–	–
β-Galacto-sidase	–	+	–	v	ND	v	+	–	*v*	+	–	v	–
Nitrate reduction	–	+	–	v	v	v	–	–	*v*	+	–	v	–
Carbohydrate utilization	v	+	–	v	ND	+	+	+	+	+	v	v	ND
Degradation of													
Agar	–	–	–	–	+	–	–	–	+	*v*	–	v	ND
Starch	v	–	–	v	+	v	ND	+	*v*	+	*v*	16[e]	–
Esculin	v	ND	–	v	ND	+	+	–	ND	+	–	17[e]	+
Gelatin	+	v	+	v	v	+	–	+	+	ND	+	15[e]	+
Resistance to penicillin G	ND	ND	–	–	ND	+[j]	ND	ND	ND	+	+	v	ND
G+C content (mol%)	33–39	37–40	35–37	36–44	32–42	33–38	35–36	35	34	37	31–33	30–37	32

Table 2. *Continued*

Characteristic	*Gelidibacter* (2)	*Mesonia algae*	*Muricauda ruestringensis*	*Myroides* (2)	*Ornithobacterium rhinotracheale*	*Polaribacter* (4)	*Psychroflexus* (2)	*Psychroserpens burtonensis*	*Riemerella* (2)	*Salegentibacter salegens*	*Tenacibaculum* (4)	*Ulvibacter litoralis*	*Vitellibacter vladivostokensis*	*Weeksella virosa*	*Zobellia* (2)
Habitat	FL (me) or S	FL (me) or S	FL (me) or S	P or S	P	FL (me) or S	FL (me) or S	FL (me) or S	P	FL (me) or S	FL (me), S or P	FL (me) or S	FL (me) or S	P or S	FL (me) or S
Cell morphology	Rods or filaments	Rods	Rods with appendages	Rods	Rods	Rods, filaments, or coils; gas vesicles	Rods, filaments, or coils	Rods, filaments, coils, or helices	Rods	Rods	Rods or filaments	Rods	Rods	Rods	Rods
Pigment production	+ C	+[f] not F	+ not F	+ F	–	+ C[d]	+ C	+ C	–/(+)[n]	+ C	+/(+) C	+ F	+ F	–	+ F
Gliding motility	+	–	–	–	–	–	v	–	–	–	+	–	–	–	+
Sea water requirement	+	–	+	–	–	+[f]	v	+	–	–	v	–	–	–	+
Capnophilic metabolism	–	–	–	–	+	–	–	–	+	–	–	–	–		
Growth at (°C)															
25	v	+	+	+	–	–	v	–	v	+	+	+	+		
37	v	–	+	+	+	–	–	–	+	–	v	–	+		
42	–	–	–	–	+	–	–	–	+	–	–	–	+		
Growth on															
MacConkey agar	–	ND	ND	+	–	ND	–[i]	–	–	ND	ND	ND	ND	+	ND
β-hydroxy-butyrate	–	ND	ND	+	ND	ND	–[i]	–	ND	ND	ND	ND	ND	+	ND
Acid production from															
Glucose	v	–	ND	–	v	+	v	–	+	v	ND	ND	–	–	+
Sucrose	–	–	ND	–	–	v	–[i]	–	–	v	ND	ND	–	–	+
Production of															
DNase	v	–	ND	+	–	ND	+	–	ND	+	+	–	+	–	+
Urease	–	–	ND	+	+	–	v	–	v	v	ND	–	–	–	–
Oxidase	–	+	+	+	+[f]	v	+	–	+	+	+	+	+	+	+
Catalase	+	+	–	+	–	+/(+)	+	+	+	+	+	+	+	+	+
Indole	–	–	–	–	–	–	–[i]	–	v	ND	ND	–	–	+	+
β-galactosi-dase	–	–	ND	–	+	v	–	v	–	+	ND	–	–	–	+
Nitrate reduction	–	–	–	–	–	–	–	–	–	+	v	+	–	–	+
Carbohydrate utilization	+	–	+	–	+	+	+	–	+	+	ND	–	+	–	+
Degradation of															
Agar	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Starch	+	–	–	–	ND	+/(+)	+	–	ND	+	v	–	–	–	v
Esculin	+	ND	ND	–	–	v	v	–	v	+	–	ND	ND	–	+
Gelatin	v	+	–	+	–	v	v	v	+	+	+	+	+	+	+
Resistance to penicillin G	ND	ND	ND	ND	v	ND	ND	ND	–	ND	–[m]	ND	ND	–	ND
G+C content (mol%)	36–40	32–34	41	30–38	37–39	31–34	32–39	27–29	29–37	37–38	30–32	36–38	41	37–38	42–43

Table 2. *Footnote Continued*

Symbols: +, positive reaction; –, negative reaction; (+), weak positive reaction; v, varies within and/or between species; *v*, varies between references; and *, data in these two boxes were erroneously inverted in Bernardet et al. (2002).
Abbreviations: FL, free-living; P, parasitic; S, saprophytic; fe, freshwater environment; me, marine environment; te, terrestrial environment; ND, not determined or determined for some species only; F, flexirubin type pigment; and C, carotenoid type pigment.

[a] The number of species is given in parentheses below the name of the genus.
[b] Type of pigment in *Capnocytophaga gingivalis*. Not determined in the six other *Capnocytophaga* species.
[c] *Chryseobacterium meningosepticum* strains either are not pigmented or produce a weak yellow pigment (e.g., the type strain; Bruun and Ursing, 1987). Members of all other *Chryseobacterium* species produce a bright yellow to orange pigment.
[d] Type of pigment in *Polaribacter glomeratus*. Not determined in the three other *Polaribacter* species.
[e] Number of species positive for this characteristic among the 22 valid *Flavobacterium* species. Only specified when 12 or more species are positive.
[f] Most strains are positive for this characteristic.
[g] Most strains are negative for this characteristic.
[h] Strain dependent for *Chryseobacterium indologenes*. Positive for other *Chryseobacterium* species except *C. scophthalmum*, "*C. proteolyticum*," and *C. defluvii*.
[i] Negative for *Psychroflexus torquis* (J. P. Bowman, personal communication); not determined for *Psychroflexus gondwanensis*.
[j] Not determined for "*Chryseobacterium proteolyticum*"; positive for all other *Chryseobacterium* species.
[k] Positive for all *Chryseobacterium* species, except *Chryseobacterium scophthalmum*.
[l] Positive for all *Capnocytophaga* species, except *Capnocytophaga canimorsus*.
[m] Negative for *Tenacibaculum maritimum* (Burchard, 1999). Not determined in the three other *Tenacibaculum* species.
[n] A greyish-white to beige pigment is produced by *Riemerella columbina* strains on Columbia blood agar (Vancanneyt et al., 1999).

Data taken from Colwell et al. (1966), Lewin (1969), Lewin and Lounsbery (1969), Holmes et al. (1977), Holmes et al. (1978), Oyaizu and Komagata (1981), Yabuuchi et al. (1983), Holmes et al. (1984a), Holmes et al. (1986a), Holmes et al. (1986b), London et al. (1985), Dees et al. (1986), Bruun and Ursing (1987), McGuire et al. (1987), Bernardet (1989a), Bernardet and Grimont (1989b), Reichenbach (1989), Ursing and Bruun (1991), Hansen et al. (1992), Dobson et al. (1993), Segers et al. (1993a), Ostland et al. (1994), Vandamme et al. (1994a), Vandamme et al. (1994b), Vandamme et al. (1999), Yamamoto et al. (1994), Bernardet et al. (1996), Bowman et al. (1997a), Bowman et al. (1998), Hugo (1997), Gosink et al. (1998), Johansen et al. (1999), Vancanneyt et al. (1999), Bowman (2000), McCammon and Bowman (2000), Yamaguchi and Yokoe (2000), Barbeyron et al. (2001), Bruns et al. (2001), Humphry et al. (2001), Ivanova et al. (2001), Suzuki et al. (2001), Bowman and Nichols (2002), Macián et al. (2002), Sakai et al. (2002), Cho and Giovannoni (2003a), Kämpfer et al. (2003), Nedashkovskaya et al. (2003a), Nedashkovskaya et al. (2003b), Nedashkovskaya et al. (2003c), Nedashkovskaya et al. (2003d), Tamaki et al. (2003), Van Trappen et al. (2003a), Van Trappen et al. (2003b), Zhu et al. (2003), J-F. Bernardet (unpublished results), J. P. Bowman (personal communication), and Nedashkovskaya (personal communication).

(2002), Cho and Giovannoni (2003a), and Nedashkovskaya et al. (Nedashkovskaya et al., 2003a; Nedashkovskaya et al., 2003b; Nedashkovskaya et al., 2003c; Nedashkovskaya et al., 2003d); and 3) the genera *Bergeyella*, *Chryseobacterium*, *Empedobacter*, *Flavobacterium*, *Myroides* and *Weeksella* are described in Bernardet et al. (1996). Differentiating characteristics for species in each genus are presented in the chapters listed in the Introduction.

In the minimal standards proposed for describing new taxa of the family Flavobacteriaceae (Bernardet et al., 2002), particular methods to determine phenotypic properties were suggested and should preferably be used. Some of these properties are of particular importance to differentiate among members of the family Flavobacteriaceae and the family from related taxa in the *Cytophaga-Flavobacterium-Bacteroides* group. Since the conditions in which the tests are performed are critical, some procedures (updated from Bernardet et al., 2002) are recommended below.

Chemotaxonomically Significant Components

All current members of the family Flavobacteriaceae exhibit menaquinone 6 as their only or major respiratory quinone (Bernardet et al., 1996), whereas menaquinone 7 is present in members of all related families and taxa that have been tested (Hanzawa et al., 1995). As stated in the description of the family, homospermidine is the major polyamine of its members, though agmatine, cadaverine, and putrescine are frequently present as minor components (Hamana and Nakagawa, 2001a; Hamana and Nakagawa, 2001b). Unlike members of the family Sphingobacteriaceae (Steyn et al., 1998), flavobacteria do not contain sphingophospholipids (Bernardet et al., 1996). Fatty acid composition provides high quality taxonomic information, mainly at the generic and specific levels (Vandamme et al., 1996). The predominant fatty acids found in members of the family Flavobacteriaceae are usually characteristic of genera, though some fatty acid profiles help to differentiate species (Vandamme et al., 1994b; Vandamme et al., 1996; Bernardet et al., 1996; Vancanneyt et al., 1996; Bowman et al., 1997; Bowman et al., 1998; Gosink et al., 1998; Barbeyron et al., 2001), provided standardized culture conditions are used. Species of the genera *Capnocytophaga* (Vandamme et al., 1996), *Myroides* (Vancanneyt et al., 1996) and *Riemerella* (Vancanneyt et al., 1999) can be readily differentiated by their protein profiles. This is particularly important for the genera *Myroides* and *Riemerella* for which few differential characteristics are available. However, for some other genera, such as *Flavobacterium*, some species can be identified by their very typical protein profiles, whereas others exhibit intraspecific heterogeneity (Bernardet et al., 1996). Again, to compare protein profiles accurately, highly standardized procedures must be used to obtain, scan, and numerically analyze sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoregrams.

Production of Enzymes

Although members of the family differ widely in their enzymatic abilities, many of them can degrade different kinds of organic macromolecules (Reichenbach, 1989; The Order Cytophagales in second edition). Interestingly, the recent description of several new taxa in the family actually resulted from enzymatic screenings performed on environmental isolates aimed at discovering potentially useful exoenzymes (Yamaguchi and Yokoe, 2000; Barbeyron et al., 2001; Humphry et al., 2001; Sakai et al., 2002). The array of enzymes produced by flavobacteria understandably depends on the major biopolymers available in their habitats (Kirchman, 2002). For instance, those living in soil and freshwater frequently synthesize cellulose-, pectin-, xylan-, and chitin-degrading enzymes that decompose dead plants, fungi, and insects (Reichenbach, 1989; Haack and Breznak, 1993; Cottrell and Kirchman, 2000; Johansen and Binnerup, 2002), while the marine flavobacteria usually produce enzymes that degrade agars, laminarin, xylan, fucoidan, and carrageenans from micro- or macroalgae (Johansen et al., 1999; Barbeyron et al., 2001; Humphry et al., 2001; Sakai et al., 2002). Proteolytic enzymes are also synthesized by flavobacteria in various environments. They probably play an important role in the virulence of some of the pathogenic members of the family. For instance, the fish-pathogenic *Flavobacterium* species produce extracellular proteases that degrade collagen, elastin, fibrinogen, and keratin (Otis, 1984; Holt, 1988). Combined with enzymes that degrade the complex polysaccharides of connective tissue, such as hyaluronic acid and chondroitin sulfate (Otis, 1984; Teska, 1993), they are responsible for the extensive necrotic lesions that occur in infected fish.

Among the complex organic substrates that some flavobacteria are able to degrade, cellulose has a particular taxonomic significance. Members of some taxa degrade soluble cellulose

derivatives such as carboxymethylcellulose or hydroxyethylcellulose, but since enzymes other than cellulases may degrade these compounds, this does not demonstrate that these taxa are cellulolytic. The decomposition of crystalline cellulose (i.e., filter paper) requires the production of a specific cellulase, and hence only strains able to degrade filter paper should be regarded as cellulose degraders (Reichenbach, 1989). The inability to degrade crystalline cellulose has been confirmed in members of most taxa included in the family Flavobacteriaceae (Bernardet, 1989a; Reichenbach, 1989; The Order Cytophagales in second edition; J.-F. Bernardet, unpublished observation), including members of the recently described genera *Aequorivita*, *Arenibacter*, *Cellulophaga*, *Croceibacter*, *Gelidibacter*, *Mesonia*, *Muricauda*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Ulvibacter*, *Vitellibacter* and *Zobellia*, as well as members of recently described *Flavobacterium* species (Gosink et al., 1998; Barbeyron et al., 2001; Bruns et al., 2001; Ivanova et al., 2001; Bernardet et al., 2002; Bowman and Nichols, 2002; Cho and Giovannoni, 2003; Nedashkovskaya et al., 2003a; Nedashkovskaya et al., 2003b; Nedashkovskaya et al., 2003c; Nedashkovskaya et al., 2003d). This characteristic distinguishes members of the family Flavobacteriaceae from those of the genus *Cytophaga*, now restricted to cellulolytic organisms (Nakagawa and Yamasato, 1996). Hence, it is essential 1) that strains be tested for their ability to degrade filter paper both on a nutrient-containing agar (presence of a cellulase) and on a mineral agar (ability to use cellulose as the only carbon source; The Order Cytophagales in second edition) and 2) that the cellulose derivatives that are used be specified.

Pigments

Members of most genera classified in the family Flavobacteriaceae produce light to bright yellow or orange pigments though nonpigmented taxa (*Bergeyella zoohelcum*, *Coenonia anatina*, *Ornithobacterium rhinotracheale* and *Weeksella virosa*) and strains (e.g., some *Chryseobacterium meningosepticum* strains are nonpigmented while others, including the type strain, exhibit a weak yellow pigmentation; Bruun and Ursing, 1987) do occur. These pigments may belong to the carotenoid or to the flexirubin types depending on the genus. However, the genus *Flavobacterium* includes carotenoid-producing and flexirubin-producing species, as well as species producing both types of pigment (Reichenbach, 1989; Bernardet et al., 1996). The localization of the two types of pigment is different (i.e., carotenoids in the cytoplasm and flexirubins in the outer membrane), and several different compounds of each type may be synthesized by one single strain (The Order Cytophagales in second edition). Carotenoid pigments are usually produced by members of marine species while flexirubin pigments are more frequently associated with clinical, freshwater or soil organisms (Reichenbach, 1989). Pigments are usually nondiffusible, but the beige pigment produced by members of *Riemerella* species is diffusible on some solid media (e.g., trypticase soy agar; J.-F. Bernardet, unpublished results).

Although chromatography and spectrophotometry are the most accurate techniques for determining the type of pigment produced by bacteria (Weeks, 1981), a simple test may be performed when these methods are unavailable: colonies having a flexirubin type of pigment exhibit an immediate color shift from yellow or orange to red, purple or brown when flooded with 20% KOH and revert to their initial color when flooded by an acidic solution once the excess of KOH has been removed (Reichenbach, 1989). Since the color shift may pass unnoticed when the KOH solution is poured directly over a thin colony on an agar plate, it is strongly recommended that the test be performed on a small mass of bacterial cells collected with a loop and deposited on a glass slide placed on a white background (Bernardet et al., 2002). Another similar mass of bacteria on which no KOH is poured may be used as a control. The color change induced by KOH is not absolutely specific for the flexirubin type of pigment (Fautz and Reichenbach, 1980), but it is still helpful when combined with the results of other tests.

Gliding Motility

The taxonomic significance of this property was once considered so high that headings of a chapter in *The Prokaryotes* (Reichenbach and Dworkin, 1981a) and of a section in *Bergey's Manual* (Larkin, 1989) were based on it. However, the fact that this type of bacterial motility is not restricted to the CFB phylum (Burchard, 1981; Reichenbach, 1989), and that gliding motility in some members of the phylum was not evident, verified Reichenbach's prediction: "Most probably, gliding motility will lose its determining character in this taxonomic group, and eventually there may be order(s), families, and perhaps even genera that comprise motile and nonmotile species" (The Order Cytophagales in second edition). Hence, although not a sound criterion to define the family Flavobacteriaceae, gliding motility is still an important characteristic

for differentiating between genera classified in the family and between species of some genera. However, precautions need to be taken, as its unequivocal recognition is not always easy. Gliding motility is highly dependent on growth conditions such as the temperature (McGrath et al., 1990) and the concentration of nutrients in the growth medium; the latter should preferably be low (Reichenbach and Dworkin, 1981a). Conversely, strains of *Gelidibacter mesophilus* appear to exhibit gliding motility only when specific carbohydrates are added to the medium (Macián et al., 2002). When gliding is strongly suspected but not readily observed by microscopic examination of a drop of liquid culture with a conventionally used coverslip, it should be tested using the hanging drop technique: the coverslip on which the drop has been deposited should be turned upside down and placed on tiny stands on a glass slide; bacteria are then observed through the coverslip. Gliding must be checked on the edge of the hanging drop and at the bottom surface of the coverslip, as this phenomenon is exclusively exhibited by bacteria in contact with a solid and wet surface, such as glass, plastic or agar. Unlike flagellar motility, gliding motility cannot occur when bacterial cells are suspended in a liquid. The movements involved in gliding motility were described previously (Henrichsen, 1972; Reichenbach and Dworkin, 1981a; Reichenbach, 1989). When gliding, bacterial cells may also bend, change direction suddenly, or rotate around one end that remains attached to the surface while the rest of the cell points away from the surface (Reichenbach, 1989). When gliding motility is very slow and hardly noticeable, it may be detected by comparing the position of bacteria in the same area at several minute intervals. Gliding can usually be suspected from the more or less rhizoidal aspect of the edge of the colonies (provided the agar is not too dry), but it cannot usually be detected in bacteria collected on agar and suspended in saline. Direct microscopic examination of the edge of a young colony, either on an open agar plate at the highest magnification possible without using immersion oil or using oil-immersion microscopy after covering the colony with a glass coverslip, may reveal either gliding itself or the slime tracks left on agar by gliding bacteria (Burchard, 1981; Bowman, 2000; The Order Cytophagales in this Volume). Chamber culture may also be used (Reichenbach and Dworkin, 1981a). The use of phase contrast microscopy is always preferable to visualize gliding motility (Bowman, 2000). Interestingly, gliding motility may be related to another characteristic exhibited by some flavobacteria, i.e., adherence of colonies to the agar and of bacterial growth on the glass flask walls in liquid culture (McGrath et al., 1990; Burchard and Sorongon, 1998; Bernardet et al., 2002). Adherence presumably facilitates the participation of flavobacteria to biofilms (see the section Habitat in this Chapter). Combined with gliding motility, this property could also be responsible for the invasion of host tissue by the fish-pathogenic *Flavobacterium* and *Tenacibaculum* species. However, only few investigations have been conducted on the adherence of flavobacteria (Burchard and Sorongon, 1998; Burchard, 1999; Decostere et al., 1999a; Decostere et al., 1999b). Both adherence and gliding motility are likely related to the production of slime by many flavobacteria (Reichenbach, 1989; The Order Cytophagales in this Volume). For instance, slime may be revealed using the Congo red test in *Flavobacterium columnare* and *Tenacibaculum maritimum*, two gliding species whose colonies adhere strongly to the agar; also, slime production increases the viscosity of liquid cultures of *T. maritimum* (see Bernardet et al. [2002] and references therein).

Salinity Requirement

The salinity requirement of members of a potentially new taxon is an important property to be investigated since several genera included in the family Flavobacteriaceae are composed of strains retrieved from seawater or ice, marine sediments, beach mud, marine animals, marine algae, hypersaline lakes, or lakes with salinity similar to that of seawater (Bowman et al., 1997; Bowman et al., 1998; Gosink et al., 1998; Barbeyron et al., 2001; Bruns et al., 2001; Humphry et al., 2001; Ivanova et al., 2001; Bowman and Nichols, 2002; Macián et al., 2002; Sakai et al., 2002; Cho and Giovannoni, 2003; Nedashkovskaya et al., 2003a; Nedashkovskaya et al., 2003b; Nedashkovskaya et al., 2003c; Nedashkovskaya et al., 2003d; see Table 1). Some bacterial strains isolated from high-salinity environments require artificial or natural seawater, either pure or diluted, for growth, whereas other isolates are able to grow on standard media which contain adequate NaCl concentration or after the addition of NaCl. For instance, the two *Chryseobacterium* species isolated from marine environments (i.e., *C. indoltheticum* and *C. scophthalmum*) grow on trypticase-soy agar (0.5 % NaCl) even better than on Bacto marine agar 2216 (1.95 % NaCl among other salts); interestingly, the six other *Chryseobacterium* species are also able to grow on both trypticase-soy agar and marine agar although they occur in non-marine environments (J.-F. Bernardet, unpublished results).

Capnophilic Metabolism

Members of most genera assigned to the family Flavobacteriaceae are composed of aerobic organisms which grow in ambient air. However, *Capnocytophaga*, *Coenonia*, *Ornithobacterium* and *Riemerella* strains require various concentrations of carbon dioxide. Culture conditions are given in detail in the corresponding chapters. After several subcultivations, some strains may be adapted to grow under aerobic conditions, although growth is always significantly better under microaerophilic conditions (Segers et al., 1993; Vandamme et al., 1994b; Vandamme et al., 1996; Vandamme et al., 1999; Vancanneyt et al., 1999). Growth should be tested under both conditions; it is usually very poor or absent under strict anaerobic conditions. Members of the genus *Muricauda* are facultative anaerobes (Bruns et al., 2001), and some *Flavobacterium* species may also grow under anaerobic conditions when certain growth factors are provided (Bernardet et al., 1996).

Genetics

Genetics of members of the family Flavobacteriaceae has been poorly investigated and few data are available. Gliding motility is the only mechanism whose genetics was extensively studied recently: mutagenesis was used to identify at least six different genes required for the gliding of *Flavobacterium johnsoniae* (Hunnicutt et al., 2002). However, mechanisms responsible for gliding motility are complex and probably differ among the organisms that exhibit this kind of motility (McBride, 2001). The same group also developed techniques for the genetic manipulation of various flavobacteria, such as a system of gene transfer, a selectable marker, a suicide vector, and a transposon (McBride and Baker, 1996; McBride and Kempf, 1996). Chromosomal DNA of flavobacteria was only studied for its taxonomic and phylogenetic applications, such as the determination of DNA/DNA homology and the G+C content of the DNA and the comparison of 16S rRNA or *gyrB* genes sequences (Bernardet et al., 2002; see the section Phylogeny in this Chapter). Several molecular techniques (e.g., rRNA gene restriction patterns [ribotyping], randomly amplified polymorphic DNA [RAPD], restriction fragment length polymorphism [RFLP], pulsed field gel electrophoresis [PFGE], etc.) were used to type collections of flavobacterial strains for epidemiological studies. Plasmid DNA was investigated for the same purpose and plasmid profiles of collections of strains have been compared. Among the 128 bacteria listed in the National Center for Biotechnology Information (NCBI) website whose whole genomes have been sequenced, *Bacteroides thetaiotaomicron* was the only member of the CFB phylum; the genomes of three more members of the phylum (i.e., *Cytophaga hutchinsonii*, *Porphyromonas gingivalis* and *Prevotella intermedia*) were being sequenced, but no member of the family Flavobacteriaceae was listed.

Pathogenicity

Members of certain species in the family Flavobacteriaceae are etiologic agents of diseases in humans, birds, fishes, and other animals (see the section Habitat in this Chapter), but the relationship to the host may differ (The Order Cytophagales in this Volume; Jooste and Hugo, 1999). Most of them are opportunistic, invading hosts already immunocompromised individually or as a result of poor farming conditions or environmental disorders. Hence, these organisms may also be isolated from environmental samples; this is the case for the potentially pathogenic members of the genera *Flavobacterium* and *Chryseobacterium* and for members of the genera *Myroides*, *Empedobacter*, *Bergeyella* and *Weeksella*. Some truly parasitic pathogens exist in the family, however, and are exclusively retrieved from diseased hosts; this is the case for the bird pathogens, i.e., members of the genera *Riemerella*, *Ornithobacterium* and *Coenonia*. Although *Capnocytophaga* species belong to the normal bacterial flora of humans, they too may become pathogenic. Human infections caused by flavobacteria are uncommon but may be serious owing to the fact that patients are immunocompromised; moreover, strains are frequently highly resistant to many antimicrobial agents. Pathogenicity is dealt with in detail in the chapters listed in the Introduction.

Applications

Understandably, practical aspects and applications of members of the family Flavobacteriaceae are related to their habitats and to their relationship to the host (see the section Habitat in this Chapter). Positive aspects include synthesis of a number of enzymes potentially useful in industry or medicine; turnover of organic matter in soil, water, and sewage plants; decomposition

of pesticides and insecticides; destruction of toxic proliferative algae; and symbiosis with various insects. Negative effects include spoilage and defects of food (meat, fish, and dairy products); infections in humans and animals; and destruction of valuable algae and vegetables (Holmes et al., 1984a; Reichenbach, 1989; The Genera Flavobacterium, Sphingobacterium and Weeksella in this Volume; The Order Cytophagales in this Volume). Practical aspects and applications are dealt with in the chapters listed in the Introduction.

Literature Cited

Agbo, J. A. C., and M. O. Moss. 1979. The isolation and characterization of agarolytic bacteria from a lowland river. J. Gen. Microbiol. 115:355–368.

Anderson, R. L., and E. J. Ordal. 1961. Cytophaga succinicans sp.n., a facultatively anaerobic, aquatic myxobacterium. J. Bacteriol. 81:130–138.

Anonymous. 2001. Notification that new names and new combinations have appeared in Volume 51, Part 3, of the IJSEM. Int. J. Syst. Evol. Microbiol. 51:1231–1233.

Bandi, C., G. Damiani, L. Magrassi, A. Grigolo, R. Fani, and L. Sacchi. 1994. Flavobacteria as intracellular symbionts in cockroaches. Proc. R. Soc. Lond. B 257:43–48.

Bano, N., and J. T. Hollibaugh. 2002. Phylogenetic composition of bacterioplankton assemblages from the Arctic Ocean. Appl. Environ. Microbiol. 68:505–518.

Barbeyron, T., S. L'Haridon, E. Corre, B. Kloareg, and P. Potin. 2001. Zobellia galactanovorans gen. nov., sp. nov., a marine species of Flavobacteriaceae isolated from a red alga, and reclassification of [Cytophaga] uliginosa (ZoBell and Upham, 1944) Reichenbach 1989 as Zobellia uliginosa gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol. 51:985–997.

Bergey, D. H., F. C. Harrison, R. S. Breed, B. W. Hammer, and F. M. Huntoon. 1923. Bergey's Manual of Determinative Bacteriology, 1st edn. Williams and Wilkins. Baltimore, MD.

Bernardet, J.-F. 1989a. Etude phénotypique et génomique des bactéries appartenant aux genres Cytophaga et Flexibacter (ordre des Cytophagales) et comparaison avec le genre Flavobacterium; application à l'identification et à la taxonomie des espèces ichtyopathogènes [PhD thesis]. Université Paris 7. Paris, France.

Bernardet, J.-F., and P. A. D. Grimont. 1989b. Deoxyribonucleic acid relatedness and phenotypic characterization of Flexibacter columnaris sp. nov., nom. rev., Flexibacter psychrophilus sp. nov., nom. rev., and Flexibacter maritimus Wakabayashi, Hikida, and Masumura 1986. Int. J. Syst. Bacteriol. 39:346–354.

Bernardet, J.-F., P. Segers, M. Vancanneyt, F. Berthe, K. Kersters, and P. Vandamme. 1996. Cutting a Gordian knot: emended classification and description of the genus Flavobacterium, emended description of the family Flavobacteriaceae, and proposal of Flavobacterium hydatis nom. nov. (basonym, Cytophaga aquatilis Strohl and Tait 1978). Int. J. Syst. Bacteriol. 46:128–148.

Bernardet, J.-F., Y. Nakagawa, and B. Holmes. 2002. Proposed minimal standards for describing new taxa of the family Flavobacteriaceae and emended description of the family. Int. J. Syst. Evol. Microbiol. 52:1049–1070.

Borg, A. F.. 1960. Studies on Myxobacteria Associated with Diseases in Salmonid Fishes. American Association for the Advancement of Science. Washington, DC. Wildlife Disease Series, No. 8.

Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. 62:1935–1943.

Bowman, J. P., S. A. McCammon, J. L. Brown, P. D. Nichols, and T. A. McMeekin. 1997a. Psychroserpens burtonensis gen. nov., sp. nov., and Gelidibacter algens gen. nov., sp. nov., psychrophilic bacteria isolated from Antarctic lacustrine and sea ice habitats. Int. J. Syst. Bacteriol. 47:670–677.

Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin. 1997b. Diversity and association of psychrophilic bacteria in Antarctic sea ice. Appl. Environ. Microbiol. 63:3068–3078.

Bowman, J. P., S. A. McCammon, T. Lewis, J. H. Skerratt, J. L. Brown, D. S. Nichols, and T. A. McMeekin. 1998. Psychroflexus torquis gen. nov., sp. nov., a psychrophilic species from Antarctic sea ice, and reclassification of Flavobacterium gondwanense (Dobson et al. 1993) as Psychroflexus gondwanense gen. nov. comb. nov. Microbiology 144:1601–1609.

Bowman, J. P., S. A. McCammon, T. Lewis, J. H. Skerratt, J. L. Brown, D. S. Nichols, and T. A. McMeekin. 1999. Psychroflexus gen. nov, Psychroflexus gondwanensis corrig., comb. nov. and Psychroflexus torquis sp. nov.: In validation of the publication of new names and new combinations previously effectively published outside the IJSB, List no. 68. Int. J. Syst. Bacteriol. 49:1–3.

Bowman, J. P. 2000. Description of Cellulophaga algicola sp. nov., isolated from the surface of Antarctic algae, and reclassification of Cytophaga uliginosa (ZoBell and Upham 1944) Reichenbach 1989 as Cellulophaga uliginosa comb. nov. Int. J. Syst. Evol. Microbiol. 50:1861–1868.

Bowman, J. P., and D. S. Nichols. 2002. Aequorivita gen. nov., a member of the family Flavobacteriaceae isolated from terrestrial and marine Antarctic habitats. Int. J. Syst. Evol. Microbiol. 52:1533–1541.

Bowman, J. P., C. Mancuso, P. D. Nichols, and J. A. E. Gibson. 2003. Algoriphagus ratkowskyi gen. nov., sp. nov., Brumimicrobium glaciale gen. nov., sp. nov., Cryomorpha ignava gen. nov., sp. nov. and Crocinitomix catalasitica gen. nov., sp. nov., novel flavobacteria isolated from various polar habitats. Int. J. Syst. Evol. Microbiol. 53:1343–1355.

Breed, R. S. 1957a. The genus Agarbacterium. *In:* R. S. Breed, E. G. D. Murray, and N. R. Smith (Eds.) Bergey's Manual of Determinative Bacteriology, 7th ed. Williams and Wilkins. Baltimore, MD. 322–328.

Breed, R. S. 1957b. The genus Pasteurella. *In:* R. S. Breed, E. G. D. Murray, and N. R. Smith (Eds.) Bergey's Manual of Determinative Bacteriology, 7th ed. Williams and Wilkins. Baltimore, MD. 395–402.

Brenner, D. J., D. G. Hollis, G. R. Fanning, and R. E. Weaver. 1989. Capnocytophaga canimorsus sp. nov. (formerly CDC group DF-2), a cause of septicemia following dog

bite, and C. cynodegmi sp. nov., a cause of localized wound infection following dog bite. J. Clin. Microbiol. 27:231–235.

Brisou, J., C. Tysset, A. Jacob, and L. Valette. 1960. Contribution à l'étude de deux germes du genre Flavobacterium saprophytes d'anguille d'eau douce (Anguilla vulgaris Turton). Arch. Inst. Pasteur Algérie 38:500–509.

Brümmer, I. H. M., W. Fehr, and I. Wagner-Döbler. 2000. Biofilm community structure in polluted rivers: abundance of dominant phylogenetic groups over a complete annual cycle. Appl. Environ. Microbiol. 66:3078–3082.

Bruner, D. W., and J. Fabricant. 1954. A strain of Moraxella anatipestifer (Pfeifferella anatipestifer) isolated from ducks. Cornell Vet. 44:461–464.

Bruns, A., M. Rohde, and L. Berthe-Corti. 2001. Muricauda ruestringensis gen. nov., sp. nov., a facultatively anaerobic, appendaged bacterium from German North Sea intertidal sediment. Int. J. Syst. Evol. Microbiol. 51:1997–2006.

Bruun, B., and J. Ursing. 1987. Phenotypic characterization of Flavobacterium meningosepticum strains identified by DNA-DNA hybridization. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 95:41–47.

Burchard, R. P. 1981. Gliding motility of prokaryotes: ultrastructure, physiology, and genetics. Ann. Rev. Microbiol. 35:497–529.

Burchard, R. P., and M. L. Sorongon. 1998. Agliding bacterium strain inhibits adhesion and motility of another gliding bacterium strain in a marine biofilm. Appl. Environ. Microbiol. 64:4079–4083.

Burchard, R. P. 1999. Beta-lactam antibiotic selection of nonswarming mutants of Flexibacter maritimus. Can. J. Microbiol. 45:786–790.

Campbell, L. L., and O. B. Williams. 1951. A study of chitin-decomposing micro-organisms of marine origin. J. Gen. Microbiol. 5:894–905.

Campbell, L. L. 1957. Genus Beneckea Campbell. *In:* R. S. Breed, E. G. D. Murray, and N. R. Smith (Eds.) Bergey's Manual of Determinative Bacteriology, 7th ed. Williams and Wilkins. Baltimore, MD. 328–332.

Cavalier-Smith, T. 2002. The neomuran origin of archaebacteria, the negibacterial root of the universal tree and bacterial megaclassification. Int. J. Syst. Evol. Microbiol. 52:7–76.

Chester, F. D. 1897. Report of the mycologist: bacteriological work. Del. Agr. Exp. Sta. Bull. 9:38–145.

Chester, F. D. 1901. A Manual of Determinative Bacteriology. Macmillan. New York, NY.

Cho, J.-C., and Giovannoni, S. J. 2003a. Croceibacter atlanticus gen. nov., sp. nov., a novel marine bacterium in the family Flavobacteriaceae. Syst. Appl. Microbiol. 26:76–83.

Cho, J.-C., and Giovannoni, S. J. 2003b. Croceibacter gen. nov., Croceibacter atlanticus sp. nov.: In validation of the publication of new names and new combinations previously effectively published outside the IJSEM, List no. 92. Int. J. Syst. Evol. Microbiol. 53:935–937.

Clark, J. W., and S. Kambhampati. 2003. Phylogenetic analysis of Blattabacterium, endosymbiotic bacteria from the wood roach, Cryptocercus (Blattodea: Cryptocercidae), including a description of three new species. Molec. Phylogen. Evol. 26:82–88.

Clayton, R. A., G. Sutton, P. S. Hinkle Jr., C. Bult, and C. Fields. 1995. Intraspecific variation in small-subunit rRNA sequences in GenBank: why single sequences may not adequately represent prokaryotic taxa. Int. J. Syst. Bacteriol. 45:595–599.

Colwell, R. R., R. V. Citarella, and P. K. Chen. 1966. DNA base composition of Cytophaga marinoflava n. sp. determined by buoyant density measurements in cesium chloride. Can. J. Microbiol. 12:1099–1103.

Cottrell, M. T., and D. L. Kirchman. 2000. Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. Appl. Environ. Microbiol. 66:5116–5122.

Crump, B. C., E. V. Armbrust, and J. A. Baross. 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. Appl. Environ. Microbiol. 65:3192–3204.

Dasch, G. A., E. Weiss, and K.-P. Chang. 1984. Genus Blattabacterium Hollande and Favre 1931. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:830–831.

Davis, H. S. 1922. A new bacterial disease of fresh-water fishes. Bull. US Bur. Fish. 38:261–280.

Decostere, A., F. Haesebrouck, J. F. Turnbull, and G. Charlier. 1999a. Influence of water quality and temperature on adhesion of high and low virulence Flavobacterium columnare strains to isolated gill arches. J. Fish Dis. 22:1–11.

Decostere, A., F. Haesebrouck, E. Van Driessche, G. Charlier, and R. Ducatelle. 1999b. Characterization of the adhesion of Flavobacterium columnare (Flexibacter columnaris) to gill tissue. J. Fish Dis. 22:465–474.

Dees, S. B., C. W. Moss, D. G. Hollis, and R. E. Weaver. 1986. Chemical characterization of Flavobacterium odoratum, Flavobacterium breve, and Flavobacterium-like groups IIe, IIh, and IIf. J. Clin. Microbiol. 23:267–273.

Dobson, S. J., R. R. Colwell, T. A. McMeekin, and P. D. Franzmann. 1993. Direct sequencing of the polymerase chain reaction-amplified 16S rRNA gene of Flavobacterium gondwanense sp.nov. and Flavobacterium salegens sp.nov., two new species from a hypersaline Antarctic lake. Int. J. Syst. Bacteriol. 43:77–83.

Dorey, M. J. 1959. Some properties of a pectinolytic soil Flavobacterium. J. Gen. Microbiol. 20:91–104.

Eilers, H., J. Pernthaler, F. O. Glöckner, and R. Amann. 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. 66:3044–3051.

Euzéby, J. P. 1997. List of bacterial names with standing in nomenclature: A folder available on the Internet. Int. J. Syst. Bacteriol. 47:590–592.

Fautz, E., and H. Reichenbach. 1980. A simple test for flexirubin-type pigments. FEMS Microbiol. Lett. 8:87–91.

Felsenstein, J. 1985. Confidence limits of phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Frankland, G. C., and P. F. Frankland. 1889. Ueber einige typische Mikroorganismen im Wasser und im Boden. Z. Hyg. 6:373–400.

Garnjobst, L. 1945. Cytophaga columnaris (Davis) in pure culture: a myxobacterium pathogenic to fish. J. Bacteriol. 49:113–128.

Gherna, R., and C. R. Woese. 1992. A partial phylogenetic analysis of the "Flavobacter-Bacteroides" phylum: Basis for taxonomic restructuring. Syst. Appl. Microbiol. 15:513–521.

Gosink, J. J., C. R. Woese, and J. T. Staley. 1998. Polaribacter gen. nov., with three new species, P. irgensii sp. nov., P. franzmannii sp. nov. and P. filamentus sp. nov., gas vacuolate polar marine bacteria of the Cytophaga-Flavobacterium-Bacteroides group and reclassification of "Flectobacillus glomeratus" as Polaribacter glomeratus comb. nov. Int. J. Syst. Bacteriol. 48:223–235.

Gupta, R. S. 2000. The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. FEMS Microbiol. Rev. 24:367–402.

Gutell, R. R., B. Weiser, C. R. Woese, and H. F. Noller. 1985. Comparative anatomy of 16S-like ribosomal RNA. Progr. Nucleic Acid Res. Molec. Biol. 32:155–216.

Haack, S. K., and J. A. Breznak. 1993. Cytophaga xylanolytica sp. nov., a xylan-degrading, anaerobic gliding bacterium. Arch. Microbiol. 159:6–15.

Hamana, K., and Y. Nakagawa. 2001a. Polyamine distribution profiles in the eighteen genera phylogenetically located within the Flavobacterium-Flexibacter-Cytophaga complex. Microbios 106:7–17.

Hamana, K., and Y. Nakagawa. 2001b. Polyamine distribution profiles in newly validated genera and species within the Flavobacterium-Flexibacter-Cytophaga-Sphingobacterium complex. Microbios 106, S2:105–116.

Hansen, G. H., Ø. Bergh, J. Michaelsen, and D. Knappskog. 1992. Flexibacter ovolyticus sp.nov., a pathogen of eggs and larvae of Atlantic halibut, Hippoglossus hippoglossus L. Int. J. Syst. Bacteriol. 42:451–458.

Hanzawa, N., S. Kanai, A.Katsuta, Y. Nakagawa, and K. Yamasato. 1995. 16S rDNA-based phylogenetic analysis of marine flavobacteria. J. Mar. Biotechnol. 3:111–114.

Harrison, F. C. 1929. The discolouration of halibut. Can. J. Res. 1:214–239.

Hendrickson, J. M., and K. F. Hilbert. 1932. A new and serious septicemic disease of young ducks with the description of the causative organism, Pfeifferella anatipestifer. Cornell Vet. 22:239–252.

Henrichsen, J. 1972. Bacterial surface translocation: a survey and a classification. Bacteriol. Rev. 36:478–503.

Hikida, M., H. Wakabayashi, S. Egusa, and K. Masumura. 1979. Flexibacter sp., a gliding bacterium pathogenic to some marine fishes in Japan. Bull. Jpn. Soc. Sci. Fish. 45:421–428.

Holmes, B., J. J. S. Snell, and S. P. Lapage. 1977. Revised description, from clinical isolates, of Flavobacterium odoratum Stutzer and Kwaschnina 1929, and designation of the neotype strain. Int. J. Syst. Bacteriol. 27:330–336.

Holmes, B., J. J. S. Snell, and S. P. Lapage. 1978. Revised description, from clinical strains, of Flavobacterium breve (Lustig) Bergey et al. 1923 and proposal of the neotype strain. Int. J. Syst. Bacteriol. 28:201–208.

Holmes, B., and R. J. Owen. 1982. Flavobacterium breve sp. nov., nom. rev. Int. J. Syst. Bacteriol. 32:233–234.

Holmes, B., R. J. Owen, and T. A. McMeekin. 1984a. Genus Flavobacterium Bergey, Harrison, Breed, Hammer and Huntoon 1923, 97AL. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:353–361.

Holmes, B., R. J. Owen, A. G. Steigerwalt, and D. J. Brenner. 1984b. Flavobacterium gleum, a new species found in human clinical specimens. Int. J. Syst. Bacteriol. 34:21–25.

Holmes, B., A. G. Steigerwalt, R. E. Weaver, and D. J. Brenner. 1986a. Weeksella virosa gen. nov., sp. nov. (formerly group IIf), found in human clinical specimens. Syst. Appl. Microbiol. 8:185–190.

Holmes, B., A. G. Steigerwalt, R. E. Weaver, and D. J. Brenner. 1986b. Weeksella zoohelcum sp.nov. (formerly group IIj), from human clinical specimens. Syst. Appl. Microbiol. 8:191–196.

Holmes, B. 1997. International Committee on Systematic Bacteriology, minutes of the meetings of the Subcommittee on the Taxonomy of Flavobacterium and Cytophaga-like bacteria in Prague. Int. J. Syst. Bacteriol. 47:593–594.

Holt, R. A. 1988. Cytophaga psychrophila, the Causative Agent of Bacterial Cold-water Disease in Salmonid Fish [PhD thesis]. Oregon State University. Corvallis, OR.

Holt, S. C., and S. A. Kinder. 1989. Genus Capnocytophaga Leadbetter, Holt and Socransky 1982. *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 3:2050–2058.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180:4765–4774.

Hugo, C. J. 1997. A Taxonomic Study of the Genus Chryseobacterium from Food and Environmental Sources [PhD thesis]. University of the Orange Free State. Bloemfontein, South Africa.

Hugo, C. J., P. Segers, B. Hoste, M. Vancanneyt, and K. Kersters. 2003. Chryseobacterium joostei sp. nov., isolated from the dairy environment. Int. J. Syst. Evol. Microbiol. 53:771–777.

Humphry, D. R., A. George, G. W. Black, and S. P. Cummings. 2001. Flavobacterium frigidarium sp. nov., an aerobic, psychrophilic, xylanolytic and laminarinolytic bacterium from Antarctica. Int. J. Syst. Evol. Microbiol. 51:1235–1243.

Hunnicutt, D. W., M. J. Kempf, and M. J. McBride. 2002. Mutations in Flavobacterium johnsoniae gldF and gldG disrupt gliding motility and interfere with membrane localization of gldA. J. Bacteriol. 184:2370–2378.

Hurst, G. D. D., T. C. Hammarton, C. Bandi, T. M. O. Majerus, D. Bertrand, and M. E. N. Majerus. 1997. The diversity of inherited parasites of insects: the male-killing agent of the ladybird beetle Coleomegilla maculata is a member of the Flavobacteria. Genet. Res., Camb. 70:1–6.

Hurst, G. D. D., C. Bandi, L. Sacchi, A. G. Cochrane, D. Bertrand, I. Karaca, and M. E. N. Majerus. 1999. Adonia variegata (Coleoptera: Coccinellidae) bears maternally inherited Flavobacteria that kill males only. Parasitology 118:125–134.

Inoue, K., and K. Komagata. 1976. Taxonomic study on obligately psychrophilic bacteria isolated from Antarctica. J. Gen. Appl. Microbiol. 22:165–176.

Ivanova, E. P., O. I. Nedashkovskaya, J. Chun, A. M. Lysenko, G. M. Frolova, V. I. Svetashev, M. V. Vysotskii, V. V. Mikhailov, A. Huq, and R. R. Colwell. 2001. Arenibacter gen. nov., new genus of the family Flavobacteriaceae and description of a new species, Arenibacter latericius sp. nov. Int. J. Syst. Evol. Microbiol. 51:1987–1995.

Jaspers, E., K. Nauhaus, H. Cypionka, and J. Overmann. 2001. Multitude and temporal variability of ecological

niches as indicated by the diversity of cultivated bacterioplankton. FEMS Microbiol. Ecol. 36:153–164.

Johansen, J. E., P. Nielsen, and C. Sjøholm. 1999. Description of Cellulophaga baltica gen. nov., sp. nov. and Cellulophaga fucicola gen. nov., sp. nov. and reclassification of [Cytophaga] lytica to Cellulophaga lytica gen. nov., comb. nov. Int. J. Syst. Bacteriol. 49:1231–1240.

Johansen, J. E., and S. J. Binnerup. 2002. Contribution of Cytophaga-like bacteria to the potential of turnover of carbon, nitrogen, and phosphorus by bacteria in the rhizosphere of barley (Hordeum vulgare L.). Microb. Ecol. 43:298–306.

Jooste, P. J. 1985. The Taxonomy and Significance of Flavobacterium-Cytophaga Strains from Dairy Sources [PhD thesis]. University of the Orange Free State. Bloemfontein, South Africa.

Jooste, P. J., and C. J. Hugo. 1999. The taxonomy, ecology and cultivation of bacterial genera belonging to the family Flavobacteriaceae. Int. J. Food Microbiol. 53:81–94.

Kaiser, P. 1961. Etude de l'activité pectinolytique du sol et d'autres substrats naturels [thesis]. Université de Paris. Paris, France.

Kämpfer, P., U. Dreyer, A. Neef, W. Dott, and H.-J. Busse. 2003. Chryseobacterium defluvii sp. nov., isolated from wastewater. Int. J. Syst. Evol. Microbiol. 53:93–97.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Molec. Evol. 16:111–120.

King, E. O. 1959. Studies on a group of previously unclassified bacteria associated with meningitis in infants. Am. J. Clin. Pathol. 31:241–247.

Kirchman, D. L. 2002. The ecology of Cytophaga-Flavobacteria in aquatic environments. FEMS Microbiol. Ecol. 39:91–100.

Krasil'nikov, N. A. 1949. Guide to the Bacteria and Actinomycetes. Akad Nauk SSSR. Moscow, Russia.

Larkin, J. M. 1989. Nonphotosynthetic, nonfruiting gliding bacteria. *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 3:2010–2138.

Leadbetter, E. R. 1974. Order II: Cytophagales Nomen novum. *In:* R. E. Buchanan and N. E. Gibbons (Eds.) Bergey's Manual of Determinative Bacteriology, 8th ed. Williams and Wilkins. Baltimore, MD. 99–122.

Leadbetter, E. R., S. C. Holt, and S. S. Socransky. 1979. Capnocytophaga: new genus of Gram-negative gliding bacteria. I: General characteristics, taxonomic considerations and significance. Arch. Microbiol. 122:9–16.

Lewin, R. A. 1969. A classification of Flexibacteria. J. Gen. Microbiol. 58:189–206.

Lewin, R. A. and D. M. Lounsbery. 1969. Isolation, cultivation and characterization of flexibacteria. J. Gen. Microbiol. 58:145–170.

London, J., R. A. Celesk, A. Kagermeier and J. L. Johnson. 1985. Emended description of Capnocytophaga gingivalis. Int. J. Syst. Bacteriol. 35:369–370.

Lustig, A. 1890. Diagnostica dei batteri delle acque con una guida alle ricerche batteriologiche e microscopiche. Rosenberg and Sellier. Torino, Italy.

Macián, M. C., M. J. Pujalte, M. C. Márquez, W. Ludwig, A. Ventosa, E. Garay, and K.-H. Schleifer. 2002. Gelidibacter mesophilus sp. nov., a novel marine bacterium in the family Flavobacteriaceae. Int. J. Syst. Evol. Microbiol. 52:1325–1329.

Maidak, B. L., J. R. Cole, C. T. Jr. Parker, G. M. Garrity, N. Larsen, B. Li, T. G. Lilburn, M. J. McCaughey, G. J. Olsen, R. Overbeek, S. Pramanik, T. M. Schmidt, J. M. Tiedje, and C. R. Woese. 1999. A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res. 27:171–173.

Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K.-H. Schleifer. 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga-Flavobacter-Bacteroides in the natural environment. Microbiology 142:1097–1106.

Manz, W. K. Wendt-Potthoff, T. R. Neu, U. Szewzyk, and J. R. Lawrence. 1999. Phylogenetic composition, spatial structure, and dynamics of lotic bacterial biofilms investigated by fluorecent in situ hybridization and confocal laser scanning microscopy. Microb. Ecol. 37:225–237.

McBride, M. J., and S. A. Baker. 1996. Development of techniques to genetically manipulate members of the genera Cytophaga, Flavobacterium, Flexibacter, and Sporocytophaga. Appl. Environ. Microbiol. 62:3017–3022.

McBride, M. J., and M. J. Kempf. 1996. Development of techniques for the genetic manipulation of the gliding bacterium Cytophaga johnsonae. J. Bacteriol. 178:583–590.

McBride, M. J. 2001. Bacterial gliding motility: multiple mechanisms for cell movement over surfaces. Ann. Rev. Microbiol. 55:49–75.

McCammon, S. A., B. H. Innes, J. P. Bowman, P. D. Franzmann, S. J. Dobson, P. E. Holloway, J. H. Skerratt, P. D. Nichols, and L. M. Rankin. 1998. Flavobacterium hibernum sp. nov., a lactose-utilizing bacterium from a freswater Antarctic lake. Int. J. Syst. Bacteriol. 48:1405–1412.

McCammon, S. A., and J. P. Bowman. 2000. The taxonomy of Antarctic Flavobacterium species: description of Flavobacterium gillisiae sp. nov., Flavobacterium tegetincola sp. nov., and Flavobacterium xanthum sp. nov., nom. rev. and reclassification of [Flavobacterium] salegens as Salegentibacter salegens gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol. 50:1055–1063.

McGrath, C. F., C. W. Moss, and R. P. Burchard. 1990. Effect of temperature shifts on gliding motility, adhesion, and fatty acid composition of Cytophaga sp. strain U67. J. Bacteriol. 172:1978–1982.

McGuire, A. J., P. D. Franzmann, and T. A. McMeekin. 1987. Flectobacillus glomeratus sp.nov., a curved, nonmotile, pigmented bacterium isolated from Antartic marine environments. Syst. Appl. Microbiol. 9:265–272.

Moore, W. E. C., E. P. Cato, and L. V. H. Moore. 1985. Index of the bacterial and yeast nomenclatural changes published in the International Journal of Systematic Bacteriology since the 1980 approved lists of bacterial names (1 January 1980 to 1 January 1985). Int. J. Syst. Bacteriol. 35:382–407.

Mudarris, M., B. Austin, P. Segers, M. Vancanneyt, B. Hoste, and J.-F. Bernardet. 1994. Flavobacterium scophthalmum sp.nov., a pathogen of turbot (Scophthalmus maximus L.). Int. J. Syst. Bacteriol. 44:447–453.

Nakagawa, Y., and K. Yamasato. 1993. Phylogenetic diversity of the genus Cytophaga revealed by 16S rRNA sequencing and menaquinone analysis. J. Gen. Microbiol. 139:1155–1161.

Nakagawa, Y., and K. Yamasato. 1996. Emendation of the genus Cytophaga and transfer of Cytophaga agaro-

vorans and Cytophaga salmonicolor to Marinilabilia gen. nov.: Phylogenetic analysis of the Flavobacterium-Cytophaga complex. Int. J. Syst. Bacteriol. 46:599–603.

Nakagawa, Y., M. Suzuki, and K. Hatano. 2001. Phylogenetic diversity of Cytophaga-like strains isolated from the subtropical zone of Japan. IFO Res. Commun. 20:61–71.

Nakagawa, Y., T. Sakane, M. Suzuki, and K. Hatano. 2002. Phylogenetic structure of the genera Flexibacter, Flexithrix, and Microscilla deduced from 16S rRNA sequence analysis. J. Gen. Appl. Microbiol. 48:155–165.

Nedashkovskaya, O. I., S. B. Kim, S. K. Han, A. M. Lysenko, M. Rohde, N. Zhukova, E. Falsen, G. M. Frolova, V. V. Mikhailov, and K. S. Bae. 2003a. Mesonia algae gen. nov., sp. nov., a novel marine bacterium of the family Flavobacteriaceae isolated from the green alga Acrosiphonia sonderi (Kütz) Kornm. Int. J. Syst. Evol. Microbiol. 53:1967–1971.

Nedashkovskaya, O. I., S. B. Kim, S. K. Han, M. S. Rhee, A. M. Lysenko, E. Falsen, G. M. Frolova, V. V. Mikhailov, and K. S. Bae. 2003b. Ulvibacter litoralis gen. nov., sp. nov., a novel member of the family Flavobacteriaceae isolated from the green alga Ulva fenestrans. Int. J. Syst. Evol. Microbiol. 54:119–123.

Nedashkovskaya, O. I., M. Suzuki, M. V. Vysotskii, and V. V. Mikhailov. 2003c. Arenibacter troitsensis sp. nov., isolated from marine bottom sediment. Int. J. Syst. Evol. Microbiol. 53:1287–1290.

Nedashkovskaya, O. I., M. Suzuki, M. V. Vysotskii, and V. V. Mikhailov. 2003d. Vitellibacter vladivostokensis gen. nov., sp. nov., a new member of the phylum Cytophaga-Flavobacterium-Bacteroides. Int. J. Syst. Evol. Microbiol. 53:1281–1286.

Olsen, G. J., C. R. Woese, and R. Overbeek. 1994. The wind of (evolutionary) change: Breathing new life into microbiology. J. Bacteriol. 176:1–6.

Olsen, I., and E. Jantzen. 2001. Sphingolipids in bacteria and fungi. Anaerobe 7:103–112.

Ordal, E. J., and R. R. Rucker. 1944. Pathogenic Myxobacteria. Proc. Soc. Exp. Biol. Med. 56:15–18.

Ostland, V. E., J. S. Lumsden, D. D. MacPhee, and H. W. Ferguson. 1994. Characteristics of Flavobacterium branchiophilum, the cause of salmonid bacterial gill disease in Ontario. J. Aquat. Anim. Health 6:13–26.

O'Sullivan, L. A., A. J. Weightman, and J. C. Fry. 2002. New degenerate Cytophaga-Flexibacter-Bacteroides-specific 16S ribosomal DNA-targeted oligonucleotide probes reveal high bacterial diversity in River Taff epilithon. Appl. Environ. Microbiol. 68:201–210.

Otis, E. J. 1984. Lesions of Coldwater Disease in Steelhead Trout (Salmo gairdneri): The Role of Cytophaga psychrophila Extracellular Products [MSc thesis]. University of Rhode Island. Kingston, RI.

Oyaizu, H., and K. Komagata. 1981. Chemotaxonomic and phenotypic characterization of the strains of species in the Flavobacterium-Cytophaga complex. J. Gen. Appl. Microbiol. 27:57–107.

Paster, B. J., W. Ludwig, W. G. Weisburg, E. Stackebrandt, R. B. Hespell, C. M. Hahn, H. Reichenbach, K. O. Stetter, and C. R. Woese. 1985. A phylogenetic grouping of the bacteroides, cytophagas, and certain flavobacteria. Syst. Appl. Microbiol. 6:34–42.

Paster, B. J., F. E. Dewhirst, I. Olsen, and G. J. Fraser. 1994. Phylogeny of Bacteroides, Prevotella, and Porphyromonas spp. and related bacteria. J. Bacteriol. 176:725–732.

Potin, P., A. Sanséau, Y. Le Gall, C. Rochas, and B. Kloareg. 1991. Purification and characterization of a new κ-carrageenase from a marine Cytophaga-like bacterium. Eur. J. Biochem. 201:241–247.

Prévot, A. R. 1961. Traité de systématique bactérienne. Dunod. Paris, France.

Raj, H. D., and S. R. Maloy. 1990. Family Spirosomaceae: Gram-negative ring-forming aerobic bacteria. Crit. Rev. Microbiol. 17:329–364.

Ravenschlag, K., K. Sahm, and R. Amann. 2001. Quantitative molecular analysis of the microbial community in marine Arctic sediments (Svalbard). Appl. Environ. Microbiol. 67:387–395.

Reichenbach, H., and M. Dworkin. 1981a. Introduction to the gliding bacteria. *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (Eds.) The Prokaryotes, 1st ed. Springer-Verlag. Berlin, Germany. 1:315–327.

Reichenbach, H., and M. Dworkin. 1981b. The Order Cytophagales (with addenda on the genera Herpetosiphon, Saprospira, and Flexithrix). *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (Eds.) The Prokaryotes, 1st ed. Springer-Verlag. Berlin, Germany. 1:356–379.

Reichenbach, H. 1989. Order I: Cytophagales Leadbetter 1974. *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 3:2011–2013.

Reichenbach, H. 1992. Flavobacteriaceae fam. nov.: Validation of the publication of new names and new combinations previously effectively published outside the IJSB, List no. 41. Int. J. Syst. Bacteriol. 42:327–329.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molec. Biol. Evol. 4:406–425.

Sakai, T., H. Kimura, and I. Kato. 2002. A marine strain of Flavobacteriaceae utilizes brown seaweed fucoidan. Mar. Biotechnol. 4:399–405.

Segers, P., W. Mannheim, M. Vancanneyt, K. De Brandt, K.-H. Hinz, K. Kersters, and P. Vandamme. 1993. Riemerella anatipestifer gen. nov., the causative agent of septicemia anserum exsudativa, and its phylogenetic affiliation within the Flavobacterium-Cytophaga rRNA homology group. Int. J. Syst. Bacteriol. 43:768–776.

Skerman, V. B. D., V. McGowan, and P. H. A. Sneath (Eds.). 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225–420.

Stanier, R. Y. 1947. Studies on non-fruiting myxobacteria. I: Cytophaga johnsonae n. sp., a chitin-decomposing myxobacterium. J. Bacteriol. 53:297–315.

Stanier, R. Y. 1957. Order VIII. Myxobacterales Jahn 1915. *In:* R. S. Breed, E. G. D. Murray, and N. R. Smith (Eds.) Bergey's Manual of Determinative Bacteriology, 7th ed. Williams and Wilkins. Baltimore, MD. 854–891.

Steyn, P. L., P. Segers, M.Vancanneyt, P. Sandra, K. Kersters, and J. J. Joubert. 1998. Classification of heparinolytic bacteria into a new genus, Pedobacter, comprising four species: Pedobacter heparinus comb. nov., Pedobacter piscium comb. nov., Pedobacter africanus sp. nov. and Pedobacter saltans sp. nov. Proposal of the family Sphingobacteriaceae fam. nov. Int. J. Syst. Bacteriol. 48:165–177.

Strohl, W. R., and L. R. Tait. 1978. Cytophaga aquatilis sp.nov., a facultative anaerobe isolated from the gills of freshwater fish. Int. J. Syst. Bacteriol. 28:293–303.

Stutzer, M., and A. Kwaschnina. 1929. Aussaaten aus den Fäzes des Menschen gelbe Kolonien bildende Bakterien (Gattung Flavobacterium u.a.). Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I. Orig. 113:219–225.

Suzuki, M., Y. Nakagawa, S. Harayama, and S. Yamamoto. 2001. Phylogenetic analysis and taxonomic study of marine Cytophaga-like bacteria: Proposal for Tenacibaculum gen. nov. with Tenacibaculum maritimum comb. nov. and Tenacibaculum ovolyticum comb. nov., and description of Tenacibaculum mesophilum sp. nov. and Tenacibaculum amylolyticum sp. nov. Int. J. Syst. Evol. Microbiol. 51:1639–1652.

Tamaki, H., S. Hanada, Y. Kamagata, K. Nakamura, N. Nomura, K. Nakano, and M. Matsumura. 2003. Flavobacterium limicola sp. nov., a psychrophilic, organic-polymer-degrading bacterium isolated from freshwater sediments. Int. J. Syst. Evol. Microbiol. 53:519–526.

Teska, J. D. 1993. Assay to evaluate the reaction kinetics of chondroitin AC lyase produced by Cytophaga columnaris. J. Aquat. Anim. Health 5:259–264.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680.

Topley, W. W. C., and G. S. Wilson. 1929. The Principles of Bacteriology and Immunity, 1st ed. Edward Arnold. London, UK.

Triyanto, and H. Wakabayashi. 1999. Genotypic diversity of strains of Flavobacterium columnare from diseased fishes. Fish Pathol. 34:65–71.

Ursing, J., and B. Bruun. 1991. Genetic heterogeneity of Flavobacterium group IIb and Flavobacterium breve demonstrated by DNA-DNA hybridization. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 99:780–786.

Vancanneyt, M., P. Segers, U. Torck, B. Hoste, J.-F. Bernardet, P. Vandamme, and K. Kersters. 1996. Reclassification of Flavobacterium odoratum (Stutzer 1929) strains to a new genus, Myroides, as Myroides odoratus comb. nov. and Myroides odoratimimus sp. nov. Int. J. Syst. Bacteriol. 46:926–932.

Vancanneyt, M., P. Vandamme, P. Segers, U. Torck, R. Coopman, K. Kersters, and K.-H. Hinz. 1999. Riemerella columbina sp. nov., a bacterium associated with respiratory disease in pigeons. Int. J. Syst. Bacteriol. 49:289–295.

Vandamme, P., J.-F. Bernardet, P. Segers, K. Kersters, and B. Holmes. 1994a. New perspectives in the classification of the flavobacteria: description of Chryseobacterium gen. nov., Bergeyella gen. nov., and Empedobacter nom. rev. Int. J. Syst. Bacteriol. 44:827–831.

Vandamme, P., P. Segers, M. Vancanneyt, K. Van Hove, R. Mutters, J. Hommez, F. Dewhirst, B. Paster, K. Kersters, E. Falsen, L. A. Devriese, M. Bisgaard, K.-H. Hinz, and W. Mannheim. 1994b. Ornithobacterium rhinotracheale gen. nov., sp.nov., isolated from the avian respiratory tract. Int. J. Syst. Bacteriol. 44:24–37.

Vandamme, P., M. Vancanneyt, A. van Belkum, P. Segers, W. G. V. Quint, K. Kersters, B. Paster, and F. Dewhirst. 1996. Polyphasic analysis of strains of the genus Capnocytophaga and Center for Disease Control group DF-3. Int. J. Syst. Bacteriol. 46:782–791.

Vandamme, P., M. Vancanneyt, P. Segers, M. Ryll, B. Köhler, W. Ludwig, and K.-H. Hinz. 1999. Coenonia anatina gen. nov., sp. nov., a novel bacterium associated with respiratory disease in ducks and geese. Int. J. Syst. Bacteriol. 49:867–874.

van der Meulen, H. J., W. Harder, and H. Veldkamp. 1974. Isolation and characterization of Cytophaga flevensis sp. nov., a new agarolytic flexibacterium. Ant. v. Leeuwenhoek 40:329–346.

Van Trappen, S., J. Mergaert, S. Van Eygen, P. Dawynt, M. C. Cnockaert, and J. Swings. 2002. Diversity of 746 heterotrophic bacteria isolated from microbial mats from ten Antarctic lakes. Syst. Appl. Microbiol. 25:603–610.

Van Trappen, S., J. Mergaert, and J. Swings. 2003a. Flavobacterium gelidilacus sp. nov., isolated from microbial mats in Antarctic lakes. Int. J. Syst. Evol. Microbiol. 53:1241–1245.

Van Trappen, S., I. Vandecandelaere, J. Mergaert and J. Swings. 2003b. Flavobacterium degerlachei sp. nov., Flavobacterium frigoris sp. nov. and Flavobacterium micromati sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes. Int. J. Syst. Evol. Microbiol. 54:85–92.

Wakabayashi, H., M. Hikida, and K. Masumura. 1986. Flexibacter maritimus sp. nov., a pathogen of marine fishes. Int. J. Syst. Bacteriol. 36:396–398.

Wakabayashi, H., G. J. Huh, and N. Kimura. 1989. Flavobacterium branchiophila sp.nov., a causative agent of bacterial gill disease of freshwater fishes. Int. J. Syst. Bacteriol. 39:213–216.

Weeks, O. B. 1974. Genus Flavobacterium Bergey et al. 1923. *In:* Buchanan and N. E. Gibbons (Eds.) Bergey's Manual of Determinative Bacteriology, 8th edition. Williams and Wilkins. Baltimore, MD. 357–364.

Weeks, O. B. 1981. Preliminary studies of the pigments of Flavobacterium breve NCTC 11099 and Flavobacterium odoratum NCTC 11036. *In:* H. Reichenbach and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga group. Gesellschaft für Biotechnologische Forschung. Weinheim, Germany. 108–114.

Weeks, A. R., F. Marec, and J. A. J. Breeuwer. 2001. A mite species that consists entirely of haploid females. Science 292:2479–2482.

Whiteley, A. S., and M. J. Bailey. 2000. Bacterial community structure and physiological state within an industrial phenol bioremediation system. Appl. Environ. Microbiol. 66:2400–2407.

Yabuuchi, E., T. Kaneko, I. Yano, C. W. Moss, and N. Miyoshi. 1983. Sphingobacterium gen. nov., Sphingobacterium spiritivorum comb. nov., Sphingobacterium multivorum comb. nov., Sphingobacterium mizutae sp. nov., and Flavobacterium indologenes sp. nov.: Glucose-nonfermenting Gram-negative rods in CDC groups IIk-2 and IIb. Int. J. Syst. Bacteriol. 33:580–598.

Yamaguchi, S. and M. Yokoe. 2000. A novel protein-deamidating enzyme from Chryseobacterium proteolyticum sp. nov., a newly isolated bacterium from soil. Appl. Environ. Microbiol. 66:3337–3343.

Yamamoto, T., S. Kajiura, Y. Hirai, and T. Watanabe. 1994. Capnocytophaga haemolytica sp. nov. and Capnocytophaga granulosa sp. nov., from human dental plaque. Int. J. Syst. Bacteriol. 44:324–329.

Yamamoto, S., and S. Harayama. 1996. Phylogenetic analysis of Acinetobacter strains based on the nucleotide sequences of gyrB genes and on the amino acids sequences of their products. Int. J. Syst. Bacteriol. 46:506–511.

Zchori-Fein, E., Y. Gottlieb, S. E. Kelly, J. K. Brown, J. M. Wilson, T. L. Karr, and M. S. Hunter. 2001. A

newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. Proc. Natl. Acad. Sci. USA 98:12555–12560.

Zhu, F., S. Wang, and P. Zhou. 2003. Flavobacterium xinjiangense sp. nov. and Flavobacterium omnivorum sp. nov., novel psychrophiles from the China no. 1 glacier. Int. J. Syst. Evol. Microbiol. 53:853–857.

ZoBell, C. E., and H. C. Upham. 1944. A list of marine bacteria including descriptions of sixty new species. Bull. Scripps Inst. Oceanogr. Univ. Calif. (Tech. Ser.) 5:329–292.

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CHAPTER 6.4

The Genus *Flavobacterium*

JEAN-FRANÇOIS BERNARDET AND JOHN P. BOWMAN

Introduction

The genus *Flavobacterium* Bergey, Harrison, Breed, Hammer, and Huntoon 1923, emend. Bernardet, Segers, Vancanneyt, Berthe, Kersters, and Vandamme 1996 (Bergey et al., 1923; Bernardet et al., 1996) is probably one of the best examples of the revolution brought to the classification of a bacterial taxon by the use of the phylogenetic techniques based on the comparison of 16S rRNA sequences. Of the seven species considered in the previous edition of *Bergey's Manual of Systematic Bacteriology* (Holmes et al., 1984), only the type species *F. aquatile* was finally retained while several other taxa, previously misclassified in other genera, were added to form an extensively emended genus *Flavobacterium* (Bernardet et al., 1996). Since then, the genus has considerably expanded owing to the description of many new species mostly originating from polar habitats. The organisms currently included in the genus *Flavobacterium* were distributed among different chapters in previous editions of *Bergey's Manual* and of *The Prokaryotes*. In *Bergey's Manual*, *Flavobacterium aquatile* was dealt with in the chapter "Genus *Flavobacterium*" (Holmes et al., 1984), while several other *Flavobacterium* species were considered (under other generic epithets) in the chapter "Order I. *Cytophagales*" (Reichenbach, 1989) in the second edition of *The Prokaryotes*. The latter species were also included in the chapter The Order Cytophagales in the second edition, while *F. aquatile* was excluded from the chapter The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* also from this Volume and not considered anywhere else! These chapters contain a wealth of information and are still well worth consulting.

The history and structure of the family Flavobacteriaceae, of which the genus *Flavobacterium* is the type genus, are presented in the chapter "An Introduction to the Family Flavobacteriaceae" in this Volume. The taxonomic and nomenclatural issues concerning the genus *Flavobacterium* are dealt with by the subcommittee on the taxonomy of *Flavobacterium* and *Cytophaga*-like bacteria of the International Committee on Systematics of Prokaryotes. This subcommittee has issued minimal standards for the description of new taxa in the genus *Flavobacterium* and other genera in the family Flavobacteriaceae (Bernardet et al., 2002).

Few species in the genus *Flavobacterium* have been extensively studied and most of them are represented by one strain (i.e., the type species *F. antarcticum* as well as *F. aquatile*, *F. flevense*, *F. frigidarium*, *F. gillisiae*, *F. granuli*, *F. hydatis*, *F. omnivorum*, *F. pectinovorum*, *F. saccharophilum*, *F. xanthum* and *F. xinjiangense*) or a limited number of strains in culture collections. Exceptions are the fish-pathogenic species, of which many strains are usually available; owing to their economic significance worldwide, they have been the subject of numerous studies (see the sections "Habitat and Ecology" and "Pathogenicity and Epidemiology" in this Chapter) and will provide many examples in this chapter.

Phylogeny

Sequences of 16S rRNA and DNA gyrase large subunit (*gyrB*) genes (Weisburg et al., 1985; Woese et al., 1990; Nakagawa and Yamasato, 1993; Suzuki et al., 2001) locate the genus *Flavobacterium* within the phylum "Bacteroidetes," clustering appropriately in a central position in the family Flavobacteriaceae (Bernardet et al., 1996) where it represents the type genus. Within the family Flavobacteriaceae, the genus *Flavobacterium* branches between two primary lineages, one consisting of mostly marine genera and the other of non-marine genera (see the chapters An Introduction to the Family Flavobacteriaceae in this Volume and The Marine Clade of the Family Flavobacteriaceae: The Genera *Aequorivita*, *Arenibacter*, *Cellulophaga*, *Croceibacter*, *Formosa*, *Gelidibacter*, *Gillisia*, *Maribacter*, *Mesonia*, *Muricauda*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Robiginitalea*, *Salegentibacter*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter* and *Zobellia* in this Volume). This

intermediate position corresponds well with the ecophysiology of the genus, which is relatively diverse (including species from freshwater and from marine ecosystems; see the section Habitat and Ecology in this Chapter). The deepest known branching members of the genus (*F. gelidilacus*, *F. columnare* and *F. aquatile*) are from freshwater ecosystems, suggesting that more halotolerant and marine-derived members of the genus (such as *F. flevense*, *F. gillisiae*, *F. frigidarium*, etc.) may have been distributed into and adapted to seawater/estuarine type environments in the past. Since practically no member of the genus is known to require Na^+ ions for growth, the adaptation to marine environments appears so far to be incomplete (see the section Physiology in this Chapter). Figure 1 presents the phylogenetic tree of the genus *Flavobacterium* based on 16S rRNA gene sequences.

Sequencing of the 16S rRNA gene has over time helped clarify the taxonomy of the genus *Flavobacterium* and is the means to definitively assign new strains within the genus. Phylogenetic data however indicate that several nomenclatural anomalies remain unresolved. Though many *Flavobacterium* species originally included in the older descriptions of the genus (Holmes et al., 1984) have been reclassified (Bernardet et al., 1996; see the section Taxonomy), the taxonomy of several validly described *Flavobacterium* species (J. P. Euzéby, List of Bacterial Names with Standing in Nomenclature) is yet to be officially corrected. For example, the species [*F.*] *devorans* and [*F.*] *yabuuchiae* are believed to be synonyms of *Sphingomonas paucimobilis* (Yabuuchi et al., 1979; Bauwens and De Ley, 1981) and *Sphingobacterium spiritovorum* (Takeuchi and Yokota, 1992), respectively. The species [*F.*] *mizutaii* and [*F.*] *ferrugineum* are clearly generically misclassified and are most closely related to the genera *Sphingobacterium* and *Chitinophaga*, respectively (Gherna and Woese, 1992; Nakagawa and Yamasato, 1993). No phylogenetic data are available for several species including [*F.*] *acidificum*, [*F.*] *acidurans*, [*F.*] *oceanosedimentum*, [*F.*] *resinovorum* or [*F.*] *thermophilum*. On the basis of DNA base composition data, all of these rather obscure species are misclassified and constitute species in completely separate bacterial phyla. For example, Holmes et al. (1984) considered [*F.*] *acidificum* to be a strain of "*Erwinia herbicola* subsp. *Ananas*" (now *Pantoea ananatis*), a member of the Gammaproteobacteria, while others such as [*F.*] *oceanosedimentum* are Gram-positives belonging to the *Bacillus* branch of the Firmicutes.

There are also discrepancies even with better-studied species. For example, sequences provided for the type strain of *F. psychrophilum* are quite different (Fig. 1). Substantial heterogeneity amongst strains classified as *F. columnare* and *F. johnsoniae* is also notable. However, in the case of *F. columnare*, this apparent heterogeneity is caused by a misidentification of the strain ATCC 43622, which more likely belongs to *F. johnsoniae*. The bona fide *F. columnare* strains are indeed grouped within a single cluster in the phylogenetic tree; the division of this cluster in three subclusters was already reported by Triyanto and Wakabayashi (1999), who based their description of three *F. columnare* genomovars on differences in 16S rRNA sequences, restriction fragment length polymorphism (RFLP), and DNA-DNA hybridization. A misidentification is not the explanation for the distance between the *F. johnsoniae* strain DSM 425 and the *F. johnsoniae* type strain, however, since their whole-cell protein and fatty acid profiles are closely related (J.-F. Bernardet, unpublished data). Some of these limitations have already been reported as early as 1984 by Reichenbach (1989) and this genetic diversity may also suggest the potential presence of additional *Flavobacterium* species amongst culture collection strains.

Taxonomy

The long and complex history of the genus *Flavobacterium* up to its emendation in 1996 has been presented in several publications (Holmes et al., 1984; Holmes et al., 1992; Bernardet et al., 1996; the chapter on The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* in this Volume) and will not be repeated here. As a result of an extensive polyphasic study (Bernardet et al., 1996), the genus *Flavobacterium* was shown to comprise the ten following valid species: *F. aquatile* (Holmes et al., 1984), *F. branchiophilum* (Wakabayashi et al., 1989), *F. columnare* (Bernardet and Grimont, 1989), *F. flevense* (Van der Meulen et al., 1974), *F. hydatis* (basonym, [*Cytophaga*] *aquatilis*; Strohl and Tait, 1978), *F. johnsoniae* (Reichenbach, 1989), *F. pectinovorum* (Reichenbach, 1989), *F. psychrophilum* (Bernardet and Grimont, 1989), *F. saccharophilum* (Agbo and Moss, 1979), and *F. succinicans* (Anderson and Ordal, 1961). (The references given here are the most relevant, although not necessarily the original descriptions.) This study also demonstrated that the invalid taxa "[*Cytophaga*] allerginae" (see the section Pathogenicity and Epidemiology in this Chapter), "[*Cytophaga*] *xantha*," "[*Flexibacter*] *aurantiacus* subsp. *excathedrus*," "[*Promyxobacterium*] *flavum*," and "[*Sporocytophaga*] *cauliformis*" belong to the genus (Bernardet et

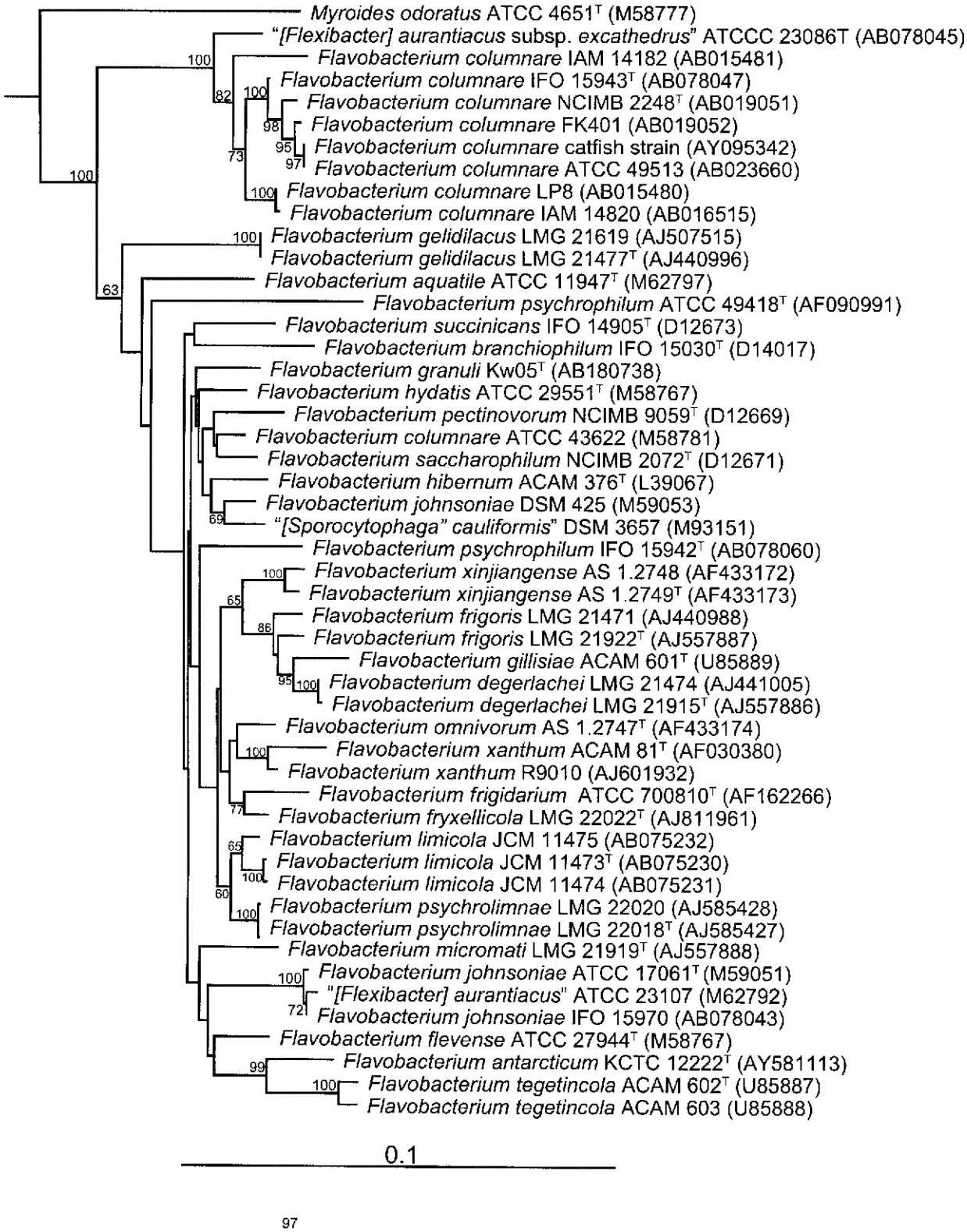

Fig. 1. A maximum likelihood distance phylogenetic tree created by the neighbor-joining procedure (Saitou and Nei, 1987) based on near complete 16S rRNA gene sequences of the genus *Flavobacterium*. The outgroup for the tree was *Myroides odoratus*. The bar indicates maximum likelihood distance.

al., 1996). Except the latter, these invalid and poorly studied taxa are each represented by a single strain. Since then, "[*Cytophaga*] *xantha*" has been validly published under the name *Flavobacterium xanthum* as a result of an extensive study of Antarctic organisms (McCammon and Bowman, 2000). The scant information available on the four remaining invalid taxa may be found in Reichenbach (1989). The determination of the 16S rRNA sequence of "[*Sporocytophaga*] *cauliformis*" and "[*Flexibacter*] *aurantiacus* subsp. *excathedrus*" has confirmed their attribution to the genus *Flavobacterium* (Fig. 1).

Since the emendation of the genus *Flavobacterium*, members of the family Flavobacteriaceae have been shown to represent an important bacterial group in Antarctic environments (Bowman et al., 1997; Van Trappen et al., 2002; see the section Habitat and Ecology in this Chapter); however, the presence of gliding "cytophagas" (which would likely be classified as *Flavobacterium* nowadays) in polar lakes had been noticed long ago (Christensen, 1974). Subsequently, extensive studies of bacterial strains retrieved from various regions of Antarctica have resulted in the description of several new *Flavobacterium* species: *F. antarcticum* (Yi et al., 2005) and *F. fryxellicola* and *F. psychrolimnae* (Van Trappen et al., 2005), *F. hibernum* (McCammon et al., 1998), *F. gillisiae* and *F. tegetincola* (McCammon and Bowman, 2000), *F. frigidarium* (Humphry et al., 2001), *F. gelidilacus* (Van Trappen et al., 2003a), and *F. degerlachei*, *F. frigoris* and *F. micromati* (Van Trappen et al., 2003a). Not from Antarctica but still from cold environments came *F. limicola* (Tamaki et al., 2003), *F. omnivorum* and *F. xinjiangense* (Zhu et al., 2003). Hence, taking into consideration the previously described *F. psychrophilum*, sixteen among the twenty-six current valid *Flavobacterium* species are distinctly psychrophilic or psychrotolerant. While *F. flevense* was the only halophilic species of the ten known in 1996, nine of those published since then may be considered halophilic or halotolerant (see the sections Habitat and Ecology, Identification, and Physiology in this Chapter). Features of the valid *Flavobacterium* species and invalid taxa are listed in Table 1.

Many other organisms, validly published or not, were included in the genus *Flavobacterium* since it was created in 1923 (Bergey et al., 1923). Most of them were subsequently attributed to other or new genera (e.g., the genera *Cellulophaga*, *Chryseobacterium*, *Empedobacter*, *Myroides*, *Pedobacter*, *Psychroflexus*, *Salegentibacter*, *Sphingobacterium* and *Zobellia*; see the corresponding chapters The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* in the second edition; The Genus *Chryseobacterium* in this Volume; The Marine Clade of the Family Flavobacteriaceae: The Genera *Aequorivita*, *Arenibacter*, *Cellulophaga*, *Croceibacter*, *Formosa*, *Gelidibacter*, *Gillisia*, *Maribacter*, *Mesonia*, *Muricauda*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Robiginitalea*, *Salegentibacter*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter* and *Zobellia*; An Introduction to the Family Flavobacteriaceae; The Genera *Bergeyella* and *Weeksella*; and The Genera *Myroides* and *Empedobacter* all in this Volume) while others (for which no 16S rRNA sequences are available) still appear in the lists of *Flavobacterium* species pending further investigation. Complete lists of organisms currently belonging to the genus *Flavobacterium* or that were attributed to it for some time may be found in the chapter "Taxonomic Outlines of the Prokaryotes" of the second edition of Bergey's Manual (Garrity and Holt, 2001) and in the *List of Bacterial Names with Standing in Nomenclature* (Euzéby, 1997; List of Bacterial Names with Standing in Nomenclature). These lists also provide the current taxonomic status and standing in nomenclature of the taxa that were subsequently excluded from the genus *Flavobacterium*.

Habitat and Ecology

Habitats known to harbor *Flavobacterium* strains are listed in Table 2.

Members of the genus *Flavobacterium* are widely distributed in nature, occurring mostly in aquatic ecosystems ranging in salinity from freshwater to seawater. *Flavobacterium* species have never been isolated from hypersaline waters: the extremely halotolerant Antarctic species formally referred to as "[*F.*] *salegens*" and "[*F.*] *gondwanense*" are now *Salegentibacter* and *Psychroflexus*, respectively (Bowman et al., 1998; McCammon and Bowman, 2000), genera which are core members of the marine clade of the family Flavobacteriaceae. By comparison, *Flavobacterium* species have a distinct predilection to low salinity, cool to cold environments and are commonly isolated from polar lakes (Van Trappen et al., 2002) and from streams, rivers, lakes and muddy soils in other cold environments. Unidentified members of the genus were also reported from marine algae (Maeda et al., 1998; Adachi et al., 2002) and mangrove environments (Nakagawa et al., 2001), although none of them actually required seawater for growth. Most *Flavobacterium* species are psychrotolerant (rather than psychrophilic, as defined by Morita 1975) and grow well at 4°C and optimally at 20–30°C. Currently, *Flavobacterium* species have been successfully isolated from temperate to polar low salinity ecosystems which on a biogeographical level correlates well with their

cold-oriented ecophysiology. Various *Flavobacterium* spp. thus have biotechnological potential and ecological interest owing to their ability to produce cold-active enzymes. Some of these enzymes may even be implicated in pathogenicity such as in coldwater disease in fish caused by *F. psychrophilum*.

As a matter of fact, several freshwater species are (or potentially are) the etiological agents of fish diseases (see the section Pathogenicity and Epidemiology in this Chapter), including *F. columnare*, *F. branchiophilum*, *F. hydatis*, *F. johnsoniae*, *F. psychrophilum* and *F. succinicans*. Strains of these species have been isolated from external lesions and internal organs of salmon and other fish, but in some cases also from the surrounding water. The growth requirements of species such as *F. branchiophilum* and *F. psychrophilum* are rather fastidious (see the section Isolation and Cultivation in this Chapter), suggesting their primary habitat could be limited to the fish tissue environment where they exist as a parasite or saprophyte depending on the immunological state of the fish and the virulence of individual strains. Recent data suggest pathogens such as *F. psychrophilum* can survive for long periods in water, with survival enhanced by sediment-derived nutrients; a salinity of 6 however drastically reduces the number of culturable *F. psychrophilum* cells (Madetoja et al., 2003). Other species such as *F. succinicans* and *F. hydatis* are less exacting and thus may be simple

Table 1. Currently recognized species classified in the genus *Flavobacterium*.[a]

Species[b]	Type strain	G+C (mol%)	Source	Reference(s)	Number of strains
Flavobacterium antarcticum	JCM 12383	38	Soil, King George Island, Antarctica	Yi et al., 2005	1
Flavobacterium aquatile [T, AL, c]	ATCC 11947	33	Deep well, United Kingdom	Holmes et al., 1984; Bernardet et al., 1996	1
Flavobacterium branchiophilum [d]	ATCC 35035	34	Gills of salmon, Japan, 1977	Wakabayashi et al., 1989; Bernardet et al., 1996	16, 2, 30
Flavobacterium columnare [e]	NCIMB 2248	32	Kidney of salmon, United States, 1955	Bernardet and Grimont, 1989c; Bernardet et al., 1996	7, 5, 20
Flavobacterium degerlachei	LMG 21915	34	Microbial mats, Lake Ace, Vestfold Hills, Antarctica	Van Trappen et al., 2003b	14, 5, 14
Flavobacterium flevense [AL, f]	ATCC 27944	35	Freshwater lake, The Netherlands	Van der Meulen et al., 1974; Bernardet et al., 1996	1
Flavobacterium frigidarium [g]	ATCC 700810	35	Marine sediment, Adelaide Island, Antarctica	Humphry et al., 2001	1
Flavobacterium frigoris	LMG 21922	34	Microbial mats, Watts Lake, Vestfold Hills, Antarctica	Van Trappen et al., 2003b	19, 5, 19
Flavobacterium fryxellicola	LMG22022	35	Microbial mats, Lake Fryxell, Dry Valley, Antarctica	Van Trappen et al., 2005	3, 3, 3
Flavobacterium gelidilacus	LMG 21477	30	Microbial mats, freshwater and saline lakes, Antarctica	Van Trappen et al., 2003a	22, 5, 22
Flavobacterium gillisiae	ACAM 601	32	Sea ice, Prydz Bay, Antarctica	McCammon and Bowman, 2000	1
Flavobacterium granuli	IAM15099	36	Wastewater treatment plant, beer brewery, South Korea	Askan et al., 2005	1
Flavobacterium hibernum [h]	ACAM 376	34	Freshwater lake, Antarctica	McCammon et al., 1998	2, 2, 2
Flavobacterium hydatis [AL, j]	ATCC 29551	34	Gills of salmon, United States, 1974	Strohl and Tait, 1978; Bernardet et al., 1996	5, 1, 5
Flavobacterium johnsoniae [AL, j]	ATCC 17061	35	Soil or mud, United Kingdom	Reichenbach, 1989; Bernardet et al., 1996	5, 11, 30
Flavobacterium limicola	JCM 11473	34	River sediment, Ibaraki Prefecture, Japan	Tamaki et al., 2003	3, 3, 3
Flavobacterium micromati	LMG 21919	33	Microbial mats, Grace Lake, Vestfold Hills, Antarctica	Van Trappen et al., 2003b	3, 3, 3
Flavobacterium omnivorum	AS 1.2747	35	Frozen soil, China No. 1 glacier, Xinjiang, China, 1999	Zhu et al., 2003	1
Flavobacterium pectinovorum [k]	NCIMB 9059	35	Soil, United Kingdom	Reichenbach, 1989; Bernardet et al., 1996	8, 1, 8
Flavobacterium psychrolimnae	LMG22018	35	Microbial mats, Lake Fryxell, Dry Valleys, Antarctica	Van Trappen et al., 2005	4, 4, 4

Table 1. *Continued*

Species[b]	Type strain	G+C (mol%)	Source	Reference(s)	Number of strains
Flavobacterium psychrophilum [l]	NCIMB 1947	33	Kidney of salmon, United States, 1955	Bernardet and Grimont, 1989c; Bernardet et al., 1996	7, 6, 200
Flavobacterium saccharophilum [m]	NCIMB 2072	36	River Wey, United Kingdom, 1976	Agbo and Moss, 1979; Reichenbach, 1989; Bernardet et al., 1996	4, 1, 4
Flavobacterium succinicans [n]	DSM 4002	37	Fin of salmon, United States, 1954	Anderson and Ordal, 1961; Reichenbach, 1989; Bernardet et al., 1996	3, 3, 3
Flavobacterium tegetincola	ACAM 602	34	Cyanobacterial mat, marine salinity lake, Antarctica	McCammon and Bowman, 2000	2, 1, 2
Flavobacterium xinjiangense	AS 1.2749	34	Frozen soil, China No. 1 glacier, Xinjiang, China, 1999	Zhu et al., 2003	1
Flavobacterium xanthum [o]	IAM 12026	36	Soil, Showa station, Antarctica, 1967	Inoue and Komagata, 1976; Reichenbach, 1989; McCammon and Bowman, 2000	1
"[*Cytophaga*] *allerginae*"	ATCC 35408	34	Water in air-cooling unit, Florida	Liebert et al., 1984; Reichenbach, 1989; Bernardet et al., 1996	1
"[*Flexibacter*] *aurantiacus* subsp. *excathedrus*"	ATCC 23086	33	Pool in cathedral, Cartago, Costa Rica	Lewin, 1969; Reichenbach, 1989; Bernardet et al., 1996	1
"[*Promyxobacterium*] *flavum*"	DSM 3577	35	Rhizosphere of tomato plant, Russia	Reichenbach, 1989; Bernardet et al., 1996	1
"[*Sporocytophaga*] *cauliformis*"	NCIMB 9488	36	Water, Lake Constance, Germany	Gräf, 1962 Reichenbach, 1989; Bernardet et al., 1996	2, 2, 2

Abbreviations: [T], type species; [AL], the species is cited on the Approved Lists of Bacterial Names (Skerman et al., 1980; Moore et al., 1985); ACAM, Australian Collection of Antarctic Microorganisms, University of Tasmania, Hobart, Tasmania, Australia; AS, culture collection of the Institute of Microbiology, Academia Sinica, Beijing, China; ATCC, American Type Culture Collection, Manassas, VA, United States; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; IAM, culture collection of the institute of Applied Microbiology, University of Tokyo, Japan; JCM, Japanese Collection of Microorganisms, Tokyo, Japan; LMG, BCCM/LMG bacteria collection, Laboratorium voor Microbiologie, University of Gent, Gent, Belgium; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

[a] Names in quotation marks have not been validly published. Brackets indicate generically misclassified bacteria. Accession number is that in the recognized culture collection in which the type strain was first deposited. The origin and G + C content are those of the type strain. When more than one strain are known, three numbers are given, i.e., the number of strains on which the original description was based, the number of strains currently available in the largest culture collections (ATCC, CIP [Collection de I'Institut Pasteur, Paris, France], DSM, LMG, and NCIMB), and the approximate number of strains which have been included in further studies and which are or may still be maintained in specialized culture collections, respectively.

[b] Previous names and corrected epithets of species (taken from Euzéby [1997]; List of Bacterial Names with Standing in Nomenclature) are as stated in footnotes c to o.

[c] "[*Bacillus*] *aquatillis*" Frankland and Frandlnad 1889, "*Bacterium aquatillis*" Chester 1897, "[*Flavobacterium*] *aquatilis*" Bergey et al. 1923, "[*Chromobacterium*] *aquatilis*" Topley and Wilson 1929, and "[*Empedobacter*] *aquatile*" Brisou et al. 1960.

[d] *Flavobacterium branchiophila* Wakabayashi et al. 1989.

[e] "[*Bacillus*] *columnaris*" Davis 1922, "[*Chondrococcus*] *columnaris*" Ordal and Rucker 1944, [*Cytophaga*] *columnaris* Garnjobst 1945, Reichenbach 1989, and [*Flexibacter*] *columnaris* Leadbetter 1974, Bernardet and Grimont 1989c.

[f] [*Cytophaga*] *flevensis* van der Meulen et al. 1974, Reichenbach 1989.

[g] "*Flavobacterium xylanivorum*," name as deposited in the 16S rRNA sequence databases and in the ATCC.

[h] "*Flavobacterium ameridies*," name as deposited in the 16S rRNA sequence databases.

[i] [*Cytophaga*] *aquatilis* Strohl and Tait 1978.

[j] [*Cytophaga*] *johnsonae* Stanier 1947, Reichenbach 1989, and "[*Cytophaga*] *johnsonii*" Stanier 1957.

[k] "*Flavobacterium pectinovorum*" Dorey 1959, "[*Empedobacter*] *pectinovorum*" Kaiser 1961, and [*Cytophaga*] *pectinovora* Reichenbach 1989.

[l] [*Cytophaga*] *psychrophila* Borg 1960, Reichenbach 1989, and [*Flexibacter*] *psychrophilus* Bernardet and Grimont 1989c.

[m] [*Cytophaga*] *saccharophila* Agbo and Moss 1979.

[n] [*Cytophaga*] *succinicans* Anderson and Ordal 1961, Reichenbach 1989, and "[*Flexibacter*] *succinicans*" Leadbetter 1974.

[o] "[*Cytophaga*] *xantha*" Inoue and Komagata 1976.

Table 2. Habitats which are known to harbor described *Flavobacterium* species.

Species	Known habitat(s)
	Freshwater fish
F. branchiophilum	Diseased gills of various fish species; United States, Canada, Hungary, Japan, and Korea
F. columnare	External lesions and internal organs of many fish species; worldwide
F. hydatis	Diseased gills of salmon; United States
F. psychrophilum	External lesions and internal organs of (mostly) salmonid fish; United States, Canada, Chile, Europe, Japan, Korea, Tasmania
F. succinicans	External lesions of salmon and aquarium water (containing salmon); United States
	Temperate freshwater and soil
F. aquatile	Well water; United Kingdom
F. flevense	Freshwater and muddy soils, e.g., from Ijsselmeer, the Netherlands
F. granuli	Wastewater treatment plant, beer brewery; South Korea
F. johnsoniae	Soil (as for *F. pectinovorum*), compost, freshwater and freshwater sediment, also external lesions of various fish species; worldwide
F. limicola	Freshwater river sediment, e.g., from Japan
F. pectinovorum	Temperate, moist, organic-rich soils, e.g., from the United Kingdom
F. saccharophilum	Freshwater sediment; United Kingdom
"[*Cytophaga*] *allerginae"*	Water in an air-cooling unit; United States
"[*Flexibacter*] *aurantiaus* subsp. *Excathedrus"*	Freshwater pool; Costa Rica
"[*Promyxobacterium*] *flavum"*	Rhizosphere of tomato plant; Russia
"[*Sporocytophaga*] *cauliformis"*	Freshwater lake (Lake Constance)
	Cold environments
F. antarcticum	Organic-rich soil (penguin habitat); Antarctica
F. degerlachei	Microbial mats, freshwater lakes; Antarctica
F. frigidarium	Marine sediment, sea-ice; Antarctica
F. frigoris	Microbial mats, freshwater lakes; Antarctica
F. fryxellicola	Microbial mats, fresh/brackish water lakes; Antarctica
F. gelidilacus	Microbial mats, freshwater and saline lakes; Antarctica
F. gillisiae	Rich basal algal assemblages of sea-ice; Antarctica
F. hibernum	Freshwater lakes fed by glacial run-off; Antarctica
F. micromati	Microbial mats, freshwater lakes; Antarctica
F. omnivorum	Frozen soil, glacier; China
F. tegetincola	Microbial mats, freshwater to marine salinity lakes; Antarctica
F. xanthum	Muddy soil, pond mud, algal mats, freshwater lakes in Antarctica; freshwater eutrophic lakes in Germany
F. xinjiangense	Frozen soil, glacier; China

freshwater commensals but may possess opportunistic pathogenic responses. Strains of *F. columnare* also can kill cysts of the protistan pathogen *Giardia lamblia* in the presence of elevated Ca^{2+} ions (Rodgers et al., 2003), suggesting that the biological interactions of pathogenic and other *Flavobacterium* species could be quite diverse (see the section Applications in this Chapter).

Other *Flavobacterium* species appear to be harmless, chemoheterotrophic species that play a role in mineralizing various types of organic matter (carbohydrates, amino acids, proteins, and polysaccharides) in aquatic ecosystems (see the sections Identification and Physiology in this Chapter), although many early reports of bacteria, fungi, dead insects, and nematods being degraded by so-called "*Flavobacterium*" in soil are questionable. A member of the genus *Flavobacterium* was recently retrieved from paper mill slime, but its role in the process was not determined (Oppong et al., 2003). The 16S rRNA gene sequence was the only clue to the attribution to the genus *Flavobacterium* of several bacterial strains retrieved from the guts of mosquitoes (Campbell et al., 2004). Some species appear to be cosmopolitan (e.g., *F. johnsoniae* and *F. xanthum*), isolated from different habitats across several continents; however, the knowledge of the distribution of most *Flavobacterium* species is severely limited and data for most species are based only on one survey. In addition, specific ecological knowledge for most species is absent, including their specific functional roles and interactions with microorganisms and metazoa. The fish pathogens are exceptions, though even for these the data are still relatively limited.

Temperate freshwater and freshwater sediments (groundwater, lakes, rivers and ponds) are known to be habitats for the species *F. johnsoniae*, *F. saccharophilum*, *F. flevense*, *F. limicola*

and *F. xanthum* (Tamaki et al., 2003), while microbial mats and water of Antarctic oligotrophic lakes harbor *F. tegetincola*, *F. xanthum*, *F. hibernum*, *F. xinjiangense*, *F. gelidilacus*, *F. degerlachei*, *F. micromati*, *F. frigoris*, *F. fryxellicola* and *F. psychrolimnae* (Jaspers et al., 2001; Van Trappen et al., 2002; Van Trappen et al., 2003a; Van Trappen et al., 2003b; Pearce, 2003; Van Trappen et al., 2005; Table 2). The latter set of species tends to be more cold-adapted than the temperate species with growth temperature limits only slightly over 25°C. Soil is also a habitat for *Flavobacterium* species including *F. pectinovorum*, *F. johnsoniae* and *F. antarcticum* (Yi et al., 2005). The soils in which they have been found were moist and organic rich; arid desert or infertile soils were not known to harbor *Flavobacterium* species until a *Flavobacterium* sp. was recently isolated from samples of arid soil (Bodour et al., 2003). Two species, *F. omnivorum* and *F. xinjiangense*, were isolated from entrained frozen soil recovered from a glacier in western China (Xinjiang Province; Zhu et al., 2003). The soil was frozen into the glacier long in the past with the freeze-resistant species probably surviving there ever since. Most bacteria found in glacial deposits are usually hardy soil-type bacteria that are probably distributed into glacial environments either by wind-dispersion or by being trapped during one of the past ice ages. The species *F. gillisiae* and *F. frigidarium* were isolated from polar sea-ice, Antarctic marine salinity lakes, and marine sediment (e.g., Bowman et al., 1997; McCammon and Bowman, 2000; Humphry et al., 2001; Brinkmeyer et al., 2003) and could have a wider distribution in cold ecosystems. Culture-independent studies using polymerase chain reaction (PCR)-amplification and sequencing of cloned 16S rRNA genes so far indicate *Flavobacterium* species are more or less limited to the environments already indicated above (see the section Identification in this Chapter). Using phenotypic characteristics and 16S rRNA gene sequences, Zdanowski et al. (2004) identified several *Flavobacterium* sp., *Chryseobacterium* sp. and *Gelidibacter* sp. strains among the extremely diverse bacterial community in penguin guano in Antarctica. Indeed, the new species *F. antarcticum* was isolated from a soil sample collected from a penguin habitat (Yi et al., 2005).

Such artificial, moist environments as air-cooling units are also colonized by members of the genus *Flavobacterium*; for instance, a *Flavobacterium* sp. strain was involved in several human cases of respiratory disease which occurred during the eighties in a United States textile facility (Flaherty et al., 1984; Liebert et al., 1984; see the section Pathogenicity and Epidemiology in this Chapter). Another, very unusual environment for *Flavobacterium* strains was discovered when amoebae were shown to harbor intracellular members of the genus (Müller et al., 1999; Horn et al., 2001; see the section Pathogenicity and Epidemiology in this Chapter). One of the most recently described *Flavobacterium* species, *F. granuli*, was isolated from anaerobic granules in the wastewater treatment plant of a beer brewing factory in South Korea (Aslam et al., 2005). These granules, composed of microorganisms, inorganic nuclei and extracellular polymers, result from the flocculation of sludge in the reactor.

The ecology and habitats of bacterial genera belonging to the family Flavobacteriaceae were reviewed by Jooste and Hugo (1999). They were also extensively described for some of the bacterial species now encompassed in the genus *Flavobacterium* by Reichenbach (1989) and in the chapter The Order Cytophagales in the second edition. Many early publications reported the degradation of food products by poorly identified "*Flavobacterium*" strains; however, these food-related members of the family Flavobacteriaceae have subsequently been moved to the genera *Bergeyella*, *Chryseobacterium*, *Empedobacter*, *Myroides* and *Weeksella* (Engelbrecht et al., 1996; Jooste and Hugo, 1999; see the three corresponding chapters in this Volume—The Genera *Bergeyella* and *Weeksella*, The Genera *Myroides* and *Empedobacter* and The Genus *Chryseobacterium*). *Flavobacterium* spp. were isolated or identified from their 16S rRNA gene sequence from poultry and the air of poultry processing establishments (Georgnaras et al., 1996; Ellerbroek, 1997), dairy products (Cousin, 1982), and traditional alcoholic beverages in Nigeria (Sanni et al., 1999) and Mexico (Escalante et al., 2004), but the identification procedure was insufficient to determine whether these isolates were true *Flavobacterium* strains or belonged to other genera in the family Flavobacteriaceae. Bacterial strains, identified as members of the genus *Flavobacterium* based on phenotypical characteristics, were isolated from the surface and muscle of fresh or ice-stored sardines, but they were not considered to play an important role in the spoilage of the fish (Gennari and Cozzolino, 1989; Elotmani et al., 2004). This was also the opinion of González et al. (2000) about the *F. aquatile* strain they isolated from muscle samples of ice-stored rainbow trout. The isolation of *Flavobacterium* strains from fish products is not surprising since they have long been known to belong to the normal bacterial flora of freshwater fish and fish eggs. From their phenotypic traits, the *Cytophaga*-like strains reported from the surface of salmonid eggs (Bell et al., 1971; Barker et al., 1989) and gills (Trust, 1975; Nieto et al., 1984) could actually well have been *Flavobacterium* isolates. Unidentified *Flavobacterium* strains were also reported from

egg, skin, gill, and intestine samples (Trust, 1975; Barker et al., 1989; Nedoluha and Westhoff, 1997; Al-Harbi and Uddin, 2003; Míguez and Combarro, 2003; Mahmoud et al., 2004), as well as from the rearing pond water and sediment (Nedoluha and Westhoff, 1997; Al-Harbi and Uddin, 2003) of various fish species, but not from the fish diet (Nedoluha and Westhoff, 1997). Members of the *Flavobacterium-Cytophaga* group represented 9% of all bacteria identified by fluorescent in situ hybridization (FISH) in the tank water of an eel intensive culture system, and even higher numbers of the same organisms occurred in the eel slime (Moreno et al., 1999).

Isolation and Cultivation

Being classic aerobic chemoheterotrophs, members of *Flavobacterium* species can be readily cultivated on several commercially available organic media. The presence of NaCl and the concentration of organic extracts strongly affect growth. This is the major reason growth of freshwater species, including the fish pathogens, is so poor on media such as marine and nutrient agars that contain substantial amounts of NaCl. The procedures available for the enrichment, isolation, and cultivation of some of the bacterial species now encompassed in the genus *Flavobacterium* were extensively described by Reichenbach (Reichenbach, 1989; Reichenbach, 1992); the corresponding information for the *Flavobacterium* species published since then was included in their individual descriptions. The cultivation of bacterial genera belonging to the family Flavobacteriaceae was reviewed by Jooste and Hugo (1999). Media and optimal incubation temperatures for known *Flavobacterium* spp. are indicated in Table 3. Although they do allow limited bacterial growth, some incubation tem-

Table 3. Media and incubation temperature for routine cultivation of *Flavobacterium* species.

Species	Anacker and Ordal's agar	Nutrient agar	Trypticase soy agar	Marine 2216 agar	Incubation temperature (°C)
Na^+-sensitive fish pathogens[a]					
F. branchiophilum	+[b]	–	–	–	20–25
F. columnare	+	–	–	–	25
F. psychrophilum	+[c]	+	–	–	15–20
Freshwater and soil species					
F. aquatile	+	–	w	–	25
F. fryxellicola	+	+	+	–	20
F. granuli	NT	+	+	NT	25–30
F. hibernum	+	+	+	–	20–25
F. hydatis	+	+	+	–	25
F. johnsoniae	+	+	+	–	25
F. limicola	+	+	+	–	20
F. omnivorum	+	+	+	–	10–15
F. pectinovorum	+	+	+	–	25
F. psychrolimnae	+	+	+	–	20
F. saccharophilum	+	+	+	–	25
F. succinicans	+	+	+	–	25
F. xinjiangense[d]	+	w	–	–	10–25
"[*Cytophaga*] *allerginae*"	+	+	NT	NT	25
"[*Flexibacter*] *aurantiacus* subsp. *excathedrus*"	+	+	NT	NT	25
"[*Promyxobacterium*] *flavum*"	+	+	NT	NT	30
"[*Sporocytophaga*] *cauliformis*"	+	+	NT	NT	25
Salt tolerant (and most polar) species					
F. antarcticum	+	+	+	+	20
F. degerlachei	+	+	+	+	20
F. flevense	+	+	+	+	25
F. frigidarium	+	+	+	+	15–20
F. frigoris	+	–	+	+	20
F. gelidilacus	+	+	+	+	20
F. gillisiae	+	+	+	+	15–20
F. micromati	+	+	w	w	20
F. tegetincola	+	+	+	+	15–20
F. xanthum	+	+	+	+	15–20

Abbreviations: +, good growth; w, weak or reduced growth; –, no or scant growth; and NT, not tested.

[a]Fish-pathogenic species will also grow well on Modified Shieh's medium (Song et al., 1988a).

[c]*Flavobacterum psychrophilum* strains grow better on Anacker and Ordal's agar containing an enriched amount of tryptone (e.g., 5 g·liter^{-1}) or on Modified Shieh's agar.

[d]For the routine cultivation of *F. xinjiangense*, PYG agar or broth can also be used (Zhu et al., 2003).

peratures and media in this table are not recommended for routine cultivation of some species.

All species can be grown conveniently on agar media although *F. columnare* strains tend to adhere strongly to the agar surface (see the section Identification in this Chapter). Many species can grow on rich media such as nutrient broth and agar and trypticase soy broth and agar (TSA); most of them can also be readily grown on media containing a lower concentration of nutrients such as R2A and other media typically used for growing freshwater bacterial chemoheterotrophic species. *Flavobacterium omnivorum* and *F. xinjiangense* grow well on rich glucose-containing media such as peptone-yeast extract-glucose (PYG; Zhu et al., 2003); this is presumably also true for other *Flavobacterium* species, although glucose does not improve the growth of *F. columnare* (Song et al., 1988b). Modified Shiehs (Song et al., 1988b) or Anacker and Ordal's media (Anacker and Ordal, 1955), with incubation at 20°C, can be used for general cultivation, as almost all *Flavobacterium* species should grow well under these conditions. Nutrient, trypticase soy, Marine 2216, and R2A broth and agar media are commercially available. The formulae for some of the other media mentioned are detailed at the end of this section.

Flavobacterium are best isolated directly from source material (water, mud, soil, fish tissue, etc.) onto agar media. Selective culturing techniques generally have not been developed for the majority of *Flavobacterium* species, except for the fish pathogens (see below). The distinctive yellow pigmentation and shiny, slightly mucoid appearance of the colonies of most *Flavobacterium* species provide a direct means for selection of putative *Flavobacterium* strains. Further identification relies then on phenotypic characteristics and 16S rRNA gene sequencing (see the section Identification in this Chapter).

The isolation procedure used can take advantage of the fact that most *Flavobacterium* species are adapted to cool to cold ecosystems: incubation temperature thus can be set to 4–20°C. Indeed, the isolation of *Flavobacterium* species from permanently cold ecosystems such as sea-ice, glacial seawater, and polar lake samples can be enhanced by a pre-enrichment step at a low temperature, typically 2–4°C for 1–2 days in a suitable medium. This boosts the populations of heterotrophic psychrophilic species, which respond rapidly to high levels of organic nutrients. The pre-enrichment culture is then spread-plated onto the corresponding agar medium. This process was used for the isolation of *F. gillisiae* and *F. tegetincola* from Antarctic ice and water samples using Marine 2216 agar (McCammon and Bowman, 2000), for the isolation of *F. xinjiangense* and *F. omnivorum* from glacial entrained soil using PYG medium (Zhu et al., 2003), and for the isolation of several species from Antarctic lake algal mat material using TSA and R2A (Van Trappen et al., 2002).

Some *Flavobacterium* species can be isolated on media containing a specific substrate, such as chitin or pectin (Reichenbach, 1989). *Flavobacterium frigidarium* can be enriched from marine sediments in a liquid xylan-containing mineral salts medium; after several weeks enrichment at 4°C, the species can be isolated and purified using the same medium solidified with agar (Humphry et al., 2001).

Understandably, the fish-pathogenic *Flavobacterium* species (see the sections Habitat and Ecology and Pathogenicity and Epidemiology in this Chapter) have received considerable attention to develop appropriate isolation media (Holt et al., 1993; Turnbull, 1993; Wakabayashi, 1993; Austin and Austin, 1999). After the Anacker and Ordal's medium (frequently called "cytophaga medium" in the fish pathology literature) was first proposed for the cultivation of *F. columnare* (Anacker and Ordal, 1955), several other media were evaluated for improved bacterial growth, including the Shieh's medium, both in its original formula (Shieh, 1980) and in a modified, more simple formula (Song et al., 1988b), and the tryptone-yeast extract-salts medium (TYES; Holt, 1988; Holt et al., 1993). *Flavobacterium columnare* is usually isolated from superficial lesions in diseased fish (see the section Pathogenicity and Epidemiology in this Chapter) in which many other bacteria also proliferate (Hawke and Thune, 1992; Tiirola et al., 2002). To prevent the overgrowth of the pathogen by saprophytic bacteria and their antagonistic effect (Chowdhury and Wakabayashi, 1989; Tiirola et al., 2002), selective media were devised on the basis of the resistance of *F. columnare* to neomycin, polymyxin B and tobramycin (Fijan, 1969; Bullock et al., 1986; Griffin, 1992; Hawke and Thune, 1992; Decostere et al., 1997) and on its ability to degrade gelatin (Bullock et al., 1986; Griffin, 1992). One such formula (Selective Flexibacter Medium; Bullock et al., 1986) is given below. Serial dilution of infected samples has also been advocated to favor the isolation of fish-pathogenic *Flavobacterium* species (Tiirola et al., 2002). No growth of *F. columnare* was reported on TSA, nutrient agar or Marine 2216 media. All *F. columnare* strains are able to grow at 18, 25 and 30°C; some of them may also grow at 15 or 37°C, depending on the temperature of the environment they were isolated from (Decostere et al., 1998; Triyanto and Wakabayashi, 1999).

Many formulae have also been proposed for the growth of *F. psychrophilum* (Holt et al., 1993; Madetoja and Wiklund, 2002; Nematollahi et al., 2003b; Cepeda et al., 2004). Anacker and Ordal's

medium has been widely used. To improve the growth of *F. psychrophilum*, several modifications of the original formula have been proposed, such as increasing the tryptone content to 2 g·liter^{-1} (Wakabayashi and Egusa, 1974) or 5 g·liter^{-1} (Bernardet and Kerouault, 1989; Crump et al., 2001) or adding 10% fetal bovine serum (Obach and Baudin-Laurencin, 1991; Brown et al., 1997) or 3% fish blood (Crump et al., 2001). Daskalov et al. (1999) also reported improved growth, gliding motility, and pigmentation of *F. psychrophilum* when grown in Anacker and Ordal's agar and broth supplemented with skimmed milk and 0.5 g·liter^{-1} each of D(+) galactose, D(+) glusose, and L-rhamnose. The Shieh's medium (Shieh, 1980; Holt et al., 1993), the tryptone-yeast extract-salts medium (TYES; Holt et al., 1993; Crump et al., 2001), and the nutrient agar and broth (Secades et al., 2001; Secades et al., 2003) also give good growth. Although the growth of *F. psychrophilum* on TSA was sometimes reported, most authors agree that no or only negligible growth occurs on this medium. The isolation of *F. psychrophilum* from blisters or internal organs usually results in the growth of pure cultures; but a tobramycin-containing medium has been proposed to prevent the overgrowth of *F. psychrophilum* by other bacteria when external lesions are sampled (Kumagai et al., 2004). This organism is not particularly fastidious, although its growth is inevitably slower than that of other fish-pathogenic *Flavobacterium* species owing to its lower range of temperature tolerance. *Flavobacterium psychrophilum* grows at temperatures between 4 and 23°C; most strains do not grow or grow very weakly at 25°C (Bernardet and Kerouault, 1989; Holt et al., 1989; Brown et al., 1997; Uddin and Wakabayashi, 1997), although growth at 25°C was reported for some strains (Ostland et al., 1997; Madetoja et al., 2001). Optimum growth occurs at 15 (Holt et al., 1989) to 20°C (Uddin and Wakabayashi, 1997). One of the main reasons for trying to improve the yield of *F. psychrophilum* in culture was to obtain large masses of bacteria that could be used for preparing a vaccine against the severe salmonid diseases it is responsible for worldwide (see the section Pathogenicity and Epidemiology in this Chapter). Recently, Crump et al. (2001) achieved large-scale production of *F. psychrophilum* in a 35-liter fermenter containing 28 liters of TYES broth supplemented with 1% maltose and 0.02% sodium acetate with stirring and aeration. Supplementation of TYES with 0.5 g·l^{-1} glucose also enhanced the growth of *F. psychrophilum*, resulting in improved recovery of the bacterium from stored cultures and infected fish and in increased production of biomass in liquid culture (Cepeda et al., 2004). Standard procedures for such investigations of *F. psychrophilum* as experimental infection, antimicrobial susceptibility testing, and virulence studies require the use of accurate numbers of viable bacteria. Improvements in the growth media and careful handling of the bacteria in isotonic suspension media resulted in predictable production of viable *F. psychrophilum* cells and allowed an absorbance/colony-forming-units relation curve to be established (Michel et al., 1999). However, as stated by Faruk (2000): "It should be noted that the growth of each *F. psychrophilum* isolate differed considerably. Ideally, a growth curve should have been prepared for each isolate to examine individual life cycles of the isolates." *F. psychrophilum* may enter a "viable but non-cultivable" state (Michel et al., 1999; Madetoja and Wiklund, 2002; Vatsos et al., 2002).

Strains of *F. johnsoniae* (originally isolated from soil samples on chitin-containing media; Stanier, 1947; Reichenbach, 1989) and *F. hydatis* grow well on media with rather low-nutrient content such as Anacker and Ordal's and modified Shieh's, as well as on more nutrient-rich nutrient media and TSA, on which they produce flat, translucent, spreading, pale yellow colonies with filamentous to rhizoid margins or raised to convex, round to undulate, opaque, bright yellow colonies with entire to irregular margins, respectively (Strohl and Tait, 1978; Reichenbach, 1989; J.-F. Bernardet, unpublished data). Hence, these media are currently used for the isolation of *F. johnsoniae* strains from fish (Carson et al., 1993), as well as for the growth of the numerous *F. johnsoniae*-like and *F. hydatis*-like strains also isolated from fish (J.-F. Bernardet, unpublished data; see the section Pathogenicity and Epidemiology in this Chapter). However, as for *F. columnare*, these organisms occur in external lesions and their recovery may be hampered by the presence of numerous saprophytic bacteria. Given the very limited impact of these organisms in fish pathology, no selective medium was devised. Incubation temperatures of 20–30°C are convenient for routine cultivation.

Flavobacterium branchiophilum is the most fastidious of all fish-pathogenic *Flavobacterium* species (Heo et al., 1990; Turnbull, 1993; Ko and Heo, 1997; see the section Pathogenicity and Epidemiology in this Chapter). Growth on Anacker and Ordal's agar is slow, with colonies appearing only after 2–5 days of incubation at 18°C. Colonies are light yellow, translucent, non-spreading, circular with regular edges; the latter characteristic is due the absence of gliding motility in *F. branchiophilum* (Wakabayashi et al., 1989; Ostland et al., 1994). Growth periods of up to seven days were used for some studies, and the enrichment of the medium with serum, gelatin or starch was suggested (Ko and Heo, 1997). For

primary isolation from gill samples, Ostland et al. (1994) plated serial 10-fold dilutions of gill tissue on Anacker and Ordal's agar and incubated the plates at 12 or 18°C for 6–12 days. The growth of several *F. branchiophilum* strains was compared on different media: no growth occurred on TSA, nutrient agar, and Mueller Hinton; only limited growth occurred on Anacker and Ordal's agar enriched to 5 g·liter^{-1} of tryptone; growth was distinctly better on original Anacker and Ordal's agar, modified Shieh's agar, *Microcyclus-Spirosoma* agar (medium no. 81 of the National Collections of Industrial, Food and Marine Bacteria [NCIMB] catalogue of strains), and Medium for Freshwater Flexibacteria (NCIMB medium no. 218; Lewin, 1969); and the best growth was achieved on casitone yeast extract agar (NCIMB medium no. 101; J.-F. Bernardet, unpublished data). Different temperatures were also compared, and the 20–25°C range was found convenient (J.-F. Bernardet, unpublished data).

The four invalid taxa which belong to the genus *Flavobacterium*, i.e., "[*Cytophaga*] *allerginae*," "[*Flexibacter*] *aurantiacus* subsp. *excathedrus*," "[*Promyxobacterium*] *flavum*" and "[*Sporocytophaga*] *cauliformis*," all grow well on nutrient, Anacker and Ordal's, and modified Shieh's media at 25°C; growth on TSA was not tested (Reichenbach, 1989; J.-F. Bernardet, unpublished data).

Anacker and Ordal's Medium (Anacker and Ordal, 1955)

Tryptone (or pancreatic digest of casein)	0.5 g
Yeast extract	0.5 g
Beef extract	0.2 g
Sodium acetate	0.2 g
Agar (optional)	10 g
Distilled water	1000 ml

Adjust pH to 7.2–7.4 and autoclave 15–20 min at 121°C.

The original recipe, used to grow *F. columnare* in agar deeps, included 0.4% agar only; in their 1959 publication, the same authors raised the agar concentration to 0.9% for streaking material from lesions of fish (Anacker and Ordal, 1959). However, an agar concentration of 1–1.5% is recommended for more comfortable streaking (Wakabayashi and Egusa, 1974; Bernardet, 1989a; Michel et al., 1999). A selective medium for the isolation of *F. columnare* was obtained by adding neomycin (5 μg·ml^{-1}) and polymyxin B (200 IU·ml^{-1}) to this medium (Hawke and Thune, 1992), while tobramycin (5 μg·ml^{-1}) was added to modified Anacker and Ordal's medium for the selective isolation of *F. psychrophilum* (Kumagai et al., 2004). The brand of beef extract used was shown to influence the growth of *F. psychrophilum* (Lorenzen, 1993).

Modified Shieh's Medium (Song et al., 1988b)

Yeast extract	0.5 g
Peptone	5 g
Sodium acetate	10 mg
$BaCl_2 \cdot H_2O$	10 mg
K_2HPO_4	0.1 g
KH_2PO_4	50 mg
$MgSO_4 \cdot 7H_2O$	0.3 g
$CaCl_2 \cdot 2H_2O$	6.7 mg
$FeSO_4 \cdot 7H_2O$	1.0 mg
$NaHCO_3$	50 mg
Distilled water	1000 ml
Agar (optional)	15 g

Autoclave 15–20 min at 121°C.

The original Shieh's recipe (Shieh, 1980) was modified by omitting the glucose, pyruvate and citrate following the demonstration that these components did not improve the growth of *F. columnare*. Decostere et al. (1997) devised a selective medium for the isolation of *F. columnare* by adding 1 μg·ml^{-1} of tobramycin to this medium.

Peptone-Yeast Extract-Glucose (PYG; Zhu et al., 2003)

Polypeptone	5 g
Tryptone	5 g
Yeast extract	10 g
Salt solution	40 ml
Agar (optional)	15–20 g
Distilled water	910 ml
Salt Solution	
$CaCl_2$	0.2 g
$MgSO_4 \cdot 7H_2O$	0.4 g
K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
$NaHCO_3$	10 g
NaCl	2.0 g
Distilled water	1000 ml

Adjust pH to 7.2 and autoclave 15–20 min at 121°C. Add 50 ml of 20% (w/v) D-glucose filter-sterilized stock solution to each 1 liter of medium following autoclaving.

Selective Flexibacter Medium (Bullock et al., 1986)

Tryptone	2.0 g
Yeast extract	0.5 g
Gelatin	3.0 g
Agar	15 g
Distilled water	1000 ml

Autoclave 15–20 min at 121°C; when cooled to 45°C, add filter-sterilized neomycin sulfate (0.004 g).

Tryptone Yeast Extract Salts (TYES; Holt, 1988)

Tryptone	4.0 g
Yeast extract	0.4 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2 \cdot 2H_2O$	0.5 g
Distilled water	1000 ml

Adjust pH to 7.2 and autoclave 15–20 min at 121°C.

Identification

Characteristics which differentiate members of the genus *Flavobacterium* from members of other genera in the family Flavobacteriaceae are listed in Table 2 of the chapter An Introduction to the Family Flavobacteriaceae in this Volume; those characteristics that differ among species in the genus *Flavobacterium* are listed in Table 4 below; see also the section Physiology.

The following description of the genus *Flavobacterium* is cited from Bernardet et al., 1996:

Emended description of the genus *Flavobacterium* Bergey, Harrison, Breed, Hammer, and Huntoon 1923. Cells are rods with parallel or slightly irregular sides and rounded or slightly tapered ends and usually are 2 to 5 μm long and 0.3 to 0.5 μm wide. Under certain growth conditions, some species may also produce shorter (1-μm) or longer (10- to 40-μm) filamentous cells. The longer rods are flexible. Motile by gliding (this characteristic has not been observed in *Flavobacterium branchiophilum*). Flagella are absent. Gram negative. Resting stages are not known. Intracellular granules of poly-β-hydroxybutyrate are absent. Colonies are circular, convex or low convex, and shiny with entire or wavy edges (sometimes sunken into the agar) on solid media containing high levels of nutrients. On solid media containing low levels of nutrients most species also produce flat or very thin, spreading, sometimes very adherent swarms with uneven, rhizoid, or filamentous margins. Colonies are typically yellow (they vary from cream to bright orange) because of nondiffusible carotenoid or flexirubin types of pigments or both, but nonpigmented strains do occur. Most species do not grow on seawater-containing media; an exception to this is *Flavobacterium flevense*. Most species are able to grow on nutrient agar and on Trypticase soy agar. Chemoorganotrophic. Aerobic with a respiratory type of metabolism. When certain growth factors are provided, *Flavobacterium hydatis* and *Flavobacterium succinicans* also grow anaerobically (Anderson and Ordal, 1961; Chase, 1965; Reichenbach, 1989; Strohl and Tait, 1978). Peptones are used as nitrogen sources, and NH_3 is released from peptones; growth occurs on peptone alone. Acid is produced from carbohydrates by all species except *Flavobacterium columnare* and *F. psychrophilum*. All species except *Flavobacterium pectinovorum* decompose gelatin and casein, and several species also hydrolyze various polysaccharides, including starch, chitin, pectin, and carboxymethyl cellulose. *Flavobacterium pectinovorum* and *Flavobacterium saccharophilum* are also agarolytic. Cellulose is never decomposed. Tributyrin and Tween compounds are decomposed. Indole is not produced. Catalase is produced. Cytochrome oxidase is produced by all species except *Flavobacterium saccharophilum*.

Menaquinone 6 is the only respiratory quinone. The predominant fatty acids are 15:0, 15:0 iso, 15:1 iso G, 15:0 iso 3OH, summed feature 4 (15:0 iso 2OH, 16:1 ω7c, or 16:1 ω7t or any combination of these fatty acids), 16:0 iso 3OH, 17:1 iso ω9c, and 17:0 iso 3OH. Sphingophospholipids are absent. Homospermidine is the major polyamine in all 10 *Flavobacterium* species; all species except *Flavobacterium branchiophilum* and *Flavobacterium saccharophilum* also contain putrescine as a minor component (Hamana and Matsuzaki, 1990; Hamana and Matsuzaki, 1991; Hamana et al., 1995). Spermidine and spermine are also minor components in *Flavobacterium branchiophilum*, while *Flavobacterium johnsoniae* is the only member of the genus that contains minor amounts of agmatine and 2-hydroxyputrescine (Hamana et al., 1995). The optimum temperature range for most species is 20 to 30°C; the optimum temperature range for *F. psychrophilum* is 15 to 18°C.

These organisms are widely distributed in soil and freshwater habitats, where they decompose organic matter. Several species are pathogenic for freshwater fish (*Flavobacterium branchiophilum*, *Flavobacterium columnare*, *F. psychrophilum*) or occasionally are isolated from diseased freshwater fish (*Flavobacterium hydatis*, *Flavobacterium johnsoniae*, *Flavobacterium succinicans*). The G+C contents of the DNAs are 32 to 37 mol%. The type species is *Flavobacterium aquatile* (Frankland and Frankland, 1889) Bergey, Harrison, Breed, Hammer, and Huntoon 1923.

The publication of sixteen additional *Flavobacterium* species since 1996 did not challenge the core of this emended description. However, additional comments are necessary on some of the characteristics included; these comments are cited below in the order of the above comments.

In some *Flavobacterium* species, spherical degenerative forms usually considered nonviable, often referred to as "spheroplasts," appear in aging liquid cultures (Reichenbach, 1989); they were long mistaken for microcysts (Ordal and Rucker, 1944; Pacha and Ordal, 1970; Bullock et al., 1971; Farkas, 1985; Fig. 2). They have been noticed in *F. columnare* (Garnjobst, 1945; Song et al., 1988a, 1988b; Tiirola et al., 2002; J.-F. Bernardet, unpublished data), *F. succinicans* (Anderson and Ordal, 1961), *F. psychrophilum* (J.-F. Bernardet, unpublished data), *F. johnsoniae* (Stanier, 1947; Liao and Wells, 1986) and *F. branchiophilum* (Ostland et al., 1994).

Few electron microscopy studies have been performed on members of the genus *Flavobacterium*. Reichenbach (1989) reviewed the early

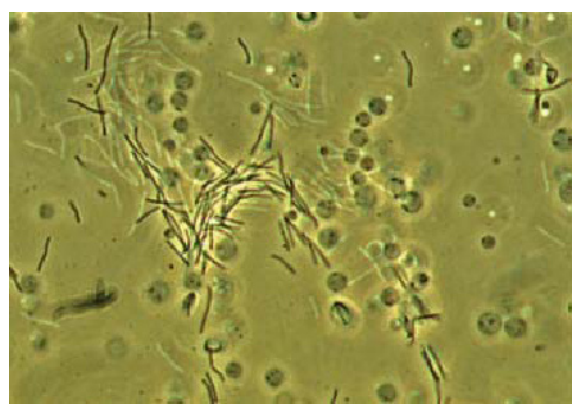

Fig. 2. Spheroplasts of *F. columnare*. Spherical degenerative forms called "spheroplasts" appeared as soon as 24 h in this shaken Anacker and Ordal's broth culture incubated at room temperature (phase-contrast microscopy, ×1000).

Table 4. Differentiating characteristics among the valid species in the genus *Flavobacterium*.

	F. antarcticum	*F. aquatile*	*F. branchiophilum*	*F. columnare*	*F. degerlachei*	*F. flevense*	*F. frigidarium*	*F. frigoris*	*F. fryxellicola*	*F. gelidilacus*	*F. gillisiae*
Habitat	Polar soil	Temperate freshwater	Temperate freshwater	Temperate freshwater	Polar saline lakes	Temperate freshwater	Polar marine sediment	Polar freshwater and saline lakes	Polar freshwater lakes	Polar freshwater and saline lakes	Sea ice
Morphology of the colonies on Anacker and Ordal's agar	Convex, round with entire margins	Low convex, round, with entrie margins	Low convex, round, with entire margins	Flat, adherent to the agar, with rhizoid margins	Flat, round, with entrie margins	Low convex, round, sunken into the agar	Flat, round, with entire margins	Flat, round, with entire margins	Flat, round, with entire margins	Flat, round, with entire margins	ND
Gliding motility	–	+	–	+	–	+	–	–	–	v	–
Congo red absorption	–	–	–	+	–	–	+[a]	–	–	–	ND
Flexirubin type pigment	–	–	–	+	–	–	–	–	–	–	–
Growth on											
Marine agar	+	–	–	–	+	+	+	+	–	+	+
Nutrient agar	+	+	–	–	+	+	+	–	+	+	+
Trypticase-soy agar	+	(+)	–	–	+	+	+	+	(+)	+	+
Growth at:											
20°C	+	+	+	+	+	+	+	+	+	+	+
25°C	(+)	+	+	+	+	+	–	(+)	(+)	+	(+)
Glucose utilization	–	ND	ND	–	+	+	+	+	+	–	+
Acid produced from carbohydrates	(+)	+	+	–	–	+	–	–	–	–	+
Degradation of											
Gelatin	+	+	+	+	–	–	+	–	–	v	–
Casein	+	+	+	+	–	–	+	+	–	+	+
Starch	–	+	+	–	+	+	–	+	–	+	+
Carboxymethyl cellulose	–	–	–	–	–	–	–	–	–	–	–
Agar	–	–	–	–	–	+	–	–	–	–	–
Alginate	–	ND	ND	ND	–	–	ND	–	–	–	ND
Pectin	–	ND	ND	ND	–	+	–	–	–	–	–
Chitin	–	–	–	–	–	–	–	–	–	–	+
Esculin	–	+	–	–	+	+	+	+	+	–	+
DNA	+	–	–	+	–	–	–	–	–	–	–
Urea	–	–	–	–	–	–	–	–	–	–	–
Tween compounds	+	+	+	+	ND	+	ND	ND	ND	ND	+
Tyrosine	–	+	+	–	–	–	–	+	–	–	–
Brown diffusible pigment on tyrosine agar	(+)	–	–	v	–	–	–	–	–	–	–
Precipitate on egg rolk agar	–	+	+	+	–	–	–	–	–	–	–
Hydrolysis of o-nitrophenyl-b-D-galactopyranoside	–	+	+	–	–	+	–	–	–	–	–
Production of											
Cytochrome oxidase	+	+	+	+	+	+	+	+	+	+	–
Hydrogen sulfide	–	–	–	+	–	–	–	–	–	–	–
Nitrate reduction	–	+	–	+	–	–	–	v	–	–	–
Susceptibility to the vibriostatic compound O/129[b]	ND	–	+	+	ND	+	ND	ND	ND	ND	–
Range of G+C content (mol%)	38	32 (33)[c]	29–31 (33–34)	30 (32–33)	34	33–35	35	34	35	30	32

Symbols: +, all strains positive; –, all strains negative, (+) weak or delayed positive response; v, variable among strains; V, variable among references; and ND, no data available.

[a]"There seemed to be absorption of Congo red by the colonies" (Humphry et al., 2001).

[b]Diffusion method, 500-mg disks.

[c]The values cited in parentheses are those determined by Bernardet et al. (1996) when different from previously published values.

From Anderson and Ordal (1961); van der Meulen et al. (1974); Christensen (1977); Strohl and Tait (1978); Agbo and Moss (1979); Oyaizu and Komagata (1981); Holmes et al. (1984); Bernardet (1989); Bernardet and Grimont (1989); Reichenbach (1989); Wakabayashi et al. (1989); Carson et al. (1993); Bernardet et al. (1996); McCammon et al. (1998); McCammon and Bowman (2000); Humphry et al. (2001); Tamaki et al. (2003); Van Trappen et al. (2003a,b); Zhu et al. (2003); Aslam et al., 2005; Van Trappen et al., 2005; Yi et al., 2005 and J.-F. Bernardet, unpublished data.

F. granuli	*F. hibernum*	*F. hydatis*	*F. johnsoniae*	*F. limicola*	*F. micromati*	*F. omnivorum*	*F. pectinovorum*	*F. psychrolimnae*	*F. psychrophilum*	*F. saccharophilum*	*F. succinicans*	*F. tegetincola*	*F. xanthum*	*F. xinjiangense*
Wastewater treatment plant	Polar freshwater lake	Temperate freshwater	Temperate soil and freshwater	Cold freshwater	Polar freshwater and saline lakes	Frozen soil	Temperate soil	Polar freshwater lakes	Temperate freshwater	Temperate freshwater	Temperate freshwater lake	Polar saline	Polar soil	Frozen soil
ND	ND	Flat, spreading, with filamentous margins	Flat, spreading, with filamentous margins	ND	Flat, round, with entire margins	ND	Low convex, round, with entire margins	Convex, round, with entire margins	Low convex, round, with entire or uneven margins	Flat, spreading, sunken into the agar	Flat, spreading, with filamentous margins	ND	ND	ND
−	+	+	+	−	−	−	+	−	(+)	+	+	+	−	−
−	−	−	−	+	−	ND	−	−	−	−	−	ND	ND	ND
−	+	+	+	−	−	−	+	−	+	+	−	−	−	−
ND	−	−	−	−	(+)	−	−	−	−	−	−	+	+	−
+	+	+	+	+	+	+	+	+	+	+	+	+	+	(+)
+	+	+	+	+	(+)	+	+	+	−	+	+	+	+	−
+	+	+	+	+	+	−	+	+	+	+	+	+	+	−
+	+	+	+	+	(+)	−	+	+	v	+	+	(+)	(+)	−
+	+	+	+	+	−	+	+	+	−	+	+	+	+	+
−	+	+	+	−	−	−	+	−	−	ND	+	+	+	−
−	+	+	+	+	−	−	+	−	+	+	(+)	−	+	+
−	+	+	+	+	−	+	+	+	+	+	+	−	+	+
−	+	+	+	+	−	+	+	+	−	+	+	−	+	−
−	−	−	+	−	−	+	+	−	−	(+)	(+)	−	−	−
−	−	−	−	+	−	−	−	−	−	+	−	−	−	−
ND	−	−	+	−	−	+	+	−	−	ND	ND	ND	ND	−
ND	−	+	+	−	−	+	+	−	−	+	ND	−	−	−
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−	−	−	−	−	−	−	−	−	−	+	−	−	+	+
−	+	+	v	−	−	+	+	−	−	+	v	−	V	−
ND	ND	−	−	ND	ND	ND	+	ND	+	+	+	−	−	ND
36	34	32–34	33 (35)	34–35	33	35	34 (35)	34	32 (33–34)	32–36 (36)	38 (35–37)	34	36	34

studies, and some of the recently described species, namely *F. branchiophilum* (Wakabayashi et al., 1989; Ostland et al., 1994; Ko and Heo, 1997), *F. frigidarium* (Humphry et al., 2001), *F. limicola* (Tamaki et al., 2003) and *F. xinjiangense* and *F. omnivorum* (Zhu et al., 2003), were also investigated using transmission electron microscopy. These studies revealed a structure of the cell wall typical of Gram-negative bacteria. *Flavobacterium branchiophilum* and *F. frigidarium* exhibit fimbria-like structures (Heo et al., 1990; Humphry et al., 2001). Nonflagellar appendages were also found in *F. aquatile*; nonfunctional pseudoflagellar structures were noticed in the same species, but this was not confirmed by further investigations (see Holmes et al. [1984] and references therein). A capsule was evidenced in *F. columnare* and *F. frigidarium* using electron microscopy and biochemical studies (Decostere et al., 1999; Humphry et al., 2001; MacLean et al., 2003); conventional microscopy also showed *F. hibernum* cells have a thick capsule when grown at 4°C (McCammon et al., 1998). Most of the newly described *Flavobacterium* species do not exhibit gliding motility; in *F. gelidilacus*, gliding motility is a strain-dependent characteristic (Van Trappen et al., 2003a). In this species and in *F. hibernum*, gliding only occurs on nutrient-poor media (McCammon et al., 1998; Van Trappen et al., 2003), which is consistent with previous reports on other *Flavobacterium* species (Reichenbach, 1989; Bernardet et al., 1996; Bernardet et al., 2002). *F. psychrophilum* is considered a poor glider, although gliding motility was indeed observed on some strains (Schmidtke and Carson, 1995; Lumsden et al., 1996; Ostland et al., 1997; Lee and Heo, 1998; Madetoja et al., 2001); however, *F. psychrophilum* usually exhibits only a slow and weak gliding motility compared to other gliding *Flavobacterium* species (Bernardet and Kerouault, 1989; Cipriano et al., 1995; Iida and Mizokami, 1996). *Flavobacterium pectinovorum* was the only species able to grow on marine media among the ten *Flavobacterium* species known in 1996. Except *F. antarcticum*, the *Flavobacterium* species which were isolated from soil or freshwater, i.e. *F. hibernum*, *F. limicola*, *F. omnivorum*, *F. xinjiangense*, *F. granuli*, *F. fryxellicola* and *F. psychrolimnae*, cannot grow on marine media. All other newly described *Flavobacterium* species (i.e., *F. degerlachei*, *F. frigidarium*, *F. frigoris*, *F. gelidilacus*, *F. gillisiae*, *F. micromati*, *F. tegetincola* and *F. xanthum*) were retrieved from marine or saline environments and are consequently able to grow on marine media (McCammon et al., 1998; McCammon and Bowman, 2000; Humphry et al., 2001; Tamaki et al., 2003; Van Trappen et al., 2003a; Van Trappen et al., 2003b; Zhu et al., 2003; Aslam et al., 2005). However, since most of them do not require NaCl for growth, they were described as merely halotolerant, not halophilic organisms. The range of NaCl concentrations they tolerate varies widely among species; in the case of *F. frigidarium*, it also varies with the composition of the growth medium used (Humphry et al., 2001). NaCl inhibits the growth of *F. limicola*, although it may tolerate up to 1.5% NaCl (Tamaki et al., 2003). Although different terms are sometimes used (e.g., chemoheterotroph [McCammon and Bowman, 2000; Tamaki et al., 2003] and heteroorganotrophic [Zhu et al., 2003]), all *Flavobacterium* species described since 1996 are chemoorganotrophic and strictly aerobic. *Flavobacterium branchiophilum* was confirmed as an obligate aerobe (Ostland et al., 1994) rather than a facultative anaerobe as previously reported (Ferguson et al., 1991). No growth factors are necessary; however, growth may be stimulated by various mineral or biological compounds, e.g., vitamins for *F. gillisiae* (McCammon and Bowman, 2000), yeast extract for *F. limicola* (Tamaki et al., 2003), and fetal bovine serum and trace mineral elements for *F. psychrophilum* (Michel et al., 1999).

In the emended description of the genus *Flavobacterium* (Bernardet et al., 1996), indole production was negative and catalase production positive for all ten species; this is still the case for the sixteen *Flavobacterium* species described since then. However, the production of catalase is weak in some *Flavobacterium* species (e.g., *F. psychrophilum*; Bernardet and Kerouault, 1989); in such cases, the production of oxygen bubbles by bacteria flooded with oxygen peroxide should be tested on fresh colonies and checked under a stereomicroscope (×20). Characteristics that are variable between *Flavobacterium* species (i.e., degradation of various polysaccharides, casein and gelatin, production of acid from carbohydrates, and production of cytochrome oxidase) are listed in Table 4. Surprisingly, even though several among the newly described *Flavobacterium* species originate from marine environments, the only new agarolytic species is *F. limicola*, isolated from freshwater (Tamaki et al., 2003). Tween 80 was decomposed by all ten *Flavobacterium* species in 1996. Although this characteristic was not tested for all newly described species, *F. limicola*, *F. omnivorum* and *F. xinjiangense* do not decompose this compound (Tamaki et al., 2003; Zhu et al., 2003), contrary to *F. antarcticum* (Yi et al., 2005). The decomposition of tributyrin, a trait for which all ten *Flavobacterium* species gave a positive reaction, was not tested in the sixteen new species. *Flavobacterium limicola*, *F. johnsoniae* and *F. granuli* were also shown to be the only urea-hydrolyzing *Flavobacterium* species among those tested (Reichenbach, 1989; Carson et al., 1993; Tamaki et al., 2003; Aslam et al., 2005). The overall fatty

acid profiles of the *Flavobacterium* species described since 1996 resemble those of the ten previously known species, with only slight discrepancies in the major and minor constituents (McCammon et al., 1998; McCammon and Bowman, 2000; Humphry et al., 2001; Tamaki et al., 2003; Van Trappen et al., 2003a; Van Trappen et al., 2003b; Zhu et al., 2003). Only *F. hibernum* and *F. xanthum* have been tested for their polyamine composition; homospermidine is still the only or major polyamine in these two species (Hamana and Nakagawa, 2001). The low optimum temperature range for *F. psychrophilum*, which was an exception in the 1996 genus description, is now shared by all new species; this is not surprising since they were all retrieved from cold environments. (*Flavobacterium granuli*, isolated from a wastewater treatment plant, is an exception; it grows at 37°C and maybe more (Aslam et al., 2005). Their optimum temperature varies from 11°C for *F. omnivorum* and *F. xinjiangense* (Zhu et al., 2003) to 26°C for *F. hibernum* (McCammon et al., 1998) and is around 15–20°C for all other species. The lowest temperature for growth is 0°C for *F. gillisiae*, *F. tegetincola*, and *F. limicola* (McCammon and Bowman, 2000; Tamaki et al., 2003), lower than 0°C for *F. frigidarium* and *F. xanthum* (McCammon and Bowman, 2000; Humphry et al., 2001), and as low as –7°C for *F. hibernum* (McCammon et al., 1998). All *F. columnare* strains tested are able to grow at 18 and 30°C, but only some of them grow at 15 or 37°C (Triyanto and Wakabayashi, 1999); strains of *F. psychrophilum* usually grow at 4–23°C (Holt et al., 1989; see the sections Isolation and Cultivation and Pathogenicity and Epidemiology in this Chapter). Cold and polar (as well as marine or saline) environments must be added to the temperate soil and freshwater habitats listed in the 1996 description, but no other fish-pathogenic *Flavobacterium* species was described. Since the G+C content of *F. gelidilacus* is around 30 mol% (Van Trappen et al., 2003b), the range of G+C content for species in the genus *Flavobacterium* is now 30–37 mol%. Although slightly lower (29 mol% for some *F. branchiophilum* strains; Wakabayashi et al., 1989) and higher (38 mol% for *F. succinicans*; Anderson and Ordal, 1961) values were reported, they have subsequently been re-evaluated (Reichenbach, 1989; Bernardet et al., 1996).

In the minimal standards proposed for describing new genera and species in the family Flavobacteriaceae (Bernardet et al., 2002), particular methods were suggested to determine some of these phenotypic properties since the conditions in which the tests are performed are critical. These procedures (updated from Bernardet et al., 2002) are listed below in the order shown in Table 4.

Colony Morphology and Gliding Motility

Provided identical growth conditions are used, colony morphology may help in differentiating between *Flavobacterium* species (Reichenbach, 1989; J.-F. Bernardet, unpublished results). Characteristics such as iridescent waves (Fig. 3A) and spreading (Figs. 3B and 3C) to rhizoidal

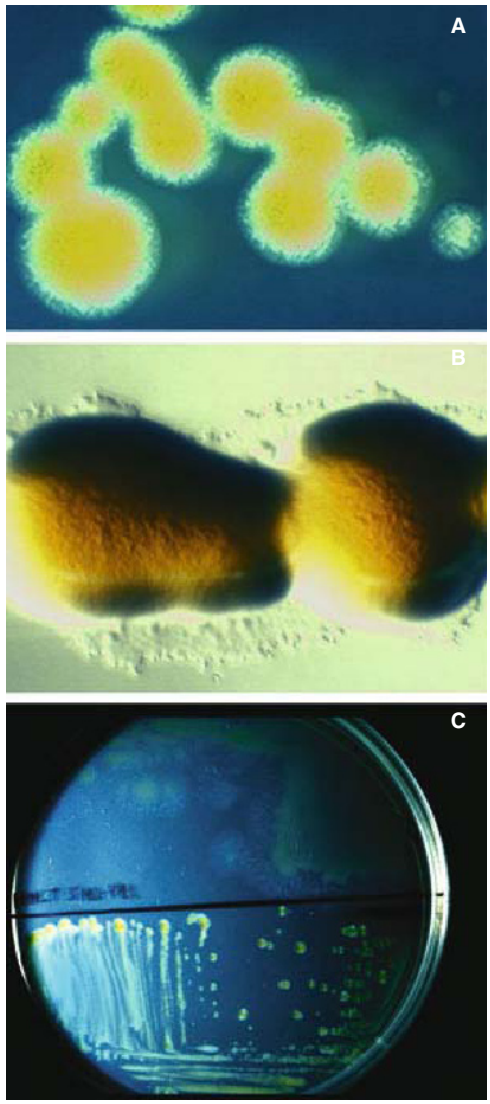

Fig. 3. Colonies of *F. psychrophilum*. Typical yellow, circular, convex, nonadherent colonies of *F. psychrophilum* after a five-day incubation on Anacker and Ordal's agar (containing 0.5% tryptone instead of 0.05%) at 18°C; under the stereomicroscope and oblique transillumination, shiny, iridescent, kaleidoscopic waves are clearly visible (A; size of the colonies is ~1–3 mm). Colonies of another strain, observed under the same conditions, exhibit zones of thin bacterial growth spreading from otherwise entire edges (B; size of the colonies ~0.5–1.5 mm). On the same Anacker and Ordal's agar plate (5 days, 18°C), two *F. psychrophilum* strains exhibit very different colony types: at the bottom of the photograph, the type strain NCIMB 1947^T produced typical circular colonies, whereas at the top another strain produced flat spreading colonies (C).

edges (Figs. 4A to 4F) are best revealed under stereomicroscopic examination (×20) through oblique transmitted light (Anderson and Ordal, 1961; Bernardet, 1989b; Bernardet and Kerouault, 1989). Different strains of *F. columnare*, *F. psychrophilum*, *F. pectinovorum* and *F. succinicans* may produce different colony types, i.e., compact with regular margins or spreading with uneven to rhizoid margins (Fig. 3C); some strains may even exhibit different colony types on the same agar plate (Anderson and Ordal, 1961; Bernardet, 1989a; Bernardet, 1989b; Bernardet and Kerouault, 1989; Schmidtke and Carson, 1995; Lee and Heo, 1998).

Anacker and Ordal's medium (see the section Isolation and Cultivation in this Chapter) is recommended to observe the typical spreading or swarming colonies exhibited by most gliding *Flavobacterium* species (Holmes et al., 1984; Bernardet, 1989a; Bernardet, 1989b; Bernardet and Kerouault, 1989; see the section Isolation and Cultivation in this Chapter). Under direct microscopic examination, their rhizoid or filamentous margin is composed of numerous bacterial cells, alone or in small groups, slowly gliding on the wet surface of the agar and progressing towards the periphery (Fig. 4F). Gliding may be readily observed; in other cases, the slime tracks left on agar by gliding cells are clearly visible (Burchard, 1981; Reichenbach, 1992; J.-F. Bernardet, unpublished data; Fig. 4G). The cells are able to glide either as single cells or as groups of cells. In this sense, their behavior is strikingly similar to the "A" (adventurous) and "S" (social) motility of *Myxoccoccus xanthus*, referred to in the chapter on The Myxobacteria in this Volume.

Information on gliding motility, methods available to observe it in liquid and solid cultures, and the relationship among gliding, production of slime, and adherence are given in Bernardet et al. (2002) and in the chapter An Introduction to the Family Flavobacteriaceae in this Volume.

Adherence of colonies to the agar is also a useful feature; it can be determined by trying to collect colonies on agar plates with a loop. Colonies of *F. pectinovorum* merely exhibit a sticky or mucoid consistency, while separating colonies from agar can be nearly impossible in some *Flavobacterium columnare* strains (Garnjobst, 1945); adherence of this pathogen to fish tissue is considered an important virulence factor (see the section Pathogenicity and Epidemiology in this Chapter). However, adherence may be lost after several subcultures: the National Collections of Industrial, Food, and Marine Bacteria (NCIMB, Aberdeen, Scotland) replaced its culture of the *F. columnare* type strain (NCMB 1038^T) by an adherent subculture (NCIMB 2248^T) after the original one lost its typical adherence. Adherence is also exhibited by *F. columnare* cells in still or slowly agitated liquid cultures: yellow filamentous clumps or tufts of bacterial cells adhere at the surface of the glass flask, forming a thick ring at the upper level of the liquid medium (Fig. 5). When the liquid culture is agitated using a magnet, the latter may be completely covered by these adherent clumps (Garnjobst, 1945; Shamsudin and Plumb, 1996; Newton et al., 1997; Decostere et al., 1998; J.-F. Bernardet, unpublished results).

Such clumps (as well as the warty center of some colonies and the "columns" of bacterial cells that appear on infected tissue; see the section Pathogenicity and Epidemiology in this Chapter) were already noticed in early studies and mistaken for fruiting bodies. This confusion, together with that of the spheroplasts for microcysts, led early authors to assign *F. columnare* to the myxobacteria, and hence the long used epithet "[*Chondrococcus*] *columnaris*" (Ordal and Rucker, 1944; Pacha and Ordal, 1970; Bullock et al., 1971). Conversely, Garnjobst correctly identified the spheroplasts and, since she did not observe fruiting bodies, she ascribed this organism to the genus *Cytophaga*, which was indeed much closer to the currently accepted taxonomic affiliation (Garnjobst, 1945).

Adsorption of Congo Red

Congo red adsorption is tested by directly flooding some colonies on the agar with a few drops of a 0.01% aqueous solution of the dye; after about two min, the dye is gently rinsed with water and the color of these colonies compared to that of control colonies not covered with the dye (Fig. 6). In the case of *Flavobacterium columnare*, the Congo red-staining material has been shown to be an extracellular galactosamine glycan in the slime (Johnson and Chilton, 1966).

Congo red may also be included in the agar; *F. columnare* colonies growing on this medium are red (P. Koski, personal communication). However, when *F. psychrophilum* was tested on a Congo red-containing agar, bacterial growth was strongly inhibited by the dye (Crump et al., 2001). Among the tested *Flavobacterium* species described since 1996, only colonies of *F. limicola* and *F. frigidarium* were shown to adsorb Congo red (Humphry et al., 2001; Tamaki et al., 2003). For the latter species, this trait was actually not definitely demonstrated since the authors carefully stated that "There seemed to be absorption of Congo red by colonies . . ."; in the same study, Congo red was also used to reveal the xylanase activity of the isolate (Humphry et al., 2001).

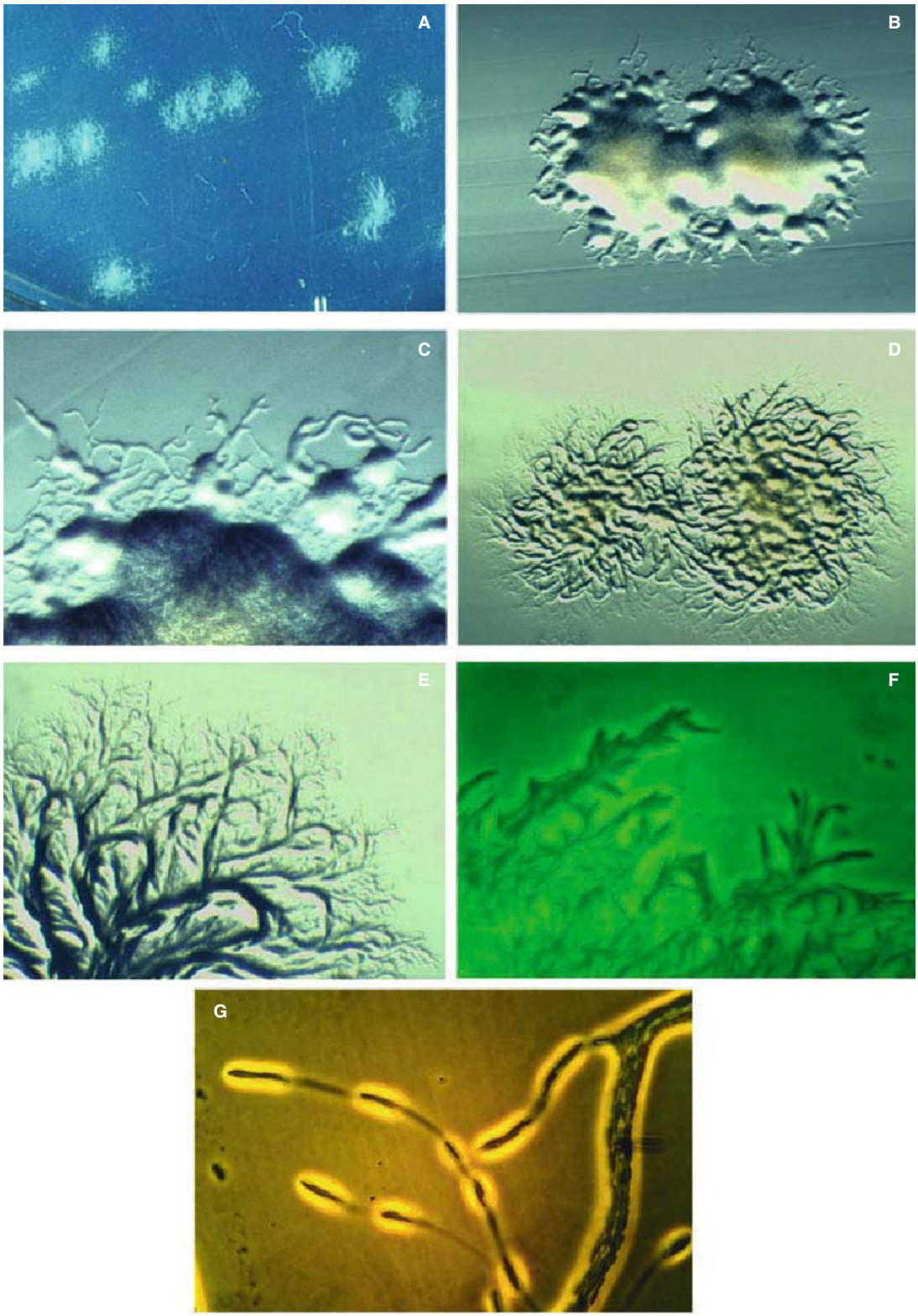

Fig. 4. Colonies of *F. columnare*. Grown on Anacker and Ordal's agar at 22°C for three days, a *F. columnare* strain produced typical pale yellow, flat, dry, rhizoid and adherent colonies (A; field of the view: approx. 5 × 3 cm). The rhizoid aspect of the edges is better seen under the stereomicroscope and oblique transmitted illumination: B and C, moderately rhizoid colonies, and D and E, very rhizoid colonies (size of the colonies: approx. 5 mm). Phase-contrast microscopy reveals groups of parallel bacterial cells spreading from the edge of a colony of the *F. columnare* type strain, NCIMB 2248^{T} (F; field of the view: approx. 0.2 × 0.14 mm). When growth and optical conditions are favorable, the observation of the edge of a *F. columnare* colony on Anacker and Ordal's agar under phase-contrast microscopy (×40) may reveal the slime tracks left by groups of bacterial cells gliding at the surface of the agar (G).

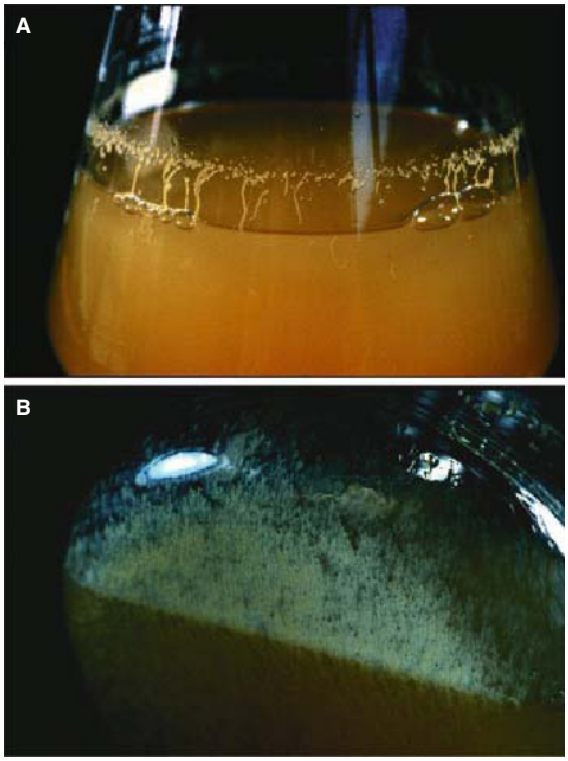

Fig. 5. Adherence of *F. columnare*. A *F. columnare* strain was cultivated for three days at 25°C in 1 liter of Anacker and Ordal's broth (enriched to 0.5% tryptone instead of 0.05%) with gentle orbital shaking. Numerous filamentous clumps of bacteria adhered to the glass, forming a ring at the top edge of the broth surface (A) and carpeting the walls of the flask (B shows the bottom of the tilted flask).

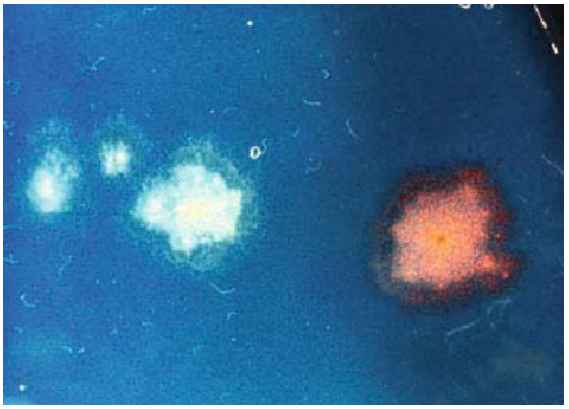

Fig. 6. Congo red test. The color of rhizoid *F. columnare* colonies was checked after flooding the colony on the right hand side with an aqueous 0.01% Congo red solution and gentle rinsing using distilled water: the colony has adsorbed the red dye, demonstrating the presence of a galactosamine glycan component in the bacterial slime (field of the view: approx. 5 × 3 cm).

Determination of Pigment

Information on the different types of pigment produced by members of the family Flavobacteriaceae and on methods to identify these pigments is given in Bernardet et al. (2002). Briefly, an easy although not absolutely specific method to demonstrate the production of flexirubin type pigments is to flood a mass of bacterial cells collected on agar with 20% KOH and to compare the resulting color with that of a control mass that has not been flooded with KOH. When the yellow color of the strain is caused by flexirubin type of pigments, the mass immediately turns dark red, purple or brown (Reichenbach, 1989; Fig. 7). When the yellow color is presumably caused by carotenoid type of pigments, no color change develops.

Several other KOH techniques were proposed. For example, KOH may be directly poured over colonies on an agar plate (e.g., Crump et al., 2001). However, the resulting color-shift may pass unnoticed on a thin layer of bacteria (Cipriano et al., 1996); this was probably why *F. psychrophilum* was first considered devoid of flexirubin type pigments by some authors (e.g., Holt, 1988). Alternatively, a loopful of bacterial cells may be streaked onto filter paper soaked in 1N NaOH (Brown et al., 1997). A "string test" was also proposed (Cipriano et al., 1996). Finally, the KOH test may be performed after methanol/chloroform extraction of the pigments from the pellet of a liquid culture (Maeda et al., 1998). The genus *Flavobacterium* includes carotenoid-producing and flexirubin-producing species, as well as species that produce both types of pigments (Reichenbach, 1989; Bernardet et al., 1996). The more frequent association of carotenoid pigments with marine species and flexirubin pigments with freshwater or soil organisms (Reichenbach, 1989) is not especially clear for members of the genus *Flavobacterium*: the yellow color of *F. frigidarium* and *F. gillisiae*, the only species originating from marine environments, is indeed caused by carotenoid pigments, but several other *Flavobacterium* species produce this type of pigment even though they occur in nonmarine environments (Table 4).

Degradation of Cellulose and Cellulose Derivatives

Some species in the family Flavobacteriaceae degrade soluble cellulose derivatives such as carboxymethylcellulose or hydroxyethylcellulose but, since enzymes other than cellulases can degrade these compounds, this does not demonstrate that these species are cellulolytic. The decomposition of crystalline cellulose (i.e., filter paper) requires the production of a specific cel-

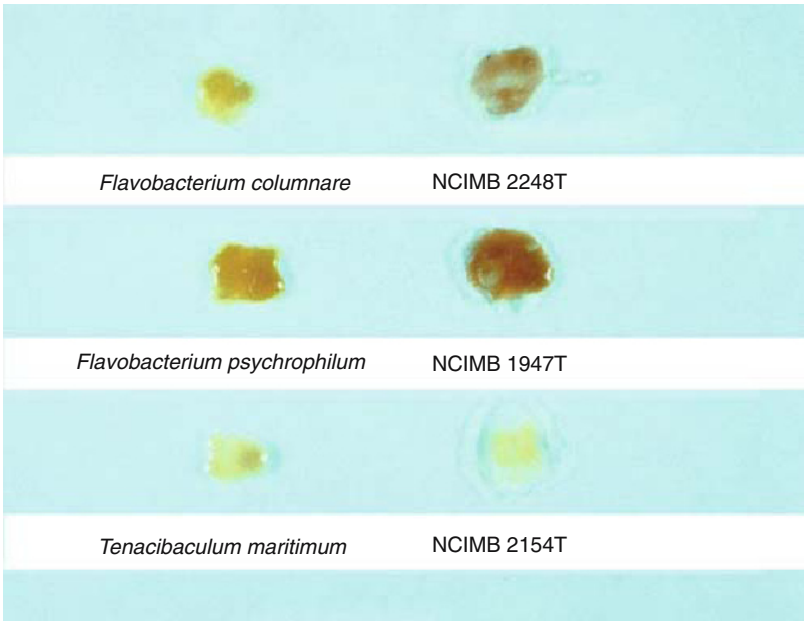

Fig. 7. KOH test for the detection of flexirubin type pigments. After two masses of bacterial cells of *Flavobacterium columnare*, *F. psychrophilum* and *Tenacibaculum maritimum* were deposited on a glass slide, those on the right hand side were flooded with a drop of 20% KOH. The immediate color shift from yellow to brownish pink of the two *Flavobacterium* strains revealed the presence of flexirubin type pigments, whereas the unchanged cream color of the *Tenacibaculum* strain suggested that it is rather due to carotenoid type pigments.

lulase, and hence only strains able to degrade filter paper should be regarded as cellulose degraders (Reichenbach, 1989). The ten *Flavobacterium* species known in 1996 were considered unable to degrade crystalline cellulose and this inability has subsequently been confirmed in members of the recently described *Flavobacterium* species (J. P. Bowman, unpublished results). Hence, this characteristic distinguishes members of the genus *Flavobacterium* from those of the genus *Cytophaga*, now restricted to cellulolytic organisms (Nakagawa and Yamasato, 1996). Two recent publications may challenge this clear-cut distinction. The degradation of cellulosic plant fibers by a number of soil organisms was investigated, and nine cellulolytic isolates were assigned to *F. johnsoniae* (Lednicka et al., 2002). However, these strains were exclusively identified through fatty acid analysis, a technique that is considered to provide taxonomic information mainly at the generic level in the family Flavobacteriaceae (Bernardet et al., 2002). A cellulose-degrading bacterial strain isolated from freshwater proved capable of killing the cyst form of the protozoan parasite *Giardia lamblia*; the determination of the sequence of its 16S rRNA showed a close relationship with those of several *F. columnare* strains, and the isolate shares a number of phenotypic traits with this species (Rodgers et al., 2003). Although some other traits as well as the G+C content were at variance with *F. columnare*, and although a high 16S rRNA homology does not guarantee conspecificity (Bernardet et al., 2002), it is still likely this isolate indeed belongs to the genus *Flavobacterium* or at least to the family Flavobacteriaceae.

Several *Flavobacterium* species are able to degrade various cellulose derivatives, carboxymethylcellulose (CMC) being the most frequently tested (Table 4), for instance, on a 0.5% CMC overlay agar (McCammon et al., 1998). Alternatively, 3% of high viscosity CMC sodium salt may be added to any liquid medium with a rather low nutrient content (e.g., Anacker and Ordal's medium; see the section Isolation and Cultivation in this Chapter); the medium is homogenized by vigorous shaking, distributed in deep glass tubes, and autoclaved. After the gelified medium was inoculated and incubated for several days, its consistency is compared to that of a control, uninoculated, tube. Depending on the ability of the strain to degrade CMC, the medium may be partially or totally liquefied (Bernardet, 1989a; Van Trappen et al., 2003a; Van Trappen et al., 2003b; Fig. 8).

Degradation of Other Biomacromolecules

The degradation of biomacromolecules other than those mentioned above, particularly polysaccharides and proteins, by bacterial species now included in the genus *Flavobacterium* was extensively reviewed by Reichenbach (1989). The degradation of various macromolecules has also been reported in recently described *Flavobacterium* species (Table 4). Most *Flavobacterium* species are able to degrade a variety of polysaccharidic components of algae, plants, fungi, or insects such as agar (Fig. 9), alginate, chitin, laminarin, pectin, xylan, etc. (Stanier, 1947; Van der Meulen et al., 1974; Liao and Wells, 1986; Reichenbach, 1989; Humphry et al., 2001).

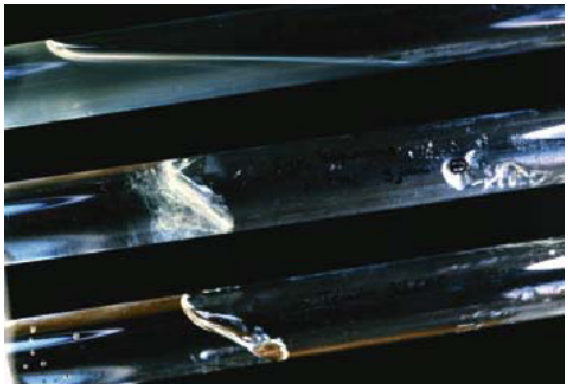

Fig. 8. Hydrolysis of carboxymethylcellulose. Each tube contained 10 ml of Anacker and Ordal's broth gelified with 3% high-viscosity CMC. The tube at the bottom of the figure was an uninoculated control, whereas the two other tubes were inoculated and incubated at 22°C for three days. The tubes were then tilted to reveal the consistency of the medium. Inoculated with *Pedobacter heparinus*, which did not hydrolyze CMC, the medium in the middle tube had the same consistency as that in the control tube. Conversely, the *F. johnsoniae* strain inoculated in the upper tube completely hydrolyzed CMC, turning the medium to liquid.

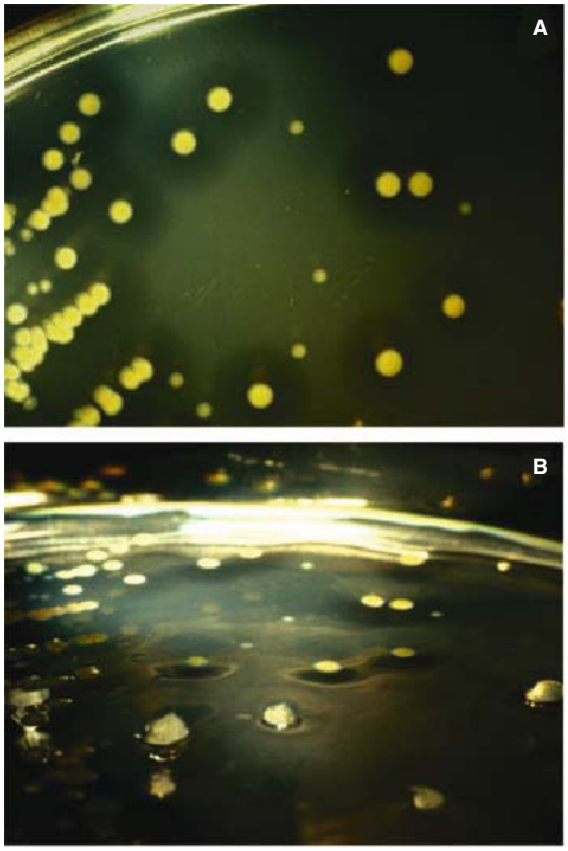

Fig. 9. Hydrolysis of agar. After a four-day incubation at 22°C on Anacker and Ordal's medium with 1.2% (w/v) agar, *F. flevense* has hydrolyzed the surrounding agar: colonies are situated in the center of shallow craters (Fig. 9B), under and around which the agar is softened and more transparent; hence the clear halos surrounding colonies (Fig. 9A).

Interestingly, the fish pathogens *F. columnare* and *F. psychrophilum* have long been considered unable to degrade any polysaccharide (Bernardet and Grimont, 1989). However, it was later discovered that they are actually able to degrade some particular complex acidic polysaccharides of connective tissue such as chondroitin sulfate and hyaluronic acid (Otis, 1984; Griffin, 1991; Teska, 1993; Stringer-Roth et al., 2002). Degradation of chondroitin sulfate was even included in an identification scheme for *F. columnare* (Griffin, 1992). Hence, these polysaccharide-degrading enzymes likely participate in the severe skin and muscular necrotic lesions that frequently occur in infected fish (see the section Pathogenicity and Epidemiology in this Chapter) in combination with the various extracellular proteases also produced by *F. columnare* and *F. psychrophilum*. These proteases, considered important virulence factors, are able to degrade components of muscle, cartilage, and connective tissue such as elastin, type IV collagen, fibrinogen, gelatin, laminin, fibronectin, actin and myosin (Otis, 1984; Holt, 1988; Bertolini and Rohovec, 1992; Bertolini et al., 1994; Nomura and Ohara, 1994; Newton et al., 1997; Ostland et al., 2000; Secades et al., 2001; Secades et al., 2003). The two metalloproteases purified from *F. psychrophilum*, produced under different physiological conditions, exhibit a broad range of hydrolytic activity and are adapted to low temperatures, being optimally produced at 12°C (Secades et al., 2001; Secades et al., 2003). Ostland et al. (2000) demonstrated that in vitro casein-, gelatin- and collagen-degrading activities of *F. psychrophilum* strains were associated with their ability to produce muscle necrosis when injected in rainbow trout. Interestingly, the optimum temperature for protease production was consistently 4–9°C below the optimum temperature for growth for all strains tested (Uddin and Wakabayashi, 1997). The low temperature of protease production may be related to the range of temperature at which outbreaks of the diseases caused by *F. psychrophilum* in salmonid fish occur (see the section Pathogenicity and Epidemiology in this Chapter). In *F. branchiophilum*, the extracellular products contain a protease, a phosphatase, and a phosphoamidase but no hemolysin or endotoxin; when juvenile rainbow trout were immersed in the extracellular products, gill lesions similar to those which occur during the natural infection were observed (Ototake and Wakabayashi, 1985; see the section Pathogenicity and Epidemiology in this Chapter). As in many microorganisms adapted to cold

environments, the production of the extracellular protease found in *F. limicola* is enhanced at low temperatures (Tamaki et al., 2003).

Production of Cytochrome Oxidase

Although the different techniques available to detect the production of cytochrome oxidase have not been compared on all *Flavobacterium* species, it has been demonstrated that the presence of cytochrome oxidase was more readily evidenced in *F. columnare* and *F. psychrophilum* using discs impregnated with dimethyl-*p*-phenylene diamine oxalate than using liquid tetramethyl-*p*-phenylene diamine dihydrochloride reagents (Koski et al., 1993; J.-F. Bernardet, unpublished results). This characteristic should be tested on fresh colonies since it becomes difficult to detect after more than 3–4 days cultivation, especially on species that yield a weak positive reaction such as *F. psychrophilum* (Bernardet and Kerouault, 1989).

Commercial Identification Galleries

In addition to (or instead of) the standard biochemical tests, commercially available kits, strips, and galleries have been extensively used over the last decade to help identify *Flavobacterium* strains. However, since most of these systems are aimed at the identification of human pathogens, the temperature and time of incubation must be adapted to test bacteria that do not grow at 37°C. API galleries (bioMérieux) have frequently been used. While API 20E galleries are not well adapted to most *Flavobacterium* strains (J.-F. Bernardet, unpublished results), API 20NE, API ZYM, API ID 32E, API 32GN and API 50CH have yielded interesting results when used at temperatures that were close to the optimum of the *Flavobacterium* species tested. As for API ZYM galleries, the time of incubation usually varied from the 4 h recommended by the manufacturer (Van Trappen et al., 2003a; Van Trappen et al., 2003b; Aslam et al., 2005) to overnight (Bernardet, 1989b; Bernardet and Grimont, 1989; Bernardet and Kerouault, 1989; Madetoja et al., 2001), although incubation time up to two or three days was sometimes used (Ostland et al., 1994; Humphry et al., 2001). API ZYM galleries were used to differentiate the most common fish pathogenic bacteria, including *F. columnare* (Sakai et al., 1993). API galleries in which bacterial growth is necessary were also incubated for two to three days (McCammon et al., 1998; Humphry et al., 2001; Van Trappen et al., 2003a; Van Trappen et al., 2003b) and up to ten days for API 50CH galleries (Bernardet, 1989b; Bernardet and Grimont, 1989; Bernardet and Kerouault, 1989). Except *F. columnare* (Bernardet, 1989b) and *F. psychrophilum* (Bernardet and Kerouault, 1989), all *Flavobacterium* species tested in API 50CH galleries were able to utilize a variety of substrates. Preliminary studies also demonstrated that *F. johnsoniae*-like fish isolates utilize a number of substrates in Biotype 100 galleries (bioMérieux; J.-F. Bernardet and C. Bizet, unpublished data). Various substrates were also utilized by *F. hibernum* (after incubation times of 4 and 24 h) and *F. limicola* in GN MicroPlates (Biolog; McCammon et al., 1998; Tamaki et al., 2003).

Besides the above-mentioned identification methods, other usually more sophisticated techniques are available to help identify *Flavobacterium* strains and species. The application of most of these techniques to the differentiation of members of the family Flavobacteriaceae was already considered by Bernardet et al. (2002) and in the chapter An Introduction to the Family Flavobacteriaceae in this Volume; some additional information primarily on the genus *Flavobacterium* is provided below.

Composition of the Cell Wall and Membrane

Reichenbach (1989) reviewed data available on the structure and composition of the cell wall and membrane in several bacterial species now attributed to the genus *Flavobacterium*. Since then, fish-pathogenic species were investigated in this regard, mostly to characterize antigens that could be targets for potential vaccines and diagnostic tests (see the section Pathogenicity and Epidemiology in this Chapter). Low- and high-molecular-mass lipopolysaccharides were identified in *F. psychrophilum*, the latter containing the O-antigen (Crump et al., 2001; MacLean et al., 2001); a very unusual sugar in the O-chain may constitute a specific diagnostic target (Crump et al., 2001). Also in *F. psychrophilum*, the outer membrane protein was tested as a possible vaccine (Rahman et al., 2002), a major glycoprotein antigen was purified and characterized (Merle et al., 2003) and a surface antigenic protein was isolated and partially characterized (Massias et al., 2004). Outer membrane proteins and the lipopolysaccharide of *F. columnare* were also studied (Shamsudin, 1994); the structure of the lipopolysaccharide O-antigen was only recently characterized and shown to differ from that of *F. psychrophilum* (MacLean et al., 2003). The structure of the glycopeptides resulting, together with the lipopolysaccharide (LPS) and the capsular polysaccharide from the phenol-water extraction of *F. columnare* cells, was determined; its role in pathogenesis and its antigenic

properties are not known yet (Vinogradov et al., 2003).

As do other members of the family Flavobacteriaceae, *Flavobacterium* species contain menaquinone 6 as their only respiratory quinone and do not contain sphingophospholipids (Bernardet et al., 1996).

The kind of chemotaxonomic information resulting from the analysis of whole-cell fatty acid methyl esters (FAMEs) is discussed in the chapter An Introduction to the Family Flavobacteriaceae in this Volume. Recently, fatty acid analysis has been used extensively for rapidly clustering large numbers of polar bacterial isolates; representative strains in each cluster were then submitted to phylogenetic analysis (see Van Trappen et al. [2002] and references therein). Those isolates that were subsequently shown to belong to the genus *Flavobacterium* mostly contained branched fatty acids (saturated, unsaturated, or saturated and hydroxylated; Van Trappen et al., 2002). These fatty acids, which contribute to the membrane fluidity at low temperature, were abundant in all psychrophilic *Flavobacterium* species (Zhu et al., 2003) and no evidence of polyunsaturated fatty acids was found (Humphry et al., 2001). The overall fatty acid compositions of newly described *Flavobacterium* species were similar to that cited in the genus description (see above), except for some discrepancies in relative proportions (e.g., Van Trappen et al., 2003, 2005; Aslam et al., 2005; Yi et al., 2005) and failure to detect 15:1 *iso* G. Among those fatty acids already listed in the genus description, 15:0, 15:0 *iso*, 15:0 *iso* 3OH, 16:0 *iso* 3OH, 17:1 *iso* 30H and particularly 16:1 ω7c were the most abundant components in most new species (McCammon et al., 1998; McCammon and Bowman, 2000; Humphry et al., 2001; Tamaki et al., 2003; Van Trappen et al., 2003a, Van Trappen et al., 2003b, 2005; Zhu et al., 2003; Aslam et al., 2005; Yi et al., 2005). Additional components which may account for a significant fraction of the total fatty acid composition of the recently described *Flavobacterium* species were 15:0 *anteiso*, 15:1, 15:1 *iso*, 15:1 ω6c, 15:1 *iso* ω10c, 16:0 *iso*, 16:1 *iso*, 17:1 ω6c, and summed feature 3. The decisive influence of growth temperature and medium on the relative amounts of the various fatty acids, although already recognized (McGrath et al., 1990), was again noticed in *F. limicola* (Tamaki et al., 2003). Therefore, although there are no species-specific fatty acid profiles in the genus *Flavobacterium*, it is still possible to define a rather typical profile for the genus itself. Given the necessary technical skill and equipment, the determination of the fatty acid profile helps assignment of new isolates to the genus.

Whole-Cell Protein Analysis

Again, provided the skill and equipment for highly standardized sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), numerical analysis of the resulting electrophoregram, and database comparison are available, the determination of whole-cell protein profiles may also help assign new isolates to published *Flavobacterium* species or delineate new species (Bernardet et al., 1996; Bernardet et al., 2002). A good correlation between high DNA homology and high similarity of whole-cell protein patterns was noticed among several members of the family Flavobacteriaceae (Vandamme et al., 1996; Bernardet et al., 2002; the chapter An Introduction to the Family Flavobacteriaceae in this Volume). Some *Flavobacterium* spp. (*F. columnare* and *F. psychrophilum*) could indeed be readily identified by their typical protein profile, but others (*F. branchiophilum*, *F. johnsoniae* and *F. succinicans*) because of intraspecific heterogeneity could not (Bertolini and Rohovec, 1992; Bernardet et al., 1996; Cipriano et al., 1996). For yet other species, no conclusion could be drawn owing to the limited number of strains tested/available. In *F. columnare* and *F. psychrophilum*, whole-cell protein patterns remained consistent regardless of the growth medium used (Cipriano and Teska, 1994). Unfortunately, none of the sixteen recently described *Flavobacterium* species was investigated for their protein profiles.

Multilocus Enzyme Electrophoresis

Multilocus Enzyme Electrophoresis (MEE) was used as an attempt to classify 106 strains of yellow, Gram-negative, nonflagellated organisms isolated from fish or freshwater environments; one of the four groups delineated by this technique was entirely composed of *F. columnare* strains (Starliper et al., 1988).

DNA-DNA Hybridization

DNA-DNA similarity, as determined using quantitative DNA-DNA hybridization, is still the acknowledged standard for species delineation, together with ΔT_m when available (Wayne et al., 1987; Stackebrandt et al., 2002). The different techniques available and a review of their application to members of the family Flavobacteriaceae were discussed extensively by Bernardet et al. (2002), and only additional information especially focused on the genus *Flavobacterium* will be given here. DNA-DNA hybridization

experiments performed on members of the species classified in the genus have revealed that the 70% cut-off value proposed by Wayne et al. (1987) does apply to *Flavobacterium* species. Strains belonging to the same species share DNA relatedness well above 70% (i.e., 75–100%), whereas DNA relatedness is distinctly below this value (i.e., 4–50%) when strains belonging to different *Flavobacterium* species are hybridized (Bernardet and Grimont, 1989; McCammon et al., 1998; McCammon and Bowman, 2000; Humphry et al., 2001; Tamaki et al., 2003; Van Trappen et al., 2003a; Van Trappen et al., 2003b; Zhu et al., 2003; Van Trappen et al., 2005). The range of DNA relatedness between different strains of the same species may be rather wide (from approximately 75% to close to 100%); this is the case for *F. columnare* (Bernardet and Grimont, 1989), *F. branchiophilum* (Wakabayashi et al., 1989), *F. limicola* (Tamaki et al., 2003) and *F. fryxellicola* (Van Trappen et al., 2005). In the particular case of *F. branchiophilum* and *F. johnsoniae*, DNA relatedness values among some strains were as low as 67% (Wakabayashi et al., 1989) and 60% (Bernardet et al., 1996), respectively; however, ΔT_m values well below 5°C have shown that such strains nevertheless belong to the same species (Bernardet et al., 1996). Three distinct hybridization groups were delineated within a collection of *F. columnare* strains; each was characterized by a particular 16S rDNA restriction pattern and a particular nucleotide signature in its 16S rRNA sequence (Triyanto and Wakabayashi, 1999). However, since no phenotypic characteristics were available to differentiate them, these groups could only be considered genospecies or genomovars. Intraspecific DNA relatedness cannot be assessed in *Flavobacterium* species for which only one bona fide strain is available, namely *F. antarcticum*, *F. aquatile*, *F. flevense*, *F. frigidarium*, *F. gillisiae*, *F. granuli*, *F. hydatis*, *F. omnivorum*, *F. pectinovorum*, *F. saccharophilum*, *F. xanthum* and *F. xinjiangense*.

Sequence Analysis of Small Subunit rRNA and *gyrB* genes

Sequences of the 16S rRNA gene of all valid *Flavobacterium* species are now available; their comparison, combined with earlier data resulting from DNA-rRNA hybridization experiments (Bauwens and De Ley, 1981; Bernardet et al., 1996), provides a distinct view of the phylogenetic relationships within the genus *Flavobacterium* (Fig. 1) and between the genus and other members of the family Flavobacteriaceae (see Figs. 1 and 2 of the chapter An Introduction to the Family Flavobacteriaceae in this Volume). Although 16S rRNA delivers valuable information to delineate taxa at the genus and family level, it should not be used as the only genomic method to delineate bacterial species, with the exception of noncultivable organisms. As shown by several studies, some of them dealing with members of the very family Flavobacteriaceae, the arbitrary cut-off value of 97% 16S rRNA sequence homology is frequently not correlated with DNA-DNA homology (see Bernardet et al. [2002] and references therein). For instance, values of 16S rRNA sequence similarity of 97.2–98.7% were recently found between several *Flavobacterium* species (Van Trappen et al., 2003b, 2005). The sequence of *gyrB* genes, encoding the subunit B protein of DNA gyrase, may also be used for phylogenetic studies. Until now, only the *gyrB* sequences of *F. aquatile*, *F. johnsoniae*, *F. psychrophilum* and "[*Sporocytophaga*] *cauliformis*" are available (Izumi and Wakabayashi, 2000; Suzuki et al., 2001); hence, an evaluation of the respective phylogenetic interest of *gyrB* and 16S rRNA is not possible yet. In the genus *Tenacibaculum*, another member of the family Flavobacteriaceae, interspecific DNA relatedness values have been shown to be more distinctly correlated to the sequence similarity of *gyrB* than to that of 16S rRNA (Suzuki et al., 2001).

A combination of two or more of the above techniques will provide a polyphasic approach to the identification of new *Flavobacterium* isolates (Vandamme et al., 1996). Indeed, it is this kind of approach that has allowed the successful identification and description of all recently published *Flavobacterium* species. Below, we list some other possible techniques for detecting *Flavobacterium* strains in the environment and in clinical samples without requiring previous culture, as well as the methods currently available to type collections of *Flavobacterium* strains.

Molecular Detection and Identification

Several culture-independent molecular methods have been used extensively over the last decade to investigate the phylogenetic composition of bacterial communities in various environments (see the section Habitat and Ecology in this Chapter and the chapter An Introduction to the Family Flavobacteriaceae in this Volume). However, although part of the panel of species used to design the rRNA-targeted oligonucleotide probes for fluorescent in situ hybridization (FISH) was *Flavobacterium* species, no probe specifically targeting members of the genus

Flavobacterium is available yet. Published probes had a broad specificity, usually including the whole *Cytophaga-Flavobacterium-Bacteroides* (CFB) group or parts of it (Manz et al., 1996; Maeda et al., 1998; Weller et al., 2000; Adachi et al., 2002; O'Sullivan et al., 2002). Hence, it is not possible to determine the proportion of *Flavobacterium* species among other members of the CFB group in the environments investigated. Other surveys of bacterial communities used the sequencing of cloned 16S rRNA gene fragments; this technique allowed the detection of several organisms distinctly belonging to the genus *Flavobacterium* in the Columbia and Delaware estuaries (Crump et al., 1999; Kirchman et al., 2003). Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments has also become a popular method to assess the diversity of bacterial flora. It was recently found, however, that *Flavobacterium* strains were the only members of the aquatic bacterioplankton studied that possessed multiple melting domains in their 16S rDNA; these domains were responsible for fuzzy bands on the gel and consequently for poor resolution of DGGE at the species level when commonly applied universal primers were used (Kisand and Wikner, 2003). This problem could lead to misinterpretation of DGGE data and thereby to incorrect estimates of the number of *Flavobacterium* species in natural bacterial communities.

A variety of PCR tests were published for the economically significant fish-pathogenic *Flavobacterium* species, i.e., *F. branchiophilum*, *F. columnare* and *F. psychrophilum* (see the section Pathogenicity and Epidemiology in this Chapter). Classical PCR assays using either 16S rRNA- or *gyrB*-targeted primers (Toyama et al., 1994; Toyama et al., 1996; Urdaci et al., 1998; Izumi and Wakabayashi, 2000) were devised, and procedures for nested PCR (Baliarda et al., 2002; Taylor and Winton, 2002), multiplex PCR (del Cerro et al., 2002), and TaqMan PCR (del Cerro et al., 2002) were also published. These PCR tests were used either to confirm the identification of cultured isolates or to specifically detect the pathogens in the tissue of diseased or apparently healthy fish (Wiklund et al., 2000; Baliarda et al., 2002), in ovarian fluid and eggs (Izumi and Wakabayashi, 1997; Kumagai and Takahashi, 1997; Baliarda et al., 2002), and in water samples (Wiklund et al., 2000; Madetoja and Wiklund, 2002). *F. psychrophilum* was also detected in water samples (Vatsos et al., 2002) and fish organs (Liu et al., 2001) by in situ hybridization using fluorescein-labeled PCR primers. Attempts were made to identify fish-pathogenic *Flavobacterium* species from samples of skin lesion by direct broad-range PCR amplification using universal primers followed by sequencing of the partial 16S rDNA PCR products; sequences similar to those of *F. columnare*, *F. psychrophilum* and *F. hibernum* were found (Tiirola et al., 2002). Presumptive identification at the generic and specific level of different fish pathogens including *F. columnare* and *F. psychrophilum* was also obtained directly from fish tissue using terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes (Nilsson and Strom, 2002).

Typing of Collections of *Flavobacterium* Strains

MOLECULAR TYPING. Several different molecular methods have been used to type collections of *Flavobacterium* strains. Understandably, because of the economic significance of the fish pathogenic species, and because collections of strains of widely different geographic origins and host species were available, typing methods have preferentially been tested on these species. Depending on the primers used, random amplified polymorphic DNA (RAPD) was shown to provide both a typing of *F. psychrophilum* and *F. columnare* strains and a clear differentiation between all *Flavobacterium* species tested (Chakroun et al., 1997); from a practical point of view, this technique was able to distinguish *F. psychrophilum* from other fish-pathogenic *Flavobacterium* species which may occur in the same environment (Crump et al., 2001). Ribotyping (or rRNA gene restriction pattern analysis) was also performed on *F. psychrophilum* strains using several different restriction endonucleases. All four strains tested by Ciprian et al. (1996) exhibited the same ribotype; however, different ribotypes were found when larger collections of strains were studied. Similar to what was obtained using RAPD, some of the ribotypes identified among 85 *F. psychrophilum* strains were distinctly correlated with the fish species from which the strains were isolated, whereas no correlation with the geographic origin was found (Chakroun et al., 1998). One ribotype was highly dominant among 299 Danish strains, and a relationship was found among this ribotype, serotype, and virulence (Madsen and Dalsgaard, 2000). Madetoja et al. (2001) also noted this relationship, but again no correlation with the geographic origin was found; interestingly, this study showed that different clones of *F. psychrophilum* could be isolated during the same outbreak. Three different restriction fragment length polymorphism (RFLP) patterns of PCR-amplified 16S rDNA were found in a collection of *F. columnare* strains, each corresponding to one of the three DNA-DNA hybridization groups identified in this species (Triyanto and

Wakabayashi, 1999; see above). In *F. psychrophilum*, RFLP of the amplified *gyrB* gene yielded different results depending on the kind of primers (i.e., universal versus *F. psychrophilum*-specific) used to amplify the gene; a certain correlation between RFLP patterns and the species of fish host was noticed (Izumi et al., 2003b). Five different plasmid profiles were found when a collection of mostly Danish *F. psychrophilum* isolates was investigated; a ~3.2-kb plasmid was present in most strains (Lorenzen et al., 1997). Chakroun et al. (1998) found that most of the 85 *F. psychrophilum* strains they tested harbored at least one plasmid and that eleven different plasmid profiles occurred; even strains belonging to the same ribotype could exhibit different plasmid profiles, and the approximate size of the plasmids varied from 2.1 to 20 kb. Thirty-seven strains originating from widely different geographic areas (i.e., the United States, Chile, Japan, and several European countries), most of them retrieved from rainbow trout, shared the same 3.5-kb plasmid (Chakroun et al., 1998). A 3.3-kb plasmid was found in 284 among 299 Danish *F. psychrophilum* strains studied, but no clear correlation was noted between the presence of this plasmid and the virulence of strains (Madsen and Dalsgaard, 2000). Among the four different plasmids found in a collection of *F. psychrophilum* isolates from various fish species and geographic areas, the 3.4 kb plasmid occurred mostly in rainbow trout isolates while two other plasmids seemed restricted to Japanese isolates; no plasmid was associated with antibiotic resistance (Izumi and Aranishi, 2004a). Given the rough method used in these studies to evaluate the plasmid size, it is most likely that the 3.2-, 3.3-, 3.4- and 3.5-kb plasmids they reported are actually identical. This plasmid, called "pCP1," was sequenced and used to develop cloning vectors for *F. johnsoniae* (McBride and Kempf, 1996; see the section Genetics in this Chapter).

Hence, the above-mentioned fingerprinting techniques may be powerful molecular tools for the epidemiological tracing of the infections caused by *Flavobacterium* species in fish, provided a sufficient number of strains representing a variety in origins is tested.

Recently, repetitive extragenic palindromic DNA-PCR (rep-PCR) fingerprinting was used to investigate the genomic diversity of clusters previously delineated by fatty acid analysis among a collection of polar isolates (Van Trappen et al., 2003, 2005). The results confirmed that strains sharing the same rep-PCR profile are closely related. Among the six new *Flavobacterium* species described by these authors, *F. degerlachei*, *F. micromati*, *F. fryxellicola* and *F. psychrolimnae* were each comprised of strains that exhibited the same rep-PCR profile, whereas *F. frigoris* and *F. gelidilacus* included strains representing two or three different rep-PCR profiles, respectively (Van Trappen et al., 2003a, Van Trappen et al., 2003b, 2005). Hence, this technique may either differentiate *Flavobacterium* species from each other or be used for intraspecific typing.

SEROLOGICAL TYPING. Fish-pathogenic *Flavobacterium* species have been extensively investigated for possible serotyping with applications to epidemiological studies and diagnostic tests in view; however, only a few studies included strains originating from various fish species and geographic areas. Early studies were reviewed by Reichenbach (1989). Many serotyping schemes were published, but it is only recently that investigators began identifying and characterizing bacterial antigens presumably responsible for the different serotypes (see the section Composition of the Cell Wall and Membrane in this Chapter). As for *F. psychrophilum*, three to seven serotypes were recognized by different authors depending on the origin and number of strains studied and on the technique used (i.e., rabbit or trout mono- or polyclonal antibodies; microtiter or slide agglutination with or without reciprocal absorption of antisera; double immunodiffusion; or enzyme linked immunosorbent assay [ELISA]; Holt, 1988; Wakabayashi et al., 1994; Lorenzen and Olesen, 1997; Izumi and Wakabayashi, 1999; Dalsgaard and Madsen, 2000; Madetoja et al., 2001; Mata et al., 2002). Recently, a new serotype was described and the different serotyping systems were compared and harmonized (Izumi et al., 2003a). No association was found between the serotypes of *F. psychrophilum* strains, their geographical origin, and the host fish species of origin (Faruk, 2000). Serological studies on *F. columnare* are scarce and early (Pacha and Ordal, 1970; Bullock, 1972; Sanders et al., 1976); again, serological relationships did not reveal a pattern based on fish host, tissue, or geographic origin (Shamsudin, 1994). Only limited numbers of *F. branchiophilum* strains have been studied; although common antigens were shared by Japanese, Canadian, and Hungarian strains, antigenic diversity was also found, even among isolates originating from the same region (Huh and Wakabayashi, 1989; Ostland et al., 1994; Ko and Heo, 1997).

BACTERIOCIN TYPING. Bacteriocins produced by some *F. columnare* strains are lethal to other strains of the same species; nine *F. columnare* types were defined according to their susceptibility to seven different bacteriocins, and a partial correlation was noted between these types and serotypes (Anacker and Ordal, 1959). Chase (1965) observed growth medium-dependent differences in the production of

these "columnaricins"; the highest production occurred during the late exponential growth phase (Needleman and Pacha, 1974).

MISCELLANEOUS. Early studies, reviewed by Reichenbach (1989), have reported the presence of phages in *F. columnare* and *F. johnsoniae*, but no practical utilization (e.g., for the typing of strains or as a control method during outbreaks of fish disease) was suggested. Recently, a *Flavobacterium* sp. was identified as a phage host in Arctic sea ice, but its highest 16S rRNA sequence homology (with *F. hibernum*) was only 94% (Borriss et al., 2003).

The only *Flavobacterium* species for which a particular odor, called "sickening," "distinct, fruity," or "pronounced and somewhat characteristic," was noted is *F. columnare* (Garnjobst, 1945; McCarthy, 1975; Shamsudin and Plumb, 1996).

The susceptibility to a range of antibiotics has been tested in only some members of the genus *Flavobacterium*. On the whole, and contrary to other members of the family Flavobacteriaceae such as the genus *Chryseobacterium* (see the corresponding chapter The Genus *Chryseobacterium* in this Volume), *Flavobacterium* strains are not considered to be highly resistant to antimicrobial drugs. Obviously, the fish pathogens have been particularly studied in this regard to determine which drugs might be used to treat infections. The drug susceptibility of *F. columnare* and *F. psychrophilum* were determined (Fijan and Voorhees, 1969; Amin et al., 1988; Hawke and Thune, 1992; Soltani et al., 1995; Shamsudin and Plumb, 1996) and their intrinsic resistance to certain antibiotics was used to propose selective media (Fijan, 1969; Decostere et al., 1997; Kumagai et al., 2004; see the section Isolation and Cultivation in this Chapter). A particular medium was proposed to test the antibiotic susceptibility of *F. columnare* (Hawke and Thune, 1992). Antimicrobial resistance patterns of bacterial strains and minimum inhibitory concentrations of commonly used antibiotics were determined for *F. columnare*, *F. johnsoniae* and *F. psychrophilum* to validate their clinical efficacy when fish were administered bath or oral treatment (Bernardet, 1989b; Bernardet and Grimont, 1989; Bernardet and Kerouault, 1989; Soltani et al., 1995; Lorenzen et al., 1997; Rangdale et al., 1997; Bruun et al., 2000, 2003; Izumi and Aranishi, 2004). Media were compared and standards were recommended to assess the minimal inhibitory concentration of chloramphenicol and florfenicol in various bacterial fish pathogens, including *F. psychrophilum* (Michel et al., 2003). In *F. psychrophilum* as in other Gram-negative bacteria, the co-occurrence of resistance to quinolones and specific mutations of the A subunit of DNA gyrase (GyrA) demonstrated that GyrA is an important target for quinolones (Izumi and Aranishi, 2004b). An extreme susceptibility to penicillin G was noted in *F. branchiophilum* (Ostland et al., 1994). Antibiotics for treatment of fish were selected from among those antibiotics that are toxic to fish-pathogenic *Flavobacterium* species but are not toxic to fish or banned from use on fish farms. Further information on the practical use of antibiotics in the treatment of fish is given in the section Pathogenicity and Epidemiology. Among the recently described *Flavobacterium* species, only *F. frigidarium* (Humphry et al., 2001) and *F. granuli* (Aslam et al., 2005) were tested for their antimicrobial resistance pattern, which proved similar to those of *F. columnare* and *F. psychrophilum*; *F. frigidarium* was found to be highly resistant to kanamycin (Humphry et al., 2001). The carbapenem-hydrolyzing β-lactamase from the *F. johnsoniae* type strain has recently been purified and characterized; the analysis of its sequence revealed that it is closely related to the β-lactamase produced by other members of the family Flavobacteriaceae such as *Chryseobacterium meningosepticum* and *C. indologenes* (Naas et al., 2003; see the chapter The Genus *Chryseobacterium* in this Volume).

Preservation

Flavobacterium species can be preserved for multiple-year periods by simple cryopreservation at –70°C or less, using 10–30% (v/v) glycerol as a cryoprotectant within a routine growth medium such as Anacker and Ordal's broth (see media formulae in the section Isolation and Cultivation). This procedure has been proposed for *F. branchiophilum* (Wakabayashi et al., 1989), *F. columnare* (Shamsudin and Plumb, 1996), *F. psychrophilum* (Madsen and Dalsgaard, 1998), and *F. antarcticum* (Yi et al., 2005) and used successfully by J.-F. Bernardet (unpublished data) for all *Flavobacterium* species using the CAS broth (i.e., 1% casitone [Difco], 0.1% $MgSO_4 \cdot 7H_2O$, pH 6.8 unadjusted) recommended by Reichenbach (1989) to which 10% sterile glycerol was added. Commercial cryopreservative media are also convenient (Cepeda et al., 2004). Storage in liquid nitrogen may also be used, the CAS medium being supplemented with 5% dimethylsulfoxide (DMSO; Reichenbach, 1989).

A study by Desolme and Bernardet (1996) indicated that lyophilization (freeze-drying) of *F. columnare* and *F. psychrophilum* (and thus of most other generally more robust *Flavobacterium* species) was effective using a suspension medium consisting of 2/3 Difco Bacto Brucella broth and 1/3 filtered and heat-inactivated (1 h at 56°C) horse serum. Lyophilization was also

used to preserve *F. branchiophilum* (Ostland et al., 1994). The frozen cells can be revived using Anacker and Ordal's agar. Better results for some of the more sensitive fish pathogenic strains, such as *F. psychrophilum*, can be obtained by reviving on Anacker and Ordal's medium enriched to 0.5% tryptone and supplemented with 5% defibrinated horse serum (Difco) and a trace element solution as described by Michel et al. (1999).

Most of the cold-adapted species can also be stored as live cultures on agar slants at low temperature. The best temperature is 2°C, which reduces the incidence of fungal contamination. However this procedure needs to be verified on a case-by-case basis, as some species may show poor survival in this manner of storage after more than 3 months. Many of the cold-adapted Antarctic species such as *F. tegetincola* can survive at least 18 months this way. Most fish-pathogenic *Flavobacterium* strains will survive for several months or even years when heavily inoculated in deep tubes containing Anacker and Ordal's medium gelified with 0.4% (w/v) agar maintained at 4°C (Anacker and Ordal, 1955; J.-F. Bernardet, unpublished data).

The influence of the preservation procedure on the virulence stability of a *F. psychrophilum* strain was evaluated using experimental infection of rainbow trout fingerlings and determination of the lethal dose 50%. Cultures were revived after 1, 8 and 23 months of storage by lyophilization, freezing at –80°C, and maintenance at 4°C in Anacker and Ordal's agar deeps: whatever the preservation procedure, virulence was maintained after 1 and 8 months of storage; after 23 months, the bacteria kept in agar deeps at 4°C had lost some of their virulence, while the lyophilized and frozen ones were only slightly attenuated (Michel and Garcia, 2003).

Physiology

Though specific aspects of the biochemical physiology of members of the genus *Flavobacterium* remain to be discovered, almost all *Flavobacterium* species are strictly aerobic and appear to lack fundamental idiosyncrasies in their carbon and energy pathways. Most species use glucose as a carbon source and probably employ the Embden-Meyerhof-Parnas pathway for their catabolism (Reichardt and Morita, 1982). Exceptions to this generalization are the species *F. succinicans* and *F. hydatis*, which can grow anaerobically when provided peptone or yeast extract, e.g., modified Shiehs medium (Anderson and Ordal, 1961; Strohl and Tait, 1978; Reichenbach, 1989; see the section Isolation and Cultivation in this Chapter). Some components of these media presumably contain so far undetermined growth factors required to drive anaerobic growth. *Flavobacterium succinicans* also requires CO_2 (which can be provided by adding 15–25 mM $NaHCO_3$ to growth media) and glucose for anaerobic growth (Chase, 1965). *Flavobacterium antarcticum* grows weakly under microaerobic conditions and poorly under anaerobic conditions (Yi et al., 2005). Capnophilic requirements, as exhibited by the related genus *Capnocytophaga* and other members of the family Flavobacteriaceae (see the chapters The Genus Capnocytophaga in this Volume and An Introduction to the Family Flavobacteriaceae in this Volume), are otherwise absent or negligible for known *Flavobacterium* spp. Anaerobic growth can occur fermentatively on carbohydrates or yeast extract or by respiration using nitrate as the electron acceptor. *Flavobacterium succinicans* can also grow by fermentation of pyruvate (converting it to mostly acetate); otherwise organic acids or amino acids are not utilized. Alternative anaerobic electron acceptors to nitrate (sulfur, ferric compounds, etc.) have yet to be tested. Fermentation products formed during growth are mostly succinate, acetate and formate. Anderson and Ordal (1961) proposed the CO_2 requirement of *F. succinicans* stems from a lack of lactate dehydrogenase; instead this species condenses CO_2 with phosphoenolpyruvate (using phosphoenolpyruvate carboxylase and guanosine 5′-diphosphate [GDP]), resulting in oxaloacetate. Oxaloacetate is then reduced further via the reduced form of nicotinamide adenine dinucleotide (NADH), forming succinate as an end product. Interesting physiological comparisons may be made between the capnophilic members of the family Flavobacteriaceae, i.e., the genera *Capnocytophaga* (see the chapter The Genus Capnocytophaga in this Volume), *Coenonia*, *Ornithobacterium* and *Riemerella* (see the chapter Capnophilic Bird Pathogens in the Family Flavobacteriaceae: Riemerella, Ornithobacterium and Coenonia in this Volume). Interestingly, *F. granuli* appears to be strictly aerobic although it was isolated from granules that had been kept under anaerobic conditions for two years (Aslam et al., 2005).

Energy metabolism can be supplemented by reduction of nitrate to nitrite by many *Flavobacterium* species, particularly freshwater species. Some strains (but not the type strains) of *F. johnsoniae* and *F. columnare* have been reported to grow by anaerobic denitrification (Stanier, 1947; Christensen, 1977; Callies and Mannheim, 1978; Nogales et al., 2002). It is possible, however, that these denitrifying strains actually belong to separate species or even genera (Reichenbach, 1989).

Carbon and nitrogen metabolism pathways are poorly understood in the genus; however, on the basis of phenotypic data, they appear quite diverse between species. This can be best seen in the capacity for carbohydrate catabolism, which varies strongly at the species level. Some species utilize a wide range of carbohydrates and polysaccharides including *F. pectinovorum*, the aptly named *F. saccharophilum*, *F. johnsoniae*, *F. hydatis* and *F. omnivorum*. On the other hand, *F. branchiophilum*, *F. columnare*, *F. psychrophilum*, *F. tegetincola* and *F. micromati* are essentially asaccharolytic or poorly saccharolytic, preferring nitrogen-containing substrates, primarily amino acids and proteins. Asaccharolytic species also lack common glycolytic enzymes (e.g., as revealed by API ZYM galleries; see Table 6 in Bernardet et al., 1996), which can be useful for their rapid discrimination from saccharolytic species. All *Flavobacterium* species except *F. branchiophilum* (which requires yeast extract) can grow on single amino acids, such as L-glutamate or L-asparagine, as sole nitrogen sources. In general, *Flavobacterium* species have little propensity to utilize other types of substrates including sugar alcohols, alcohols, organic acids, hydrocarbons and aromatics. The ability to utilize xenobiotics occasionally ascribed to *Flavobacterium* sp. (e.g., Topp et al., 1988) is almost certainly erroneous and the strains concerned are likely instead *Sphingomonas* spp., which can be confused with *Flavobacterium* owing to superficial morphological similarities.

Several other aspects pertaining to the physiology of members of the genus *Flavobacterium* are dealt with in other sections in this Chapter, particularly the section Identification.

Genetics

Besides investigations targeting the whole genome (DNA-DNA hybridization and determination of the G+C content) or particular genes (sequencing of 16S rRNA and *gyrB* genes) for taxonomic and phylogenetic purposes or for the molecular typing of bacterial strains (see the section Identification in this Chapter), genetics of members of the genus *Flavobacterium* has been poorly investigated to date. However, a group of scientists studied extensively the genetics of gliding motility in *F. johnsoniae*. Tools for the genetic manipulation of this organism were first developed: a *Bacteroides* transposon was shown to function in *F. johnsoniae* and cloning vectors based on a cryptic plasmid of *F. psychrophilum* were devised (McBride and Kempf, 1996). The system of gene transfer, the selectable marker, the suicide vector, and the transposon developed to genetically manipulate *F. johnsoniae* were also used successfully on *F. succinicans*, on other members of the family Flavobacteriaceae (i.e., *Chryseobacterium meningosepticum*), and on other members of the *Cytophaga-Flavobacterium-Bacteroides* phylum (i.e., [*Flexibacter*] *canadensis* and *Cytophaga hutchinsonii*; McBride and Baker, 1996). Recently, methods for the genetic manipulation of *F. psychrophilum* were also developed; selectable markers, plasmid cloning vectors, a *lacZY* reporter construct, and a transposon are now available and should allow for the analysis of virulence mechanisms and the development of vaccine strains (Alvarez et al., 2004). The most common plasmid in *F. psychrophilum* strains, pCP1 (see the section Molecular Typing in this Chapter), was entirely sequenced (M. J. McBride, seq. no. NC004811). Mutagenesis was used to address the genetic mechanisms of gliding motility in *F. johnsoniae*; at least eight different genes were shown to be required (Kempf and McBride, 2000; Hunnicutt and McBride, 2001; Hunnicutt et al., 2002; McBride et al., 2003, McBride and Braun, 2004). The most recently discovered genes, *gldH* and *gldI*, are required for gliding motility, chitin utilization, and infection by bacteriophages; these properties are lost in *gldH* or *gldI* mutants and restored when *gldH* or *gldI* is reintroduced in the genome via a plasmid (McBride et al., 2003). However, mechanisms responsible for gliding motility are complex and probably differ among the organisms that exhibit this kind of motility (McBride, 2001). Recently, the nucleotide sequence of the *F. aquatile* operon coding for the *Fau*I restriction-modification system was determined (Abdurashitov et al., 2003).

Pathogenicity and Epidemiology

Plant Pathogens

The decay of various fresh plants and vegetables called "soft rot" has been attributed to *F. johnsoniae*, among other bacterial species (Liao and Wells, 1986; Lelliott and Stead, 1987). The pathogenic strains were shown to produce more pectate lyase in broth medium than the non-pathogenic ones; however, *F. johnsoniae* was mostly considered an opportunistic pathogen (Liao and Wells, 1986).

Human Medicine

After several human cases of a lung disease similar to hypersensitivity pneumonitis occurred in a United States textile facility, a bacterial endotoxin was isolated from the biomass growing in

the air humidification system and shown serologically to be the cause of the clinical disease; the main representatives of the bacterial flora were isolated from the system and a *Cytophaga* strain was identified as the source of the endotoxin (Flaherty et al., 1984). Further studies showed that the bacterium was related to several *Cytophaga*, *Flexibacter*, and *Flavobacterium* reference strains; although DNA relatedness with *F. hydatis* was 78%, several biochemical discrepancies with this species led the authors to propose the name "[*Cytophaga*] *allerginae*" (Liebert et al., 1984). Fatty acid analysis, whole-cell protein profile, and DNA-rRNA hybridization (Bernardet et al., 1996), as well as a rather high DNA relatedness with *F. johnsoniae* (J.-F. Bernardet, unpublished data), confirmed the relationships of "[*C.*] *allerginae*" with members of the genus *Flavobacterium* (see the section Taxonomy in this Chapter). To our knowledge, no other case of lung disease in humans related to such organisms was reported since then. However, since bacterial strains closely affiliated to *F. johnsoniae* and *F. succinicans* were shown to occur intracellularly in amoebae retrieved from drinking water in hospitals and from a human corneal sample (Müller et al., 1999; Horn et al., 2001), other human pathology may possibly result from the close contact with such endocytobionts.

Veterinary Medicine

Although some members of the genus *Flavobacterium* are part of the normal bacterial flora in the mucus at the surface of fish and fish eggs (see the section Habitat and Ecology in this Chapter), several *Flavobacterium* species exhibit various degrees of pathogenicity for fish. To date, freshwater fish are the only animals known to be extensively affected by flavobacterial diseases, although infections in newt (Brown et al., 1997) and frog tadpole (Bullock et al., 1971) were exceptionally reported. Wild fish may be infected (Lehmann et al., 1991; Wiklund et al., 1994; Iida and Mizokami, 1996), but farmed fish are particularly exposed to these diseases owing to the high density of fish in farms and to the extensive national and international trade in fish and fish eggs. The fish-pathogenic *Flavobacterium* species are listed below in the order of decreasing economic significance. Data on cultural, phenotypical, serological, and molecular characteristics of these pathogens, as well as on their antibiotic susceptibility, are provided in other sections in this chapter and in Tables 2, 3 and 4; additional information may also be found in recent reviews (Austin and Austin, 1999; Shotts and Starliper, 1999). The terms used by fish farmers and fish pathologists to designate these bacteria reflect the great taxonomic and nomenclatural confusion that long characterized this bacterial group. Owing to the fact that spheroplasts were mistaken for microcysts and bacterial clumps for fruiting bodies (see the section Identification in this Chapter), fish pathogens were wrongly attributed to the fruiting gliding bacteria and the global term "myxobacteria" was consequently used (and still frequently is). Such generic epithets as *Chondrococcus* or *Cytophaga* were also commonly used for the most important fish pathogens until they were classified in the genus *Flexibacter* on the basis of phenotypic characteristics (Bernardet and Grimont, 1989) and finally in the emended genus *Flavobacterium* after extensive genomic and phylogenetic studies (Bernardet et al., 1996; Bernardet et al., 2002).

FLAVOBACTERIUM PSYCHROPHYLUM. Following the description of "peduncle disease" (see below) by Davis (1946), the responsible bacterium was first isolated by Borg (1948), who named it [*Cytophaga*] *psychrophila* and studied it extensively (Borg, 1960). For the following decades, the disease seemed restricted to the United States and Canada (Borg, 1960; Holt, 1988). *F. psychrophilum* was first recognized in Europe during the mid-eighties (Von Weis, 1987; Bernardet et al., 1988) and subsequently appeared in all other major areas of salmonid aquaculture (Japan, Tasmania, and Chile) during the nineties; its distribution is now considered worldwide. It is suspected that the pathogen extended its geographical range through the international trade in fish and fish eggs (Wakabayashi et al., 1994; Kumagai and Takahashi, 1997). Nevertheless, *F. psychrophilum* may also have occurred in some areas long ago, passing unnoticed because it was not responsible for serious outbreaks before intensive farming was put into practice and because media and temperature appropriate to its isolation were not commonly used. In contrast to *F. columnare* (see below), *F. psychrophilum* mostly infects salmonid fish (as well as the related species called "ayu," *Plecoglossus altivelis*, reared in Japan and Korea), although it may occasionally be isolated from eel, various cyprinids, and other fish species (Lehmann et al., 1991; Iida and Mizokami, 1996; Amita et al., 2000; Izumi et al., 2003b) in which it usually causes less severe infections. Hence, resident non-salmonid fish species as well as salmonid fish having survived an outbreak (Dalsgaard and Madsen, 2000; Madetoja et al., 2000) likely act as carriers or reservoirs of *F. psychrophilum* for salmonids in the same river. The pathogen can survive starvation outside fish hosts: although its culturability and viability declined when maintained in stream water, growth was still possible after 36 weeks provided

a resuscitation step was performed (Vatsos et al., 2003). Similarly, Madetoja et al. (2003) reported a survival capacity of more than 300 days in sterilized fresh water at 15°C even though the virulence declined much more rapidly. Consequently, *F. psychrophilum* was detected in fish-farm water, river water, and algae growing at the surface of stones on riverbeds (Amita et al., 2000; Madetoja and Wiklund, 2002; Vatsos et al., 2002). However, the main sources of infection are the high numbers of bacterial cells that are released into the water by infected and dead fish; disruption of the skin is likely a major invasion route into the fish, whereas oral contamination trials using live feed as a vector did not succeed (Madetoja et al., 2000). *F. psychrophilum* is currently one of the main bacterial pathogens in reared and wild salmonids in temperate to cold water and is responsible for considerable economic losses in salmonid culture worldwide (Izumi et al., 2003b). Besides the above-mentioned general reviews of fish-pathogenic *Flavobacterium* species, much information on the diseases caused by *F. psychrophilum* may be found in reviews focused on this pathogen (Dalsgaard, 1993; Holt et al., 1993; Nematollahi et al., 2003b).

Several different pathological entities have been described depending on the fish species and size (Holt et al., 1993; Nematollahi et al., 2003b). In the classical form observed in Northern America since the forties in the two most susceptible species, coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*), the area around the adipose fin becomes dark and eroded; pathological changes progressively extend to the whole caudal peduncle and to the tail, usually resulting in a deep ulcerative lesion exposing the spinal cord (Davis, 1946; Pacha and Ordal, 1970; Holt et al., 1993), and hence the name "peduncle disease" (Fig. 10). Similar lesions may occur around the dorsal fin; this particular location won the condition the name "saddleback disease."

The terms "bacterial cold-water disease" and "low temperature disease" are also frequently used since outbreaks usually occur when water temperature is below 10°C. When groups of experimentally infected salmonids were kept at 3–23°C, the shortest time from infection to death was reported in the group kept at 12–15°C (Holt et al., 1989). In contrast to *F. columnare* infections (see below), those caused by *F. psychrophilum* rapidly become septicemic and the bacterium may be isolated from internal organs such as the spleen and kidney. Mortality may reach up to 50% in coho salmon fingerlings and 20% in larger fish (Holt, 1988). Gill damage due to *F. psychrophilum* (which is less severe than that due to *F. columnare* or *F. branchiophilum*

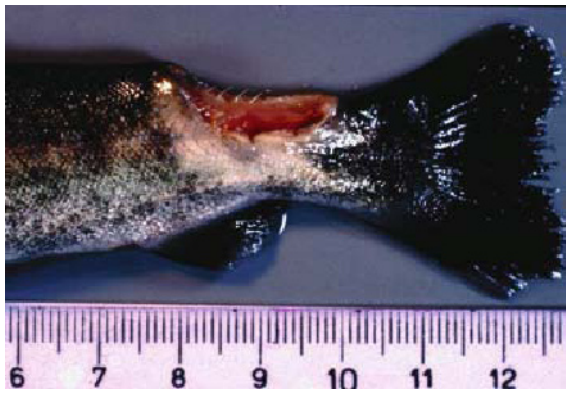

Fig. 10. "Peduncle disease" caused by *F. psychrophilum*. Extensive necrotic lesions may appear in various locations on the body of infected fish, including the caudal peduncle. On this young rainbow trout, extracellular enzymes of the pathogen have destroyed the skin, connective tissue and muscle, revealing the spinal column.

infections [see below]) has been reported in several salmonid species in Ontario (Ostland et al., 1999). Nervous forms of this disease are also known in the United States: young salmon and trout having recovered from acute infection displayed ataxia and spiral swimming behavior stemming from compression of nervous tissue by cranial lesions and spinal deformities; in such cases, osteitis, osteochondritis, meningitis and myeloencephalitis were noted (Kent et al., 1989; Meyers, 1989; Ostland et al., 1997). Such clinical signs as anemia, exophthalmia, necrotic scleritis, and skin bullae or blisters evolving into cavernous ulcers due to necrotic myositis were also reported (Lumsden et al., 1996; Ostland et al., 1997; Ostland et al., 2000). In Europe, although cases of typical cold-water disease were occasionally reported in table-size rainbow trout (Dalsgaard, 1993; Wiklund et al., 1994; Madetoja et al., 2001), *F. psychrophilum* infection is mostly a concern in rainbow trout fry and fingerlings; hence the terms "rainbow trout fry syndrome" (RTFS) and "fry mortality syndrome" (FMS; see Nematollahi et al. [2003b] and references therein). These conditions may result in high to very high mortality (i.e., 20–70%). Infection is septicemic and fish usually display only limited and nonspecific clinical signs such as anemia, lethargy, loss of appetite, dark color, and exophthalmia (Lorenzen, 1994), although fin erosion, blisters (from which *F. psychrophilum* may be isolated in pure culture; J.-F. Bernardet, unpublished data), ulcers, as well as mouth and eye lesions may also occur in fingerlings (Von Weis, 1987; Bernardet and Kerouault, 1989; Lehmann et al., 1991; Dalsgaard, 1993; Lorenzen, 1994; Martínez et al., 2004). The most consistent

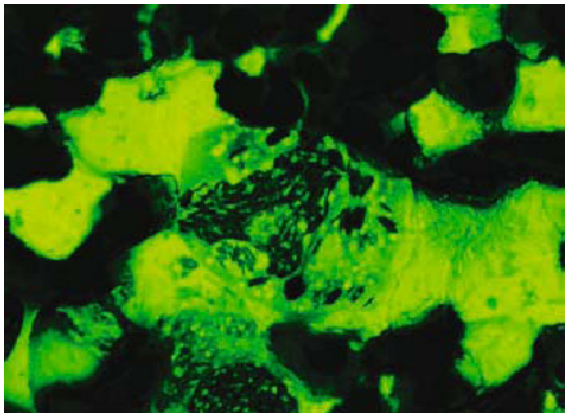

Fig. 11. *Flavobacterium psychrophilum* in a fish spleen macrophage. A spleen imprint from a rainbow trout with hemorrhagic septicemia caused by *F. psychrophilum* reveals numerous bacterial cells in the vacuoles of a macrophage (May-Grunwald-Giemsa, ×450).

internal sign is a severe splenic hypertrophy; in spleen imprints or histological preparations, numerous bacterial rods are dispersed in the parenchyma throughout the tissue (Rangdale et al., 1999; Ekman and Norrgren, 2003; Ekman et al., 2003) as well as located within phagocytes and endothelial cells in the spleen, liver, heart, and kidney (Lehmann et al., 1991; Ekman and Norrgren, 2003; Ekman et al., 2003; Fig. 11).

The presence of bacteria in the spleen tissue is considered pathognomonic for RTFS together with the replacement of the border of the spleen by a loose eosinophilic layer, fibrinous inflammation, and intercellular edema (Rangdale et al., 1999). Clinical signs and pathological findings were similar in the three salmonid species experimentally infected (Ekman and Norrgren, 2003). Following intraperitoneal injection of *F. psychrophilum* cells to rainbow trout, epifluorescence microscopy revealed that the intracellularly located bacteria in the spleen of fry were still viable and increased in number with time, suggesting that the ability of bacteria to escape from the bactericidal mechanisms of phagocytes and to be protected against the humoral defense mechanisms of the host could explain, at least partly, why fry display clinical signs and suffer mortality. Conversely, older fish displayed no sign of infection and no bacteria were detected in their spleen phagocytes (Decostere et al., 2001). Wiklund and Dalsgaard (2003) showed that 1) the *F. psychrophilum* cells associated with kidney phagocytes of large rainbow trout in vitro were rapidly destroyed, 2) the differences in association with the phagocytes were not clearly related to the serotype or virulence of the strains, and 3) a phagocyte toxin was not necessary for the virulence.

Besides the resistance to the defense mechanisms of the fish, several other factors have been suggested to explain the virulence of *F. psychrophilum* (see Dalsgaard [1993] and Nematollahi et al. [2003a] and references therein) and its wide variation among strains (Holt, 1988; Faruk, 2000). Adherence is likely the initial step of infection. The adherence of *F. psychrophilum*, studied on a gill perfusion model, was influenced by water temperature and chemical composition; also, a high virulence strain attached more readily than a low virulence one (Nematollahi et al., 2003a). The adhesion of *F. psychrophilum* to the eggs of rainbow trout (Vatsos et al., 2001) and to the body surface of ayu (Kondo et al., 2002) was also studied; initial attachment of the bacterium was shown to occur preferentially on the jaw and caudal peduncle, which are precisely the locations of some of the most typical lesions. However, the role of the slime layer of *F. psychrophilum* in the adhesion process (unlike that of *F. columnare* [see below]) has not yet been demonstrated (Nematollahi et al., 2003b). Surprisingly, when the adhesion of *F. psychrophilum* to mucus preparations from rainbow trout esophagus, stomach, intestine, gills and skin was compared to that of other fish-pathogenic bacteria, it proved to exhibit the lowest adhesion ability, a detectable adhesion being only reported to mucus from stomach and intestine (Nikoskelainen et al., 2001). The ability of *F. psychrophilum* to agglutinate and hemolyze rainbow trout erythrocytes (Lorenzen, 1994; Lorenzen et al., 1997) may explain, at least partially, the severe anemia that occurs in infected fish. Recently, a sialic acid-binding lectin was shown to be involved in the hemagglutinating properties exhibited by *F. psychrophilum* strains belonging to some serotypes (Møller et al., 2003). The extracellular proteases (Otis, 1984; Holt, 1988; Bertolini and Rohovec, 1992; Bertolini et al., 1994; Nomura and Ohara, 1994; Newton et al., 1997; Faruk, 2000; Ostland et al., 2000; Secades et al., 2001; Secades et al., 2003) and chondroitin lyase (Otis, 1984) produced by *F. psychrophilum* are certainly important virulence factors which may explain to some degree the extensive necrotic lesions displayed by diseased fish (see the section Identification in this Chapter). Scanning electron microscopy investigations of infected fin rays of Atlantic salmon revealed that erosion of the ray axis resulted from the formation of grooves and tubular boreholes likely caused by *F. psychrophilum* extracellular proteases (Martínez et al., 2004). Among the various proteolytic capacities of *F. psychrophilum*, the elastin-degrading capacity in particular has been suggested as a virulence factor, since the mortality rates in groups of rainbow trout experimentally infected

with an elastin-positive strain were significantly higher compared to those of groups infected with an elastin-negative strain; a certain correlation was also found between the elastin-degrading capacity and the serotype of the strains studied (Madsen and Dalsgaard, 1998). However, the opposite result was observed when rainbow trout eggs were injected with *F. psychrophilum* strains with or without elastin-degrading capacity, suggesting that other virulence factors are involved in the virulence of the pathogen (Ekman et al., 2003). It has also been suggested that the lytic activity displayed by *F. psychrophilum* strains against other bacterial species may constitute an advantage, indirectly contributing to its virulence (Nematollahi et al., 2003a). Wiklund and Dalsgaard (2002) showed that virulent as well as nonvirulent strains of *F. psychrophilum* were resistant to the action of the complement present in the serum of rainbow trout. Although the structure of the lipopolysaccharide of *F. psychrophilum* was recently determined (Crump et al., 2001; MacLean et al., 2001; see the section Identification in this Chapter), its role in pathogenesis has not been evaluated. A certain relationship between serotype, ribotype, and virulence was found in *F. psychrophilum* isolates; however, virulence was not clearly correlated to the plasmid content (Madsen and Dalsgaard, 2000; see the section Identification in this Chapter).

The diagnosis of *F. psychrophilum* infections usually relies on the isolation of the pathogen, preferably from internal organs (see the section Isolation and Cultivation in this Chapter), and on its identification using phenotypical and serological characteristics (see the section Identification in this Chapter). The molecular techniques available to specifically detect the bacterium in infected tissue and to identify isolates are listed in the section Identification. Serological methods such as ELISA (Rangdale and Way, 1995; Faruk, 2000), immunohistochemistry (Evensen and Lorenzen, 1996; Ekman and Norrgren, 2003), and immunofluorescence (Lorenzen and Karas, 1992; Amita et al., 2000; Faruk, 2000) have been devised to detect *F. psychrophilum* in fish tissue and to reveal antigenic differences between isolates.

F. psychrophilum has long been suspected to be vertically transmitted from brood fish to progeny, i.e., within fish eggs. If confirmed, vertical transmission associated with the international trade in fish eggs would help explain the dramatic worldwide spread of rainbow trout fry syndrome within only a few years despite disinfection procedures routinely applied to eggs (Izumi and Wakabayashi, 1997; Kumagai and Takahashi, 1997). The detection using cultivation and/or molecular methods of *F. psychrophilum* in the coelomic fluid and at the surface of the ova of infected female salmonids, as well as in the milt of certain males, is well documented (Holt, 1988; Lorenzen, 1994; Rangdale et al., 1996; Brown et al., 1997; Izumi and Wakabayashi, 1997; Kumagai and Takahashi, 1997; Ekman et al., 1999; Amita et al., 2000; Baliarda et al., 2002; Taylor, 2004). However, since *F. psychrophilum* is embedded in the mucus at the surface of the egg, a complete disinfection is difficult to achieve (Brown et al., 1997; Kumagai and Takahashi, 1997) and thus transmission within disinfected eggs rather than on their still-infected or re-infected surfaces is difficult to demonstrate. The strongest evidence for in ovo transmission of *F. psychrophilum* came from Brown et al. (1997), who reported the isolation of the pathogen from crushed steelhead trout eggs after they were disinfected using iodine compounds and after the sterility of their surface was verified by a 72-h incubation in an appropriate broth medium. The same authors also demonstrated that *F. psychrophilum* is resistant to concentrations of lysozyme greater than those found within salmonid eggs and that the pathogen is able to survive within the content of the eggs. Injection of infected females with erythromycin or tetracycline prior to fecundation did not significantly reduce the proportion of infected eggs (Brown et al., 1997; Roberts, 1998). Recent investigations by Kumagai et al. (2000) using immunofluorescence on frozen sections of experimentally infected coho salmon eggs and by Taylor (2004) using a nested PCR assay on yolk material from four different naturally infected Pacific salmonid species also succeeded in revealing *F. psychrophilum* cells within the eggs. Nanoinjection of *F. psychrophilum* in rainbow trout eggs was recently used to mimic vertical transmission and to compare the virulence of two strains (Ekman et al., 2003).

The study of the pathogenesis of *F. psychrophilum* infections and the evaluation of candidate vaccines (see below) were made possible by various experimental infection and challenge models reviewed by Nematollahi et al. (2003b). Clinical disease was successfully reproduced using subcutaneous, intramuscular, or intraperitoneal injection of bacterial suspensions, but these routes were criticized for not being natural and for bypassing the surface defense mechanisms of the fish. To better approximate the natural route of infection, other infection models were devised, using cohabitation with diseased fish or immersion in bacterial suspensions. These procedures usually failed to induce infection, except when fish were previously stressed somehow (see Madetoja et al. [2000], Busch et al. [2003], and Nematollahi et al. [2003b] and references therein); yet, skin injury or osmotic stress

did not significantly increase the mortality of rainbow trout submitted to a bath infection (Garcia et al., 2000). Presence of ectoparasites was expected to favor the penetration of *F. psychrophilum* in fish through the microlesions they cause on the skin; however, no distinct enhancement of infection could be demonstrated in rainbow trout fry bathed in a bacterial suspension and exposed to a monogenean ectoparasite compared to fry only exposed to the bacterium (Busch et al., 2003).

Although the mucus and serum of infected rainbow trout were shown to contain specific antibodies against *F. psychrophilum*, the number of bacterial cells incubated in the serum decreased only slightly, suggesting that high levels of antibodies alone do not provide protection against the pathogen and that stimulation of nonspecific immune factors is necessary to achieve nearly complete protection (LaFrentz et al., 2002; Wiklund and Dalsgaard, 2002). The inability of antibodies to provide complete protection was also demonstrated by the partial success of passive immunization experiments (LaFrentz et al., 2003). Among the vaccination attempts to prevent *F. psychrophilum* infections (see the reviews by Bernardet [1997] and Nematollahi et al. [2003b]), injection of or immersion in formalin-killed bacterial cells has met with varying degrees of success. Recently, Kondo et al. (2003) have claimed to induce protection of juvenile ayu using an oral vaccination procedure. Mortality of ayu injected intraperitoneally with formalin-killed bacterin mixed with water-soluble adjuvants was significantly lower than that of un-vaccinated fish (Nagai et al., 2003; Rahman et al., 2003). Improved vaccines could result from the identification of several immunogenic cell surface molecules (Crump et al., 2001) and from the characterization of the O-antigen of the *F. psychrophilum* lipopolysaccharide (MacLean et al., 2001). Recently, a good protection was obtained using the outer membrane protein of *F. psychrophilum* as a vaccine (Rahman et al., 2002). The major glycoprotein antigen purified from the bacterial cell membrane is another candidate for a subunit vaccine against *F. psychrophilum* (Merle et al., 2003). Following the identification of several immunogenic fractions of the bacterium using western-blot analysis of rainbow trout immune sera, immunization with the 70–100 kDa fraction resulted in a very good protection of fish (LaFrentz et al., 2004). Since antibodies in the serum of protected fish recognized high molecular weight proteins and the O-polysaccharide component of the lipopolysaccharide, these antigens may be responsible for the protective immune response and could serve as vaccine candidates. Methods for the genetic manipulation of *F. psychrophilum* are now available (Alvarez et al., 2004; see the section Genetics in this Chapter); they should facilitate the rational design of vaccine strains.

Holt et al. (1993), Austin and Austin (1999), and Nematollahi et al. (2003b) have reviewed the different antibacterial drugs (e.g., sulfonamides, furans, oxytetracycline, and florfenicol) that may be used in the control of *F. psychrophilum* infections. Although antibacterial therapy remains the most effective control method, the progressive development of resistance to oxytetracycline, oxolinic acid and amoxicillin, as well as the importance of performing in vitro antimicrobial susceptibility testing, are well documented (Soltani et al., 1995; Bruun et al., 2000; Bruun et al., 2003; Dalsgaard and Madsen, 2000; Schmidt et al., 2000; see the section Identification in this Chapter). However, a recent survey of the susceptibility of fish-pathogenic bacteria isolated in France to chloramphenicol and florfenicol found no resistant strain of *F. psychrophilum* (Michel et al., 2003). Manipulation of water temperature or salinity has also been suggested to reduce the morbidity due to *F. psychrophilum* infections in farmed fish (Soltani and Burke, 1994).

FLAVOBACTERIUM COLUMNARE. Although different generic epithets were successively used to designate it (see above and the comments to Table 1 in the chapter An Introduction to the Family Flavobacteriaceae in this Volume), *F. columnare* has long been recognized as an important fish pathogen in relatively warm freshwater worldwide, causing dramatic losses in a considerable variety of farmed and wild, food and ornamental fish. The list of fish species which may be infected and the history of "columnaris disease," as it is called by fish farmers and fish pathologists since its discovery by Davis (1922), may be found in the extensive literature devoted to this organism, particularly in recent reviews (Wakabayashi, 1993; Austin and Austin, 1999; Shotts and Starliper, 1999). Early reports of *F. columnare* from seawater or marine fish (see Bullock et al. [1971] and references therein) were subsequently invalidated following the demonstration that the bacterium was unable to survive in seawater (Chowdhury and Wakabayashi, 1988); in fact, the organism responsible for "marine columnaris" was likely *Tenacibaculum maritimum* (see the chapter The Marine Clade of the Family Flavobacteriaceae: The Genera Aequorivita, Arenibacter, Cellulophaga, Croceibacter, Formosa, Gelidibacter, Gillisia, Maribacter, Mesonia, Muricauda, Polaribacter, Psychroflexus, Psychroserpens, Robiginitalea, Salegentibacter, Tenacibaculum, Ulvibacter, Vitellibacter and Zobellia in this Volume). Even though the overall economic losses due to *F. psychrophilum* worldwide are now far higher

than those caused by *F. columnare*, the latter remains one of the most serious bacterial pathogens in commercially reared channel catfish (*Ictalurus punctatus*) in the United States (Hawke and Thune, 1992; Newton et al., 1997) and in several tropical and/or ornamental fish (Decostere et al., 1998; Michel et al., 2002; Tripathi et al., 2005).

Many environmental and host-related factors (such as water temperature and composition, skin or gill damage, stress due to handling and high stocking density, and feed or oxygen deprivation) may influence the impact of columnaris disease on fish populations (Pacha and Ordal, 1970; Becker and Fujihara, 1978; Austin and Austin, 1999). Columnaris disease is usually problematic only in the warmer periods of the year: the optimum temperature for an outbreak is 20–30°C and mortality usually does not occur below 15°C, although highly virulent strains may still be pathogenic at lower temperatures (see Wakabayashi [1991] and references therein). Field studies demonstrated that various species of feral fish serve as reservoir of infection (Pacha and Ordal, 1970; Becker and Fujihara, 1978; Wakabayashi, 1991). The survival of *F. columnare* in water depends on the concentration of various ions, on the presence of organic matter (such as particulate fish feeds on which the bacterium grows well), and on the temperature (Wakabayashi, 1991).

The pathology of columnaris disease has been extensively described (e.g., Pacha and Ordal, 1970; Amin et al., 1988; Shotts and Starliper, 1999; Decostere et al., 2002) and gross clinical signs may help achieve diagnosis (Austin and Austin, 1999). The gross pathology in young fish infected by highly virulent *F. columnare* strains is usually very limited; the gill is the major site of damage, but the body, mouth (Fig. 12), fins and tail are also frequently damaged. In adult fish, yellowish areas of necrotic tissue appear in the gills, eventually destroying completely the gill filament (Fig. 13).

Lesions on the body begin as small eroded and hyperemic areas which progressively extend to large ulcerative and hemorrhagic necrotic lesions exposing the underlying muscle tissue. A particular aspect of columnaris disease in salmonids is named "saddleback" because the lesion is typically located around the dorsal fin (Cone et al., 1980; Morrison et al., 1981). In severe cases of *F. columnare* infection, a septicemia may also occur. In tropical ornamental fish, columnaris disease is frequently called "cotton-wool" disease or "mouth fungus" since the filaments composed of numerous bacterial cells emerge from the lesions; however, recent descriptions of columnaris disease in three different tropical fish species only mentioned local skin discoloration, with or without ulceration, and degeneration of muscle fibers (Decostere et al., 1998; Michel et al., 2002). When fragments of infected fish tissue are examined under the microscope in wet mounts, great numbers of bacterial cells are

Fig. 12. Mouth lesion in fish infected by *F. columnare*. Bacteria have destroyed the floor of the oral cavity in a young rainbow trout; such necrotic lesions ("mouth rot") are common and may also affect other parts of the body ("fin rot," "tail rot," skin ulcers, etc.).

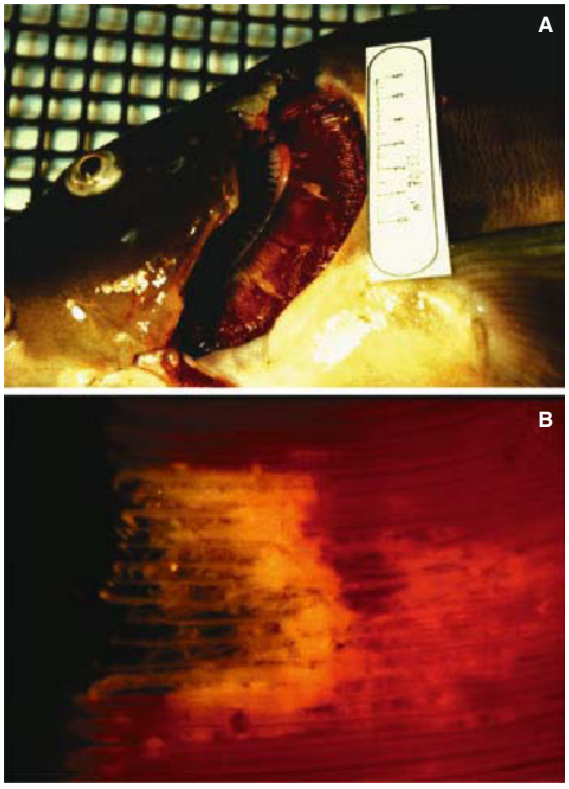

Fig. 13. Gill necrosis in carp caused by *F. columnare*. Yellowish necrotic lesions in the gills of a heavily infected common carp (A). Under the stereomicroscope (×10), gill tissue appears completely destroyed by the extracellular enzymes of *F. columnare*, and yellow bacterial growth progressively invades the gills (B).

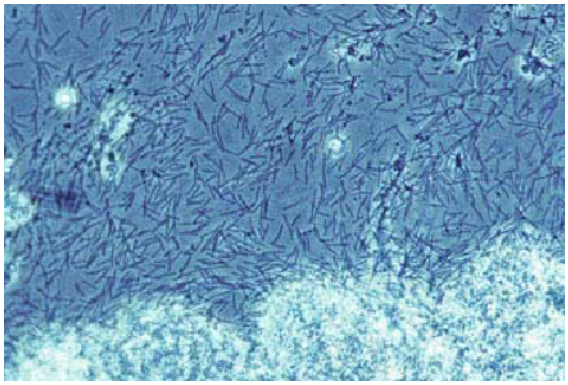

Fig. 14. Direct microscopic examination of a fragment of *F. columnare*-infected tissue. Under phase-contrast microscopy (×1000), a fragment of infected skin tissue from neon tetra (*Paracheirodon innesi*) shows numerous actively gliding bacterial cells; the dense, column-like masses of gliding bacterial cells frequently found on the surface of infected tissue are at the origin of the specific epithet "*columnare*."

observed, slowly gliding and aggregating in columnar masses, from which the specific epithet of *F. columnare* is derived (Fig. 14). Histopathology of columnaris disease was described in several fish species (Pacha and Ordal, 1967; Cone et al., 1980; Morrison et al., 1981; Amin et al., 1988; Shamsudin, 1994; Decostere et al., 1999; Tripathi et al., 2005).

Flavobacterium columnare may usually be readily isolated from external lesions, provided samples are taken from the edge of recent lesions and streaked on convenient media (see the section Isolation and Cultivation in this Chapter). When columnaris disease reaches a septicemic phase, the bacterium may also be isolated from internal organs, preferably spleen or kidney (Morrison et al., 1981; Hawke and Thune, 1992; Decostere et al., 1998; Tripathi et al., 2005). Although different colony types may occur (Song et al., 1988a; Shamsudin and Plumb, 1996), most *F. columnare* strains may be readily identified from their typical colonies on Anacker and Ordal's agar (Anacker and Ordal, 1955). These are pale greenish yellow, flat, spreading, with more or less rhizoid margins, are adherent to the agar, and may appear warty or elevated at the center (Pacha and Ordal, 1970). The gliding motility in liquid culture is also easy to observe and those who know this organism well recognize its odor. Of course, additional tests are necessary for an accurate identification (see other sections in this chapter and Table 4). Various PCR techniques have been proposed to detect *F. columnare* in the tissue of diseased fish or to identify isolates (Toyama et al., 1996; Nilsson and Strom, 2002; Tiirola et al., 2002; Darwish et al., 2004; Tripathi et al., 2005; see also the section Isolation and Cultivation in this Chapter). The typing of *F. columnare* isolates may be achieved using biochemical, serological and molecular techniques; early descriptions of bacteriocin and phage typing were also published (see the section Identification in this Chapter).

Columnaris disease was experimentally reproduced using different routes. These experiments showed *F. columnare* strains exhibit various degrees of virulence: highly virulent strains killed all experimental fish within 24 h while strains exhibiting a low virulence required over 96 h to reach 100% mortality; some of the least virulent *F. columnare* strains failed to infect the fish unless their skin was previously scarified or did not kill the fish at all (Pacha and Ordal, 1970; Amin et al., 1988; Shamsudin, 1994; Chowdhury, 1995; Decostere et al., 1999; Tripathi et al., 2005). Extensive external lesions only appear when the disease lasts several days, i.e., when it is caused by strains with low or moderate virulence. Although the virulence mechanisms of *F. columnare* are far from completely elucidated, several putative virulence factors have been studied. No correlation was found between the degree of virulence and such epidemiological markers as serotypes (Pacha and Ordal, 1970), plasmid or RAPD profiles, and ribotypes (see the section Identification in this Chapter). The various enzymes, such as extracellular proteases (Newton et al., 1997) and chondroitin AC lyase (Stringer-Roth et al., 2002), produced by *F. columnare* are presumably responsible for the extensive necrotic lesions (see the section Identification in this Chapter), and a correlation was indeed found between the degree of virulence and that of chondroitin AC lyase activity (Stringer-Roth et al., 2002). The adherence of *F. columnare* was extensively studied in vivo (Decostere et al., 1999) and ex vivo using a model of isolated gill arches (Decostere et al., 1999; Decostere et al., 1999); these studies demonstrated that 1) the adhesion of bacterial cells to the gill tissue was an important step in pathogenesis, 2) adhesion was favored by the presence of nitrite or organic matter in the water and by high water temperatures, and 3) a high virulence strain adhered more readily than a low virulence one. The role of adherence in pathogenesis was further substantiated when a decrease in virulence was observed in fish challenged with an adhesion-defective mutant of *F. columnare* obtained by serial passages on an ampicillin-enriched medium (Bader et al., 2005). Adherence ability of *F. columnare* was correlated to its hemagglutination capacity, and a lectin-like component of the capsule was responsible for the attachment of the bacterium to gill tissue; moreover, the capsule was thicker and denser in highly virulent strains than is low virulence ones (Decostere et al., 1999). Nevertheless, according

to MacLean et al. (2003), the adhesins involved have not been definitely identified; possible candidates are the lipopolysaccharide, the capsule, the fimbriae, or other appendages of the bacterium.

The control of columnaris disease is difficult to achieve and outbreaks frequently occur in the same river, lake or fish farm when environmental conditions are adequate; healthy fish may be infected by resident carrier fish, by bacteria surviving in the environment, or by recently introduced infected fish. The facts that rainbow trout surviving a columnaris disease outbreak were resistant to a re-exposure the following year (Becker and Fujihara, 1978) and that channel catfish, rainbow trout and tilapia injected with suspensions of heat- or formalin-killed virulent strains produced specific antibodies in plasma and skin mucus (Fujihara and Nakatani, 1971; Schachte and Mora, 1973, Grabowski et al., 2004) have led to various vaccination trials. Although bath or parenteral vaccination of fish against columnaris disease with killed whole-cell bacterins have shown some promise (Fujihara and Nakatani, 1971; Moore et al., 1990; Bernardet, 1997), the protection is neither strong nor long lasting, and better vaccines are needed. As suggested by Newton et al. (1997), detoxified proteases of *F. columnare* could possibly be more effective antigens. Seven outer membrane proteins, some of them common to all *F. columnare* isolates, were strongly recognized by antibodies in the serum of convalescent channel catfish; they may also prove useful in developing an improved vaccine (Davidson, 1996). Rabbit antisera raised against the outer membrane proteins of *F. columnare* were used to screen an expression library of the bacterium; two genes, encoding a zinc metalloprotease (possibly representing a new family of zincins) and a prolyl oligopetidase (Pop), both membrane-associated, were identified (Xie et al., 2004). Comparison of immunodominant antigens in whole-cell lysates of *F. columnare* showed that formalin treatment (but not pressure treatment) inactivates one of the protein antigens (Bader et al., 1997). The recently characterized lipopolysaccharide *O*-polysaccharide antigen and the glycopeptides of *F. columnare* may also provide target molecules for vaccines (MacLean et al., 2003; Vinogradov et al., 2003). The modification of water temperature and salinity has been proposed as a control method during outbreaks of columnaris disease, when possible (Holt et al., 1993; Soltani and Burke, 1994). Chemotherapy remains the most widely used control method in fish farms, but very few antibiotics are at the same time authorized for use in fish, nontoxic to fish, and actually effective in vivo despite demonstration of in vitro activity against *F. columnare* (Soltani et al., 1995; see the section Identification in this Chapter). Oxytetracycline is commonly used in feed or as a bath for external infections; oral administration of oxolinic acid, sulfonamides or florfenicol is also recommended (Wakabayashi, 1993; Austin and Austin, 1999; Shotts and Starliper, 1999). Bath, flush or dip treatments with various disinfectants (sodium chloride, copper sulfate, potassium permanganate, hydrogen peroxide, chloramine-T, quaternary ammonium compounds, Diquat, etc.) have met with varying degrees of success (Altinok, 2004; Thomas-Jinu and Goodwin, 2004). After having been advocated as a strategy to control infectious diseases of channel catfish, withholding feed has been shown to actually reduce the resistance of fish to *F. columnare* (Shoemaker et al., 2003).

FLAVOBACTERIUM BRANCHIOPHILUM. Although *F. columnare*, *F. psychrophilum* and presumably *F. hydatis* are able to cause bacterial gill disease in various fish species (see the corresponding paragraphs), a different, nongliding, yellow filamentous organism first recognized from gill disease in salmonids in Japan (Kimura et al., 1978) is actually the main causative agent. Following the identification of other, serologically different strains from similar cases in Oregon (Wakabayashi et al., 1980) and from gill disease in rainbow trout, sheatfish, and silver carp in Hungary (Farkas, 1985), comparative studies of representative isolates originating from these three geographic areas resulted in the description of the new species *Flavobacterium branchiophila* (Wakabayashi et al., 1989), which was rapidly corrected to *branchiophilum* (Von Graevenitz, 1990). The new pathogen was also recognized in Ontario, Canada, as the causative agent of gill disease, one of the most important conditions affecting the salmonid industry in this region (Ferguson et al., 1991; Turnbull, 1993; Ostland et al., 1994). More recently, cases were also identified in Korea (Ko and Heo, 1997). As for *F. psychrophilum*, it is difficult to know whether what appears to be a fast, worldwide diffusion of a new pathogen could actually be only related to improvements in the isolation and identification of a long-established bacterial species after fish pathologists became aware of the disease in other parts of the world. Bacterial gill disease was reproduced experimentally in various fish species (Wakabayashi et al., 1980; Ferguson et al., 1991; Ostland et al., 1995). Histopathological changes in infected gills were evaluated and the ability of all *F. branchiophilum* strains to attach to the gills was demonstrated, although only virulent strains were able to further colonize the gills and cause mortality (Turnbull, 1993; Ostland et al., 1995). Contrary to the two above-mentioned fish pathogens, *F. branchiophilum* is considered noninvasive and is

usually not isolated from internal organs; it is a relatively fastidious organism, easily overgrown by other bacteria (Turnbull, 1993). The virulence for gills was connected to the various enzymatic and hemagglutinating activities detected in the extracellular products (Ototake and Wakabayashi, 1985) which affect respiratory functions of rainbow trout (Wakabayashi and Iwado, 1985). Serological studies (see the section Identification in this Chapter) made possible the use of immunofluorescence and ELISA to specifically detect and quantify *F. branchiophilum* at the gill surface (Heo et al., 1990; MacPhee et al., 1995). Indirect enzyme immunoassay showed that infected fish produce serum and gill-surface antibodies (Lumsden et al., 1993); however, intermittent occurrence of gill disease outbreaks in the same fish population suggested that surviving fish are not protected against the pathogen (Heo et al., 1990). Nevertheless, immunization trials (reviewed by Bernardet, 1997) showed that the local gill immune response was probably responsible for the significant reduction in gill-associated *F. branchiophilum* and for the partial protection seen in bath-vaccinated fish challenged with relatively low numbers of bacteria (Lumsden et al., 1994). The use of NaCl (Heo et al., 1990), H_2O_2 (Derksen et al., 1999), formalin and chloramine-T (Ostland et al., 1995), and nifurpirinol (now banned; Ostland et al., 1989) was evaluated for the bath treatment of bacterial gill disease and has met with varying degrees of success (Turnbull, 1993; Shotts and Starliper, 1999).

FLAVOBACTERIUM JOHNSONIAE. [*Cytophaga*] *johnsonae* was long known as a common soil organism involved in the degradation of various biomacromolecules (Stanier, 1947; Reichenbach, 1989). It was later transferred to the genus *Flavobacterium* and its specific epithet was corrected (Bernardet et al., 1996). After strains isolated from diseased fish were first mentioned by Christensen (1977), similar strains were frequently recognized in external lesions of different fish species worldwide (Rintamäki-Kinnunen et al., 1997; J.-F. Bernardet, unpublished data). The best description of a fish disease caused by *F. johnsoniae* was published following its isolation in pure culture from extensive skin lesions in juvenile farmed barramundi (*Lates calcarifer*) in Australia (Carson et al., 1993). However, the fact that this outbreak occurred in intensive farming conditions after an increase in suspended solids in the water and a sudden drop in water temperature indicated that special conditions had to be met for *F. johnsoniae* to become pathogenic. This was confirmed when experimental infection trials in various fish species were shown to be effective only in barramundi and then only when fish were challenged after being submitted to a drop in water temperature (Soltani et al., 1994). Hence, these studies confirmed *F. johnsoniae* as an opportunistic pathogen. Although phenotypic and chemotaxonomic (i.e., whole-cell protein profiles and fatty acid analysis) investigations of a collection of fish isolates showed they were indeed highly related to *F. johnsoniae*, only rather low DNA relatedness was found between the isolates tested and the type strain (J-F. Bernardet, unpublished data). Hence, pending further research, such strains should preferably be referred to as "*F. johnsoniae*-like." Since a certain degree of heterogeneity was noticed between the strains available in culture collections (Bernardet et al., 1996), the splitting of *F. johnsoniae* into two or several species may be necessary in the future.

OTHER *FLAVOBACTERIUM* SPECIES. Several other so-called *Flavobacterium* species have been either retrieved from fish or described as fish pathogens; however, most of them have subsequently been moved to other or new genera, and they have not been involved in any outbreak since their original description.

Several *Flavobacterium*-like organisms were described from diseased marine fish; for instance, "[*Flavobacterium*] *piscicida*," isolated from various fish species in Florida, was subsequently reclassified as "[*Pseudomonas*] *piscicida*" and finally as *Pseudoalteromonas piscicida* (see Bernardet [1998] and references therein; the chapter The Genus *Alteromonas* and Related Proteobacteria in Volume 6).

[*Flavobacterium*] *balustinum* was originally isolated from the surface of dead fish; the current opinion is that this organism was a fish spoilage agent rather than a pathogen (see Austin and Austin [1999] and references therein). It was moved to the genus *Chryseobacterium* as *C. balustinum* after further taxonomic investigations (Vandamme et al., 1994).

A new bacterial species isolated from turbot (*Scophthalmus maximus*) and seawater in Scotland was first named "[*Flavobacterium*] *scophthalmum*" (Mudarris et al., 1994); this bacterium, responsible for gill disease and hemorrhagic septicemia, was also transferred to the genus *Chryseobacterium* as *C. scophthalmum* (Vandamme et al., 1994).

A related bacterium, although not isolated from fish, should be mentioned: an organism responsible for hemorrhagic septicemia in farmed bullfrog (*Rana catesbeiana*) in Taiwan was characterized and named "[*Flavobacterium*] *ranacida*" (see Faung et al. [1996] and references therein); recently, this organism was identified as *Chryseobacterium meningosepticum* (J.-F. Bernardet, unpublished data; for the three above-mentioned organisms, see

the chapter The Genus *Chryseobacterium* in this Volume).

Three strains of a facultatively anaerobic gliding bacterium were isolated from skin lesions of salmon and from a water sample in a Washington state hatchery, characterized, and named "[*Cytophaga*] *succinicans*" (Anderson and Ordal, 1961) in spite of phenotypic differences between the three strains (Reichenbach, 1989). Whole-cell protein profile and fatty acid content confirmed that one strain differs from the two others (Bernardet et al., 1996). No other strain of this bacterial species was ever described, and it was subsequently transferred to the genus *Flavobacterium* as *F. succinicans* (Bernardet et al., 1996). Also isolated from external (i.e., gill) lesions in salmon in a Michigan hatchery, another facultatively anaerobic gliding bacterium was described as the new species [*Cytophaga*] *aquatilis* (Strohl and Tait, 1978). This species was later transferred to the genus *Flavobacterium* under the new epithet *F. hydatis* (Bernardet et al., 1996). Similar strains have frequently been isolated from external lesions (i.e., gill or fin necrosis, skin ulcers, etc.) of fish since then (e.g., Austin and Austin, 1999; J.-F. Bernardet, unpublished data). As in the case of *F. johnsoniae*, the fish isolates were phenotypically and chemotaxonomically similar to *F. hydatis* in spite of rather low DNA relatedness between those that were tested and the type and only bona fide *F. hydatis* strain available in culture collections (J.-F. Bernardet, unpublished data); consequently, these strains should be referred to as "*F. hydatis*-like." The pathogenicity of *F. succinicans* and *F. hydatis* for fish was never actually demonstrated (Austin and Austin, 1999); these organisms likely act as opportunistic pathogens, invading fish already weakened by other pathogens, poor farming conditions, or environmental disorders.

Bacterial strains whose 16S rRNA gene sequence matched that of *F. frigidarium* were recently identified among the bacterial community in amoebae-infested gill tissue of salmon, but the relative implication of bacteria and parasites in the disease is not known (Bowman and Nowak, 2004).

Applications

Flavobacterium species produce a variety of enzymes (see the section Identification in this Chapter) that have potential biotechnological applications in processes involving the degradation of such biomacromolecules as agar, alginate, chitin, laminarin, pectin, xylan, etc. For instance, a keratinase-producing *Flavobacterium* sp. was recently isolated from a poultry industry (Riffel and Brandelli, 2002). This organism was able to completely degrade raw feathers and could be used to hydrolyze keratin; however, its affiliation to the genus *Flavobacterium* was only based on a scant phenotypic study. Some bona fide members of the genus have particular biotechnological potential and ecological interest because they can produce cold-active enzymes (see the section Habitat and Ecology in this Chapter).

Over the last few years, a number of algicidal bacteria were identified, especially in Japan; they have attracted much attention as possible tools to regulate the population of phytoplankton responsible for the "red tide" blooms, which frequently cause serious damage to the aquaculture industry in coastal seawaters. Some of these bacteria were attributed to the genus *Flavobacterium* thanks to their 16S rRNA sequence; though isolated from seawater, these isolates did not require NaCl for growth (Maeda et al., 1998). A whole-cell hybridization method using fluorescently labeled probes was developed for the specific detection and enumeration of another algicidal *Flavobacterium* sp. (Adachi et al., 2002).

A *F. columnare*-like organism capable of killing the cyst form of the protozoan parasite *Giardia lamblia* was recently isolated from freshwater (Rodgers et al., 2003). Provided the concentration of Ca^{2+} in the medium was high and the bacteria and cyst were in direct contact, up to 80% of the cysts were destroyed within 48 h. Hence, such strains could be used as biological control agents against *Giardia* cysts in drinking water treatment plants.

A biosurfactant-producing *Flavobacterium* sp. was isolated from samples of arid soil (Bodour et al., 2003). It produces a mixture of at least 37 compounds representing a new class of biosurfactants, named "flavolipids," exhibiting a unique polar moiety and strong and stable emulsifying and solubilizing activities; they have a variety of potential biotechnological and industrial applications, for instance, in the remediation of contaminated soils (Bodour et al., 2004).

Studying the dynamics of bacterial community in a soil contaminated with petroleum hydrocarbons, Kaplan and Kitts (2004) noticed that organisms attributed to the genera *Flavobacterium* and *Pseudomonas* from their 16S rRNA gene sequence were most abundant during the fast degradation phase. This observation supported the importance of *Flavobacterium* spp. in the bioremediation of hydrocarbon-contaminated soils already reported by previous publications (Kaplan and Kitts, 2004 and references therein), although the identification of bacterial strains may not have been based on firm grounds in all these studies.

The degradation potential of a *Flavobacterium* sp. strain on paper mill effluents was investi-

gated; degradation appeared more effective after the bacterium was pre-treated with sinusoidal magnetic fields (Aarthi et al., 2004).

A *Flavobacterium* sp. strain producing antifungal compounds may have some potential for the biocontrol of fungi responsible for the banana crown rot (Gunasinghe et al., 2004).

Literature Cited

Aarthi, T. Leelapriya, P. T. Kalaichelvan, K. S. Dhilip, and P. V. S. Narayan. 2004. Application of weak sinusoidal magnetic field on Flavobacterium species in the treatment of paper mill effluent. Electromag. Biol. Med. 23:215–227.

Abdurashitov, M. A., S. S. Okhapkina, N. A. Netesova, L. N. Golikova, D. A. Gonchar, and S. K. Degtyarev. 2003. The unique FauI restriction-modification system: Cloning and protein sequence comparisons. Molec. Biol. 37:524–528.

Adachi, M., K. Fukami, R. Kondo, and T. Nishijima. 2002. Identification of marine algicidal Flavobacterium sp. 5N-3 using multiple probes and whole-cell hybridization. Fish. Science 68:713–720.

Agbo, J. A. C., and M. O. Moss. 1979. The isolation and characterization of agarolytic bacteria from a lowland river. J. Gen. Microbiol. 115:355–368.

Al-Harbi, A. H., and N. Uddin. 2003. Quantitative and qualitative studies on bacterial flora of hybrid tilapia (Oreochromis niloticus x O. aureus) cultured in earthen ponds in Saudi Arabia. Aquacult. Res. 34:43–48.

Alvarez, B., P. Secades, M. J. McBride, and J. A. Guijarro. 2004. Development of genetic techniques for the psychrophilic fish pathogen Flavobacterium psychrophilum. Appl. Environ. Microbiol. 70:581–587.

Amin, N. E., I. S. Abdallah, M. Faisal, M. EL-S. Easa, T. Alaway, and S. A. Alyan. 1988. Columnaris infection among cultured Nile tilapia Oreochromis niloticus. Ant. v. Leeuwenhoek 54:509–520.

Amita, K., M. Hoshino, T. Honma, and H. Wakabayashi. 2000. An investigation on the distribution of Flavobacterium psychrophilum in the Umikawa River [in Japanese, with abstract, tables and figures in English]. Fish Pathol. 35:193–197.

Anacker, R. L., and E. J. Ordal. 1955. Study of a bacteriophage infecting the myxobacterium Chondrococcus columnaris. J. Bacteriol. 70:738–741.

Anacker, R. L., and E. J. Ordal. 1959. Studies on the myxobacterium Chondrococcus columnaris. II: Bacteriocins. J. Bacteriol. 78:33–40.

Anderson, R. L., and E. J. Ordal. 1961. Cytophaga succinicans sp.n., a facultatively anaerobic, aquatic myxobacterium. J. Bacteriol. 81:130–138.

Aslam, Z., W.-T. Im, M. K. Kim, and S.-T. Lee. 2005. Flavobacterium granuli sp. nov., isolated from granules used in a wastewater treatment plant. Int. J. Syst. Evol. Microbiol. 55:747–751.

Austin, B., and D. A. Austin. 1999. Bacterial Fish Pathogens: Disease of Farmed and Wild Fish, 3rd (revised) ed. Praxis Publishing. Chichester, UK.

Bader, J. A., P. H. Klesius, and S. Vinitnantharat. 1997. Comparison of whole-cell antigens of pressure- and formalin-killed Flexibacter columnaris from channel catfish (Ictalurus punctatus). Am. J. Vet. Res. 58:985–988.

Bader, J. A., C. A. Shoemaker, and P. H. Klesius. 2005. Production, characterization and evaluation of virulence of an adhesion defective mutant of Flavobacterium columnare produced by β-lactam selection. Let. Appl. Microbiol. 40:123–127.

Baliarda, A., D. Faure, and M. C. Urdaci. 2002. Development and application of a nested PCR to monitor brood stock salmonid ovarian fluid and spleen for detection of the fish pathogen Flavobacterium psychrophilum. J. Appl. Microbiol. 92:510–516.

Barker, G. A., S. M. Smith, and N. R. Bromage. 1989. The bacterial flora of rainbow trout, Salmo gairdneri Richardson, and brown trout, Salmon trutta L., eggs and its relationship to developmental success. J. Fish Dis. 12:281–293.

Bauwens, M., and J. De Ley. 1981. Improvements in the taxonomy of Flavobacterium by DNA:rRNA hybridization. *In:* H. Reichenbach and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group. Gesellschaft für Biotechnologische Forschung. Weinheim, Germany. 27–31.

Becker, C. D., and M. P. Fujihara. 1978. The Bacterial Pathogen Flexibacter columnaris and its Epizootiology among Columbia River Fish: A Review and Synthesis. American Fisheries Society. Washington, DC. Monograph no. 2.

Bell, G. R., G. E. Hoskins, and W. Hodgkiss. 1971. Aspects of the characterization, identification, and ecology of the bacterial flora associated with the surface of stream-incubating Pacific salmon (Oncorhynchus) eggs. J. Fish. Res. Board Canada 28:1511–1525.

Bergey, D. H., F. C. Harrison, R. S. Breed, B. W. Hammer, and F. M. Huntoon. 1923. Bergey's Manual of Determinative Bacteriology, 1st ed. Williams & Wilkins. Baltimore, MD.

Bernardet, J.-F., F. Baudin-Laurencin, and G. Tixerant. 1988. First identification of "Cytophaga psychrophila" in France. Bull. Eur. Ass. Fish Pathol. 8:104–105.

Bernardet, J.-F. 1989a. Etude phénotypique et génomique des bactéries appartenant aux genres Cytophaga et Flexibacter (ordre des Cytophagales) et comparaison avec le genre Flavobacterium; application à l'identification et à la taxonomie des espèces ichtyopathogènes [PhD thesis]. Université Paris 7. Paris, France.

Bernardet, J.-F. 1989b. "Flexibacter columnaris": First description in France and comparison with bacterial strains from other origins. Dis. Aquat. Org. 6:37–44.

Bernardet, J.-F., and P. A. D. Grimont. 1989. Deoxyribonucleic acid relatedness and phenotypic characterization of Flexibacter columnaris sp. nov., nom. rev., Flexibacter psychrophilus sp. nov., nom. rev., and Flexibacter maritimus Wakabayashi, Hikida, and Masumura 1986. Int. J. Syst. Bacteriol. 39:346–354.

Bernardet, J.-F., and B. Kerouault. 1989. Phenotypic and genomic studies of "Cytophaga psychrophila" isolated from diseased rainbow trout (Oncorhynchus mykiss) in France. Appl. Environ. Microbiol. 55:1796–1800.

Bernardet, J.-F., P. Segers, M. Vancanneyt, F. Berthe, K. Kersters, and P. Vandamme. 1996. Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family Flavobacteriaceae, and proposal of Flavobacterium hydatis nom. nov. (basonym, Cytophaga aquatilis Strohl and Tait 1978). Int. J. Syst. Bacteriol. 46:128–148.

Bernardet, J.-F. 1997. Immunization with bacterial antigens: Flavobacterium and Flexibacter infections. Devel. Biol. Stand. 90:179–188.

Bernardet, J.-F. 1998. Cytophaga, Flavobacterium, Flexibacter and Chryseobacterium infections in cultured marine fish. Fish Pathol. 33:229–238.

Bernardet, J.-F., Y. Nakagawa, and B. Holmes. 2002. Proposed minimal standards for describing new taxa of the family Flavobacteriaceae and emended description of the family. Int. J. Syst. Evol. Microbiol. 52:1049–1070.

Bertolini, J. M., and J. S. Rohovec. 1992. Electrophoretic detection of proteases from different Flexibacter columnaris strains and assessment of their variability. Dis. Aquat. Org. 12:121–128.

Bertolini, J. M., H. Wakabayashi, V. G. Watral, M. J. Whipple, and J. S. Rohovec. 1994. Electrophoretic detection of proteases from selected strains of Flexibacter psychrophilus and assessment of their variability. J. Aquat. Anim. Health 6:224–233.

Bodour, A. A., K. P. Drees, and R. M. Maier. 2003. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. Appl. Environ. Microbiol. 69:3280–3287.

Bodour, A. A., C. Guerrero-Barajas, B. V. Jiorle, M. E. Malcomson, A. K. Paull, A. Somogyi, L. N. Trinh, R. B. Bates, and R. M. Maier. 2004. Structure and characterization of flavolipids, a novel class of biosurfactants produced by Flavobacterium sp. strain MTN11. Appl. Environ. Microbiol. 70:114–120.

Borg, A. F. 1948. Studies on Myxobacteria Associated with Diseases in Salmonid Fishes [Ph D thesis]. University of Washington. Seattle, WA.

Borg, A. F. 1960. Studies on Myxobacteria Associated with Diseases in Salmonid Fishes. American Association for the Advancement of Science. Washington, DC. Wildlife Disease, no. 8.

Borriss, M., E. Helmke, R. Hanschke, and T. Schweder. 2003. Isolation and characterization of marine psychrophilic phage-host systems from Arctic sea ice. Extremophiles 7:377–384.

Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin. 1997. Diversity and association of psychrophilic bacteria in Antarctic sea ice. Appl. Environ. Microbiol. 63:3068–3078.

Bowman, J. P., S. A. McCammon, T. Lewis, J. H. Skerratt, J. L. Brown, D. S. Nichols, and T. A. McMeekin. 1998. Psychroflexus torquis gen. nov., sp. nov., a psychrophilic species from Antarctic sea ice, and reclassification of Flavobacterium gondwanense (Dobson et al. 1993) as Psychroflexus gondwanense gen. nov., comb. nov. Microbiology 144:1601–1609.

Bowman, J. P., and B. Nowak. 2004. Salmonid gill bacteria and their relationship to amoebic gill disease. J. Fish Dis. 27:483–492.

Brinkmeyer, R., K. Knittel, J. Jürgens, H. Weyland, R. Amann, and E. Helmke. 2003. Diversity and structure of bacterial communities in Arctic versus Antarctic pack ice. Appl. Environ. Microbiol. 69:6610–6619.

Brisou, J., C. Tysset, A. Jacob, and L. Valette. 1960. Contribution à l'étude de deux germes du genre Flavobacterium saprophytes d'anguille d'eau douce (Anguilla vulgaris Turton). Arch. Inst. Pasteur Algérie 38:500–509.

Brown, L. L., W. T. Cox, and R. P. Levine. 1997. Evidence that the causal agent of bacterial cold-water disease Flavobacterium psychrophilum is transmitted within salmonid eggs. Dis. Aquat. Org. 29:213-218.

Bruun, M. S., A. S. Schmidt, L. Madsen, and I. Dalsgaard. 2000. Antimicrobial resistance patterns in Danish isolates of Flavobacterium psychrophilum. Aquaculture 187:201–212.

Bruun, M. S., L. Madsen, and I. Dalsgaard. 2003. Efficiency of oxytetracycline treatment in rainbow trout experimentally infected with Flavobacterium psychrophilum strains having different in vitro antibiotic susceptibilities. Aquaculture 215:11–20.

Bullock, G. L., D. A. Conroy, and S. F. Snieszko. 1971. Bacterial diseases of fish: Myxobacterioses. *In:* S. F. Snieszko and H. R. Axelrod (Eds.)Diseases of Fish. TFH Publication. Jersey City, NJ. 60–87.

Bullock, G. L. 1972. Studies on Selected Myxobacteria Pathogenic for Fishes and on Bacterial Gill Disease in Hatchery-reared Salmonids. U.S. Bureau of Sport Fisheries and Wildlife Technical Papers 60.

Bullock, G. L., T. C. Hsu, and E. B. Shotts. 1986. Columnaris Disease of Fishes. US Department of the Interior, Fish and Wildlife Service, Division of Fisheries and Wetlands Research. Washington, DC. Fish Disease Leaflet no. 72.

Burchard, R. P. 1981. Gliding motility of prokaryotes: ultrastructure, physiology, and genetics. Ann. Rev. Microbiol. 35:497–529.

Busch, S., I. Dalsgaard, and K. Buchmann. 2003. Concomitant exposure of rainbow trout fry to Gyrodactylus derjavini and Flavobacterium psychrophilum: Effects on infection and mortality of host. Veterinary Parasitology 117:117–122.

Callies, E., and W. Mannheim. 1978. Classification of the Flavobacterium-Cytophaga complex on the basis of respiratory quinones and fumarate respiration. Int. J. Syst. Bacteriol. 28:14–19.

Campbell, C. L., D. L. Mummey, E. T. Schmidtmann, and W. C. Wilson. 2004. Culture-independent analysis of midgut microbiota in the arbovirus vector Culicoides sonorensis (Diptera: Ceratopogonidae). J. Med. Entomol. 41:340–348.

Carson, J., L. M. Schmidtke, and B. L. Munday. 1993. Cytophaga johnsonae: a putative skin pathogen of juvenile farmed barramundi, Lates calcarifer Bloch. J. Fish Dis. 16:209–218.

Chakroun, C., M. C. Urdaci, D. Faure, F. Grimont, and J.-F. Bernardet. 1997. Random Amplified Polymorphic DNA analysis provides rapid differentiation among isolates of the fish pathogen Flavobacterium psychrophilum and among Flavobacterium species. Dis. Aquat. Org. 31:187–196.

Chakroun, C., F. Grimont, M. C. Urdaci, and J.-F. Bernardet. 1998. Fingerprinting of Flavobacterium psychrophilum isolates by ribotyping and plasmid profiling. Dis. Aquat. Org. 33:167–177.

Chase, J. M. 1965. *In:* Nutrition of Some Aquatic Myxobacteria [MSc thesis]. University of Washington. Washington, DC.

Chester, F. D.. 1897. Report of the mycologist: bacteriological work. Del. Agric. Exp. Sta. Bull. 9:38–145.

Chowdhury, M. B. R., and H. Wakabayashi. 1988. Effects of sodium, potassium, calcium and magnesium ions on the survival of Flexibacter columnaris in water. Fish Pathol. 23:231–235.

Chowdhury, M. B. R., and H. Wakabayashi. 1989. Effects of competitive bacteria on the survival and infectivity of Flexibacter columnaris. Fish Pathol. 24:9–15.

Chowdhury, M. B. R. 1995. Study on the virulence of Flexibacter columnaris strains. *In:* M. Shariff, J. R. Arthur, and R. P. Subasinghe (Eds.) Diseases in Asian

Aquaculture. Asian Fisheries Society, Fish Health Section. Quezon City, The Phillipines. II:269–274.

Christensen, P. J. 1974. A microbiological study of some lake waters and sediments from the Mackenzie Valley with special reference to cytophagas. Arctic 27:309–312.

Christensen, P. J. 1977. The history, biology, and taxonomy of the Cytophaga group. Can. J. Microbiol. 23:1599–1653.

Cipriano, R. C., and J. D. Teska. 1994. Effects of medium composition on the growth of two fish pathogens, Cytophaga columnaris and Cytophaga psychrophila. Biomed. Lett. 49:7–12.

Cipriano, R. C., L. A. Ford, and J. D. Teska. 1995. Association of Cytophaga psychrophila with mortality among eyed eggs of Atlantic salmon (Salmo salar). J. Wildl. Dis. 31:166–171.

Cipriano, R. C., W. B. Schill, J. D. Teska, and L. A. Ford. 1996. Epizootiological study of bacterial cold-water disease in Pacific salmon and further characterization of the etiologic agent, Flexibacter psychrophila. J. Aquat. Anim. Health 8:28–36.

Cone, D. K., J. D. Miller, and W. K. Austin. 1980. The pathology of "saddleback" disease of underyearling Atlantic salmon (Salmo salar). Can. J. Zool. 58:1283–1287.

Cousin, M. A. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. J. Food Protect. 45:172–207.

Crump, B. C., E. V. Armbrust, and J. A. Baross. 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. Appl. Environ. Microbiol. 65:3192–3204.

Crump, E. M., M. B. Perry, S. C. Clouthier, and W. W. Kay. 2001. Antigenic characterization of the fish pathogen Flavobacterium psychrophilum. Appl. Environ. Microbiol. 67:750–759.

Dalsgaard, I. 1993. Virulence mechanisms in Cytophaga psychrophila and other Cytophaga-like bacteria pathogenic for fish. Ann. Rev. Fish Dis. 1993:127–144.

Dalsgaard, I., and L. Madsen. 2000. Bacterial pathogens in rainbow trout, Oncorhynchus mykiss (Walbaum), reared at Danish freswater farms. J. Fish Dis. 23:199–209.

Darwish, A. M., A. A. Ismaiel, J. C. Newton, and J. Tang. 2004. Identification of Flavobacterium columnare by a species-specific polymerase chain reaction and renaming of ATCC 43622 strain to Flavobacterium johnsoniae. Mol. Cell. Probes 18:421–427.

Daskalov, H., D. A. Austin, and B. Austin. 1999. An improved growth medium for Flavobacterium psychrophilum. Lett. Appl. Microbiol. 28:297–299.

Davidson, M. L.. 1996. Characterization of the Outer Membrane Proteins of Flavobacterium columnare and Examination of their Antigenicity to Channel Catfish Ictalurus punctatus [MSc thesis]. Auburn University. Auburn, AL.

Davis, H. S. 1922. A new bacterial disease of fresh-water fishes. Bull. US Bur. Fish. 38:261–280.

Davis, H. S. 1946. Care and Diseases of Trout. US Government Printing Office. Washington, DC. US Department of Interior Research Report no. 12.

Decostere, A., F. Haesebrouck,and L. A. Devriese. 1997. Shieh medium supplemented with tobramycin for selective isolation of Flavobacterium columnare (Flexibacter columnaris) from diseased fish. J. Clin. Microbiol. 35:322–324.

Decostere, A., F. Haesebrouck, and L. A. Devriese. 1998. Characterization of four Flavobacterium columnare (Flexibacter columnaris) strains isolated from tropical fish. Vet. Microbiol. 62:35–45.

Decostere, A., F. Haesebrouck, G. Charlier, and R. Ducatelle. 1999. The association of Flavobacterium columnare strains of high and low virulence with gill tissue of black mollies (Poecilia sphenops). Vet. Microbiol. 67:287–298.

Decostere, A., F. Haesebrouck, J. F. Turnbull, and G. Charlier. 1999. Influence of water quality and temperature on adhesion of high and low virulence Flavobacterium columnare strains to isolated gill arches. J. Fish Dis. 22:1–11.

Decostere, A., F. Haesebrouck, E. Van Driessche, G. Charlier, and R. Ducatelle. 1999. Characterization of the adhesion of Flavobacterium columnare (Flexibacter columnaris) to gill tissue. J. Fish Dis. 22:465–474.

Decostere, A., E. D'Haese, M. Lammens, H. Nelis, and F. Haesebrouck. 2001. In vivo study of phagocytosis, intracellular survival and multiplication of Flavobacterium psychrophilum in rainbow trout, Oncorhynchus mykiss (Walbaum), spleen phagocytes. J. Fish Dis. 24:481–487.

Decostere, A., R. Ducatelle, and F. Haesebrouck. 2002. Flavobacterium columnare (Flexibacter columnaris) associated with severe gill necrosis in koi carp (Cyprinus carpio L.). Vet. Rec. 150:694–695.

del Cerro, A., M. C. Mendoza, and J. A. Guijarro. 2002. Usefulness of a TaqMan-based polymerase chain reaction assay for the detection of the fish pathogen Flavobacterium psychrophilum. J. Appl. Microbiol. 92:1–8.

del Cerro, A., I. Marquez, and J. A. Guijarro. 2002. Simultaneous detection of Aeromonas salmonicida, Flavobacterium psychrophilum, and Yersinia ruckeri, three major fish pathogens, by multiplex PCR. Appl. Environ. Microbiol. 68:5177–5180.

Derksen, J. A., V. E. Ostland, and H. W. Ferguson. 1999. Effects of hydrogen peroxide on clearance of formalin-killed Flavobacterium branchiophilum from the gills of rainbow trout, Oncorhynchus mykiss (Walbaum). J. Fish Dis. 22:59–67.

Desolme, B., and J.-F. Bernardet. 1996. Freeze-drying of Flavobacterium columnare, Flavobacterium psychrophilum and Flexibacter maritimus. Dis. Aquat. Org. 27:77–80.

Dorey, M. J. 1959. Some properties of a pectinolytic soil Flavobacterium. J. Gen. Microbiol. 20:91–104.

Ekman, E., H. Börjeson, and N. Johansson. 1999. Flavobacterium psychrophilum in Baltic salmon Salmo salar brood fish and their offspring. Dis. Aquat. Org. 37:159–163.

Ekman, E., and L. Norrgren. 2003. Pathology and immunohistochemistry in three species of salmonids after experimental infection with Flavobacterium psychrophilum. J. Fish Dis. 26:529–538.

Ekman, E., G. Åkerman, L. Balk, and L. Norrgren. 2003. Nanoinjection as a tool to mimic vertical transmission of Flavobacterium psychrophilum in rainbow trout Oncorhynchus mykiss. Dis. Aquat. Org. 55:93–99.

Ellerbroek, L. 1997. Airborne microflora in poultry slaughtering establishments. Food Microbiol. 14:527–531.

Elotmani, F., O. Assobhei, A. M. Revol-Junelles, and J. B. Milliere. 2004. Microflora of fresh and ice-stored sardines (Sardina pilchardus) from the Moroccan Atlantic coast. Ciencias Marinas 30:627–635.

Engelbrecht, K., P. J. Jooste, and B. A. Prior. 1996. Spoilage characteristics of Gram-negative genera and species

isolated from Cape marine fish. S. Afr. J. Food Sci. Nutr. 8:66–71.

Escalante, A., M. E. Rodrigez, A. Martinez, A. Lopez-Munguia, F. Bolivar, and G. Gosset. 2004. Characterization of bacterial diversity in Pulque, a traditional Mexican alcoholic beverage, as determined by 16S rDNA analysis. FEMS Microbiol. Let. 235:273–279.

Euzéby, J. P. 1997. List of Bacterial Names with Standing in Nomenclature: A folder available on the Internet. Int. J. Syst. Bacteriol. 47:590–592.

Evensen, Ø., and E. Lorenzen. 1996. An immunohistochemical study of Flexibacter psychrophilus infection in experimentally and naturally infected rainbow trout (Oncorhynchus mykiss) fry. Dis. Aquat. Org. 25:53–61.

Farkas, J. 1985. Filamentous Flavobacterium sp. isolated from fish with gill diseases in cold water. Aquaculture 44:1–10.

Faruk, M. A. R. 2000. Characterization of Flavobacterium psychrophilum, the Causative Agent of Rainbow Trout Fry Syndrome [PhD thesis]. University of Stirling. Stirling, UK.

Faung, S.-T., L. Chiu, and C.-T. Wang. 1996. Platelet lysis and functional perturbation by 13-methyl myristate, the major fatty acid in Flavobacterium ranacida. Thrombosis Res. 81:91–100.

Ferguson, H. W., V. E. Ostland, P. Byrne, and J. S. Lumsden. 1991. Experimental production of bacterial gill disease in trout by horizontal transmission and by bath challenge. J. Aquat. Anim. Hlth. 3:118–123.

Fijan, N. N. 1969. Antibiotic additives for the isolation of Chondrococcus columnaris from fish. Appl. Microbiol. 17:333–334.

Fijan, N. N., and P. R. Voorhees. 1969. Drug sensitivity of Chondrococcus columnaris. Vet. Arhiv, Zagreb 39:259–267.

Flaherty, D. K., F. H. Deck, M. A. Hood, C. Liebert, F. Singleton, P. Winzenburger, K. Bishop, L. R. Smith, L. M. Bynum, and W. B. Witmer. 1984. A Cytophaga species endotoxin as a putative agent of occupation-related lung disease. Infect. Immun. 43:213–216.

Frankland, G. C., and P. F. Frankland. 1889. Über einige typische Mikroorganismen im Wasser und im Boden. Z. Hyg. 6:373–400.

Fujihara, M. P., and R. E. Nakatani. 1971. Antibody production and immune responses of rainbow trout and coho salmon to Chondrococcus columnaris. J. Fish. Res. Board Can. 28:1253–1258.

Garcia, C., F. Pozet, and C. Michel. 2000. Standardization of experimental infection with Flavobacterium psychrophilum, the agent of rainbow trout Oncorhynchus mykiss fry syndrome. Dis. Aquat. Org. 42:191–197.

Garnjobst, L. 1945. Cytophaga columnaris (Davis) in pure culture: A myxobacterium pathogenic to fish. J. Bacteriol. 49:113–128.

Garrity, G. M., and J. G. Holt. 2001. The road map to the manual: Taxonomic outlines of the Archaea and Bacteria. *In:* D. R. Boone and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY 1.

Gennari, M., and C. Cozzolino. 1989. Observations on Flavobacterium, Cytophagaceae and other pigmented bacteria isolated from fresh and ice stored sardines. Arch. Vet. Ital. 40:372–384.

Georgnaras, I., A. E. de Jesus, E. van Zyl, and A. von Holy. 1996. Bacterial populations associated with poultry processing in a South African abattoir. Food Microbiol. 13:457–465.

Gherna, R., and C. R. Woese. 1992. A partial phylogenetic analysis of the "Flavobacter-Bacteroides" phylum: Basis for taxonomic restructuring. System. Appl. Microbiol. 15:513–521.

González, C. J., J. A. Santos, M.-L. García-López, and A. Otero. 2000. Psychrobacters and related bacteria in freshwater fish. J. Food Protect. 63:315–321.

Griffin, B. R. 1991. Characteristics of a chondroitin AC lyase produced by Cytophaga columnaris. Trans. Am. Fish. Soc. 120:391–395.

Griffin, B. R. 1992. As simple procedure for identification of Cytophaga columnaris. J. Aquat. Anim. Health 4:63–66.

Gunasinghe, R. N., C. J. Ikiriwatte, and A. M. Karunaratne. 2004. The use of Pantoea agglomerans and Flavobacterium sp. to control banana pathogens. J. Horticult. Sci. Biotechnol. 79:1002–1006.

Hamana, K., and S. Matsuzaki. 1990. Occurrence of homospermidine as a major polyamine in the authentic genus Flavobacterium. Can. J. Microbiol. 36:228–231.

Hamana, K., and S. Matsuzaki. 1991. Polyamine distribution in the Flavobacterium-Cytophaga-Sphingobacterium complex. Can. J. Microbiol. 37:885–888.

Hamana, K., Y. Nakagawa, and K. Yamasato. 1995. Chemotaxonomic significance of polyamine distribution patterns in the Flavobacterium-Cytophaga complex and related genera. Microbios 81:135–145.

Hamana, K., and Y. Nakagawa. 2001. Polyamine distribution profiles in newly validated genera and species within the Flavobacterium-Flexibacter-Cytophaga-Sphingobacterium complex. Microbios 106 (S2):105–116.

Hawke, J. P., and R. L. Thune. 1992. Systemic isolation and antimicrobial susceptibility of Cytophaga columnaris from commercially reared channel catfish. J. Aquat. Anim. Health 4:109–113.

Heo, G.-J., K. Kasai, and H. Wakabayashi. 1990. Occurrence of Flavobacterium branchiophila associated with bacterial gill disease at a trout hatchery. Fish Pathol. 25:99–105.

Heo, G.-J., H. Wakabayashi, and S. Watabe. 1990. Purification and characterization of pili from Flavobacterium branchiophila. Fish Pathol. 25:21–27.

Holmes, B., R. J. Owen, and T. A. McMeekin. 1984. Genus Flavobacterium Bergey, Harrison, Breed, Hammer and Huntoon 1923, 97^{AL}. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins Baltimore MD. 1:353–361.

Holmes, B. 1992. The Genera Flavobacterium, Sphingobacterium, and Weeksella. *In:* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 3620–3630.

Holt, R. A. 1988. Cytophaga psychrophila, the Causative Agent of Bacterial Cold-water Disease in Salmonid Fish [PhD thesis]. Oregon State University. Corvallis, OR.

Holt, R. A., A. Amandi, J. S. Rohovec, and J. L. Fryer. 1989. Relation of water temperature to bacterial cold-water disease in coho salmon, chinook salmon, and rainbow trout. J. Aquat. Anim. Health 1:94–101.

Holt, R. A., J. S. Rohovec, and J. L. Fryer. 1993. Bacterial cold-water disease. *In:* V. Inglis, R. J. Roberts, and N. R. Bromage (Eds.) Bacterial Diseases of Fish. Blackwell Scientific Publications. Oxford, UK. 3–22.

Horn, M., M. D. Harzenetter, T. Linner, E. N. Schmid, K.-D. Müller, R. Michel, and M. Wagner. 2001. Members of the Cytophaga-Flavobacterium-Bacteroides phylum as intracellular bacteria of acanthamoebae: Proposal of "Candidatus Amoebophilus asiaticus." Environ. Microbiol. 3:440–449.

Huh, G.-J., and H. Wakabayashi. 1989. Serological characteristics of Flavobacterium branchiophila isolated from gill diseases of freshwater fishes in Japan, USA, and Hungary. J. Aquat. Anim. Health 1:142–147.

Humphry, D. R., A. George, G. W. Black, and S. P. Cummings. 2001. Flavobacterium frigidarium sp. nov., an aerobic, psychrophilic, xylanolytic and laminarinolytic bacterium from Antarctica. Int. J. Syst. Evol. Microbiol. 51:1235–1243.

Hunnicutt, D. W., and M. J. McBride. 2001. Cloning and characterization of the Flavobacterium johnsoniae gliding motility genes gldD and gldE. J. Bacteriol. 183:4167–4175.

Hunnicutt, D. W., M. J. Kempf, and M. J. McBride. 2002. Mutations in Flavobacterium johnsoniae gldF and gldG disrupt gliding motility and interfere with membrane localization of gldA. J. Bacteriol. 184:2370–2378.

Iida, Y., and A. Mizokami. 1996. Outbreaks of coldwater disease in wild ayu and pale chub. Fish Pathol. 31:157–164.

Inoue, K., and K. Komagata. 1976. Taxonomic study on obligately psychrophilic bacteria isolated from Antarctica. J. Gen. Appl. Microbiol. 22:165–176.

Izumi, S., and H. Wakabayashi. 1997. Use of PCR to detect Cytophaga psychrophila from apparently healthy juvenile ayu and coho salmon eggs. Fish Pathol. 32:169–173.

Izumi, S., and H. Wakabayashi. 1999. Further study on serotyping of Flavobacterium psychrophilum. Fish Pathol. 34:89–90.

Izumi, S., and H. Wakabayashi. 2000. Sequencing of gyrB and their application in the identification of Flauobacterium psychrophilum by PCR. Fish Pathol. 35:93–94.

Izumi, S., F. Aranishi, and H. Wakabayashi. 2003a. A novel serotype of Flavobacterium psychrophilum detected using antiserum against an isolate from amago, Oncorhynchus masou rhodurus Jordan & Gilbert, in Japan. J. Fish Dis. 26:677–680.

Izumi, S., F. Aranishi, and H. Wakabayashi. 2003b. Genotyping of Flavobacterium psychrophilum using PCR-RFLP analysis. Dis. Aquat. Org. 56:207–214.

Izumi, S., and F. Aranishi. 2004b. Relationship between gyrA mutations and quinolone resistance in Flavobacterium psychrophilum isolates. Appl. Environ. Microbiol. 70:3968–3972.

Jaspers, E., K. Nauhaus, H. Cypionka, and J. Overmann. 2001. Multitude and temporal variability of ecological niches as indicated by the diversity of cultivated bacterioplankton. FEMS Microbiol. Ecol. 36:153–164.

Johnson, J. L., and W. S. Chilton. 1966. Galactosamine glycan of Chondrococcus columnaris. Science 152:1247–1248.

Jooste, P. J., and C. J. Hugo. 1999. The taxonomy, ecology and cultivation of bacterial genera belonging to the family Flavobacteriaceae. Int. J. Food Microbiol. 53:81–94.

Kaiser, P. 1961. Etude de l'activité pectinolytique du sol et d'autres substrats naturels [thesis]. Université de Paris. Paris, France.

Kaplan, C. W., and C. L. Kitts. 2004. Bacterial succession in a petroleum land treatment unit. Appl. Environ. Microbiol. 70:1777–1786.

Kempf, M. J., and M. J. McBride. 2000. Transposon insertions in the Flavobacterium johnsoniae ftsX gene disrupt gliding motility and cell division. J. Bacteriol. 182:1671–1679.

Kent, M. L., J. M. Groff, J. K. Morrison, W. T. Yasutake, and R. A. Holt. 1989. Spiral swimming behavior due to cranial and vertabral lesions associated with Cytophaga psychrophila infections in salmonid fishes. Dis. Aquat. Org. 6:11–16.

Kimura, N., H. Wakabayashi, and S. Kudo. 1978. Studies on bacterial gill disease in salmonids. I: Selection of bacterium transmitting gill disease [in Japanese with English abstract]. Fish Pathol. 12:233–242.

Kirchman, D. L., L. Y. Yu, and M. T. Cottrell. 2003. Diversity and abundance of uncultured Cytophaga-like bacteria in the Delaware estuary. Appl. Environ. Microbiol. 69:6587–6596.

Kisand, V., and J. Wikner. 2003. Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. J. Microbiol. Meth. 54:183–191.

Ko, Y.-M., and G.-J. Heo. 1997. Characteristics of Flavobacterium branchiophilum isolated from rainbow trout in Korea. Fish Pathol. 32:97–102.

Kondo, M., K. Kawai, K. Kurohara, and S. Oshima. 2002. Adherence of Flavobacterium psychrophilum on the body surface of the ayu Plecoglossus altivelis. Microbes and Infection 4:279–283.

Kondo, M., K. Kawai, M. Okabe, N. Nakano, and S. Oshima. 2003. Efficacy of oral vaccine against bacterial coldwater disease in ayu Plecoglossus altivelis. Dis. Aquat. Org. 55:261–264.

Koski, P., V. Hirvelä-Koski, and J.-F. Bernardet. 1993. Flexibacter columnaris infection in Arctic char (Salvelinus alpinus L.): First isolation in Finland. Bull. Eur. Ass. Fish Pathol. 13:66–69.

Kumagai, A., and K. Takahashi. 1997. Imported eggs responsible for the outbreaks of cold-water disease among cultured coho salmon in Japan. Fish Pathol. 32:231–232.

Kumagai, A., S. Yamakoa, K. Takahashi, H. Fukuda, and H. Wakabayashi. 2000. Waterborne transmission of Flavobacterium psychrophilum in coho salmon eggs. Fish Pathol. 35:25–28.

LaFrentz, B. R., S. E. LaPatra, G. R. Jones, J. L. Congleton, B. Sun, and K. D. Cain. 2002. Characterization of serum and mucosal antibody responses and relative per cent survival in rainbow trout, Oncorhynchus mykiss (Walbaum), following immunization and challenge with Flavobacterium psychrophilum. J. Fish Dis. 25:703–713.

LaFrentz, B. R., S. E. LaPatra, G. R. Jones, and K. D. Cain. 2003. Passive immunization of rainbow trout, Oncorhynchus mykiss (Walbaum), against Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease and rainbow trout fry syndrome. J. Fish Dis. 26:377–384.

Leadbetter, E. R.. 1974. Order II: Cytophagales Nomen novum. *In:* R. E. Buchanan and N. E. Gibbons (Eds.) Bergey's Manual of Determinative Bacteriology, 8th ed. Williams & Wilkins. Baltimore, MD. 99–122.

Lednicka, D., J. Mergaert, M. C. Cnockaert, and J. Swings. 2002. Isolation and identification of cellulolytic bacteria involved in the degradation of natural cellulosic fibres. Syst. Appl. Microbiol. 23:292–299.

Lee, K.-B., and G.-J. Heo. 1998. First isolation and identification of Cytophaga psychrophila from cultured ayu in Korea. Fish Pathol. 33:37–38.

Lehmann, J., D. Mock, F.-J. Stürenberg, and J.-F. Bernardet. 1991. First isolation of Cytophaga psychrophila from a

systemic disease in eel and cyprinids. Dis. Aquat. Org. 10:217–220.

Lelliott, R. A., and D. E. Stead. 1987. Methods for the Diagnosis of Bacterial Diseases in Plants. Blackwell Scientific Publications. Oxford, UK.

Lewin, R. A. 1969. A classification of Flexibacteria. J. Gen. Microbiol. 58:189–206.

Liao, C.-H., and J. M. Wells. 1986. Properties of Cytophaga johnsonae strains causing spoilage of fresh produce at food markets. Appl. Environ. Microbiol. 52:1261–1265.

Liebert, C. A., M. A. Hood, F. H. Deck, K. Bishop, and D. K. Flaherty. 1984. Isolation and characterization of a new Cytophaga species implicated in a work-related lung disease. Appl. Environ. Microbiol. 48:936–943.

Liu, H., S. Izumi, and H. Wakabayashi. 2001. Detection of Flavobacterium psychrophilum in various organs of ayu Plecoglossus altivelis by in situ hybridization. Fish Pathol. 36:7–11.

Lorenzen, E., and N. Karas. 1992. Detection of Flexibacter psychrophilus by immunofluorescence in fish suffering from fry mortality syndrome: A rapid diagnostic method. Dis. Aquat. Org. 13:231–234.

Lorenzen, E. 1993. The importance of the brand of the beef extract in relation to the growth of Flexibacter psychrophilus in Anacker and Ordal's medium. Bull. Eur. Ass. Fish Pathol. 13:64–65.

Lorenzen, E. 1994. Studies on Flexibacter psychrophilus in Relation to Rainbow Trout Fry Syndrome (RTFS) [PhD thesis]. National Veterinary Laboratory, Århus, and Royal Veterinary and Agriculture University. Copenhagen, Denmark.

Lorenzen, E., I. Dalsgaard, and J.-F. Bernardet. 1997. Characterization of isolates of Flavobacterium psychrophilum associated with cold-water disease or rainbow trout fry syndrome I: phenotypic and genomic studies. Dis. Aquat. Org. 31:197–208.

Lorenzen, E., and N. J. Olesen. 1997. Characterization of isolates of Flavobacterium psychrophilum associated with cold-water disease or rainbow trout fry syndrome. II: Serological studies. Dis. Aquat. Org. 31:209–220.

Lumsden, J. S., V. E. Ostland, P. J. Byrne, and H. W. Ferguson. 1993. Detection of a distinct gill-surface antibody response following horizontal infection and bath challenge of brook trout Salvelinus fontinalis with Flavobacterium branchiophilum, the causative agent of bacterial gill disease. Dis. Aquat. Org. 16:21–27.

Lumsden, J. S., V. E. Ostland, D. D. MacPhee, J. Derksen, and H. W. Ferguson. 1994. Protection of rainbow trout from experimentally induced bacterial gill disease caused by Flavobacterium branchiophilum. J. Aquat. Anim. Health 6:292–302.

Lumsden, J. S., V. E. Ostland, and H. W. Ferguson. 1996. Necrotic myositis in cage cultured rainbow trout, Oncorhynchus mykiss (Walbaum), caused by Flexibacter psychrophilus. J. Fish Dis. 19:113–119.

MacLean, L. L., E. Vinogradov, E. M. Crump, M. B. Perry, and W. W. Kay. 2001. The structure of the lipopolysaccharide O-antigen produced by Flavobacterium psychrophilum (259–93). Eur. J. Biochem. 268:2710–2716.

MacLean, L. L., M. B. Perry, E. M. Crump, and W. W. Kay. 2003. Structural characterization of the lipopolysaccharide O-polysaccharide antigen produced by Flavobacterium columnare ATCC 43622. Eur. J. Biochem. 270:3440–3446.

MacPhee, D. D., V. E. Ostland, J. S. Lumsden, and H. W. Ferguson. 1995. Development of an enzyme-linked immunosorbent assay (ELISA) to estimate the quantity of Flavobacterium branchiophilum on the gills of rainbow trout Oncorhynchus mykiss. Dis. Aquat. Org. 21:13–23.

Madetoja, J., P. Nyman, and T. Wiklund. 2000. Flavobacterium psychrophilum, invasion into and shedding by rainbow trout Oncorhynchus mykiss. Dis. Aquat. Org. 43:27–38.

Madetoja, J., M.-L. Hänninen, V. Hirvelä-Koski, I. Dalsgaard, and T. Wiklund. 2001. Phenotypic and genotypic characterization of Flavobacterium psychrophilum from Finnish fish farms. J. Fish Dis. 24:469–479.

Madetoja, J., and T. Wiklund. 2002. Detection of the fish pathogen Flavobacterium psychrophilum in water from fish farms. System. Appl. Microbiol. 25:259–266.

Madetoja, J., S. Nystedt, and T. Wiklund. 2003. Survival and virulence of Flavobacterium psychrophilum in water microcosms. FEMS Microbiol. Ecol. 43:217–223.

Madsen, L., and I. Dalsgaard. 1998. Characterization of Flavobacterium psychrophilum: comparison of proteolytic activity and virulence of strains isolated from rainbow trout (Oncorhynchus mykiss). *In:* A. C. Barnes, G. A. Davidson, M. P. Hiney, and D. McIntosh (Eds.) Methodology in Fish Diseases Research. Fisheries Research Services. Aberdeen, UK. 45–52.

Madsen, L., and I. Dalsgaard. 2000. Comparative studies of Danish Flavobacterium psychrophilum isolates: Ribotypes, plasmid profiles, serotypes and virulence. J. Fish Dis. 23:211–218.

Maeda, T., M. Murakami, S. Ohsugi, M. Furushita, A. Mitsutani, and T. Shiba. 1998. Perspectives of the development of 16S rDNA probe specific for algicidal and/or algallytic bacteria. Fish. Science 64:861–865.

Mahmoud, B. S. M., K. Yamazaki, K. Miyashita, S. Il-Shik, C. Dong-Suk, and T. Suzuki. 2004. Bacterial microflora of carp (Cyprinus carpio) and its shelf-life extension by essential oil compounds. Food Microbiol. 21:657–666.

Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K.-H. Schleifer. 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiology 142:1097–1106.

Mata, M., A. Skarmeta, and Y. Santos. 2002. A proposed serotyping system for Flavobacterium psychrophilum. Lett. Appl. Microbiol. 35:166–170.

McBride, M. J., and S. A. Baker. 1996. Development of techniques to genetically manipulate members of the genera Cytophaga, Flavobacterium, Flexibacter, and Sporocytophaga. Appl. Environ. Microbiol. 62:3017–3022.

McBride, M. J., and M. J. Kempf. 1996. Development of techniques for the genetic manipulation of the gliding bacterium Cytophaga johnsonae. J. Bacteriol. 178:583–590.

McBride, M. J. 2001. Bacterial gliding motility: multiple mechanisms for cell movement over surfaces. Ann. Rev. Microbiol. 55:49–75.

McBride, M. J., T. F. Braun, and J. L. Brust. 2003. Flavobacterium johnsoniae gldH is a lipoprotein that is required for gliding motility and chitin utilization. J. Bacteriol. 185:6648–6657.

McBride, M. J., and T. F. Braun. 2004. GldI is a lipoprotein that is required for Flavobacterium johnsoniae gliding

motility and chitin utilization. J. Bacteriol. 186:2295–2302.

McCammon, S. A., B. H. Innes, J. P. Bowman, P. D. Franzmann, S. J. Dobson, P. E. Holloway, J. H. Skerratt, P. D. Nichols, and L. M. Rankin. 1998. Flavobacterium hibernum sp. nov., a lactose-utilizing bacterium from a freswater Antarctic lake. Int. J. Syst. Bacteriol. 48:1405–1412.

McCammon, S. A., and J. P. Bowman. 2000. The taxonomy of Antarctic Flavobacterium species: description of Flavobacterium gillisiae sp. nov., Flavobacterium tegetincola sp. nov., and Flavobacterium xanthum sp. nov., nom. rev. and reclassification of [Flavobacterium] salegens as Salegentibacter salegens gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol. 50:1055–1063.

McCarthy, D. H. 1975. Columnaris disease. J. Inst. Fish. Mgmt. 6:44–47.

McGrath, C. F., C. W. Moss, and R. P. Burchard. 1990. Effect of temperature shifts on gliding motility, adhesion, and fatty acid composition of Cytophaga sp. strain U67. J. Bacteriol. 172:1978–1982.

Merle, C., D. Faure, M.-C. Urdaci, and M. Le Hénaff. 2003. Purification and characterization of a membrane glycoprotein from the fish pathogen Flavobacterium psychrophilum. J. Appl. Microbiol. 94:1120–1127.

Meyers, T. R. 1989. Apparent chronic bacterial myeloencephalitis in hatchery-reared juvenile coho salmon Oncorhynchus kisutch in Alaska. Dis. Aquat. Org. 6:217–219.

Michel, C., D. Antonio, and R. P. Hedrick. 1999. Production of viable cultures of Flavobacterium psychrophilum: Approach and control. Res. Microbiol. 150:351–358.

Michel, C., S. Messiaen, and J.-F. Bernardet. 2002. Muscle infections in imported neon tetra, Paracheirodon innesi Myers: Limited occurrence of microsporidia and predominance of severe forms of columnaris disease caused by an Asian genomovar of Flavobacterium columnare. J. Fish Dis. 25:253–263.

Michel, C., and C. Garcia. 2003. Virulence stability in Flavobacterium psychrophilum after storage and preservation according to different procedures. Vet. Res. 34:127–132.

Michel, C., B. Kerouault, and C. Martin. 2003. Chloramphenicol and florfenicol susceptibility of fish-pathogenic bacteria isolated in France: Comparison of minimum inhibitory concentration, using recommended provisory standards for fish bacteria. J. Appl. Microbiol. 95:1008–1015.

Míguez, B., and M. P. Combarro. 2003. Bacteria associated with sardine (Sardina pilchardus) eggs in a natural environment (Ría de Vigo, Galicia, northwestern Spain). FEMS Microbiol. Ecol. 44:329–334.

Møller, J. D., J. L. Larsen, L. Madsen, and I. Dalsgaard. 2003. Involvement of a sialic acid-binding lectin with hemagglutination and hydrophobicity of Flavobacterium psychrophilum. Appl. Environ. Microbiol. 69:5275–5280.

Moore, W. E. C., E. P. Cato, and L. V. H. Moore. 1985. Index of the bacterial and yeast nomenclatural changes published in the International Journal of Systematic Bacteriology since the 1980 Approved Lists of Bacterial Names (1 January 1980 to 1 January 1985). Int. J. Syst. Bacteriol. 35:382–407.

Moore, A. A., M. E. Eimers, and M. A. Cardella. 1990. Attempts to control Flexibacter columnaris epizootics in pond-reared channel catfish by vaccination. J. Aquat. Anim. Health 2:109–111.

Moreno, Y., C. R. Arias, H. Meier, E. Garay, and R. Aznar. 1999. In situ analysis of the bacterial communities associated to farmed eel by whole-cell hybridization. Lett. Appl. Microbiol. 29:160–165.

Morita, R. Y. 1975. Psychrophilic bacteria. Bacteriol. Rev. 39:144–167.

Morrison, C., J. Cornick, G. Shum, and B. Zwicker. 1981. Microbiology and histopathology of "saddleback" disease of underyearling Atlantic salmon, Salmo salar L. J. Fish Dis. 4:243–258.

Mudarris, M., B. Austin, P. Segers, M. Vancanneyt, B. Hoste, and J.-F. Bernardet. 1994. Flavobacterium scophthalmum sp. nov., a pathogen of turbot (Scophthalmus maximus L.). Int. J. Syst. Bacteriol. 44:447–453.

Müller, K.-D., E. N. Schmid, and R. Michel. 1999. Intracellular bacteria of Acanthamoebae resembling Legionella spp. turned out to be Cytophaga sp. Zbl. Bakteriol. 289:389–397.

Naas, T., S. Bellais, and P. Nordmann. 2003. Molecular and biochemical characterization of a carbapenem-hydrolyzing beta-lactamase from Flavobacterium johnsoniae. J. Antimicrob. Chemother. 51:267–273.

Nakagawa, Y., and K. Yamasato. 1993. Phylogenetic diversity of the genus Cytophaga revealed by 16S rRNA sequencing and menaquinone analysis. J. Gen. Microbiol. 139:1155–1161.

Nakagawa, Y., and K. Yamasato. 1996. Emendation of the genus Cytophaga and transfer of Cytophaga agarovorans and Cytophaga salmonicolor to Marinilabilia gen. nov.: Phylogenetic analysis of the Flavobacterium-Cytophaga complex. Int. J. Syst. Bacteriol. 46:599–603.

Nakagawa, Y., M. Suzuki, and K. Hatano. 2001. Phylogenetic diversity of Cytophaga-like strains isolated from the subtropical zone of Japan. IFO Res. Commun. 20:61–71.

Nedoluha, P. C., and D. Westhoff. 1997. Microbiological analysis of striped bass (Morone saxatilis) grown in a recirculating system. J. Food Protect. 60:948–953.

Needleman, D. S., and R. E. Pacha. 1974. Isolation and partial characterization of a bacteriocin from Chondrococcus columnaris. Abstr. Ann. Meet. Am. Soc. Microbiol. 74:21.

Nematollahi, A., A. Decostere, F. Pasmans, R. Ducatelle, and F. Haesebrouck. 2003a. Adhesion of high and low virulence Flavobacterium psychrophilum strains to isolated gill arches of rainbow trout Oncorhynchus mykiss. Dis. Aquat. Org. 55:101–107.

Nematollahi, A., A. Decostere, F. Pasmans, and F. Haesebrouck. 2003b. Flavobacterium psychrophilum infections in salmonid fish. J. Fish Dis. 26:563–574.

Newton, J. C., T. M. Wood, and M. M. Hartley. 1997. Isolation and partial characterization of extracellular proteases produced by isolates of Flavobacterium columnare derived from channel catfish. J. Aquat. Anim. Health 9:75–85.

Nieto, T. P., A. E. Toranzo, and J. L. Barja. 1984. Comparison between the bacterial flora associated with fingerling rainbow trout cultured in two different hatcheries in the North-West of Spain. Aquaculture 42:193–206.

Nikoskelainen, S., S. Salminen, G. Bylund, and A. C. Ouwehand. 2001. Characterization of the properties of human- and dairy-derived probiotics for prevention of infectious diseases in fish. Appl. Environ. Microbiol. 67:2430–2435.

Nilsson, W. B., and M. S. Strom. 2002. Detection and identification of bacterial pathogens of fish in kidney tissue

using terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes. Dis. Aquat. Org. 48:175–185.

Nogales, B., K. N. Timmis, D. B. Nedwell, and A. M. Osborn. 2002. Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription-PCR amplification from mRNA. Appl. Environ. Microbiol. 68:5017–5025.

Nomura, S., and N. Ohara. 1994. Production and some properties of the extracellular proteases of Cytophaga columnaris. Fish. Science 60:97–102.

Obach, A., and F. Baudin-Laurencin. 1991. Vaccination of rainbow trout (Oncorhynchus mykiss) against the visceral form of cold-water disease. Dis. Aquat. Org. 12:13–15.

Oppong, D., V. M. King, and J. A. Bowen. 2003. Isolation and characterization of filamentous bacteria from paper mill slimes. Int. Biodeter. Biodegrad. 52:53–62.

Ordal, E. J., and R. R. Rucker. 1944. Pathogenic Myxobacteria. Proc. Soc. Exp. Biol. Med. 56:15–18.

Ostland, V. E., H. W. Ferguson, and R. M. W. Stevenson. 1989. Case report: bacterial gill disease in goldfish Carassius auratus. Dis. Aquat. Org. 6:179–184.

Ostland, V. E., J. S. Lumsden, D. D. MacPhee, and H. W. Ferguson. 1994. Characteristics of Flavobacterium branchiophilum, the cause of salmonid bacterial gill disease in Ontario. J. Aquat. Anim. Health 6:13–26.

Ostland, V. E., P. J. Byrne, D. J. Speare, M. A. Thornburn, A. Cook, M. Morrison, and H. W. Ferguson. 1995. Comparison of formalin and chloramine-T for control of a mixed infection (bacterial gill disease and ichthyobodiasis) in rainbow trout. J. Aquat. Anim. Health 7:118–123.

Ostland, V. E., D. D. MacPhee, J. S. Lumsden, and H. W. Ferguson. 1995. Virulence of Flavobacterium branchiophilum in experimentally infected salmonids. J. Fish Dis. 18:249–262.

Ostland, V. E., D. G. McGrogan, and H. W. Ferguson. 1997. Cephalic osteochondritis and necrotic scleritis in intensively reared salmonids associated with Flexibacter psychrophilus. J. Fish Dis. 20:443–451.

Ostland, V. E., P. J. Byrne, J. S. Lumsden, D. D. MacPhee, J. A. Derksen, M. Haulena, K. Skar, E. Myhr, and H. W. Ferguson. 1999. Atypical bacterial gill disease: A new form of bacterial gill disease affecting intensively reared salmonids. J. Fish Dis. 22:351–358.

Ostland, V. E., P. J. Byrne, G. Hoover, and H. W. Ferguson. 2000. Necrotic myositis of rainbow trout, Oncorhynchus mykiss (Walbaum): proteolytic characteristics of a crude extracellular preparation from Flavobacterium psychrophilum. J. Fish Dis. 23:329–336.

O'Sullivan, L. A., A. J. Weightman, and J. C. Fry. 2002. New degenerate Cytophaga-Flexibacter-Bacteroides-specific 16S ribosomal DNA-targeted oligonucleotide probes reveal high bacterial diversity in River Taff epilithon. Appl. Environ. Microbiol. 68:201–210.

Otis, E. J. 1984. Lesions of Coldwater Disease in Steelhead Trout (Salmo gairdneri): The Role of Cytophaga psychrophila Extracellular Products [MSc thesis]. University of Rhode Island. Kingston, RI.

Ototake, M., and H. Wakabayashi. 1985. Characteristics of extracellular products of Flavobacterium sp., a pathogen of bacterial gill disease. Fish Pathol. 20:167–171.

Oyaizu, H., and K. Komagata. 1981. Chemotaxonomic and phenotypic characterization of the strains of species in the Flavobacterium-Cytophaga complex. J. Gen. Appl. Microbiol. 27:57–107.

Pacha, R. E., and E. J. Ordal. 1967. Histopathology of experimental columnaris disease in young salmon. J. Comp. Pathol. 77:419–423.

Pacha, R. E., and E. J. Ordal. 1970. Myxobacterial Diseases of Salmonids. American Fisheries Society, Diseases of Fishes and Shellfishes. Bethesda, Maryland. 5:243–257.

Pearce, D. A. 2003. Bacterioplankton community structure in a maritime Antarctic oligotrophic lake during a period of holomixis, as determined by denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization (FISH). Microb. Ecol. 46:92–105.

Rahman, H., A. Kuroda, J. M. Dijkstra, I. Kiryu, T. Nakanishi, and M. Ototake. 2002. The outer membrane fraction of Flavobacterium psychrophilum induces protective immunity in rainbow trout and ayu. Fish Shell. Immunol. 12:169–179.

Rangdale, R. E., and K. Way. 1995. Rapid identification of Cytophaga psychrophila from infected spleen tissue using an enzyme-linked-immunosorbent assay (ELISA). Bull. Eur. Ass. Fish Pathol. 15:213–216.

Rangdale, R. E., R. H. Richards, and D. J. Alderman. 1996. Isolation of Cytophaga psychrophila, causal agent of rainbow trout fry syndrome (RTFS) from reproductive fluids and egg surfaces of rainbow trout (Oncorhynchus mykiss). Bull. Eur. Ass. Fish Pathol. 16:63–67.

Rangdale, R. E., R. H. Richards, and D. J. Alderman. 1997. Minimum inhibitory concentrations of selected antimicrobial compounds against Flavobacterium psychrophilum the causal agent of rainbow trout fry syndrome (RTFS). Aquaculture 158:193–201.

Rangdale, R. E., R. H. Richards, and D. J. Alderman. 1999. Histopathological and electron microscopical observations on rainbow trout fry syndrome. Vet. Rec. 144:251–254.

Reichardt, W., and R. Y. Morita. 1982. Influence of temperature adaptation on glucose metabolism in a psychrotrophic strain of Cytophaga johnsonae. Appl. Environ. Microbiol. 44:1282–1288.

Reichenbach, H. 1989. Order I: Cytophagales Leadbetter 1974. *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 3:2011–2013.

Reichenbach, H. 1992. The Order Cytophagales. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. 2:3631–3675.

Riffel, A., and A. Brandelli. 2002. Isolation and characterization of a feather-degrading bacterium from the poultry processing industry. J. Indust. Microbiol. Biotechnol. 29:255–258.

Rintamäki-Kinnunen, P., J.-F. Bernardet, and A. Bloigu. 1997. Yellow pigmented filamentous bacteria connected with farmed salmonid fish mortality. Aquaculture 149:1–14.

Roberts, S. D. 1998. Ineffective control of bacterial coldwater disease after pre-spawning erythromycin injections. Fish Health Newslett. 26:10–11.

Rodgers, M., D. Flanigan, S. Pfaller, W. Jakubowski, and B. Kinkle. 2003. Identification of a flavobacterium strain virulent against Giardia lamblia cysts. World J. Microbiol. Biotechnol. 19:703–709.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molec. Biol. Evol. 4:406–425.

Sakai, M., M. K. Soliman, T. Yoshida, and M. Kobayashi. 1993. Identification of pathogenic fish bacteria using the API ZYM system. Can. J. Fish. Aquat. Sci. 50:1137–1141.

Sanders, J. E., R. A. Holt, and J. L. Fryer. 1976. Serological comparison of Flexibacter columnaris isolates using rabbit and rainbow trout (Salmo gairdneri) antisera. J. Fish. Res. Board Can. 33:1386–1388.

Sanni, A. I., A. A. Onilude, I. F. Fadahunsi, and R. O. Afolabi. 1999. Microbial deterioration of traditional alcoholic beverages in Nigeria. Food Res. Int. 32:163–167.

Schachte Jr., J. H., and E. C. Mora. 1973. Production of agglutinating antibodies in the channel catfish (Ictalurus punctatus) against Chondrococcus columnaris. J. Fish. Res. Board Can. 30:116–118.

Schmidt, A. S., M. S. Bruun, I. Dalsgaard, K. Pedersen, and J. L. Larsen. 2000. Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Appl. Environ. Microbiol. 66:4908–4915.

Schmidtke, L. M., and J. Carson. 1995. Characteristics of Flexibacter psychrophilus isolated from Atlantic salmon in Australia. Dis. Aquat. Org. 21:157–161.

Secades, P., B. Alvarez, and J. A. Guijarro. 2001. Purification and characterization of a psychrophilic, calcium-induced, growth-phase-dependent metalloprotease from the fish pathogen Flavobacterium psychrophilum. Appl. Environ. Microbiol. 67:2436–2444.

Secades, P., B. Alvarez, and J. A. Guijarro. 2003. Purification and properties of a new psychrophilic metalloprotease (Fpp2) in the fish pathogen Flavobacterium psychrophilum. FEMS Microbiol. Lett. 226:273–279.

Shamsudin, M. N. 1994. Pathogenesis of Flexibacter columnaris and Immunity in Channel Catfish [PhD thesis]. Auburn University. Auburn, AL.

Shamsudin, M. N., and J. A. Plumb. 1996. Morphological, biochemical, and physiological characterization of Flexibacter columnaris isolates from four species of fish. J. Aquat. Anim. Health 8:335–339.

Shieh, H. S. 1980. Studies on the nutrition of a fish pathogen, Flexibacter columnaris. Microbios Lett. 13:129–133.

Shoemaker, C. A., P. H. Klesius, C. Lim, and M. Yildirim. 2003. Feed deprivation of channel catfish, Ictalurus punctatus (Rafinesque), influences organosomatic indices, chemical composition and susceptibility to Flavobacterium columnare. J. Fish Dis. 26:553–561.

Shotts, E. B., and C. E. Starliper. 1999. Flavobacterial diseases: Columnaris disease, cold-water disease and bacterial gill disease. *In:* P. T. K. Woo and D. W. Bruno (Eds.) Fish Diseases and Disorders, Volume 3: Viral, Bacterial and Fungal Infections. CABI Publishing. Oxford, UK. 559–576.

Skerman, V. B. D., V. McGowan, and P. H. A. Sneath. 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225–420.

Soltani, M., and C. M. Burke. 1994. Responses of fish-pathogenic Cytophaga/Flexibacter-like bacteria (CFLB) to environmental conditions. Bull. Eur. Ass. Fish Pathol. 14:185–187.

Soltani, M., B. Munday, and J. Carson. 1994. Susceptibility of some freshwater species of fish to infection by Cytophaga johnsonae. Bull. Eur. Ass. Fish Pathol. 14:133–135.

Soltani, M., S. Shanker, and B. L. Munday. 1995. Chemotherapy of Cytophaga/Flexibacter-like bacteria (CFLB) infections in fish: studies validating clinical efficacies of selected antimicrobials. J. Fish Dis. 18:555–565.

Song, Y.-L., J. L. Fryer, and J. S. Rohovec. 1988a. Comparison of gliding bacteria isolated from fish in North America and other areas of the Pacific rim. Fish Pathol. 23:197–202.

Song, Y.-L., J. L. Fryer, and J. S. Rohovec. 1988b. Comparison of six media for the cultivation of Flexibacter columnaris. Fish Pathol. 23:91–94.

Stackebrandt, E., W. Frederiksen, G. M. Garrity, P. A. D. Grimont, P. Kämpfer, M. C. J. Maiden, X. Nesme, R. Rosselló-Mora, J. Swings, H. G. Trüper, L. Vauterin, A. C. Ward, and W. B. Whitman. 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. 52:1043–1047.

Stanier, R. Y. 1947. Studies on non-fruiting myxobacteria. I: Cytophaga johnsonae n. sp., a chitin-decomposing myxobacterium. J. Bacteriol. 53:297–315.

Stanier, R. Y. 1957. Order VIII: Myxobacterales Jahn 1915. *In:* R. S. Breed, E. G. D. Murray, and N. R. Smith (Eds.) Bergey's Manual of Determinative Bacteriology, 7th ed. Williams & Wilkins. Baltimore, MD. 854–891.

Starliper, C. E., E. B. Schotts, T. C. Hsu, and W. B. Schill. 1988. Genetic relatedness of some Gram-negative yellow pigmented bacteria from fishes and aquatic environments. Microbios 56:181–198.

Stringer-Roth, K. M., W. Yunghans, and L. F. Caslake. 2002. Differences in chondroitin AC lyase activity of Flavobacterium columnare isolates. J. Fish Dis. 25:687–691.

Strohl, W. R., and L. R. Tait. 1978. Cytophaga aquatilis sp.nov., a facultative anaerobe isolated from the gills of freshwater fish. Int. J. Syst. Bacteriol. 28:293–303.

Suzuki, M., Y. Nakagawa, S. Harayama, and S. Yamamoto. 2001. Phylogenetic analysis and taxonomic study of marine Cytophaga-like bacteria: proposal for Tenacibaculum gen. nov. with Tenacibaculum maritimum comb. nov. and Tenacibaculum ovolyticum comb. nov., and description of Tenacibaculum mesophilum sp. nov. and Tenacibaculum amylolyticum sp. nov. Int. J. Syst. Evol. Microbiol. 51:1639–1652.

Takeuchi, M., and A. Yokota. 1992. Proposals of Sphingobacterium faecium sp. nov., Sphingobacterium piscium sp. nov., Sphingobacterium heparinum comb. nov., Sphingobacterium thalpophilum, comb. nov. and two genospecies of the genus Sphingobacterium, and synonymy of Flavobacterium yabuuchiae and Sphingobacterium spiritivorum. J. Gen. Appl. Microbiol. 38:465–482.

Tamaki, H., S. Hanada, Y. Kamagata, K. Nakamura, N. Nomura, K. Nakano, and M. Matsumura. 2003. Flavobacterium limicola sp. nov., a psychrophilic, organic-polymer-degrading bacterium isolated from freshwater sediments. Int. J. Syst. Evol. Microbiol. 53:519–526.

Taylor, P. W., and J. R. Winton. 2002. Optimization of nested polymerase chain reaction assays for identification of Aeromonas salmonicida, Yersinia ruckeri, and Flavobacterium psychrophilum. J. Aquat. Anim. Health 14:216–224.

Teska, J. D. 1993. Assay to evaluate the reaction kinetics of chondroitin AC lyase produced by Cytophaga columnaris. J. Aquat. Anim. Health 5:259–264.

Tiirola, M., E. T. Valtonen, P. Rintamäki-Kinnunen, and M. S. Kulomaa. 2002. Diagnosis of flavobacteriosis by direct amplification of rRNA genes. Dis. Aquat. Org. 51:93–100.

Topley, W. W. C., and G. S. Wilson. 1929. The Principles of Bacteriology and Immunity, 1st ed. Edward Arnold. London, UK.

Topp, E., R. L. Crawford, and R. S. Hanson. 1988. Influence of readily metabolizable carbon on pentachlorophenol metabolism by a pentachlorophenol-degrading Flavobacterium sp. Appl. Environ. Microbiol. 54:2452–2459.

Toyama, T., K. Kita-Tsukamoto, and H. Wakabayashi. 1994. Identification of Cytophaga psychrophila by PCR targeted 16S ribosomal RNA. Fish Pathol. 29:271–275.

Toyama, T., K. Kita-Tsukamoto, and H. Wakabayashi. 1996. Identification of Flexibacter maritimus, Flavobacterium branchiophilum and Cytophaga columnaris by PCR targeted 16S ribosomal DNA. Fish Pathol. 31:25–31.

Tripathi, N. K., K. S. Latimer, C. R. Gregory, B. W. Ritchie, R. E. Wooley, and R. L. Walker. 2005. Development and evaluation of an experimental model of cutaneous columnaris disease in koi carp (Cyprinus carpio). J. Vet. Diag. Invest. 17:45–54.

Triyanto, and H. Wakabayashi. 1999. Genotypic diversity of strains of Flavobacterium columnare from diseased fishes. Fish Pathol. 34:65–71.

Trust, T. J. 1975. Bacteria associated with the gills of salmonid fishes in freshwater. J. Appl. Bacteriol. 38:225–233.

Turnbull, J. F. 1993. Bacterial gill disease and fin rot. *In:* V. Inglis, R. J. Roberts, and N. R. Bromage (Eds.) Bacterial Diseases of Fish. Blackwell Scientific Publications. Oxford, UK. 40–57.

Uddin, M. N., and H. Wakabayashi. 1997. Effects of temperature on growth and protease production of Cytophaga psychrophila. Fish Pathol. 32:225–226.

Urdaci, M. C., C. Chakroun, D. Faure, and J.-F. Bernardet. 1998. Development of a polymerase chain reaction assay for identification and detection of the fish pathogen Flavobacterium psychrophilum. Res. Microbiol. 149:519–530.

Vandamme, P., J.-F. Bernardet, P. Segers, K. Kersters, and B. Holmes. 1994. New perspectives in the classification of the flavobacteria: description of Chryseobacterium gen. nov., Bergeyella gen. nov., and Empedobacter nom. rev. Int. J. Syst. Bacteriol. 44:827–831.

Vandamme, P., B. Pot, M. Gillis, P. De Vos, K. Kersters, and J Swings. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60:407–438.

Van der Meulen, H. J., W. Harder, and H. Veldkamp. 1974. Isolation and characterization of Cytophaga flevensis sp. nov., a new agarolytic flexibacterium. Ant. v. Leeuwenhoek 40:329–346.

Van Trappen, S., J. Mergaert, S. Van Eygen, P. Dawynt, M. C. Cnockaert, and J. Swings. 2002. Diversity of 746 heterotrophic bacteria isolated from microbial mats from ten Antarctic lakes. System. Appl. Microbiol. 25:603–610.

Van Trappen, S., J. Mergaert, and J. Swings. 2003. Flavobacterium gelidilacus sp. nov., isolated from microbial mats in Antarctic lakes. Int. J. Syst. Evol. Microbiol. 53:1241–1245.

Van Trappen, S., I. Vandecandelaere, J. Mergaert and J. Swings. 2003. Flavobacterium degerlachei sp. nov., Flavobacterium frigoris sp. nov. and Flavobacterium micromati sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes. Int. J. Syst. Evol. Microbiol. 54:85–92.

Van Trappen, S., I. Vandecandelaere, J. Mergaert, and J. Swings. 2005. Flavobacterium fryxellicola sp. nov. and Flavobacterium psychrolimnae sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes. Int. J. Syst. Evol. Microbiol. 55:769–772.

Vatsos, I. N., K. D. Thompson, and A. Adams. 2001. Adhesion of the fish pathogen Flavobacterium psychrophilum to unfertilized eggs of rainbow trout (Oncorhynchus mykiss) and n-hexadecane. Lett. Appl. Microbiol. 33:178–182.

Vatsos, I. N., K. D. Thompson, and A. Adams. 2002. Development of an immunofluorescent antibody technique (IFAT) and insitu hybridization to detect Flavobacterium psychrophilum in water samples. Aquacult. Res. 33:1087–1090.

Vatsos, I. N., K. D. Thompson, and A. Adams. 2003. Starvation of Flavobacterium psychrophilum in broth, stream water and distilled water. Dis. Aquat. Org. 56:115–126.

Vinogradov, E., M. B. Perry, and W. W. Kay. 2003. The structure of the glycopeptides from the fish pathogen Flavobacterium columnare. Carbohydr. Res. 338:2653–2658.

Von Graevenitz, A. 1990. Revised nomenclature of Campylobacter laridis, Enterobacter intermedium, and "Flavobacterium branchiophila." Int. J. Syst. Bacteriol. 40:211.

Von Weis, J. 1987. Über das Vorkommen einer Kaltwasserkrankheit bei Regenbogenforellen, Salmo gairdneri. Tierärztl. Umschau 42:575–577.

Wakabayashi, H., and S. Egusa. 1974. Characteristics of myxobacteria associated with some freshwater fish diseases in Japan. Bull. Jap. Soc. Sci. Fish. 40:751–757.

Wakabayashi, H., S. Egusa, and J. L. Fryer. 1980. Characteristics of filamentous bacteria isolated from a gill disease of salmonids. Can. J. Fish. Aquat. Sci. 37:1499–1507.

Wakabayashi, H., and T. Iwado. 1985. Effects of a bacterial gill disease on the respiratory functions of juvenile rainbow trout. *In:* A. E. Ellis (Ed.)Fish and Shellfish Pathology. Academic Press. London, UK. 153–160.

Wakabayashi, H., G. J. Huh, and N. Kimura. 1989. Flavobacterium branchiophila sp.nov., a causative agent of bacterial gill disease of freshwater fishes. Int. J. Syst. Bacteriol. 39:213–216.

Wakabayashi, H. 1991. Effects of environmental conditions on the infectivity of Flexibacter columnaris to fish. J. Fish Dis. 14:279–290.

Wakabayashi, H. 1993. Columnaris disease. *In:* V. Inglis, R. J. Roberts, and N. R. Bromage (Eds.) Bacterial Diseases of Fish. Blackwell Scientific Publications. Oxford, UK. 23–39.

Wakabayashi, H., T. Toyama, and T. Iida. 1994. A study on serotyping of Cytophaga psychrophila isolated from fishes in Japan. Fish Pathol. 29:101–104.

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, & H. G. Trüper. 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37:463–464.

Weisburg, W. G., Y. Oyaizu, H. Oyaizu, and C. R. Woese. 1985. Natural relationship between bacteroides and flavobacteria. J. Bacteriol. 164:230–236.

Weller, R., F. O. Glöckner, and R. Amann. 2000. 16S rRNA-targeted oligonucleotide probes for the in situ detection

of members of the phylum Cytophaga-Flavobacterium-Bacteroides. System. Appl. Microbiol. 23:107–114.

Wiklund, T., K. Kaas, L. Lönnström, and I. Dalsgaard. 1994. Isolation of Cytophaga psychrophila (Flexibacter psychrophilus) from wild and farmed rainbow trout (Oncorhynchus mykiss) in Finland. Bull. Eur. Ass. Fish Pathol. 14:44–46.

Wiklund, T., L. Madsen, M. S. Bruun, and I. Dalsgaard. 2000. Detection of Flavobacterium psychrophilum from fish tissue and water samples by PCR amplification. J. Appl. Microbiol. 88:299–307.

Wiklund, T., and I. Dalsgaard. 2002. Survival of Flavobacterium psychrophilum in rainbow trout (Oncorhynchus mykiss) serum in vitro. Fish Shell. Immunol. 12:141–153.

Wiklund, T., and I. Dalsgaard. 2003. Association of Flavobacterium psychrophilum with rainbow trout (Oncorhynchus mykiss) kidney phagocytes in vitro. Fish Shell. Immunol. 15:387–395.

Woese, C.R., D. Yang, L. Mandelco, and K. O. Stetter. 1990. The flexibacter-flavobacter connection. System. Appl. Microbiol. 13:161–165.

Xie, H. X., P. Nie, and B. J. Sun2004. Characterization of two membrane-associated protease genes obtained from screening out-membrane protein genes of Flavobacterium columnare G4. J. Fish Dis. 27:719–729.

Yabuuchi, E., E. Tanimura, and I. Ohyama. 1979. Flavobacterium devorans ATCC 10829: A strain of Pseudomonas paucimobilis. J. Gen. Appl. Microbiol. 25:95–107.

Yi, H., H.-M. Oh, J.-H. Lee, S.-J. Kim, and J. Chun. 2005. Flavobacterium antarcticum sp. nov., a novel psychrotolerant bacterium isolated from the Antarctic. Int. J. Syst. Evol. Microbiol. 55:637–641.

Zdanowski, M. K., P. Weglenski, P. Golik, J. M. Sasin, P. Borsuk, M. J. Zmuda, and A. Stankovic. 2004. Bacterial diversity in Adélie penguin, Pygoscelis adeliae, guano: molecular and morpho-physiological approaches. FEMS Microbiol. Ecol. 50:163–173.

Zhu, F., S. Wang, and P. Zhou. 2003. Flavobacterium xinjiangense sp. nov. and Flavobacterium omnivorum sp. nov., novel psychrophiles from the China no. 1 glacier. Int. J. Syst. Evol. Microbiol. 53:853–857.

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CHAPTER 6.5

The Genera *Bergeyella* and *Weeksella*

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Introduction

Bergeyella and *Weeksella* have a history of being a separate group of organisms in the flavobacteria-grouping. They were originally treated as one genus (*Weeksella*) with two species, namely, *W. virosa* (Holmes et al., 1986a) and *W. zoohelcum* (Holmes et al., 1986b). Since 1994, however, they were separated into two genera in the Flavobacteriaceae family (Vandamme et al., 1994) as discussed in the introductory chapter about this family (see the chapter An Introduction to the Family Flavobacteriaceae in this Volume). Strains of both of these genera, especially *Bergeyella* with its frequent association with animal bite wounds, are found in clinical specimens.

The aim of this chapter is to provide more detail on the taxonomy, phylogeny, habitat, isolation and preservation, identification, pathogenicity and applications of these two genera.

Taxonomy and Phylogeny

Formerly known as Centers for Disease Control (CDC) groups IIf (*Weeksella*) and IIj (*Bergeyella*), these two genera were originally isolated and grouped by the late Elizabeth O. King and her successors (Tatum et al., 1974). These organisms have also been referred to as *Flavobacterium* Group 3 (Pickett and Manclark, 1970a), Group 1 (Olsen and Ravn, 1971) and "*Flavobacterium genitale*" (Price and Pickett, 1981). After Holmes and Owen (1979) emended the description of *Flavobacterium*, they found Groups IIf and IIj conformed to the emended description and later concluded that they probably should be placed in a separate genus (1981). Dees et al. (1981) were of the opinion that Group IIj and Group IIf (1986) should be accommodated in *Flavobacterium* on the basis of cellular fatty acid composition. Other authors, however, regarded Group IIj as intermediate in terms of its characteristics between the genera *Brucella* and *Moraxella* (Owen and Snell, 1973; Saphir and Carter, 1976; Bailie et al., 1978).

In 1981, Holmes and Owen allocated the flavobacteria into four groups (the recent correct names of the organisms are given in square brackets): Group A (*F. balustinum* [*Chryseobacterium balustinum*; Vandamme et al., 1994], *F. breve* [*Empedobacter brevis*; Vandamme et al., 1994], *F. indoltheticum* [*Chryseobacterium indoltheticum*; Vandamme et al., 1994], *F. meningosepticum* [*Chryseobacterium meningosepticum*; Vandamme et al., 1994], and remaining CDC Group IIb strains), Group B (*F. odoratum* [*Myroides odoratus*; Vancanneyt et al., 1996]), Group C (CDC Group IIk, type 2 and "Group IIk, type 3" [*Sphingobacterium* species; Yabuuchi et al., 1983; Takeuchi and Yokota, 1992]), and Group D (CDC Groups IIf and IIj). The organisms in Group D were then frequently referred to as *Flavobacterium*-like.

In 1986, Holmes et al. proposed the names "*Weeksella virosa*" (1986a) for strains belonging to CDC Group IIf and "*Weeksella zoohelcum*" (1986b) for strains belonging to CDC Group IIj, respectively. They also included these two species in the family Cytophagaceae, as had also been suggested earlier for the genus *Flavobacterium* (Callies and Mannheim, 1980). A number of subsequent studies (Holmes, 1992; Segers et al., 1993), however, found *W. zoohelcum* sufficiently different from *W. virosa* to constitute a different genus. After extensive genotypic, chemotaxonomic, and phenotypic studies, *Weeksella zoohelcum* was transferred to a new genus, namely *Bergeyella zoohelcum* (Vandamme et al., 1994). Although both genera currently consist only of one species each, it has been suggested by other studies that more than one species, including environmental strains, could be allocated to these two genera (Botha et al., 1989; Botha et al., 1998a; Botha et al., 1998b).

Habitat

Clinical Isolates

Both genera have had a clinical origin. They are not considered to be free-living but are regarded as constituents of the normal microflora of

mucous membranes of humans and other warm-blooded animals (Holmes et al., 1986a; Holmes et al., 1986b).

The majority of strains of *Bergeyella zoohelcum* examined by Tatum et al. (1974) was isolated from infected lesions from dog or cat bites or scratches. Other sources were blood, spinal fluid, and sputa of humans with or without a history of animal bites or scratches. The organism has also been isolated from gingival scrapings of dogs (Saphir and Carter, 1976) as well as oral and nasal fluids of dogs (Bailie et al., 1978). In a recent review, *Bergeyella zoohelcum* is considered a component of the normal oral flora of dogs and other animals (Griego et al., 1995).

Weeksella virosa has been isolated from human urine, cervix, vagina, Bartholin's gland cysts, blood, umbilical stump, ears, urethra, and spinal fluid (Tatum et al., 1974). This organism, however, seems to be predominantly associated with the female genital tract (Holmes et al., 1986a; Reina et al., 1989), and it appears to be more prevalent in sexually promiscuous women (Mardy and Holmes, 1988). There is no clear evidence of the pathogenic role of this organism.

Environmental Isolates

These two genera are not considered to be free-living in the environment, as stated above (Holmes et al., 1986a; 1986b). CDC Group IIf- and IIj-like strains have, however, been isolated from meat (Hayes, 1977) and dairy (Jooste et al., 1985) sources. In subsequent studies, these strains could not be identified as either *Bergeyella zoohelcum* or *Weeksella virosa*, but it was suggested that they could be included in one or more new species within these two genera (Botha et al., 1989; Botha et al., 1998a). *Bergeyella*- and *Weeksella*-like organisms have been isolated from raw beef, pork, chicken and lamb portions, and it was suggested that three species may be necessary to accommodate the environmental *Bergeyella*- and *Weeksella*-like bacteria (Botha et al., 1998b). *Weeksella*-like organisms were isolated from South African marine fish and were considered to be spoilage organisms because they were associated with the production of stale or pungent odors (Engelbrecht et al., 1996). A *Weeksella* strain was also isolated from petroleum-contaminated soils (see the section Applications for more details in this Chapter; Xia et al., 2000).

Isolation and Preservation

The general (nonselective) isolation, maintenance and preservation media used for culturing members of the Flavobacteriaceae are discussed in detail by Jooste and Hugo (1999) and Hugo and Jooste (2003).

Heart infusion medium containing 5% rabbit blood and other standard selective media may be used to isolate and cultivate the *Bergeyella* and *Weeksella* species (Dees et al., 1986; Jooste et al., 1985; Weyant et al., 1996). Plate count agar containing 0.5% NaCl was used to isolate *Weeksella*-like organisms from South African marine fish (Engelbrecht et al., 1996). Cultivation of *B. zoohelcum* has been reported on 5% horse blood agar (Holmes et al., 1986b).

Although no specific medium is known for the selective isolation of *Bergeyella* strains, β-hydroxybutyrate agar may be used for the enumeration of *Weeksella*, *Myroides*, *E. brevis* and *Chryseobacterium* (Bernardet et al., 1996; Jooste and Hugo, 1999). MacConkey agar has also been used for the enumeration of *Weeksella* (Bernardet et al., 1996; Jooste and Hugo, 1999), but according to Hollis et al. (1995), no *Weeksella* strains and only 2% of *Bergeyella* strains grew on MacConkey agar. This difference could presumably be ascribed to different composition of media. Selective media on which *Bergeyella* strains will not grow include β-hydroxybutyrate agar and MacConkey agar, and they exhibit only weak growth on nutrient agar (Holmes et al., 1986b; Bernardet et al., 1996; Jooste and Hugo, 1999).

For the medium-term preservation of the *Bergeyella* and *Weeksella* species, the general media used for flavobacterial maintenance may be used. These media include PMYA II (peptonized milk yeast extract agar; Christensen and Cook, 1972) and also Dorset egg medium in a screw-capped bijou bottle stored at 4°C (Cowan, 1974; Holmes et al., 1984). For longer-term preservation, isolates may be freeze dried (Holmes, 1992), or agar slant culture growth (18-h old) can be suspended in defibrinated rabbit blood placed in ampoules or other suitable containers and frozen in a mixture of dry ice and alcohol prior to storage at –50°C (Holmes et al., 1984).

Identification

When *Weeksella* and *Bergeyella* were still referred to as CDC Groups IIf and IIj, respectively, some investigators were of the opinion that they belonged to the *Flavobacterium* genus (Pedersen et al., 1970; Pickett and Manclark, 1970a; Pickett and Pedersen, 1970b; Pickett and Pedersen, 1970c; Olsen and Ravn, 1971; Price and Pickett, 1981). Others considered them intermediate in characteristics between *Flavobacterium* and *Moraxella* (Owen and Snell, 1973). In any case, these taxa may be confused with each other in the laboratory. The differen-

tial characteristics of the four groups of flavobacteria (Holmes and Owen, 1981) are given in Table 1.

For the identification of members of the Flavobacteriaceae, specific methods have been suggested (Bernardet et al., 2002). As some of these methods and characteristics are of special importance in differentiating among taxa of Flavobacteriaceae and related taxa, an update of the methods is given and discussed in detail in the introductory chapter An Introduction to the Family Flavobacteriaceae in this Volume. Only the morphological and phenotypical characteristics of importance for the differentiation of *Bergeyella* and *Weeksella* will be discussed in this chapter.

Morphology

Both genera are Gram-negative, nonsporeforming rods that are 0.6 μm wide and 2–3 μm long with parallel sides and rounded ends. They are nonmotile in hanging-drop preparations after overnight incubation in nutrient broth at either 37°C or room temperature (18–22°C). No cellular gliding movement is present (Holmes et al., 1986a; Holmes et al., 1986b; Bernardet et al., 2002).

Bergeyella and *Weeksella* colonies are nonpigmented, nonfluorescent and nonhemolytic on 5% (v/v) horse blood agar after 24 h. *Bergeyella* strains do not produce a brown, diffusible, melanin-like pigment on tyrosine agar in contrast to *Weeksella* strains. Colonies of both genera are circular, 0.5–2 mm in diameter, low convex, smooth and shiny with entire edges on nutrient agar. According to Tatum et al. (1974) and von Graevenitz (1995), *Bergeyella* colonies are sticky and, in color, are tan to yellow, while *Weeksella* colonies are butyrous to mucoid, adherent, and tan to brown. The authors did not discuss which media were used to determine these characteristics.

Phenotypic Identification and Physiology

Both genera are strictly aerobic with menaquinone 6 as the major respiratory quinone. Both genera will grow at 25°C and 37°C, but not at 5°C. The majority of *Weeksella* strains will grow at 42°C and on β-hydroxybutyrate agar, but this does not apply to *Bergeyella* strains (Holmes et al., 1986a; Holmes et al., 1986b). Divergence exists regarding the ability of the two genera to grow on MacConkey agar (Holmes et al., 1986a; Holmes et al., 1986b; Hollis et al., 1995; Weyant et al., 1996).

Both genera produce indole (Ehrlich's reagent), oxidase, catalase and phosphatase, and both degrade gelatin. Neither of the genera will produce DNase or β-galactosidase, reduce nitrate, utilize carbohydrates, or degrade starch, esculin or agar. *Bergeyella* will, however, produce urease while *Weeksella* will not (Holmes et al., 1986a; Holmes et al., 1986b; Weyant et al., 1996; Bernardet et al., 2002). Both the genera are nonsaccharolytic and will not produce acid from glucose, adonitol, arabinose, cellobiose, dulcitol, ethanol, fructose, glycerol, inositol, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose or xylose. The general phenotypic and differential characteristics of *Bergeyella* and *Weeksella* are given in Table 2. For full descriptions and more details, the reader is referred to Holmes et al. (Holmes et al., 1986a; Holmes et al., 1986b), Hollis et al. (1995), and Weyant et al. (1996).

In contrast to the other closely related genera in the Flavobacteriaceae family (*Chryseobacterium*, *Empedobacter* and *Myroides*), *Bergeyella*

Table 1. Differential characteristics of the four groups of flavobacteria.[a]

Characteristic	Group A *Chryseobacterium*	Group B *Myroides*	Group C *Sphingobacterium*	Group D *Bergeyella* + *Weeksella*
Habitat	Free-living	Free-living	Free-living	Parasitic or saprophytic
Resistant to antimicrobials	+	+	+	–
Pigment production	+	+	+	–
Saccharolytic activity	+	–	+	–
Indole production	+	–	–	+
Oxidase production	+	+	+	+
Nitrate reduction	V	–	–	–
Degradation of gelatin	+	+	–	+
G+C content (mol%)	33–38	30–38	39–42	35–38

Symbols and abbreviations; +, positive reaction; –, negative reaction and V, varies within and/or between strains.
[a]From Holmes and Owen (1981), Yabuuchi et al. (1983), Rubin et al. (1985), Holmes et al. (1986a, b), Holmes (1992), and Bernardet et al. (2002).

Table 2. General phenotypic and differential characteristics of *Bergeyella* and *Weeksella*.[a]

Characteristic	*Bergeyella zoohelcum*	CDC *Bergeyella* n = 87	*Weeksella virosa*	CDC *Weeksella* n = 41
Growth at				
25°C	+[a]	V	+	V
37°C	+	+	+	+
42°C	–[a]	–	+	V
Growth on				
MacConkey agar	–	–	+	–
β-Hydroxybutyrate	–	ND	+	ND
Cetrimide agar	–	ND	–	ND
Acid production from				
Glucose	–	–	–	–
Maltose	–	–	–	–
Production of				
DNase	–	ND	–	ND
Hydrogen sulfide	–	V	–	+
Alkali on Christensen's citrate	–	–	–	ND
Arginine desimidase	–	ND	–	ND
Arginine dihydrolase	–	–	–	–
Lysine decarboxylase	–	–	–	–
Ornithine decarboxylase	–	–	–	–
3-Ketolactose	–	ND	–	ND
β-Galactosidase	–	ND	–	ND
Urease	+	+	–	–
Catalase	+	+	+	+
Indole	+	+	+	+
Reduction of				
Nitrate	–	–	–	–
Nitrite	–	ND	–	ND
Selenite	–	ND	–	ND
Utilization of				
Citrate	–	ND	–	ND
Malonate	–	ND	–	ND
Degradation/hydrolysis of				
Agar	–	ND	–	ND
Starch	–	ND	–	ND
Esculin	–	–	–	–
Gelatin	+	+	+	+
Tween 20	ND	ND	+	ND
Tween 80	–	ND	–[a]	ND
Tyrosine	–	ND	V	ND
Casein	ND	ND	+	ND
Gluconate oxidation	–	ND	–	ND
Phenylalanine deamination	–	–	–	–
KCN (0.0075% w/v) toleration	–	ND	–	ND
Resistance to Pen G	–	ND	–	ND
G+C content (mol%)	35–37	ND	37–38	ND

Symbols and abbreviations: a, most strains are positive or negative for this characteristic; +, positive reaction; –, negative reaction; and V, varies within and/or between strains.

[a]From Holmes et al. (1986a, b), Weyant et al. (1996), and Bernardet et al. (2002).

and *Weeksella* are susceptible to most antibiotics such as β-lactam antibiotics, tetracycline, chloramphenicol, nalidixic acid, erythromycin and sulfamethoxazole-trimethoprim (Pedersen et al., 1970; Schell et al., 1985). They are, however, resistant to aminoglycosides (von Graevenitz, 1995; Fass et al., 1996; Goldstein et al., 1998; Goldstein et al., 1999a; Goldstein et al., 1999b; Goldstein et al., 2001; Goldstein et al., 2002).

The dominant fatty acids in *Bergeyella* are 15:0 iso, 15:0 iso 2 OH, 17:1 iso, and 17:0 iso 3OH (Vandamme et al., 1994). The DNA G+C is 35–37 mol% (Vandamme et al., 1994; Bernardet et al., 2002).

The dominant fatty acids of *Weeksella* species are 15:0 iso, 15:0 iso 2OH, 16:0, 16:0 iso 3OH, 17:1(1) iso, 17:1(2) iso, and 17:0 iso 3OH (Dees et al., 1986). The DNA G+C is 37–38 mol% (Bernardet et al., 2002).

Pathogenicity

Bergeyella zoohelcum has most frequently been isolated from the upper respiratory tract of dogs and from human wounds caused by dog and cat bites. In a study of bacteriological etiologies of 50 infected dog bites and 57 infected cat bites, *B. zoohelcum* was isolated from 4% and 7% of wounds, respectively (Talan et al., 1999). At first, there was no real evidence for its pathogenic role in humans except for a report of meningitis in a 5-year old child following a dog bite (Bracis et al., 1979) and another report of septicemia (Noell et al., 1989). In the past 10–15 years, however, the following cases were reported: leg abscesses following a dog bite (Reina and Borrell, 1992), pneumonia (Grimault et al., 1996), infectious tenosynovitis developed in a man after a Siberian tiger bite (Isotalo et al., 2000), and bacteremia after a dog bite (Montejo et al., 2001). Recently, there has been a report where *B. zoohelcum* has been associated with a necrotizing infection of the respiratory tract in a cat (Decostere et al., 2002).

Although *Weeksella virosa* has been most frequently isolated from the urogenital tract, there is little evidence for its pathogenicity (von Graevenitz, 1981; Holmes et al., 1986a). There are, however, two cases of peritonitis caused by *W. virosa* reported in the literature: one associated with peritoneal dialysis (Faber et al., 1991) and one spontaneous case (Boixeda et al., 1998).

Applications

Although Xia et al. (2000) did not clearly indicate how the organisms were identified, a *Weeksella* strain and a *Pseudomonas* strain were isolated from petroleum contaminated soils. Both strains possessed petroleum-degrading and surfactant-degrading properties. It was suggested that after the largest portion of petroleum in the contaminated zone had been removed with surfactants, these organisms could be used to degrade the residual surfactants and petroleum (Xia et al., 2000).

Literature Cited

Bailie, W. E., E. C. Stow, and A. M. Schmitt. 1978. Aerobic bacterial flora of oral and nasal fluids of canines with reference to bacteria associated with bites. J. Clin. Microbiol. 7:223–231.

Bernardet, J.-F., P. Segers, M. Vancanneyt, F. Berthe, K. Kersters, and P. Vandamme. 1996. Cutting a Gordian knot: emended classification and description of the genus Flavobacterium, emended description of the family Flavobacteriaceae, and proposal of Flavobacterium hydatis nom. nov. (basonym, Cytophaga aquatilis Strohl and Tait 1978). Int. J. Syst. Bacteriol. 46:128–148.

Bernardet, J.-F., Y. Nakagawa, and B. Holmes. 2002. Proposed minimal standards for describing new taxa of the family Flavobacteriaceae and emended description of the family. Int. J. Syst. Evol. Microbiol. 52:1049–1070.

Boixeda, D., D. A. de Luis, M. A. Meseguer, R. Aller, C. Martin de Argila, and A. Lopez Sanroman. 1998. A case of spontaneous peritonitis caused by Weeksella virosa. Eur. J. Gastroenterol. Hepatol. 10:897–898.

Botha, W. C., P. J. Jooste, and T. J. Britz. 1989. The taxonomic relationship of certain environmental flavobacteria to the genus Weeksella. J. Appl. Bacteriol. 67:551–559.

Botha, W. C., P. J. Jooste, and C. J. Hugo. 1998a. Taxonomic interrelationship of Weeksella- and Bergeyella-like strains from dairy sources using electrophoretic protein profiles, DNA base composition and non-polar fatty acid composition. Food Microbiol. 15:479–489.

Botha, W. C., P. J. Jooste, and C. J. Hugo. 1998b. The incidence of Weeksella- and Bergeyella-like bacteria in the food environment. J. Appl. Microbiol. 84:349–356.

Bracis, R., K. Seibers, and R. M. Julien. 1979. Meningitis caused by Group IIj following a dog bite. West. J. Med. 131:438–440.

Callies, E., and W. Mannheim. 1980. Deoxyribonucleic acid relatedness of some menaquinone-producing Flavobacterium and Cytophaga strains. Ant. v. Leeuwenhoek 46:41–49.

Christensen, P.J. and F.D. Cook. 1972. The isolation and enumeration of cytophagas. Can. J. Microbiol. 18:1933–1940.

Cowan, S.T.. 1974. Cowan and Steel's Manual for the identification of medical bacteria, 2nd edition. Cambridge University Press. Cambridge, UK.

Decostere, A., L. A. Devriese, R. Ducatelle, and F. Haesebrouck. 2002. Bergeyella (Weeksella) zoohelcum associated with respiratory disease in a cat. Vet. Rec. 151:392.

Dees, S. B., J. Powell, C. W. Moss, D. G. Hollis, and R. E. Weaver. 1981. Cellular fatty acid composition of organisms frequently associated with human infections resulting from dog bites: Pasteurella multocida and Groups EF-4, IIj, M-5, and DF-2. J. Clin. Microbiol. 14:612–616.

Dees, S. B., C. W. Moss, D. G. Hollis, and R. E. Weaver. 1986. Chemical characterization of Flavobacterium odoratum, Flavobacterium breve, and Flavobacterium-like groups IIe, IIh, and IIf. J. Clin. Microbiol. 23:267–273.

Engelbrecht, K., P. J. Jooste, and B. A. Prior. 1996. Spoilage characteristics of Gram-negative genera and species isolated from Cape marine fish. S. Afr. J. Food Sci. Nutr. 8:66–71.

Faber, M. D., R. del Busto, C. Cruz, and E. Mezger. 1991. Response of Weeksella virosa peritonitis to imipenem cilastin. Adv. Perit. Dial. 7:133–134.

Fass, R. J., J. Barnishan, M. C. Solomon, and L. W. Ayers. 1996. In vitro activities of quinolones, β-lactams, tobramycin, and trimethoprim-sulfamethoxazole against nonfermentative Gram-negative bacilli. Antimicrob. Agents Chemother. 40:1412–1418.

Goldstein, E. C., D. M. Citron, S. H. Gerardo, M. Hudspeth, and C. V. Merriam. 1998. Activities of HMR 3004 (RU 64004) and HMR 3647 (RU 66647) compared to those of erythromycin, azithromycin, clarithromycin, roxithromycin, and eight other antimicrobial agents against unusual aerobic and anaerobic human and animal bite pathogens isolated from skin and soft tissue infections

in humans. Antimicrob. Agents Chemother. 42:1127–1132.

Goldstein, E. C., D. M. Citron, and C. V. Merriam. 1999a. Linezolid activity compared to those of selected macrolides and other agents against aerobic and anaerobic pathogens isolated from soft tissue bite infections in humans. Antimicrob. Agents Chemother. 43:1469–1474.

Goldstein, E. C., D. M. Citron, C. V. Merriam, K. Tyrrell, and Y. Warren. 1999b. Activity of gatifloxacin compared to those of five other quinolones versus aerobic and anaerobic isolated from skin and soft tissue samples of human and animal bite wound infections. Antimicrob. Agents Chemother. 43:1475–1479.

Goldstein, E. C., D. M. Citron, C. V. Merriam, Y. A. Warren, K. Tyrrell, and H. Fernandez. 2001. Comparitive in vitro activity of ertapenem and 11 other antimicrobial agents against aerobic and anaerobic pathogens isolated from skin and soft tissue animal and human bite wound infections. J. Antimicrob. Chemother. 48:641–651.

Goldstein, E. C., D. M. Citron, C. V. Merriam, Y. A. Warren, K. L. Tyrrell, and H. Fernandez. 2002. In vitro activities of the des-fluoro(6) quinolone BMS-284756 against aerobic and anaerobic pathogens isolated from skin and soft tissue animal and human bite wound infections. Antimicrob. Agents Chemother. 46:866–870.

Griego, R. D., T. Rosen, I. F. Orengo, and J. E. Wolf. 1995. Dog, cat, and human bites: a review. J. Am. Acad. Dermatol. 33:1019–1029.

Grimault, E., J. C. Glerant, P. Aubry, G. Laurans, J. P. Poinsot, and V. Jounieaux. 1996. Uncommon site of Bergeyella zoohelcum: a propos of a case. Rev. Pneumol. Clin. 52:387–389.

Hayes, P. R. 1977. A taxonomic study of flavobacteria and related Gram-negative yellow pigmented rods. J. Appl. Bacteriol. 43:345–367.

Hollis, D. G., M. I. Daneshvar, C. W. Moss, and C. N. Baker. 1995. Phenotypic characteristics, fatty acid composition, and isoprenoid quinone content of CDC Group IIg bacteria. J. Clin. Microbiol. 33:762–764.

Holmes, B., and R. J. Owen. 1979. Proposal that Flavobacterium breve be substituted as the type species of the genus in place of Flavobacterium aquatile and emended description of the genus Flavobacterium: status of the named species of Flavobacterium. Int. J. Syst. Bacteriol. 29:416–426.

Holmes, B., and R. J. Owen. 1981. Emendation of the genus Flavobacterium and the status of the genus: Developments after the 8th edition of Bergey's Manual. *In:* H. Reichenbach and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group, Proceedings of the International Symposium on Yellow-Pigmented Gram-negative Bacteria of the Flavobacterium-Cytophaga Group, Braunschweig, July 8 to 11, 1980. Verlag Chemie. Weinheim, Germany. 17–26.

Holmes, B., R. J. Owen, and T.A. McMeekin. 1984. Genus Flavobacterium Bergey, Harrison, Breed and Huntoon, 1923. *In*: N. R. Krieg and J. G. Holt (Eds.). Bergey's Manual of Systematic Bacteriology Williams and Wilkins, Co. Baltimore, MD. 1:353–361.

Holmes, B., A. G. Steigerwalt, R. E. Weaver, and D. J. Brenner. 1986a. Weeksella virosa gen. nov., sp. nov. (formerly Group IIf), found in human clinical specimens. Syst. Appl. Microbiol. 8:185–190.

Holmes, B., A. G. Steigerwalt, R. E. Weaver, and D. J. Brenner. 1986b. Weeksella zoohelcum sp. nov. (formerly Group IIj), from human clinical specimens. Syst. Appl. Microbiol. 8:191–196.

Holmes, B. 1992. The genera Flavobacterium, Sphingobacterium and Weeksella. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. Berlin, Germany. 4:3620–3630.

Hugo, C. J. and P. J. Jooste. 2003. Culture media for genera in the family Flavobacteriaceae. *In:* J. E. L. Corry, G. D. W. Curtis, and R. M. Baird (Eds.) Handbook of Culture Media for Food Microbiology, 2nd ed. Elsevier Science Publishers. London, UK. 355–367.

Isotalo, P. A., D. Edgar, and B. Toye. 2000. Polymicrobial tenosynovitis with Pasteurella multocida and other Gram-negative bacilli after a Siberian tiger bite. J. Clin. Pathol. 53:871–872.

Jooste, P. J., T. J. Britz, and J. De Haast. 1985. A numerical taxonomic study of Flavobacterium-Cytophaga strains from dairy sources. J. Appl. Bacteriol. 59:311–323.

Jooste, P. J., and C. J. Hugo. 1999. The taxonomy, ecology and cultivation of bacterial genera belonging to the family Flavobacteriaceae. Int. J. Food Microbiol. 53:81–94.

Mardy, C., and B. Holmes. 1988. Incidence of vaginal Weeksella virosa (formerly group IIf). J. Clin. Pathol. 41:211–214.

Montejo, M., K. Aguirrebengoa, J. Ugalde, L. Lopez, J. A. S. Nieto, and J. L. Hernández. 2001. Bergeyella zoohelcum bacteremia after a dog bite. Clin. Infect. Dis. 33:1608–1609.

Noell, F., M. F. Gorce, C. Garde, and C. Bizet. 1989. Isolation of Weeksella zoohelcum in septiceamia. Lancet 2(8658):332.

Olsen, H., and Ravn, T. 1971. Flavobacterium meningosepticum isolated from the genitals. Acta Path. Microbiol. Immunol. Scand., Sect. B 79:102–106.

Owen, R. J., and J. J. S. Snell. 1973. Comparison of group IIf with Flavobacterium and Moraxella. Ant. v. Leeuwenhoek 39:473–480.

Pedersen, M. M., E. Marso, and M. J. Pickett. 1970. Nonfermentative bacilli associated with man. III: Pathogenicity and antibiotic susceptibility. Am. J. Clin. Pathol. 54:178–192.

Pickett, M. J., and C. R. Manclark. 1970a. Non-fermentative bacilli associated with man. I: Nomenclature. Am. J. Clin. Pathol. 54:155–163.

Pickett, M. J., and M. M. Pedersen. 1970b. Nonfermentative bacilli associated with man: II. Detection and identification. Am. J. Clin. Pathol. 54:164–177.

Pickett, M. J., and M. M. Pedersen. 1970c. Salient features of non-saccharolytic and weakly saccharolytic nonfermentative rods. Can. J. Microbiol. 16:401–409.

Price, K. W., and M. J. Pickett. 1981. Studies of clinical isolates of flavobacteria. *In:* H. Reichenbach and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group. Proceedings of the International Symposium on Yellow-Pigmented Gram-negative Bacteria of the Flavobacterium-Cytophaga Group, Braunschweig, July 8 to 11, 1980. Verlag Chemie. Weinheim, Germany. 63–77.

Reina, J., J. Gil, and P. Alomar. 1989. Isolation of Weeksella virosa (formerly CDC Group IIf) from a vaginal sample. Eur. J. Clin. Microbiol. Inf. Dis. 8:569–570.

Reina, J., and N. Borrell. 1992. Leg abscess caused by Weeksella zoohelcum following a dog bite. Clin. Infect. Dis. 14:1162–1163.

Rubin, S. J., P. A. Granato, and B. L. Wasilauskas. 1985. Glucose-nonfermenting Gram-negative bacteria. *In:* E. H. Lennette, A. Balows, W. J. Hausler, and H. J. Shadomy (Eds.) Manual of Clinical Microbiology, 4th ed.. American Society for Microbiology. Washington, DC. 330–349.

Saphir, D. A., and G. R. Carter. 1976. Gingival flora of the dog with special reference to bacteria associated with bites. J. Clin. Microbiol. 3:344–349.

Schell, R. F., M. Francisco, J. A. Bihl, and J. L. Le Frock. 1985. The activity of ceftazidime compared with those of aztreonam, newer cephalosporins and Sch 29482 against nonfermentative Gram negative bacilli. Chemother. 31:181–190.

Segers, P., W. Mannheim, M. Vancanneyt, K. De Brandt, K.-H. Hinz, K. Kersters, and P. Vandamme. 1993. Riemerella anatipestifer gen. nov., comb. nov., the causative agent of septicemia anserum exsudativa, and its phylogenetic affiliation within the Flavobacterium-Cytophaga rRNA homology group. Int. J. Syst. Bacteriol. 43:768–776.

Takeuchi, M., and A. Yokota. 1992. Proposals of Sphingobacterium faecium sp. nov., Sphingobacterium piscium sp. nov., Sphingobacterium heparinum comb. nov., Sphingobacterium thalpophilum comb. nov., and two genospecies of the genus Sphingobacterium, and synonymy of Flavobacterium yabuuchiae and Sphingobacterium spiritivorum. J. Gen. Appl. Microbiol. 38:465–482.

Talan, D. A., D. M. Citron, F. M. Abrahamian, G. J. Moran, and E. J. C. Goldstein. 1999. Bacteriological analysis of infected dog and cat bites. New Engl. J. Med. 340:85–92.

Tatum, H. W., W. H. Ewing, and R. E. Weaver. 1974. Miscellaneous Gram-negative bacteria. *In:* E. H. Lennette, E. H. Spaulding, and J. P. Truant (Eds.) Manual of Clinical Microbiology, 2nd ed. American Society for Microbiology. Washington, DC. 270–294.

Vancanneyt, M., P. Segers, U. Torck, B. Hoste, J.-F. Bernardet, P. Vandamme, and K. Kersters. 1996. Reclassification of Flavobacterium odoratum (Stutzer 1929) strains to a new genus, Myroides, as Myroides odoratus comb. nov. and Myroides odoratimimus sp. nov. Int. J. Syst. Bacteriol. 46:926–932.

Vandamme, P., J.-F. Bernardet, P. Segers, K. Kersters, and B. Holmes. 1994. New perspectives in the classification of the flavobacteria: description of Chryseobacterium gen. nov., Bergeyella gen. nov., and Empedobacter nom. rev. Int. J. Syst. Bacteriol. 44:827–831.

Von Graevenitz, A. 1981. Clinical significance and antimicrobial susceptibility of flavobacteria. *In:* H. Reichenbach and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group. Proceedings of the International Symposium on Yellow-Pigmented Gram-negative Bacteria of the Flavobacterium-Cytophaga Group, Braunschweig, July 8 to 11, 1980. Verlag Chemie. Weinheim, Germany. 153–164.

Von Graevenitz, A. 1995. Acinetobacter, Alcaligenes, Moraxella and other nonfermentative Gram-negative bacteria. *In:* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (Eds.) Manual of Clinical Microbiology, 6th ed.. ASM Press. Washington, DC. 522–532.

Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1996. Identification of Unusual Pathogenic Gram-negative Aerobic and Facultatively Anaerobic Bacteria, 2nd ed. CDC. Atlanta, GA. 544–547.

Xia, X.-H., L. Lin, and J.-L. Xu. 2000. Mixed surfactant washing of petroleum contaminants from the soils and sediments in the unsaturated zone. J. Environ. Sci. 12:108–114.

Yabuuchi, E., T. Kaneko, I. Yano, C. W. Moss, and N. Miyoshi. 1983. Sphingobacterium gen. nov., Sphingobacterium spiritivorum comb. nov., Sphingobacterium multivorum comb. nov., Sphingobacterium mizutae sp. nov., and Flavobacterium indologenes sp. nov.: Glucose-nonfermenting Gram-negative rods in CDC groups IIK-2 and IIb. Int. J. Syst. Bacteriol. 33:580–598.

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CHAPTER 6.6

The Genera *Flavobacterium*, *Sphingobacterium* and *Weeksella*

BARRY HOLMES

The name *Flavobacterium* was proposed in 1923 for a genus of the family Bacteriaceae encompassing the rod-shaped, nonendosporeforming, chemoorganotrophic bacteria (Bergey et al., 1923). Most of the pigmented bacteria of the family were segregated in the tribe Chromobactereae, which contained four genera of aerobic bacteria separated from each other by differences in color. These genera were *Chromobacterium*, *Flavobacterium*, *Pseudomonas*, and *Serratia*, for the purple, yellow, green fluorescent, and red strains, respectively. This emphasis on pigmentation (a character shared by genetically diverse bacteria [Weeks, 1969]) for taxonomic assignment to *Flavobacterium* has given the genus a dubious reputation in the past (McMeekin et al., 1972; Weeks, 1969), and as a consequence the genus has served too frequently as a repository for pigmented bacteria that possess the general attributes of *Flavobacterium* but had not been subjected to detailed classification studies. Taxonomic heterogeneity and general uncertainty have characterized *Flavobacterium* from its inception, and its history is a record of proposals to achieve credibility for the genus.

Stanier (1947) recognized that the cytophagas have more than a casual phenetic resemblance to pigmented, Gram-negative eubacteria such as *Flavobacterium* and the relationship to the cytophagas has dominated the taxonomic consideration of *Flavobacterium*. Differentiation of flavobacteria from cytophagas has depended primarily upon demonstration of the gliding movement and colonial translocation characteristic of the latter bacteria, but absence of these features has not deterred assignment of flavobacterial species to *Cytophaga* (Mitchell et al., 1969). Freshly isolated cytophagas have the unusual ability to use a great variety of complex natural polymers, e.g., agar, alginates, cell walls, cellulose, chitin, DNA, keratins, lipids, pectin, porphyrins, proteins, RNA, and starch, as nutrients. This ability is not a general property of flavobacterial species, although strains of some species may hydrolyze casein, chitin, gelatin, or starch.

This chapter was taken unchanged from the second edition.

The problem of differentiating *Flavobacterium* from *Cytophaga* has been discussed extensively (Christensen, 1977a; McMeekin et al., 1972; Mitchell et al., 1969; Weeks, 1969). An outcome would be more nearly possible if two issues were resolved. These are the heterogeneity of *Flavobacterium* and the differentiation of nonmotile flavobacteria from cytophagas. A primary requirement for the resolution of both issues is an acceptable definition of *Flavobacterium*. The concept of *Flavobacterium* was hardly altered in successive editions of *Bergey's Manual of Determinative Bacteriology* until the fifth (Bergey et al., 1939), which eliminated from the genus the least well-described species and the polarly flagellated strains. Those known to be Gram-positive were excluded in the seventh edition (Weeks and Breed, 1957). In the eight edition (Weeks, 1974) the genus remained heterogeneous, as evidenced by the two disparate reported ranges of GC content of the DNA: 30–42 and 63–70 mol%. Only in *Bergey's Manual of Systematic Bacteriology* (Holmes et al., 1984a) did the genus at last become reasonably homogeneous by including only nonmotile low GC strains. Interspecies DNA-DNA hybridization studies of the latter organisms showed a background level of hybridization of about 20%, which may represent a common DNA complement (Callies and Mannheim, 1980; Owen and Holmes, 1981).

Despite sharing several features, the low GC flavobacteria nevertheless show some heterogeneity and fall into four natural groups (A–D, Table 1), as previously suggested by Holmes and Owen (1981). The strains of group D, so far found only on mammalian mucus membranes, are susceptible to penicillin and are nonpigmented; they now comprise the genus *Weeksella*. Strains of the remaining groups are free-living, show resistance to a wide range of antimicrobial agents and are yellow-pigmented (Table 1). These organisms constitute the genus *Flavobacterium* and they can be divided into groups A–C on the basis of differences in indole production, oxidation of carbohydrates, and proteolytic activity (Table 1). A detailed taxonomic study of these organisms by techniques such as DNA-

Table 1. Differentiation of the flavobacteria.

Character	Habitat	Resistant to antimicrobial agents	Yellow pigment	Indole production	Oxidation of carbohydrates	Proteolytic activity
Group A: *Flavobacterium balustinum, F. breve, Flavobacterium* species group IIb *(F. gleum, F. indologenes), F. indoltheticum, F. meningosepticum*	Free-living	+	+	+	+	+
Group B: *Flavobacterium odoratum*	Free-living	+	+	–	–	+
Group C:[a] *Flavobacterium mizutaii, F. multivorum, F. spiritivorum, F. thalpophilum, F. yabuuchiae*	Free-living	+	+	–	+	–
Group D: *Weeksella virosa, W. zoohelcum*	Strict saprophyte	–	–	+	–	+

+, character present; –, character absent.
[a]Yabuuchi et al. (1983) have proposed that *F. mizutaii*, *F. multivorum*, and *F. spiritivorum* should be placed in a separate genus, *Sphingobacterium*.

rRNA hybridization is necessary to determine their phylogenetic relationships and therefore whether they all belong in the same genus. Pending such a study, it seems undesirable to divide the genus further or to admit to it taxa of uncertain affiliation, otherwise the genus is in danger once more of becoming heterogeneous. The limited data available from DNA-rRNA hybridizations (Bauwens and De Ley, 1981) indicate that *F. breve*, *F. meningosepticum*, and *F. odoratum* may not be closely related and that *F. aquatile*, the type species of the genus (and itself represented by a single strain), is not closely related to the above three species. For other reasons, *F. aquatile* had been considered unrepresentative of the genus. Consequently, Holmes and Owen (1979) requested that *F. breve* be made the type species instead. Their request was subsequently denied, and thus if the genus is further subdivided in the future the name *Flavobacterium* will remain with *F. aquatile* and the majority of organisms currently recognized as *Flavobacterium* will be transferred to one or more new genera. For the above reasons, *F. aquatile* is not considered in the tables in this paper (its characters are given by Holmes et al., 1984a). Perhaps prematurely, the genus *Sphingobacterium* has been proposed by Yabuuchi et al. (1983) for organisms in group C (Table 1) based on the fact that most of these organisms have been shown to possess novel sphingolipids in their cell walls. As this character is not easily determined routinely, and pending further study and possible revision of the genus as a whole, the group C strains are here retained in *Flavobacterium*. (Although all strains in group C have valid combinations of their specific epithets with *Flavobacterium*, there has as yet been no proposal to transfer *F. thalpophilum* or *F. yabuuchiae* to *Sphingobacterium*.)

With *Flavobacterium* better defined, it is now possible to reassess the differentiation of this genus from *Cytophaga.* It has been suggested that organisms of both these genera, which share a distinctive cellular fatty acid composition, a characteristic menaquinone system, and a low GC content, should all be included in *Cytophaga* (Oyaizu and Komagata, 1981). However, this view does not take into account the heterogeneity of the *Flavobacterium-Cytophaga* group as revealed by the preliminary DNA-rRNA hybridization results of Bauwens and De Ley (1981). More recent DNA-DNA hybridization studies (Bernardet, 1989) reveal no appreciable homology between *Cytophaga johnsonae* and *Flavobacterium* species of groups A, B, and C. Oligonucleotide cataloging (Paster et al., 1985) places *C. johnsonae* and *F. aquatile* in the same group while finding that *F. breve* is only peripherally related to the same group. Cataloging also places *Cytophaga* and *Flavobacterium* in the same eubacterial "phylum" as *Bacteroides*.

In summary, while *Flavobacterium* is now more homogeneous than it has ever been, further work is necessary to group the species into additional genera so as to reflect their phylogenetic relationships. Among the flavobacteria of clinical origin at least, these generic groupings may well be reflected by the groups A–C as defined in Table 1. While these organisms would appear to be distinct from *Cytophaga,* other flavobacteria, including *F. aquatile*, may be more closely related to *Cytophaga.* The latest arrangement of including the families Bacteroidaceae and Cytophagaceae plus a newly proposed family "Flavobacteriaceae" in the order Cytoph-

agales (Reichenbach, 1989) is a good reflection of our present knowledge of the phylogenetic relationships of these organisms.

Habitats

Yellow-pigmented, nonfermentative, Gram-negative, nonmotile, rod-shaped bacteria, which have been placed in the genus *Flavobacterium* or have been termed flavobacteria, have been isolated from fresh and marine waters, soil and ocean sediments, foods and food-processing plants, and clinical materials. These bacteria are widely distributed in nature and are especially common in water, which would explain their seeming omnipresence.

Nonclinical Sources

Flavobacterial strains have been isolated most commonly from freshwater and marine environments, and many general bacteriological surveys of such habitats have reported their presence (for example, ZoBell and Upham, 1944). The numerical taxonomic studies of Hayes (1963) and of Floodgate and Hayes (1963) used marine strains isolated from fish surfaces, sea water, and marine mud; the samples were taken from the north Atlantic region, western North American coast, and Florida. Comprehensive investigations of dairy and meat-processing industries have shown numerous flavobacteria (McMeekin et al., 1971, 1972; Jooste et al., 1985), including *Weeksella*-like organisms (Botha et al., 1989). The bacteria have also been found in chlorinated cooling water of vegetable canning plants and were the cause of characteristic spoilage following post-sterilization contamination (Bean and Everton, 1969). *F. multivorum* has been isolated from soil (Hayward and Sly, 1984; Pichinoty et al., 1985).

Clinical Sources

Flavobacteria have consistently been found among the nonfermentative, Gram-negative bacteria isolated from clinical specimens such as blood, urine, infected wounds, and feces (Pickett and Manclark, 1970; Pickett and Pedersen, 1970a, 1970b; Tatum et al., 1974). Their frequency of occurrence is usually low, 1% or less, and their pathogenicity is low-grade or doubtful. They are, however, extremely resistant to many antimicrobial agents (and so may well colonize the patient on intensive chemotherapy for an infection caused by some other organism). Such resistant strains are found in each of groups A–C: *F. breve* (Holmes et al., 1978); *F. meningosepticum* (King, 1959); Group IIb of King (Tatum et al., 1974; this taxon includes *F. gleum* [Holmes et al., 1984b] and *F. indologenes* [Yabuuchi et al., 1983]); *F. odoratum* (Holmes et al., 1977, 1979); *F. multivorum* (Holmes et al., 1981); *F. spiritivorum* (Holmes et al., 1982) and *F. thalpophilum* (Holmes et al., 1983).

F. meningosepticum, the best-known pathogen in the genus, is associated with a sometimes fatal meningitis of infants and has been isolated from their throats, spinal fluid, and blood as well as from throats of normal adults (King, 1959; Holmes, 1987). *F. odoratum* has also been a cause of ventriculitis (Macfarlane et al., 1985). Both *F. multivorum* and *F. odoratum* have been reported as a cause of bacteremia (Freney et al., 1987; Potvliege et al., 1984; Prieur et al., 1988), while both *F. odoratum* and *F. thalpophilum* have been described in wound case reports (Davis et al., 1979; Hansen et al., 1988). *Weeksella virosa* appears to be almost exclusively associated with the female genital tract (Holmes et al., 1986a; Reina et al., 1989), and it seems more prevalent in sexually promiscuous women (Mardy and Holmes, 1988). *W. zoohelcum* is commonly found in the canine oral cavity and has been isolated from human dog-bite wounds (Holmes et al., 1986b). However, one strain has been reported as a cause of meningitis in a child following a dog-bite (Bracis et al., 1979) and another as a cause of septicemia (Noell et al., 1989).

Isolation

Nonclinical *Flavobacterium*

Flavobacteria are chemoorganotrophic and not difficult to isolate, although maintenance of the cultures sometimes presents a problem.

General studies do not require enrichment procedures and usually nutrient agar-type media are used. Studies of marine, pigmented bacteria, for example, have used seawater-agar media, such as the following:

Marine *Flavobacterium* Medium (Hayes, 1963)

Beef extract (Lab Lemco)	10 g
Peptone (Evans)	10 g
Agar (Difco)	15 g
Aged seawater	750 ml
Distilled water	250 ml

Adjust pH to 7.2–7.3.

Isolation of yellow-pigmented bacteria from food and food-processing equipment used a similar medium:

Food *Flavobacterium* Medium (McMeekin et al., 1971)

Beef extract (Oxoid L20)	10 g
Peptone (Oxoid L37)	10 g
NaCl	5 g
Agar (Oxoid No. 3)	12 g
Distilled water	1 liter

Incubation temperatures were similar to that of the environment, i.e., 20–25°C, and incubation times were about 4 days.

Weeks (1955) used medium M1, which contains lesser amounts of nutrients, for both isolation and maintenance of flavobacterial cultures.

Medium M1 (Weeks, 1955)

Proteose peptone (Difco)	5 g
Yeast extract (Difco)	1 g
Beef extract (Difco)	2 g
NaCl	3 g
Agar	12 g
Distilled water	1 liter

pH 7.2–7.4.

It is not unusual to find that media containing relatively large amounts of the individual nutrients, such as those used by Hayes (1963) and McMeekin et al. (1971), are not as well suited to maintenance of flavobacterial cultures as are media with lower concentrations. A study on maintenance by Christensen and Cook (1972) dealt primarily with the isolation of cytophagas but flavobacteria were included. In general, media such as PMYA II containing small amounts of nutrients were superior.

PMYA II (Christensen and Cook, 1972)

Peptonized milk	1 g
Yeast extract	0.2 g
Sodium acetate	0.02 g
Agar	15 g
Distilleµd water	1 liter

This medium was excellent for detection of cytophagal gliding and colonial swarming, which is useful in differentiating between flavobacteria and cytophagas.

Flint (1985), when developing a selective agar medium for the enumeration of *Flavobacterium* species in water, found that on nutrient agar alone less than 10% of colonies recovered were yellow-pigmented; however, when a nutrient agar medium containing kanamycin at 50 µg/ml was used, this figure increased to 70%.

Clinical *Flavobacterium*

Tatum et al. (1974) have described in detail the procedures for isolation of flavobacteria from clinical sources, such as those of *F. meningosepticum* and Group IIb. Primary plating of a clinical specimen is usually onto blood, chocolate, eosin-methylene blue, or MacConkey agar, but other media may be used. Incubation temperature is 35–37°C, but the bacteria will grow at room temperatures (20–25°C). Other workers have found ordinary nutrient agar quite satisfactory (for example, Holmes et al., 1977, 1978).

Preservation of Cultures

Pickett and his colleagues have used cystine-trypticase agar (BBL) for maintenance of cultures of the clinical flavobacteria. Long-term preservation was carried out using brucella broth (BBL) containing 10% glycerol and the cultures were stored at –50°C (Pickett and Pedersen, 1970a). Isolates can also be stored freeze-dried.

Identification

Assignment of a culture to *Flavobacterium* now rests primarily upon the attributes ascribed to the genus (Holmes et al., 1984a). Strains are strictly aerobic, Gram-negative, nonmotile, yellow-pigmented, free-living nonfermenters with low GC content and do not show gliding motility; clinical strains at least are highly resistant to a wide range of antimicrobial agents. Since these features are quite general, the personal judgment of the investigator is a major contribution, especially for the nonclinical strains which have not been studied as thoroughly as clinical strains.

Clinical isolates can usually be identified by assigning them initially to one of the groups A–D on the basis of the characters given in Table 1 and then using the appropriate table for species determination. Methods for determining some characters useful in the differentiation of flavobacteria (indole formation, hydrolysis of DNA, starch, and urea, and pigment production) have been compared by Pickett (1989). Just as the flavobacteria may eventually comprise several genera, so new species are set to emerge from the existing recognized species. It is apparent from Tables 2–3–4 that many species are heterogeneous in phenotypic characters and such is the genomic diversity among the flavobacteria that many recognized species comprise very few known strains. Indeed, several are represented by a single strain.

Among the taxa in group A, *F. balustinum* (from heart blood of fish) and *F. indoltheticum* (from the marine environment) are represented by single strains and extensive DNA-DNA hybridization studies of phenotypically similar strains have ascribed no additional strains to the former and only three to the latter (A. G. Steigerwalt and D. J. Brenner, personal communication). Similar DNA relatedness studies reveal that in *F. breve* there is a "core" of closely related strains with other strains showing lower levels of relatedness to the "core" strains (Owen

Table 2. Characteristics for practical identification and differentiation of the *Flavobacterium* taxa of group A.

Test	*F. balustinum* (1 strain)	*F. breve* (7 strains)	*F. indoltheticum* (1 strain)	*F. meningosepticum* (49 strains)	*Flavobacterium species group* IIb (55 strains)
Acid from ammonium salt-sugar medium containing:					
Glucose	+	6/7[a]	+	42/49	+
Arabinose	–	–	–	1/49	13/55
Cellobiose	–	–	–	4/49	3/55
Ethanol	+	–	–	28/49	9/55
Glycerol	–	–	–	38/49	35/55
Lactose	–	–	–	27/49	–
Maltose	–	6/7	+	46/49	+
Mannitol	–	–	–	31/49	3/55
Sucrose	–	–	–	–	12/55
Trehalose	–	–	–	42/49	48/55
Xylose	–	–	–	3/49	9/55
Esculin hydrolysis	+	–	+	47/49	52/55
Growth at 42°C	–	–	–	6/49	15/55
Indole production (Ehrlich reagent)	+	+	+	24/49	53/55
Nitrate reduction	+	–	–	2/49	16/55
Nitrite reduction	–	–	–	18/49	14/55
Starch hydrolysis	–	–	–	–	36/55
Urease production	–	–	–	16/49	11/55
β-D-Galactosidase production	–	–	–	48/49	15/55
GC content (mol%)	33.1	32.4 ± 0.6[b]	33.8	37.0 ± 0.5	35 to 38.5
Number of strains tested for GC content	1	10	1	8	13

+, all strains tested positive; –, all strains tested negative.

[a]Number of strains showing characteristic/number of strains tested.

[b]Mean ± standard deviation.

The phenotypic results were derived from previous work at the National Collection of Type Cultures, as follows: *F. balustinum, F. indoltheticum, Flavobacterium* species Group IIb (Holmes, 1983), *F. breve* (Holmes et al., 1978), and *F. meningosepticum* (Holmes, 1987). The GC values were derived as follows: *F. balustinum* and *F. breve* (Owen and Holmes, 1980), *F. meningosepticum* (Owen and Lapage, 1974; Owen and Snell, 1976), *F. indoltheticum* and *Flavobacterium* species group IIb (B. Holmes and R. J. Owen, unpublished observations).

and Holmes, 1980). Owen and Snell (1976) showed that of the strains representing the then six serovars of *F. meningosepticum*, the type strain of the species, which represented serovar A, showed much lower levels of relatedness to strains of the other serovars (B–F) than did these strains to each other. Work on this species was extended by Ursing and Bruun (1987), who found only three other strains that showed appreciable homology to the type strain; the remaining 48 strains could be divided into four subgroups. Despite the genomic differentiation of these 52 strains into two major groups, no correlation of these groups could be found with antimicrobial susceptibility (Bruun, 1987), crossed immunoelectrophoresis (Bruun and Høiby, 1987), or phenotypic characters (Bruun and Ursing, 1987). The name *F. indologenes* was proposed for all strains of group IIb (Yabuuchi et al., 1983), despite the fact that it is genomically heterogeneous (Owen and Snell, 1976). The name *F. gleum* was proposed for several group IIb strains that show a high level of DNA-DNA relatedness (Holmes et al., 1984b). More recent DNA-DNA hybridization studies of group IIb strains (A. G. Steigerwalt and D. J. Brenner, personal communication) show that, while several strains display high levels of relatedness to the respective type strains of either *F. gleum* or *F. indologenes*, as many strains again remain to be assigned to one or more genomic groups. Until this problem is further investigated, it would seem prudent to continue referring to the taxon by its original designation of *Flavobacterium* species group IIb.

Group B comprises only *F. odoratum*, easily recognized by its fruity odor (Holmes et al., 1977). Although only ten strains have been studied by DNA-DNA hybridization, there is again genomic heterogeneity in the taxon, with the strains falling into three genomic groups (Owen and Holmes, 1978).

With the exception of *F. multivorum*, all the other species in groups C and D have been examined for DNA-DNA relatedness (*F. mizutaii*, *F. spiritivorum*, and *F. yabuuchiae* [Holmes et al., 1988], *F. thalpophilum* [Holmes et al., 1983], *Weeksella virosa* [Holmes et al., 1986a], *W. zoo-*

Table 3. Characteristics for practical identification and differentiation of the group C flavobacteria.[a]

Test	*F. mizutaii* (3 strains)	*F. multivorum* (28 strains)	*F. spiritivorum* (11 strains)	*F. thalpophilum* (7 strains)	*F. yabuuchiae* (2 strains)
Acid from ammonium salt-sugar medium containing:					
Adonitol	2/3[b]	–	–	+	–
Arabinose	1/3	+	3/11	+	–
Ethanol	–	–	+	–	+
Glycerol	–	27/28	+	+	+
Mannitol	–	–	+	–	+
Rhamnose	+	13/28	1/11	+	–
Gelatinase production (plate method)	1/3	4/28	+	6/7	+
Growth at 42°C	–	–	–	+	–
Hydrolysis of 2-napththyl phosphate at pH 5.4[c]	NT	+	+	+	–
Nitrate reduction	–	–	–	+	–
Nitrite reduction	+	–	–	–	–
Urease production	1/3	27/28	+	+	+
Extracellular deoxyribonuclease production	–	17/28	+	6/7	+
GC content (mol%)	41.0 ± 0.8[d]	39.6 ± 0.5	41.4 ± 0.4	45.0 ± 0.8	41.4 ± 0.5
Number of strains tested for GC content	3	11	6	7	2

+, all strains positive; –, all strains negative; NT, not tested.
[a]These organisms are also known as the sphingobacteria.
[b]Number of strains showing characteristic/number of strains tested.
[c]Tested in standard API ZYM gallery.
[d]Mean ± standard deviation.
The phenotypic results for *F. mizutaii* were taken from National Collection of Type Cultures records (B. Holmes, unpublished observations), and the GC contents for these strains were obtained from Yabuuchi et al. (1983). The results for *F. multivorum* were derived from Holmes et al. (1981), those for *F. thalpophilum* from Holmes et al. (1983), and those for *F. spiritivorum* and *F. yabuuchiae* from Holmes et al. (1988).

Table 4. Characteristics for practical identification and differentiation of *Weeksella* species.

Test	*W. virosa* (29 strains)	*W. zoohelcum* (30 strains)
Urease production	–	+
Growth at 42°C	+	1/30[a]
Growth on MacConkey agar	+	–
Growth on β-hydroxybutyrate	+	–
GC content (mol%)	37.3 ± 0.5[b]	35.7 ± 0.8
Number of strains tested for GC content	4	4

+, all strains tested positive; –, all strains tested negative.
[a]Number of strains showing characteristic/number of strains tested.
[b]Mean ± standard deviation.
The phenotypic results and GC values for *W. virosa* were derived from Holmes et al. (1986a) and those for *W. zoohelcum* from Holmes et al. (1986b).

helcum [Holmes et al., 1986b]). These studies showed that the species were genomically homogeneous except for *F. spiritivorum*, which contained two strains showing high levels of relatedness to each other but lower levels to other strains of *F. spiritivorum*; these two strains now constitute *F. yabuuchiae* (Holmes et al., 1988).

Although there is clear evidence for additional genospecies in *Flavobacterium* (perhaps in *Weeksella* also [Botha et al., 1989]), some of these may not be recognized formally as new species unless phenotypic characters can be found to differentiate them. In addition, there are phenotypically discernible taxa which remain unnamed pending a full taxonomic study. These organisms are groups IIc, IIe, IIh, and IIi; they are all saccharolytic and the last three, at least, are indole producers (Rubin et al., 1985). They may therefore well be additional members of *Flavobacterium* group A, especially as strains of Groups IIe and IIh are known to contain menaquinone 6 and lack sphingolipids (Dees et al., 1986), which would also exclude them from group C (the sphingobacteria). Some differentiation of the flavobacteria can be achieved by gas liquid chromatographic analysis of volatile fatty acids produced in culture (Rasoamananjara et al., 1988). Serology has only been applied to *F. meningosepticum*, where types A–O are recognized; however, strains representing some of these serotypes are biochemically atypical of the species and may be strains of *F. breve* (Holmes et al., 1984a).

Identification of the nonclinical flavobacteria seems to be essentially a matter of deciding whether nonmotile flavobacteria are cytophagas. In this connection, it is a requirement to know whether or not an isolate demonstrates gliding movement and colonial translocation as defined by Henrichsen (1972). It is also necessary to know the DNA base composition and whether the culture can utilize a variety of complex macromolecules in its nutrition. Cultures that display properties of typical cytophagas do not present a problem since these would not be classified as flavobacteria; cultures not having such properties could be considered flavobacteria. Isolates that do not display cytophagal gliding but do demonstrate colonial translocation would not be cytophagas according to Henrichsen (1972). Cultures that are believed to display gliding motion but which do not show colonial translocation probably should not be assigned to the Cytophagaceae. Christensen (1977b) believed that colonial spreading would occur in such cultures if the correct experimental conditions were used.

It has already been stated that *F. aquatile* (from water) is not included in the identification scheme given in this chapter. Also not included are the more recently described species *F. thermophilum* (Loginova and Egorova, 1978) and *F. branchiophila* (Wakabayashi et al., 1989), the latter of which causes bacterial gill disease in freshwater fishes. The former is a thermophile, probably a *Thermus* species (see The Genus *Thermus* and Relatives in this Volume), and the latter, like *F. aquatile*, neither grows at 37°C nor grows well on nutrient agar. Two further new species, both halophiles from an Antarctic Lake, "*F. gondwanense*" and "*F. salegens*," may be proposed (T. A. McMeekin, personal communication). Whether these more recently described organisms truly belong in the genus remains to be determined. DNA-DNA hybridization studies (Bernardet, 1989) reveal that *F. branchiophila* has higher levels of relatedness to *Cytophaga* species than to *Flavobacterium* species. The other validly published species in the genus that are not mentioned here are of uncertain taxonomic position and do not belong in *Flavobacterium* for the reasons given by Holmes et al. (1984a).

Physiological Properties

Within the group A flavobacteria, the main cellular fatty acid components in *F. breve* are iso-$C_{15:0}$ and 3-OH iso-$C_{17:0}$ acids (as they are also present in *F. aquatile*); *F. meningosepticum* contains in addition significant amounts of 2-OH iso-$C_{15:0}$ and is similar to *Flavobacterium* species group IIb (Moss and Dees, 1978; Oyaizu and Komagata, 1981). *F. odoratum* (group B) differs from members of group A in containing iso-$C_{15:0}$, iso-$C_{17:1}$, 3-OH iso-$C_{15:0}$ and 3-OH iso-$C_{17:0}$ acids (Oyaizu and Komagata, 1981). Among the group C flavobacteria (or sphingobacteria), *F. multivorum* at least contains iso-$C_{15:0}$ and 2-OH iso-$C_{15:0}$ as major fatty acid components while the main cellular lipids are sphingophospholipids (Yabuuchi et al., 1981), as indeed they also are in *F. mizutaii*, *F. spiritivorum*, and *F. thalpophilum* (Dees et al., 1985; Yabuuchi et al., 1983). The group C organisms also contain major quantities of menaquinone 7 but no menaquinone 6, in contrast to the flavobacteria of groups A and B which contain major amounts of menaquinone 6 but no menaquinone 7 (Dees et al., 1985). These characters further support the case for classifying the group C organisms in the separate genus *Sphingobacterium* but they rely on characters not easily determined routinely.

Cells of all *Flavobacterium* species are nonmotile in both hanging drop preparations and in soft agar; it is generally accepted that they lack flagella. However, Weeks (1955) examined the type strain of *F. aquatile* by electron microscopy and observed structures that he thought were like "pseudoflagella." By light microscopy, Webster and Hugh (1979) observed what they thought to be nonfunctional flagella on both the type strain of *F. aquatile* and that of *F. meningosepticum*, but this was not confirmed by Thomson et al. (1981) using electron microscopy (although extracellular appendages of a nonflagellar nature, like those on gliding bacteria, were seen in *F. aquatile*).

The yellow pigments produced by these organisms are nonfluorescent and insoluble in growth media; in *F. aquatile* the pigment is carotenoid (principally zeaxanthin; O. B. Weeks, unpublished observations) whereas in *F. breve* and *F. odoratum* they are not carotenoid but are probably of the flexirubin type.

Clinical strains are generally resistant to many antimicrobial agents, including amikacin, ampicillin, carbenicillin, gentamicin, kanamycin, polymyxin B, streptomycin, and tobramycin. Although an R plasmid conferring resistance to ampicillin, carbenicillin, and erythromycin has been reported in a strain of *F. odoratum* (Kono et al., 1980), detectable plasmids have been rarely reported (Owen and Holmes, 1981) and resistance in these organisms is therefore possibly associated with chromosomal genes.

Applications

No applications for strains of the genera *Flavobacterium*, *Sphingobacterium*, or *Weeksella*

are yet known. However, suitable strains of "*Flavobacterium keratolyticus*" (isolated from soil in Japan), an organism not only without standing in nomenclature but possibly also not even a member of the genus *Flavobacterium*, naturally produce an endo-β-galactosidase. This enzyme exposes the human red blood cell cryptantigen Tk and is therefore potentially useful in blood transfusion laboratories for determining the type of end-link sugar on the red cell membrane that carries the blood group antigen (Liew et al., 1982). Growth measurements with a supposed *Flavobacterium* strain have proved useful in determining the concentration of maltose- and starch-like compounds in drinking water, where these compounds may contribute to undesirable bacterial growth in distribution systems (Van Der Kooij and Hijnen, 1985).

Literature Cited

Bauwens, M., De Ley, J. 1981. Improvements in the taxonomy of *Flavobacterium* by DNA:rRNA hybridizations. 27–31. Reichenbach, H. and Weeks, O. B. (ed.) The *Flavobacterium-Cytophaga* group, Proceedings of the International Symposium on Yellow-Pigmented Gram-negative Bacteria of the *Flavobacterium-Cytophaga* Group, Braunschweig July 8 to 11, 1980. Weinheim, Verlag Chemie.

Bean, P. G., Everton, J. R. 1969. Observations on the taxonomy of chromogenic bacteria isolated from cannery environments. Journal of Applied Bacteriology 32:51–59.

Bergey, D. H., Breed, R. S., Murray, E. G. D., Hitchens, A. P. 1939. Bergey's manual of determinative bacteriology, 6th ed. Baltimore, Williams and Wilkins.

Bergey, D. H., Harrison, F. C., Breed, R. S., Hammer, B. W., Huntoon, F. M. 1923. Bergey's manual of determinative bacteriology. Baltimore, Williams and Wilkins.

Bernardet, J.-F. 1989. Etude phenotypique et genomique des bacteries appartenant aux genres *Cytophaga* et *Flexibacter* (ordre des Cytophagales) et comparaison avec le genre *Flavobacterium*; application a l'identification et a la taxonomie des especes ichtyopathogenes. Doctoral thesis, University of Paris.

Botha, W. C., Jooste, P. J., Britz, T. J. 1989. The taxonomic relationship of certain environmental flavobacteria to the genus *Weeksella*. Journal of Applied Bacteriology 67:551–559.

Bracis, R., Seibers, K., Julien, R. M. 1979. Meningitis caused by Group IIj following a dog bite. Western Journal of Medicine 131:438–440.

Bruun, B. 1987. Antimicrobial susceptibility of *Flavobacterium meningosepticum* strains identified by DNA-DNA hybridization. Acta Pathologica, Microbiologica et Immunologica Scandinavica, Section B 95:95–101.

Bruun, B., Høiby, N. 1987. Crossed immunoelectrophoretic analysis of *Flavobacterium meningosepticum* and allied *Flavobacterium* taxa. Acta Pathologica, Microbiologica et Immunologica Scandinavica, Section B 95:245–252.

Bruun, B., Ursing, J. 1987. Phenotypic characterization of *Flavobacterium meningosepticum* strains identified by DNA-DNA hybridization. Acta Pathologica, Microbiologica et Immunologica Scandinavica, Section B 95:41–47.

Callies, E., Mannheim, W. 1980. Deoxyribonucleic acid relatedness of some menaquinone-producing *Flavobacterium* and *Cytophaga* strains. Antonie van Leeuwenhoek Journal of Microbiology 46:41–49.

Christensen, P. 1977a. The history, biology, and taxonomy of the *Cytophaga* group. Canadian Journal of Microbiology 23:1599–1653.

Christensen, P. 1977b. Synonymy of *Flavobacterium pectinovorum* Dorey with *Cytophaga johnsonae* Stanier. International Journal of Systematic Bacteriology 27:122–132.

Christensen, P. J., Cook, F. D. 1972. The isolation and enumeration of cytophagas. Canadian Journal of Microbiology 18:1933–1940.

Davis, J. M., Peel, M. M., Gillians, J. A. 1979. Colonization of an amputation site by *Flavobacterium odoratum* after gentamicin therapy. Medical Journal of Australia 2:703–704.

Dees, S. B., Carlone, G. M., Hollis, D., Moss, C. W. 1985. Chemical and phenotypic characteristics of *Flavobacterium thalpophilum* compared with those of other *Flavobacterium* and *Sphingobacterium* species. International Journal of Systematic Bacteriology 35:16–22.

Dees, S. B., Moss, C. W., Hollis, D. G., Weaver, R. E. 1986. Chemical characterization of *Flavobacterium odoratum*, *Flavobacterium breve*, and *Flavobacterium*-like groups IIe, IIh, and IIf. Journal of Clinical Microbiology 23:267–273.

Flint, K. P. 1985. A note on a selective agar medium for the enumeration of *Flavobacterium* species in water. Journal of Applied Bacteriology 59:561–566.

Floodgate, G. D., Hayes, P. R. 1963. The Adansonian taxonomy of some yellow pigmented marine bacteria. Journal of General Microbiology 30:237–244.

Freney, J., Hansen, W., Ploton, C., Meugnier, H., Madier, S., Bornstein, N., Fleurette, J. 1987. Septicemia caused by *Sphingobacterium multivorum*. Journal of Clinical Microbiology 25:1126–1128.

Hansen, W., Freney, J., Ploton, C., Seynave, D., Holmes, B. 1988. Isolation of *Flavobacterium thalpophilum* from a wound. European Journal of Clinical Microbiology and Infectious Diseases 7:818–819.

Hayes, P. R. 1963. Studies on marine flavobacteria. Journal of General Microbiology 30:1–19.

Hayward, A. C., Sly, L. I. 1984. Characterization of a dextranolytic biotype of *Flavobacterium multivorum* from soil. Journal of Applied Bacteriology 57:505–516.

Henrichsen, J. 1972. Bacterial surface translocation: A survey and a classification. Bacteriological Reviews 36:478–503.

Holmes, B. 1983. The taxonomy of the genus *Flavobacterium*. Leclerc, H. (ed.) Gram-negative bacteria of medical and public health importance: Taxonomy-Identification-Applications. (Proceedings of symposium, Lille, May 25 to 27, 1983). Les Editions INSERM 114:273–94.

Holmes, B. 1987. Identification and distribution of *Flavobacterium meningosepticum* in clinical material. Journal of Applied Bacteriology 62:29–41.

Holmes, B., Hollis, D. G., Steigerwalt, A. G., Pickett, M. J., Brenner, D. J. 1983. *Flavobacterium thalpophilum*, a new species recovered from human clinical material. International Journal of Systematic Bacteriology 33:677–682.

Holmes, B., Owen, R. J. 1979. Proposal that Flavobacterium breve be substituted as the type species of the genus in place of Flavobacterium aquatile and emended description of the genus Flavobacterium: Status of the named species of Flavobacterium. International Journal of Systematic Bacteriology 29:416–426.

Holmes, B., Owen, R. J. 1981. Emendation of the genus *Flavobacterium* and the status of the genus. *In*: Reichenbach, H. and Weeks, O. B. (ed.)The *Flavobacterium-Cytophaga* Group, Proceedings of the International Symposium on Yellow-Pigmented Gram-negative Bacteria of the *Flavobacterium-Cytophaga* Group, Braunschweig July 8 to 11, 1980. Weinheim, Verlag Chemie. 17–26.

Holmes, B., Owen, R. J., Hollis, D. G. 1982. *Flavobacterium spiritivorum*, a new species isolated from human clinical specimens. International Journal of Systematic Bacteriology 32:157–165.

Holmes, B., Owen, R. J., McMeekin, T. A. 1984a. Genus *Flavobacterium* Bergey, Harrison, Breed, Hammer and Huntoon 1923, 97. 353–361. Krieg, N. R. (ed.) Bergey's manual of systematic bacteriology, vol. 1. Baltimore, Williams and Wilkins.

Holmes, B., Owen, R. J., Steigerwalt, A. G., Brenner, D. J. 1984b. *Flavobacterium gleum*, a new species found in human clinical specimens. International Journal of Systematic Bacteriology 34:21–25.

Holmes, B., Owen, R. J., Weaver, R. E. 1981. *Flavobacterium multivorum*, a new species isolated from human clinical specimens and previously known as Group IIk, biotype 2. International Journal of Systematic Bacteriology 31:21–34.

Holmes, B., Snell, J. J. S., Lapage, S. P. 1977. Revised description, from clinical isolates, of *Flavobacterium odoratum* Stutzer and Kwaschnina 1929, and designation of the neotype strain. International Journal of Systematic Bacteriology 27:330–336.

Holmes, B., Snell, J. J. S., Lapage, S. P. 1978. Revised description, from clinical strains, of *Flavobacterium breve* (Lustig) Bergey et al. 1923 and proposal of the neotype strain. International Journal of Systematic Bacteriology 28:201–208.

Holmes, B., Snell, J. J. S., Lapage, S. P. 1979. *Flavobacterium odoratum*: a species resistant to a wide range of antimicrobial agents. Journal of Clinical Pathology 32:73–77.

Holmes, B., Steigerwalt, A. G., Weaver, R. E., Brenner, D. J. 1986a. *Weeksella virosa* gen. nov., sp. nov. (formerly Group IIf), found in human clinical specimens. Systematic and Applied Microbiology 8:185–190.

Holmes, B., Steigerwalt, A. G., Weaver, R. E., Brenner, D. J. 1986b. *Weeksella zoohelcum* sp. nov. (formerly Group IIj), from human clinical specimens. Systematic and Applied Microbiology 8:191–196.

Holmes, B., Weaver, R. E., Steigerwalt, A. G., Brenner, D. J. 1988. A taxonomic study of *Flavobacterium spiritivorum* and *Sphingobacterium mizutae*: Proposal of *Flavobacterium yabuuchiae* sp. nov. and *Flavobacterium mizutaii* comb. nov. International Journal of Systematic Bacteriology 38:348–353.

Jooste, P. J., Britz, T. J., De Haast, J. 1985. A numerical taxonomic study of *Flavobacterium-Cytophaga* strains from dairy sources. Journal of Applied Bacteriology 59:311–323.

King, E. O. 1959. Studies on a group of previously unclassified bacteria associated with meningitis in infants. American Journal of Clinical Pathology 31:241–247.

Kono, M., Sasatsu, M., Makino, K. 1980. R-plasmid conferring resistance to ampicillin, carbenicillin and erythromycin in a *Flavobacterium odoratum* strain. Microbios Letters 14:55–58.

Liew, Y. W., Bird, G. W. G., King, M. J. 1982. The human erythrocyte cryptantigen Tk: exposure by an endo-beta-galactosidase from Flavobacterium keratolycus. Blood Transfusion and Immunohaematology 25:639–641.

Loginova, G. B. U., Egorova, L. A. 1978. A new species of obligate thermophilic bacteria, *Flavobacterium thermophilum*. Mikrobiologia 47:1081–1084.

Macfarlane, D. E., Baum-Thureen, P., Crandon, I. 1985. *Flavobacterium odoratum* ventriculitis treated with intraventricular cefotaxime. Journal of Infection 11:233–238.

Mardy, C., Holmes, B. 1988. Incidence of vaginal *Weeksella virosa* (formerly group IIf). Journal of Clinical Pathology 41:211–214.

McMeekin, T. A., Patterson, J. T., Murray, J. G. 1971. An initial approach to the taxonomy of some Gram-negative yellow pigmented rods. Journal of Applied Bacteriology 34:699–716.

McMeekin, T. A., Stewart, D. B., Murray, J. G. 1972. The Adansonian taxonomy and the deoxyribonucleic acid base ratio composition of some Gram-negative, yellow pigmented rods. Journal of Applied Bacteriology 35:129–137.

Mitchell, T. G., Hendrie, M. S., Shewan, J. M. 1969. The taxonomy, differentiation and identification of *Cytophaga* species. Journal of Applied Bacteriology 32:40–50.

Moss, C. W., Dees, S. B. 1978. Cellular fatty acids of *Flavobacterium meningosepticum* and *Flavobacterium* species Group IIB. Journal of Clinical Microbiology 8:772–774.

Noell, F., Gorce, M. F., Garde, C., Bizet, C. 1989. Isolation of *Weeksella zoohelcum* in septicaemia. Lancet ii:332.

Owen, R. J., Holmes, B. 1978. Heterogeneity in the characteristics of deoxyribonucleic acid from *Flavobacterium odoratum*. FEMS Microbiology Letters 4:41–46.

Owen, R. J., Holmes, B. 1980. Differentiation between strains of *Flavobacterium breve* and allied bacteria by comparisons of deoxyribonucleic acids. Current Microbiology 4:7–11.

Owen, R. J., Holmes, B. 1981. Identification and classification of *Flavobacterium* species from clinical sources. 39–50. Reichenbach, H. and Weeks, O. B. (ed.) The *Flavobacterium-Cytophaga* group. Proceedings of the International Symposium on Yellow-Pigmented Gram-Negative Bacteria of the *Flavobacterium-Cytophaga* Group, Braunschweig July 8 to 11, 1980. Weinheim, Verlag Chemie.

Owen, R. J., Lapage, S. P. 1974. A comparison of King's group IIb of *Flavobacterium* with *Flavobacterium meningosepticum*. Antonie van Leeuwenhoek Journal of Microbiology and Serology 40:255–264.

Owen, R. J., Snell, J. J. S. 1976. Deoxyribonucleic acid reassociation in the classification of flavobacteria. Journal of General Microbiology 93:89–102.

Oyaizu, H., Komagata, K. 1981. Chemotaxonomic and phenotypic characterization of the strains of species in the *Flavobacterium-Cytophaga* complex. Journal of General and Applied Microbiology 27:57–107.

Paster, B. J., Ludwig, W., Weisburg, W. G., Stackebrandt, E., Hespell, R. B., Hahn, C. M., Reichenbach, H., Stetter, K. O., Woese, C. R. 1985. A phylogenetic grouping of the *Bacteroides*, cytophagas, and certain flavobacteria. Systematic and Applied Microbiology 6:34–42.

Pichinoty, F., Baratti, J., Kammoun, S., Allais, J.-J, Asselineau, J., Richard, C., Denis, M., Richaud, P., Mutaftschiev, S. 1985. Isolement et description de souches de *Flavobacterium multivorum* d'origine tellurique. Annales de l'Institut Pasteur/ Microbiologie 136A:359–370.

Pickett, M. J. 1989. Methods for identification of flavobacteria. Journal of Clinical Microbiology 27:2309–2315.

Pickett, M. J., Manclark, C. R. 1970. Nonfermentative bacilli associated with man: I. Nomenclature. American Journal of Clinical Pathology 54:155–163.

Pickett, M. J., Pedersen, M. M. 1970a. Nonfermentative bacilli associated with man: II. Detection and identification. American Journal of Clinical Pathology 54:164–177.

Pickett, M. J., Pedersen, M. M. 1970b. Characterization of saccharolytic nonfermentative bacteria associated with man. Canadian Journal of Microbiology 16:351–362.

Potvliege, C., Dejaegher-Bauduin, C., Hansen, W., Dratwa, M., Collart, F., Tielemans, C., Yourassowsky, E. 1984. *Flavobacterium multivorum* septicemia in a hemodialyzed patient. Journal of Clinical Microbiology 19:568–569.

Prieur, D., Colombani, J. C., Michelon, G. 1988. Bacteriemie a *Flavobacterium odoratum*. Médecine et Maladies Infectieuses 10:466–467.

Rasoamananjara, D., Turlot, J. C., Monteil, H. 1988. Identification of *Flavobacterium* strains by gas liquid chromatographic analysis of volatile fatty acids produced in culture. Annales de l'Institut Pasteur/Microbiologie 139:411–419.

Reichenbach, H. 1989, 1974. Order 1. Cytophagales Leadbetter. *In*: Staley, J. T., Bryant, M. P., Pfennig, N., and Holt, J. G. (ed.) Bergey's manual of systematic bacteriology, 3rd ed. Williams and Wilkins Co.. Baltimore, MD. 99:2011–2013.

Reina, J., Gil, J., Alomar, P. 1989. Isolation of *Weeksella virosa* (formerly CDC Group IIf) from a vaginal sample. European Journal of Clinical Microbiology and Infectious Diseases 8:569–570.

Rubin, S. J., Granato, P. A., Wasilauskas, B. L. 1985. Glucose-nonfermenting Gram-negative bacteria. 330–349. Lennette, E. H., Balows, A., Hausler Jr., W. J., and Shadomy, H. J. (ed.) Manual of clinical microbiology, 4th ed. Washington DC, American Society for Microbiology.

Stanier, R. Y. 1947. Studies on non-fruiting myxobacteria. I. *Cytophaga johnsonae* n. sp. a chitin-decomposing myxobacterium. Journal of Bacteriology 53:297–315.

Tatum, H. W., Ewing, W. H., Weaver, R. E. 1974. Miscellaneous Gram-negative bacteria. 270–294.Lennette, E. H., Spaulding, E. H., Truant, J. P. (ed.) Manual of clinical microbiology, 2nd ed. Washington DC, American Society for Microbiology.

Thomson, K. S., McMeekin, T. A., Thomas, C. J. 1981. Electron microscopic observations of *Flavobacterium aquatile* NCIB 8694 (= ATCC 11947) and *Flavobacterium meningosepticum* NCTC 10016 (= ATCC 13253). International Journal of Systematic Bacteriology 31:226–231.

Ursing, J., Bruun, B. 1987. Genetic heterogeneity of *Flavobacterium meningosepticum* demonstrated by DNA-DNA hybridization. Acta Pathologica, Microbiologica et Immunologica Scandinavica, Section B 95:33–39.

Van Der Kooij, D., Hijnen, W. A. M. 1985. Determination of the concentration of maltose- and starch-like compounds in drinking water by growth measurements with a well-defined strain of a *Flavobacterium* species. Applied and Environmental Microbiology 49:765–771.

Wakabayashi, H., Huh, G. J., Kimura, N. 1989. *Flavobacterium branchiophila* sp. nov., a causative agent of bacterial gill disease of freshwater fishes. International Journal of Systematic Bacteriology 39:213–216.

Webster, J. A., Hugh, R. 1979. *Flavobacterium aquatile* and *Flavobacterium meningosepticum*: glucose nonfermenters with similar flagellar morphologies. International Journal of Systematic Bacteriology 29:333–338.

Weeks, O. B. 1955. *Flavobacterium aquatile* (Frankland and Frankland) Bergey et al., type species of the genus *Flavobacterium*. Journal of Bacteriology 69:649–658.

Weeks, O. B. 1969. Problems concerning the relationships of cytophagas and flavobacteria. Journal of Applied Bacteriology 32:13–18.

Weeks, O. B. 1974. *Flavobacterium*. *In*: Buchanan, R. E., and Gibbons, N. E. (ed.) Bergey's manual of determinative bacteriology. Williams and Wilkins. Baltimore, MD. 357–364.

Weeks, O. B., Breed, R. S. 1957. Genus Flavobacterium. Breed, R. S., Murray, E. G. D., and Smith, N. R. (ed.) Bergey's manual of determinative bacteriology. Williams and Wilkins. Baltimore, MD. 309–322.

Yabuuchi, E., Kaneko, T., Yano, I., Moss, C. W., Miyoshi, N. 1983. *Sphingobacterium* gen. nov., *Sphingobacterium spiritivorum* comb. nov., *Sphingobacterium multivorum* comb. nov., *Sphingobacterium mizutae* sp. nov., and *Flavobacterium indologenes* sp. nov.: Glucose-nonfermenting Gram-negative rods in CDC Groups IIk-2 and IIb. International Journal of Systematic Bacteriology 33:580–598.

Yabuuchi, E., Yano, I., Kaneko, T., Ohyama, A. 1981. Classification of Group IIK-2 and related bacteria. 79–90.Reichenbach, H. and Weeks, O. B. (ed.) The *Flavobacterium-Cytophaga* Group. Proceedings of the International Symposium on Yellow-Pigmented Gram-negative Bacteria of the *Flavobacterium-Cytophaga* Group, Braunschweig July 8 to 11, 1980. Weinheim, Verlag Chemie.

ZoBell, C. E., Upham, H. C. 1944. A list of marine bacteria including descriptions of sixty new species. Bulletin of the Scripps Institute of Oceanography 5:239–292.

Prokaryotes (2006) 7:549–590
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CHAPTER 6.7

The Order Cytophagales

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In the past decade much has been learnt about the order Cytophagales and about some of the organisms belonging to it, although the majority of its members are as unfamiliar as ever. On the basis of 16S rRNA studies, we can now delimit the group with some confidence and have a well-founded idea of its phylogenetic position (Paster et al., 1985; Woese et al., 1985). Accordingly, the Cytophagales appear to be distantly related to the *Bacteroides* group, and these two together comprise one of the main branches, perhaps a phylum, in the bacterial phylogenetic system. The substructure of the *Cytophaga* branch of the phylum is more difficult to evaluate. There is a main line on which we find unicellular gliders—*Cytophaga* (*Cy.**) *johnsonae, Cy. lytica, Cy. aquatilis* = *Flavobacterium (Fv.) aquatile*, and *Sporocytophaga (Sp.) myxococcoides*—but at a lower level unicellular nonmotile bacteria (*Fv. breve*, i.e., low GC, true flavobacteria) are also found. At a still lower level, a cluster branches off which comprises the unicellular gliders—*Flexibacter (Fx.) filiformis* = *Fx. elegans* Fx el, *Cy. heparina*, and *Taxeobacter* = Myx 2105) unicellular nongliding flavobacteria (*Fv. ferugineum*), but also filamentous, multicellular, gliding (*Saprospira*) and nonmotile bacteria (*Haliscomenobacter*). It is obvious from these data that our present definition of genera does not reflect the phylogenetic situation and also that the grouping in families and perhaps orders needs to be reconsidered. Before that is done, however, 16S rRNA sequences of further species should be determined.

From what has been said above it is clear that only a preliminary characterization of the order is possible at present. In this chapter, the order Cytophagales is restricted to unicellular gliding bacteria; the relevant genera are listed in Table 1. Filamentous, gliding bacteria of the genus *Saprospira* may or may not belong to the order, and they are sufficiently different to justify a separate treatment (see The Order Cytophagales in this Volume); contrary to earlier suggestions, these organisms are not apochlorotic cyanobacteria (Reichenbach et al., 1986). Unicellular, gliding bacteria of the genus *Lysobacter* have been found to be closely linked to the xanthomonads and thus belong to the gamma branch of the Proteobacteria (Woese et al., 1985); consequently they are discussed separately (see The Genus *Lysobacter* in Volume 6) It should be mentioned that, in the past, lysobacters have often erroneously been classified as cytophagas; this is discussed in The Genus *Lysobacter* in Volume 6. On the other hand, the cytophagas have been regarded as myxobacteria for some time and accordingly named; *Cy. columnaris* has even been classified in the myxobacterial genus *Chondrococcus* (now *Corallococcus*). However, as pointed out above, the two groups are not phylogenetically related. The nonmobile bacteria that cluster with the Cytophagales are discussed in The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* and The Genus *Haliscomenobacter* in this Volume.

The Cytophagales as outlined above are all unicellular, gliding, Gram-negative bacteria. All have rod-shaped cells, which may differ substantially in shape. They may be short or long, delicate or stout, with tapering or rounded ends (Fig. 1). Two genera exhibit a controlled and cyclic shape change: *Sporocytophaga* produces resting cells in the form of spherical microcysts, and some *Flexibacter* species alternate between very long and extremely agile thread cells and very short, almost spherical and nonmotile rod cells (Fig. 2). Many of the other species are more or less pleomorphic, with cell populations that consist of short and very long rods and chains of cells, particularly in older cultures.

The typical colonies are spreading swarms (Fig. 3). Sometimes they are filmlike and may cover the whole culture plate within a few days. In other cases, they only expand slowly or remain more or less compact. In a few instances there is also rhizoid growth. Many Cytophagales produce brightly colored colonies in shades of yellow, orange, or brick red. The yellow and orange colonies often change immediately into red, purple, or brown if covered with a 10% KOH solution. This color change is due to flexirubin-type pigments (Fig. 4), which have so far only been found in organisms of this group (including flavobacteria). The organisms belonging to the Cytophagales may be aerobic, microaerophilic, capnophilic (CO_2-requiring), or facultatively anaerobic. They are all organotrophs, many of them able to degrade biomacromolecules like proteins, chitin, pectin, agar, starch, or cellulose. They are ubiquitous, are abundant, and probably play a major role in

Table 1. Survey of the taxonomy of the order Cytophagales of the *Bacteriodes-Flavobacterium-Cytophaga* branch.

Order: Cytophagales
Family: Cytophagaceae
Genera: *Cytophaga*
Sporocytophaga
Unnamed *Cytophaga*-like bacteria (serveral genera)
Flexibacter
Microscilla
Flexithrix
Capnocytophaga
Taxeobacter
(Saprospira)[a]

[a]Saprospira may or may not belong to the order. It is discussed separately in The Order Cytophagales in this Volume.

In this chapter, the following abbreviations sometimes are used for the genera of the order Cytophagales: CLB, *Cytophaga*-like bacteria; *Cp., Capnocytophaga*; *Cy., Cytophaga; Ft., Flexithrix*; *Fv., Flavobacterium*; *Fx., Flexibacter*, *Mc., Microscilla; Sa., Saprospira*; *Sp., Sporocytophaga*; *Tx., Taxeobacter.*

This chapter was taken unchanged from the second edition.

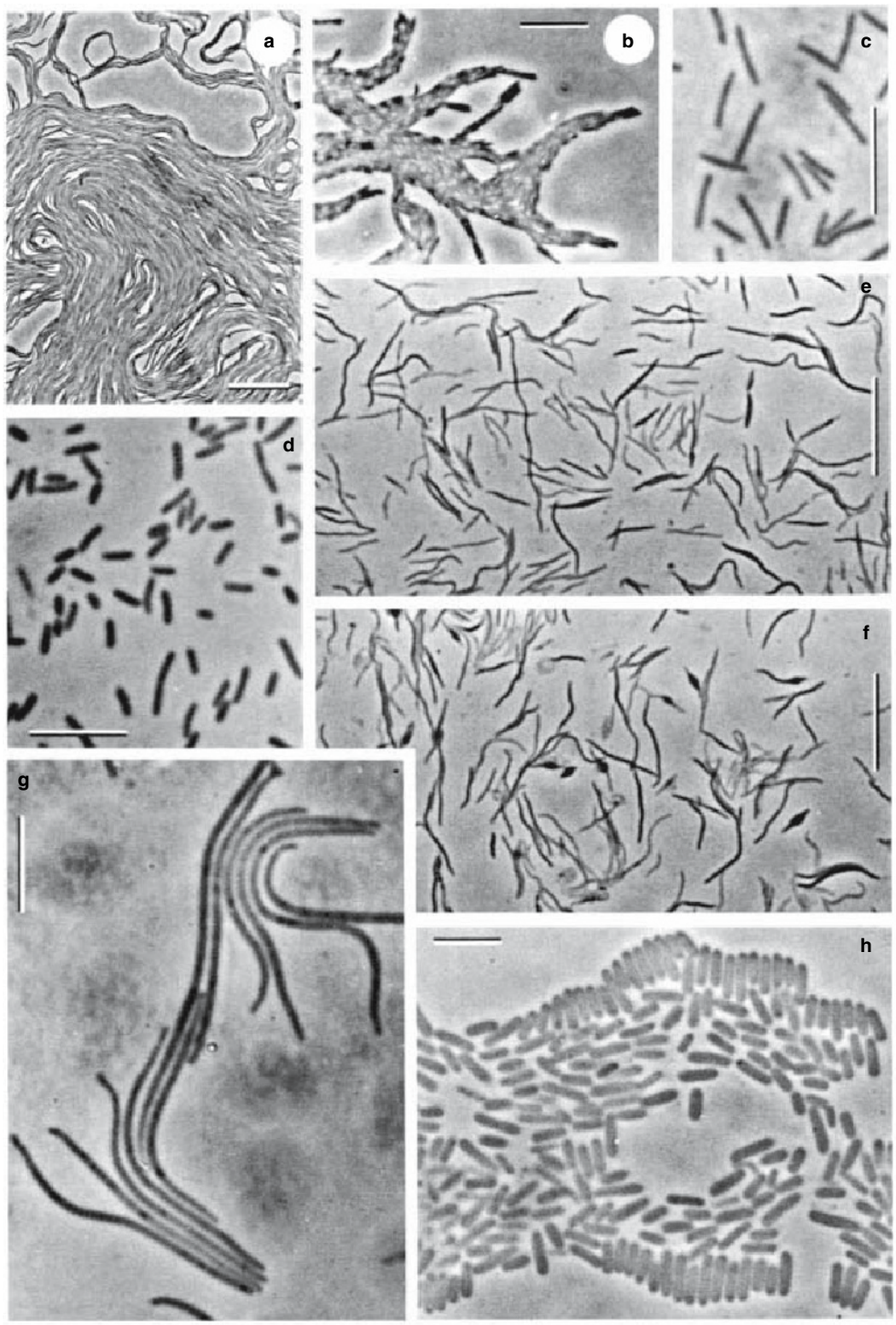

Fig. 1. Cell shape of the Cytophagales in phase contrast. (a) A brick red, marine, agar decomposer, probably a *Microscilla* species, in chamber culture; the long, flexible, thread cells have tapered ends. Bar = 25 µm. (b) CLB from soil, in chamber culture, showing the typical arrangement of cells at the swarm edge. Bar = 10 µm. (c) *Cytophaga lytica*, a yellow, marine, agar-digesting CLB, from SP2 liquid medium. Bar = 5 µm. (d) *Cytophaga succinicans*, a facultatively anaerobic freshwater CLB, from AO agar. Bar = 5 µm. (e and f) *Cytophaga aurantiaca* type strain, a cellulose-degrading true *Cytophaga*, from glucose-glutamate agar. The population in (e) consists of delicate, flexible rods, which are pleomorphic with very long and with slightly swollen cells; in (f), cells from older parts of the colony begin to produce dark, lemon-shaped inflations, which later degenerate to spheroplasts. Bar = 10 µm in both pictures. (g) *Flexibacter flexilis* type strain, from starch agar; one of the thread cells (top edge of the photograph) shows the beginning of branching at one end. Such branching is not uncommon among the Cytophagales. Bar = 5 µm. (h) *Taxeobacter* species, cells in situ on water agar with a streak of living *E. coli*, showing a characteristic palisade pattern. Bar = 25 µm.

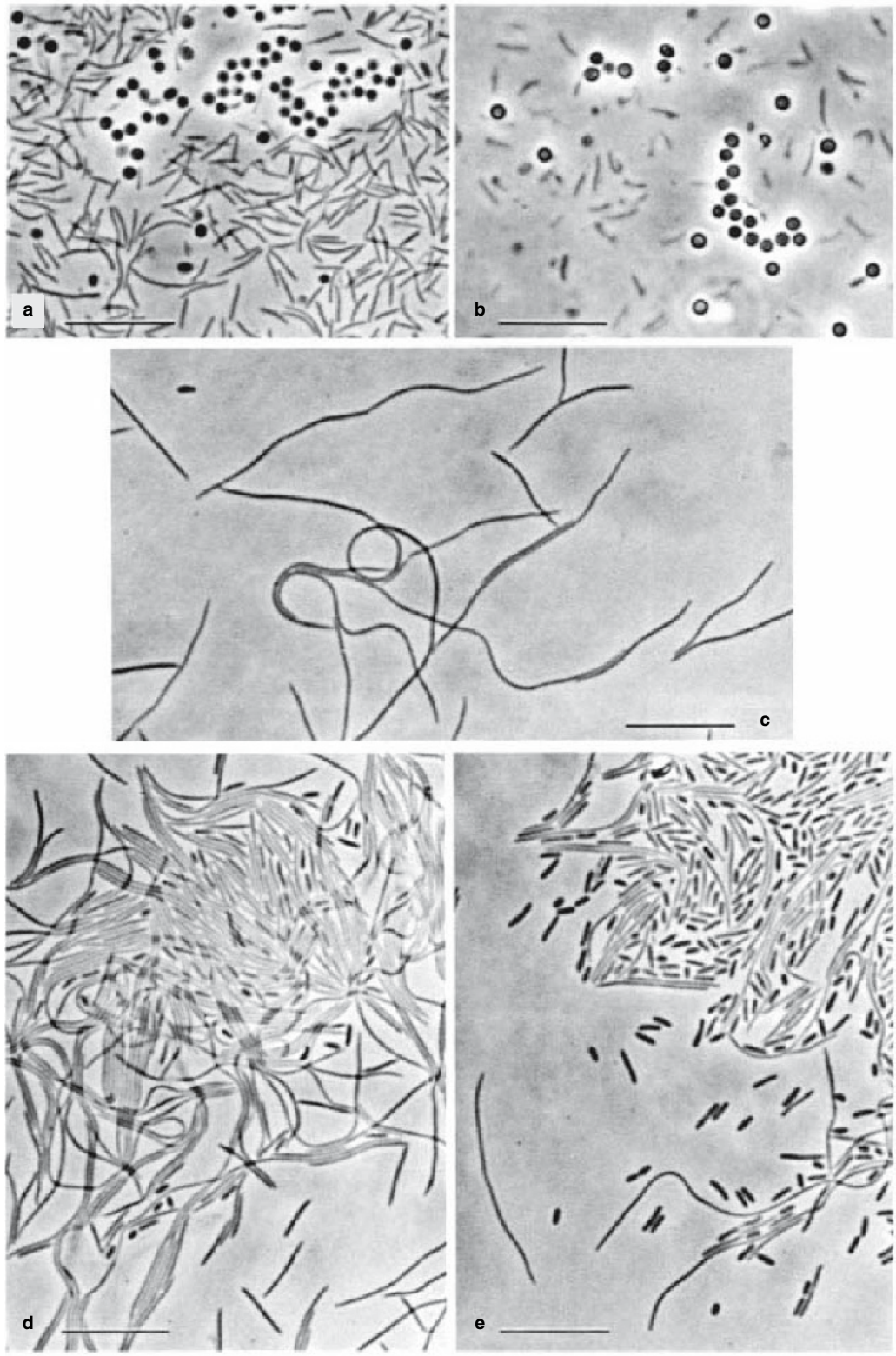

Fig. 2. Cytophagales with a cyclic change in cell shape, shown in phase contrast. (a and b) *Sporocytophaga myxococcoides* from a membrane of regenerated cellulose on ST6 agar; in (a), most cells are still vegetative rods, but young microcysts and intermediary stages of cell conversion can already be seen; in (b), mature, otpically refractile microcysts are present. (c to e) *Flexibacter filiformis* (formerly called *Fx. elagans* Fx el) from VY/2 agar; (c) a slide mount from the very edge of the swarm colony shows very long, delicate, flexible, and extremely agile thread cells without, or with only very few, cross walls; (d) at some distance from the edge, the thread cells have become much shorter by fragmentation; (e) finally, the cell population consists mainly of very short rods which are also clearly fatter and darker and are no longer motile. Bars = 10 μm.

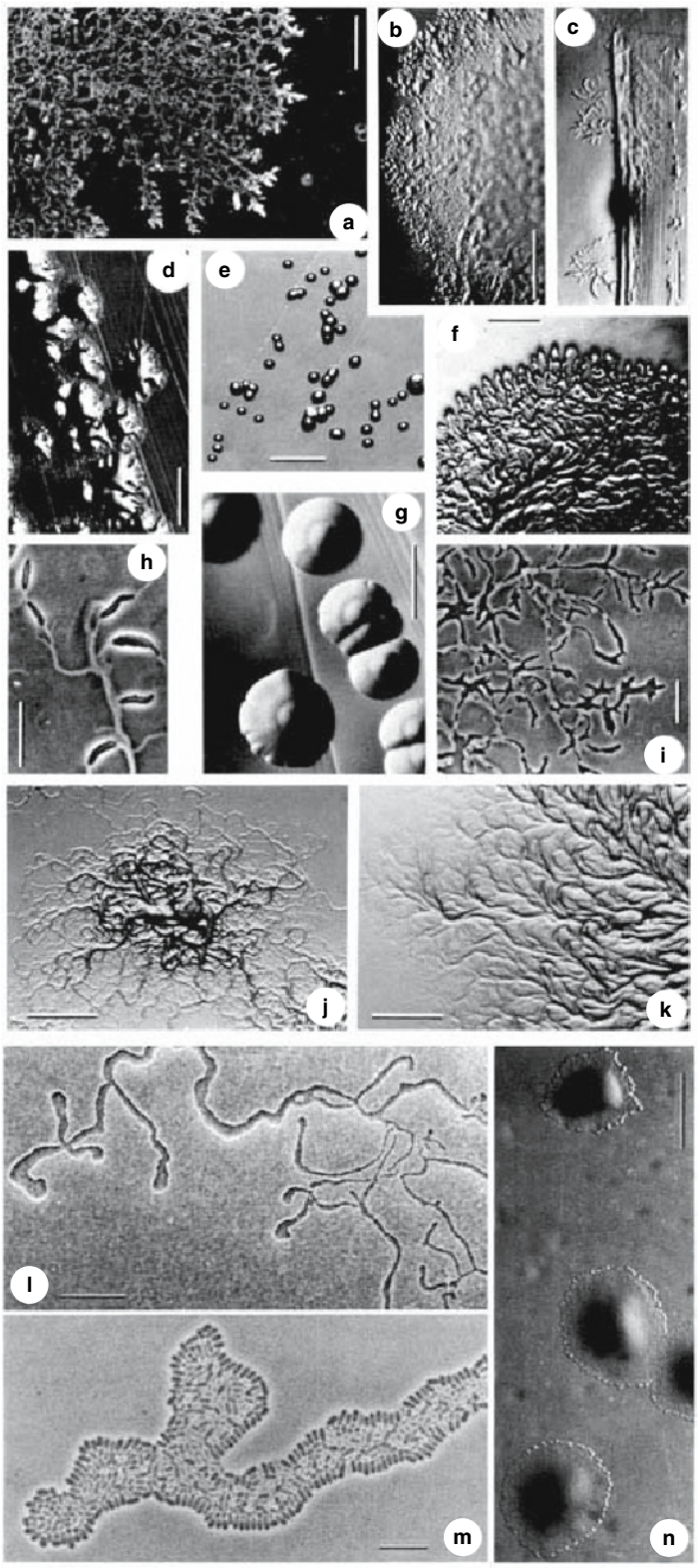

Fig. 3. Colonies of various diverse Cytophagales. (a) Spreading colony of a CLB from soil, growing in chamber culture, dark field. Bar = 100 µm. (b) *Flexibacter filiformis* Fx el, swarm colony on agar plate. Bar = 3 mm. (c) CLB from soil; typical swarm colonies emerge at the side of a streak on an agar surface. Bar = 1 mm. (d and e) CLB from soil growing on two different agar media. (d) On the poorer substrate, the colonies are relatively large and show some spreading; (e) they remain small and compact on the rich medium. Bar = 1 mm in both. (f) CLB from soil, swarm colony on CY agar showing a distinct surface pattern. Bar = 1 mm. (g) *Cytophaga flevensis*, an agar-digesting CLB from freshwater; as a relatively rich agar substrate was used, the colonies remained rather compact. Bar = 2 mm. (h) *Flexibacter filiformis* Fx el, chamber culture; dense clusters of cells are sitting in conspicuous slime tracks; phase contrast. Bar = 30 µm. (i) CLB from soil, chamber culture, edge of a swarm colony with a network of slim tracks; phase contrast. Bar = 30 µm. (j and k) *Cytophaga columnaris*, a fish-pathogenic CLB from freshwater; swarm colonies on MYX agar (j) and AO agar (k), showing the typical growth pattern of the organism. Bar = 1 mm in both. (l to n) *Taxeobacter ocellatus* Tx ol (= Myx 2105): (l and m) on water agar with a streak of living *E. coli*, the organism typically spreads out, producing long, tendril-like strips; (m) at a higher magnification, the unusual palisadelike arrangement of the cells becomes apparent; both phase contrast; (n) after plating on CY agar, relatively compact, brick red colonies begin to spread out. Bar = 100 µm in (l), 10 µm in (m), and 2 mm in (n).

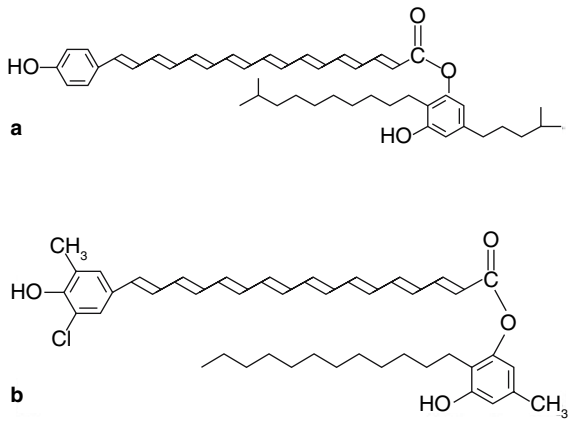

Fig. 4. Flexirubin-type pigments characteristic for members of the Cytophagales. (a) *Cytophaga*-flexirubin from CLB. (b) Chloroflexirubin from a *Flexibacter* species.

the turnover of matter in nature (e.g., Ruschke, 1968; Ruschke and Rath, 1966). Some may also be of practical interest.

A few reviews on the Cytophagales and their subgroups have been published since the first edition of *The Prokaryotes* and can be recommended here for further information (several chapters in *Bergey's Manual of Systematic Bacteriology*, vol. 3, 1989, for detailed discussions of the taxonomic problems; also, Reichenbach and Weeks, 1981; Shewan and McMeekin, 1983; and on practical aspects: Reichenbach, 1988; see also: Bernardet, 1989; Jooste, 1985; Kath, 1990).

Habitats

The Cytophagales are by far the most common of all gliding bacteria. They are found in enormous numbers in certain habitats, mainly those rich in organic material. They can adapt, however, to rather low nutrient levels (Höfle, 1982, 1983) and probably usually subsist on biomacromolecules, which they are specialized to degrade. The main habitats of the Cytophagales, and thus convenient sources for their isolation, are soils at or close to neutral pH, decaying plant material, and dung of animals, particularly that of herbivores. In freshwater environments, they are found on river banks and lake shores, in estuaries, in aerobic bottom sediments, and in algal mats. Certain *Cytophaga*-like bacteria (CLB) have also been found in large numbers freely floating in water, especially during the cold season, e.g., in Lake Constance, the Mindelsee, and the Feldsee in southwestern Germany (Gräf, 1962a; Reichardt, 1974; Ruschke and Rath, 1966). The principal cellulose decomposers in southern Canadian lakes appear to be cytophagas (Hoeniger, 1985). CLB are common in sewage plants where, in the aerobic regions, CLB may constitute 5 to 10% of the total cultivatable bacterial population (Güde, 1980). Here, too, a seasonal variation in the number of CLB can be observed, with a maximum in the winter months. Typically, the CLB are particularly enriched in those stages at the end of the purification process, where only recalcitrant organic molecules remain.

In marine environments, the Cytophagales are abundant near the shores: on living and dead seaweeds, in aerobic and anaerobic bottom sediments, and on decaying sea animals, such as crustaceans with their chitin exoskeleton. Marine cellulose degraders have been found on fishing nets in Japan, where they once were a serious problem in the deterioration of the fabric (Kadota, 1956). Cellulose degraders seem, however, not to be common in marine habitats; at least we have failed so far to isolate any from European coasts. Little is known about the distribution of CLB in the open sea, and a careful investigation of this point would be desirable. As CLB are not likely to form spreading colonies on rich media, the "flavobacteria" that are often observed in ecological studies of various aquatic environments perhaps may frequently really be CLB instead.

In estuarine habitats in the upper Chesapeake Bay, CLB were found to constitute a substantial proportion of the chitin-degrading bacterial flora (Reichardt et al., 1983), among them many facultatively anaerobic, flexirubin-positive strains resembling *Cy. johnsonae* and *Cy. aquatilis*. These organisms tolerated various levels of salt, but did not depepnd on elevated salt concentrations.

However, the boundaries between land and sea appear in general to be rather sharp for CLB. This may be concluded from the observation that virtually all strains of CLB and *Flexibacter* isolated by us from terrestrial and freshwater habitats contain, in addition to carotenoids, flexirubin-type pigments, while strains isolated from the sea coast usually produce carotenoids exclusively and only exceptionally produce flexirubin-type pigments (Reichenbach et al., 1981). Since enormous numbers of terrestrial CLB must constantly be washed or blown into the sea, this can only mean that terrestrial strains cannot easily adapt to and become established in the marine environment. An interesting exception may be *Cy. flevensis*. This is an agar-decomposing organism that does not produce flexirubin-type pigments, both characteristics which would speak for a marine origin. It has, however, been isolated from fresh water of the Isselmeer in the Netherlands (van der Meulen et al., 1974). Since the Isselmeer is a part of the North Sea that has been separated from the open sea for decades and has gradually become constituted with fresh water, marine bacteria would have had ample time to adapt to gradually changing conditions and thus may have had a chance to survive. These

observations also suggest that marine and terrestrial CLB may be less closely related than was thought before; this is indeed supported by 16S rRNA analyses (Paster et al., 1985).

In recent years, it has been discovered that CLB may also occur in milk and dairy products and perhaps occasionally contribute to deterioration (Jooste, 1985; Jooste et al., 1985). Even more serious are sporadic reports of CLB as human pathogens. CLB in the air humidifier system of a textile plant were found to be responsible for a hypersensitivity pneumonitis of workers; they were later described as a new species, *Cy. allerginae* (Flaherty et al., 1984; Liebert et al., 1984). The *Capnocytophaga* species live in the tooth pockets of humans (Leadbetter et al., 1979). Although a role for them in periodontic disorders has been discussed, it seems that the contribution of capnocytophagas to that disease is only a minor one at the most. But life-threatening septicemias and other kinds of general infections have been observed repeatedly and not only in immunocompromised patients as has originally been thought (e.g., Forlenza et al., 1980; Paerregaard and Gutschik, 1987; Shlaes et al., 1982). Fortunately, the organisms respond well to antibiotics (Forlenza et al., 1981; Gräaf and Morhard, 1966).

An economically serious problem are the fish-pathogenic CLB. These organisms are gaining even more importance as fish cultivation is intensified. First discovered in aquarium fishes in the USA (Davis, 1922), infections with CLB became a major concern in the 1940s in the western USA, where they caused severe losses among salmonid fishes in rivers and lakes and in fish hatcheries. The infections were then intensively studied, and it was discovered that more than one species of CLB were involved, causing several different diseases with diverse symptoms. The two most important diseases were columnaris disease, brought about by *Cy. columnaris* (= *Fx. columnaris* = *Chondrococcus columnaris*), and cold water disease by *Cy. psychrophila.* One factor that may have contributed to these outbreaks may have been the warming of the water by electric power plants, mainly along the Columbia River. It appears that, as a rule, unfavorable living conditions for the fish, like confinement, crowding, and suboptimal water temperature, favor infections by CLB (e.g., Anacker and Ordal, 1959; Becker and Fujihara, 1978; Bullock, 1972; Colgrove and Wood, 1966; Collins, 1970; Davis, 1949; Fujihara and Hungate, 1972; Fujihara et al., 1971; Garnjobst, 1945; Ordal and Rucker, 1944; Pacha, 1968; Pacha and Ordal, 1970; Pacha and Porter, 1968; Rucker et al., 1953; Snieszko and Bullock, 1976). Fish diseases caused by CLB are now known worldwide, and they often are a serious threat to fish culturists (e.g., Ajmal and Hobbs, 1967; Bootsma and Clerx, 1976; Deufel, 1974; Ghittion, 1972; Lehmann, 1978; Song et al., 1988; Wakabayashi and Egusa, 1974; Wakabayashi et al., 1970a, 1970b). In Europe, CLB diseases have been observed only since about 1965, but it is not clear whether the pathogens were there before but had previously been overlooked or whether infections have finally become established on a larger scale because environmental conditions have deteriorated. It appears that in the past few years *Cy. psychrophila* has begun to spread through Europe; again, the reasons are not known (Bernardet, 1989; Bernardet and Kerouault, 1989). Very recently, flavobacteria have also been found to be involved in fish diseases (*Fv. branchiophila*), and it will be interesting to see if they are related to fish pathogenic CLB. The data on DNA-DNA hybridization with *Cy. columnaris* were insignificant (Wakabayashi et al., 1989). Further, CLB diseases have been encountered in marine environments (e.g., Anderson and Conroy, 1969; Bullock, 1972; Campbell and Buswell, 1982; Hikida et al., 1979), apparently due to infections by *Fx. maritimus* (Bernardet et al., 1990; Wakabayashi et al., 1986). CLB and flavobacteria obviously belong to the bacterial flora that colonize the eggs of cod and halibut, but in this case detrimental effects have not been demonstrated (Hansen and Olafsen, 1989). However, CLB may have a role in disorders of other marine animals; they have been linked to the destruction of the hinge ligaments of cultivated oysters (Dungan et al., 1989). Based on what is known so far, it may be concluded that the infection mechanism of the CLB pathogenic for fish is connected to their very strong protease activity and, in some, also DNase activity.

Isolation

There are no selective methods that are universally applicable for the enrichment and isolation of the diverse members of the Cytophagales. The general strategy for their isolation is to provide conditions that promote spreading growth on surfaces and then to pick organisms that produce spreading colonies or swarms (Fig. 3) for subcultivation. The nutrient level is usually kept low in the medium used for crude and enrichment cultures in order to stimulate gliding motility and delay development of contaminants. By combining this approach with the use of special substrates in the isolation medium, nutritional specialists among the Cytophagales may be enriched more specifically, e.g., cellulose and chitin degraders. The isolation procedure thus always requires the use of plate cultures. Liquid

media have only been used successfully for baiting cellulose decomposers (see below).

With some experience, it will often be possible to recognize members of the Cytophagales microscopically, because many have a characteristic cell morphology (Fig. 1 and 2). If that is not the case, the appearance of the colonies may provide a clue. The property of swarming presents some difficulties since some organisms cannot be or, only with difficulty, can be induced to form spreading colonies, even when gliding motility can be recognized unequivocally by microscopic examination of wet mounts or by the occurrence of slime trails in chamber cultures. This is why quite a few CLB were originally classified as *Flavobacterium* species. On the other hand, spreading growth is, of course, shared by other gliding bacteria. But there are only two other groups of organotrophic, unicellular gliders: the myxobacteria, which usually show a totally different swarm morphology and typically produce fruiting bodies in crude cultures (see The Genus *Pelobacter* in this Volume), and the lysobacters, which have a peculiar cell morphology, which is, however, shared by some CLB (see The Genus *Lysobacter* in Volume 6). In many cases, pigmentation may be a useful criterion. As already mentioned, many, although not all, members of the Cytophagales are bright yellow, orange, or red. These pigments are always cell-bound (in contrast to some or all of the *Lysobacter* pigments, which, in addition, are usually much paler). If growth is heavy, pigmentation can already be easily recognized in the colonies; alternatively it may be observed after the cells have been concentrated by scraping them together. A large group of soil and freshwater strains of *Cytophaga*, *Sporocytophaga*, CLB, and *Flexibacter* produce flexirubin-type pigments that, upon addition of alkali, turn from orange to purple (Achenbach et al., 1978; Reichenbach et al., 1974). This test can easily be applied to culture plates. In this case, the yellow or orange colonies will turn a much deeper color, either red, purple-violet, or brown, when spotted with 10% KOH solution. The color change differs from that of the pure pigments because of the presence of other pigments, usually carotenoids, which alter the flexirubin absorption spectrum. The color change reaction is reversible upon addition of HCl. If a gliding bacterium gives a positive result with this color test, one can be certain that it belongs to one of the above-mentioned groups of organisms. The only nongliding bacteria known to contain flexirubin-type pigments are the phylogenetically related, low GC (true) flavobacteria (Reichenbach et al., 1980; Weeks, 1981). It should be understood, however, that the described color change is a phenolate reaction that can also be observed with other pigment types. In fact, a similar color reaction has been found in *Brevibacterium linens*, which contains the phenolic carotenoid 3,3′-dihydroxy-isorenieratene (Kohl et al., 1983). Nonmotile, Gram-positive *Brevibacterium*, of course, cannot easily be confused with the organisms discussed here.

Sources for the isolation of Cytophagales will be found in the section on "Habitats." If a broad representation of those organisms is desired, the samples should be processed soon after collection, especially if the samples are from aquatic or humid environments, because in such samples, oxygen depletion and desiccation in particular can quickly lead to the elimination of sensitive species. Thus, in my experience, the cellulose-degrading *Cytophaga* species are easily lost in this way. On the other hand, many species of Cytophagales are completely desiccation resistant; I have been able to isolate strains of *Sporocytophaga*, *Flexibacter*, CLB, and *Taxeobacter* from samples that had been air-dried and stored for 5 to 10 years at room temperature.

Methods for the Isolation of Cytophagales

Small samples of promising natural material are inoculated either as individual spots or as one to three well-separated streaks onto the surface of dry water agar (WAT agar) plates. For marine samples, seawater agar (MS1 or SW2 agar) is used. Alternatively, media with a very low nutrient content may be used (CYT, MS4, SP2, SP6 agar). Finely divided samples such as soil may also be sprinkled over the plate, as a way of multi-spot inoculation. This can be advantageous because the organisms tend to stick to tiny particles and thus may be distributed in the sample very unevenly. To reduce the growth of fungi, cycloheximide (2.5 mg/100 ml) should be added to the isolation medium (not necessary with marine samples).

WAT Agar and WCX Agar

$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved. After autoclaving, 25 µg cycloheximide may be added per ml from a filter-sterilized stock solution for WCX agar.

MS1 Agar

Agar	1.5%

In natural seawater.
The pH is not adjusted. The medium is autoclaved.

SW2 Agar

Na acetate	0.002%
NH_4Cl	0.1%
Agar	1.5%

In artificial seawater (see below).
The pH is adjusted to 7.2. The medium is autoclaved.

Artificial Seawater (Dawson et al., 1969)

NaCl	24.7 g
KCl	0.7 g
$MgSO_4 \cdot 7H_2O$	6.3 g
$MgCl_2 \cdot 6H_2O$	4.6 g
$CaCl_2$ anhydrous	1.0 g
$NaHCO_3$	0.2 g

Dissolve in 1 liter water. To prevent precipitation, $CaCl_2$ and $NaHCO_3$ are autoclaved as separate stock solutions.

CYT Agar

Casitone (Difco)	0.1%
Yeast extract (Difco)	0.05%
$CaCl_2 \cdot 2H_2O$	0.05%
$MgCl_2 \cdot 7H_2O$	0.05%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved.

MS4 Agar

$(NH_4)_2SO_4$	0.1%	(autoclaved separately)
Glucose	0.2%	(autoclaved separately)
Agar	1.5%	

In natural seawater.
The pH is not adjusted. The medium is autoclaved.

SP2 Agar

Casitone (Difco)	0.1%
Yeast extract (Difco)	0.02%
Na acetate	0.002%
Agar	1.5%

In artificial seawater.
The pH is adjusted to 7.2. The medium is autoclaved.

SP6 Agar

Casitone (Difco)	0.03%
Yeast extract (Difco)	0.01%
Agar	1.5%

In artificial seawater.
The pH is adjusted to 7.2. The medium is autoclaved.

The plates are incubated at 30°C, at room temperature, or at 12°C, depending on the origin of the samples. Cultures started from aquatic sources are usually incubated at the lower temperature range because many aquatic CLB will not grow above 25 to 27°C.

The cultures are checked at regular intervals of 1 to 2 days for spreading colonies. It takes between 24 h and several weeks for spreading organisms to appear, depending on the organism, the incubation temperature, and the nutrient concentration in the culture, which may have been substantially augmented by the introduced sample material. As the spreading swarms are often filmlike and extremely delicate, the plates must be examined with a dissecting microscope equipped with a tiltable mirror so that side illumination can be used from below. Some experience is required before gliding organisms can be reliably distinguished from flagellated bacteria, which occasionally also spread out (Fig. 3b and 3d). A good distinguishing characteristic is usually the pattern of the swarm edge which, for gliding bacteria, is usually dentate with fine flamelike projections, while for flagellated organisms, it tends to be entire or coarsely lobed. However, certain *Bacillus* strains that form long filaments may also spread out with flamelike tongues at the edge of their colonies. Amebae or, in marine samples, gliding diatoms like *Nitzschia putida* may mislead the investigator. Usually a look through the microscope will quickly dispel all doubts; thus, e.g., no gliding bacterium is known that is able to swim actively (that is, move without being in contact with a surface).

The baiting technique used for the isolation of myxobacteria (see The Genus *Pelobacter* in this Volume) also often yields members of the Cytophagales. Living cells of *Escherichia coli* are streaked cross-wise or in three parallel lines onto water agar and inoculated with a suitable sample. The organisms develop along the *E. coli* streaks, often without lysing them, and spread as an extremely thin film, usually a monolayer, far out over the agar surface between the streaks. The technique is in essence nothing else than an enrichment in a low nutrient medium.

If the Cytophagales are present in sufficiently high numbers, the samples can be suspended in sterile water, diluted, and plated. Media with low nutrient concentrations, like CYT and SP6 agar, are well suited. Media with a somewhat higher nutrient concentration, like CY, SP2, and SAP2 agar, may also be used. They result in higher colony counts, but spreading of the gliding bacteria may be reduced or suppressed completely (Fig. 3i and 3j). Here, the color of the colonies may provide a useful lead. It should be kept in mind, however, that dilution plating is often not very successful, because the organisms tend to stick to particles and may be lost during the first few dilution steps.

CY Agar, CYG2 Agar, and CYCX Agar

Casitone (Difco)	0.3%
Yeast extract (Difco)	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved. The medium may be supplemented with 0.3% glucose, which is separately autoclaved, for CYG2 agar. If the medium is used for plating of dilution steps, 25 µg cycloheximide may be added per ml after autoclaving from a filter-sterilized stock solution, for CYCX agar.

SAP2 Agar

Tryptone (Difco)	0.1%
Yeast extract (Difco)	0.1%
Agar	1.5%

In artificial seawater.
The pH is adjusted to 7.2. The medium is autoclaved.

Water samples may be plated directly or upon dilution. Often, however, it will be necessary to concentrate the bacteria by passing the sample through a membrane filter. The filter is then incubated right-side up on a suitable culture plate, either as a whole or after having been cut into several sectors; the latter approach increases the chance of isolating several different organisms. If media with somewhat higher nutrients are used, like CY or SAP2 agar, the incubation time is shortened and, thus, the danger of overgrowth reduced. But spreading of the organisms may become more or less suppressed, so that members of the Cytophagales can only be recognized by their color and cell shape. The strategy that is best applied depends on the load of contaminants expected. Some of the smallest gliding bacteria can move through the filter pores (at 0.3- to 0.5-µm pore size) and form colonies on the agar surface below. This can be used as a selective technique. In this case, the filter should be removed from the agar surface after about one day to prevent contaminants from also moving through the filter.

Isolation of Nutritionally Specialized Organisms

AGAR DECOMPOSERS. Agar decomposers are very common in marine environments and can be obtained by the methods outlined above. They are easily recognized, because their colonies rest in shallow craters or in deep pits, and are surrounded by large, uncolored, "gelase" zones if the plate is flooded with iodine solution (Stanier, 1941; Veldkamp, 1961). Some organisms liquify the agar very quickly so that they can only be isolated if widely separated colonies are obtained after plating. Here, the filter migration technique described above is sometimes useful. A few agar-decomposing CLB have also been found in freshwater habitats near the sea coast, viz. *Cy. flevensis* (van der Meulen et al., 1974) and *Cy. saccharophila* (Agbo and Moss, 1979).

CHITIN DECOMPOSERS. Chitinolytic bacteria are very common among the Cytophagales. Samples are streaked out on chitin agar, e.g., CT6 or CT7 agar (for marine strains, on CT8 or CT9 agar). The chitin is only added in a thin top layer in order to conserve chitin, which is relatively laborious to prepare, and to prevent the chitin from settling on the bottom of the dish out of reach of the organisms; in addition, superior results are obtained in this way because the cleared zones in the opaque chitin layer appear sooner and become more pronounced (Stanier, 1947; Veldkamp, 1955, 1965). Good sources for chitin decomposers are soil, material from sewage plants, and the shells of dead crustaceans. All known chitin-degrading Cytophagales can also grow very well without chitin, e.g., on CY agar, and they can be isolated by the general techniques described above. Thus, they can be purified by plating on, e.g., CY or SAP2 agar.

CT6 Agar

Top layer:

$MgSO_4 \cdot 7H_2O$	0.1%
K_2HPO_4	0.02%
Agar	1.5%

The pH is adjusted to 7.5. After autoclaving, enough chitin is added to give an easily recognizable turbidity, which should be achieved with about 0.5% chitin. The chitin is taken from an autoclaved stock suspension of reprecipitated material; the suspension must be dense enough so that the volume to be added to the medium does not exceed 30%. The preparation of the chitin is described in The Genus *Lysobacter* in this Volume 6. The medium is poured as a thin layer on top of the following:

Base agar:

Casitone (Difco)	0.1%
Yeast extract (Difco)	0.05%
$MgSO_4 \cdot 7H_2O$	0.1%
Agar	1.2%

The pH is adjusted to 7.2. The medium is autoclaved.

CT7 Agar

Top layer as for CT6 agar. The base agar is WAT agar. On this medium only organisms that can use chitin as the only carbon and nitrogen source are able to grow.

CT8 Agar

Top layer:

K_2HPO_4 (autoclaved separately)	0.02%
Agar	1.5%

In artificial seawater.

The pH is adjusted to 7.5. After autoclaving, chitin is added, as for CT6 agar. The medium is poured on SW2 agar plates.

CT9 Agar

Top layer as for CT8 agar; base agar is SP2 agar.

CELLULOSE DECOMPOSERS. Decaying plant material or soil is sprinkled on filter paper that has been placed on mineral salts agar, e.g., ST6 agar (for marine organisms, SW5 agar may be used).

ST6 Agar and ST6CX Agar

$(NH_4)_2SO_4$	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
$MnSO_4 \cdot 7H_2O$	0.01%
$FeCl_3 \cdot 6H_2O$	0.02%
Yeast extract (Difco)	0.002%
Agar	1%
K_2HPO_4	0.1% (autoclaved separately)

After autoclaving, a filter-sterilized trace element solution is added, and, for crude cultures, 25 µg cycloheximide per ml from a filter-sterilized stock solution is added, for ST6CX agar. A recipe for a trace element solution is found in The Genus *Pelobacter* in this Volume.

SW5 Agar

$(NH_4)_2SO_4$	0.1%
Yeast extract (Difco)	0.02%
Agar	1%
K_2HPO_4 (autoclaved separately)	0.1%

In artificial seawater.

The medium is autoclaved.

It appears that the type of filter paper used does not make much difference; we get good results with thin, moderately dense, high-quality filter paper (68 g/m^2, ash 0.1 to 0.2%, more than 95% α-cellulose). Growth of cytophagas on mineral salts agar with cellulose is usually considerably stimulated by Ca^{2+}, Mn^{2+}, and $Fe^{2+/3+}$ (Bortels, 1956). While media with NO_3^- as the nitrogen source are often recommended in the literature, in my experience, media with NH_4^+ are far superior for the isolation of cytophagas and sporocytophagas, at least on agar media.

As an alternative, enrichment cultures may be started with strips of filter paper immersed in a mineral salts solution, e.g., Dubos medium or ST5 medium, in test tubes, so that part of the filter paper remains above the surface of the liquid. The tubes are then inoculated. After incubation for 1 to 3 weeks, glassy, translucent yellow to orange or red spots on the paper at the air-liquid interface indicate growth of cellulose-decomposing cytophagas and sporocytophagas. The yellow and orange spots usually give the flexirubin reaction when a drop of 10% KOH solution is added, but some (terrestrial) *Cytophaga* strains are flexirubin-negative. It appears that, in general, enrichment on plates gives better results because it allows the use of a heavier inoculum. However, an enrichment in liquid medium may be useful when habitats are investigated that are poor in cellulose degraders, such as marine environments. For the latter, good results have been reported with KM7 mineral medium and incubation at 25°C (Kadota, 1956). As already mentioned, fresh samples should be used for isolating *Cytophaga* strains.

Dubos Mineral Medium (Dubos, 1928)

$NaNO_3$	0.05%
K_2HPO_4 (autoclaved separately)	0.1%
$MgSO_4 \cdot 7H_2O$	0.05%
KCl	0.05%
$FeSO_4 \cdot 7H_2O$	0.001%

The pH is adjusted to 7.2. The medium is autoclaved.

ST5 Mineral Medium (Stanier, 1942)

$(NH_4)_2SO_4$	0.1%
K_2HPO_4 (autoclaved separately)	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
$CaCl_2 \cdot 7H_2O$	0.01%
$FeCl_3$	0.002%

The pH is adjusted to 7.0 to 7.5. The medium is autoclaved. For enrichment cultures, 25 µg cycloheximide is added per ml from a filter-sterilized stock solution.

KM7 Mineral Medium (Kadota, 1956)

$NaNO_3$	0.05%
K_2HPO_4	0.1%
$MgSO_4 \cdot 7H_2O$	0.05%
$FeSO_4 \cdot 7H_2O$	0.01%

In natural seawater.

The pH is adjusted to 7.2. The medium is autoclaved. This medium was originally developed for a different purpose, which explains the inclusion of relatively small amounts of $MgSO_4$ in a seawater medium.

The purification of cellulose decomposers may be a tedious procedure that requires skill and considerable patience, and it almost always takes several weeks or months. The first subcultures are best started on filter paper placed on mineral salts agar. Now, however, small (about 10×15 mm) rectangular filter strips are usually used and inoculated independently from different sites of the crude culture. The filter paper pieces, three or four per plate, are kept well separated to prevent the passage of contaminants from one to the other. Of course, organisms such as flagellated bacteria, amebae, ciliates, and nematodes will spread quickly within the water-soaked filter pad. Before further purification is attempted, they should first be eliminated. To get rid of amebae, which are almost always abundant in cultures started from soil, the plate is exposed to the vapors of a 5% ammonia solution for 2 min (M. Aschner, personal communication). After another one to two minutes of incubation, the cytophagas are transferred to a fresh plate. If there are very many amebae, the procedure may have to be repeated after the cellulose decomposers have grown up. If nematodes are still there, the plate is frozen at –80°C for 2 days. The cellulose decomposers are transferred to a fresh plate as soon as possible after thawing. Next, contaminating bacteria have to be removed, which is the most difficult step. After the load of contaminants has been reduced by repeated transfers to filter paper on mineral salts agar, the cellulose decomposer may be transferred to cellulose overlay agar; we have often had good results with CEL1 agar.

CEL1 Agar

Top layer:

$(NH_4)_2SO_4$	0.1%
Cellulose powder	0.5%
Agar	1.0%

Cellulose powder MN300 from Macherey and Nagel (Düren, Germany) works well.

The pH is adjusted to 7.2. The medium is autoclaved and then poured as a thin layer on top of ST6 agar plates.

The cellulose decomposers penetrate the agar medium and spread within the cellulose layer, concommitantly producing a clear lysis zone. Most or all of the contaminants stay close to the inoculation site, so that after 1 to 3 weeks, a pure, or at least improved, inoculum can be obtained from the edge of the lysis zone.

Favorable results are sometimes obtained by inoculating a small rectangle of filter paper that has been placed on top of cellulose overlay agar, preferably close to one side of the plate. In this case, the cellulose decomposers grow profusely on the filter paper, and from there they spread within the cellulose agar, while the contaminants tend to remain in the macerating filter paper where they find higher nutrient concentrations.

We also find carboxymethyl cellulose media, like ST10 agar, useful for purification of cellulose decomposers. On this medium, too, they spread within the substrate; this can be recognized from a slowly increasing, shallow depression in the surface of the plate. Growth is usually very thin and can barely be seen, if at all; but bacterial cells are found under the microscope if slide mounts are made from the depressed area. Transfers are made from the edge of the depression zone after some time.

ST10 Agar

$(NH_4)_2SO_4$	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
$FeCl_3$	0.02%
K_2HPO_4 (autoclaved separately)	0.1%
Casitone (Difco)	0.2%
Carboxymethyl cellulose	1.5%
Agar	0.6%

Carboxymethyl cellulose Sigma no. C-5013, sodium salt, high viscosity, works well.

After autoclaving, standard trace elements (see The Genus *Pelobacter* in this Volume) and, if used for contaminated strains, 25 µg cycloheximide per ml are added from filter-sterilized stock solutions.

Another technique for purification is to transfer the cellulose decomposers to a membrane filter (cellulose nitrate, 0.3 to 0.4 µm pore size) on cellulose overlay agar or, better, on a membrane of regenerated cellulose (e.g., no. 11604/50/N, pore size 0.8 µm, from Sartorius, Göttingen, Germany) on ST6 agar. The cytophagas will migrate through the membrane filter and produce colonies on the substrate below. If regenerated cellulose is used, these colonies appear particularly early and show a sharp margin. It may, however, still take from 1 to 3 weeks before colonies develop. They are often bright yellow and spread very slowly. With this technique, the time of removal of the top membrane is crucial, because other bacteria may soon grow through the membrane and contaminate the cellulose degraders again. Therefore, it is best to start several parallel cultures and to remove the top membrane at various different times, e.g., after incubation at 30°C overnight, for 1 day, and for 2 days.

With sporocytophagas, final purification is often possible by cautious heating of microcysts suspended in water. The heat resistance seems to vary substantially from strain to strain and probably also depends on the maturation stage. For example, the thermal death point was reached after 10 min at 68°C with one strain of *Sp. myxococcoides*, while another strain survived 10 min at 70°C; a strain of *Sp. ellipsospora* was killed after 10 min at 58°C (Imshenetsky and Solntseva, 1936; Sijpesteijn and Fåhraeus, 1949). We found that occasionally a strain may even survive boiling for 10 min. Results are usually satisfactory in our laboratory if we incubate microcysts at 58°C and take samples after 10, 20, and 40 min. The heated samples may be streaked on CEL1 agar, on filter paper on CY agar, or on CA2, CA13, or HP18 agar.

CA2 Agar (Mullings and Parish, 1984)

Base agar:

Agar	1.5%

Stock solution A:

KNO_3	7.5 g
K_2HPO_4	7.5 g

Dissolved to give 100 ml.

Stock solution B:

$MgSO_4 \cdot 7H_2O$	1.5 g

Dissolved to give 100 ml.

Stock solution C:

$CaCl_2 \cdot 2H_2O$	0.27 g
$FeCl_3$	0.15 g

Dissolved to give 100 ml.

After autoclaving, the base agar is supplemented with: (a) 1% (v/v) of each of the three stock solutions; (b) 1% (w/v) of glucose (from an autoclaved 20% stock solution); and (c) standard trace elements (see, e.g., The Genus *Pelobacter* in this Volume).

CA13 Agar

As for CA2 agar, but the KNO_3 in stock solution A is replaced by 0.5 g of $(NH_4)_2SO_4$.

HP18 Agar

Na glutamate	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
Agar	1.5%

The pH is adjusted to 7.2. After autoclaving, 0.1% glucose (from an autoclaved stock solution) and standard trace elements are added.

Of course, plating of diluted cell suspensions would seem to be the method of choice for the purification of cellulose-degrading cytophagas. However, this can only be done if the organisms are already enriched to near purity since colonies composed of single cells are apparently not produced by these organisms, not even on glucose-containing media, such as those just mentioned, that allow massive growth of pure cultures. Whenever isolated colonies are obtained upon plating, these colonies probably always start from cell clusters, and these are normally contaminated when heavily contaminated cultures are used to prepare the suspension. Further, the cellulose degraders produce a lot of slime which makes it difficult to suspend the cells uniformly in a liquid. After plating on cellulose-overlay agar, colonies which are surrounded by a lysis zone appear only after weeks of incubation, and this gives contaminants ample time to develop and overgrow the cellulose degraders. Gliding and spreading bacteria become a particular nuisance under such conditions. It may be mentioned here that, when glucose is used in a medium, care has to be taken to sterilize the glucose separately, either by filtration or by autoclaving, since cellulose-degrading cytophagas are extremely sensitive to toxic products arising when glucose is autoclaved together with other medium compounds (Stanier, 1942). Cellulose-degrading sporocytophagas and cytophagas have been known for many years (Hutchinson and Clayton, 1919; Winogradsky, 1929), but relatively few pure strains are available. They are very common in soil and decaying plant material (for their occurrence, isolation, and cultivation, see Fåhraeus, 1947; Imschenezki, 1959; Imshenetski and Solntseva, 1936; Kadota, 1956; Krzemieniewska, 1930; Krzemieniewska, 1933; Stanier, 1942; Stapp and Bortels, 1934; Veldkamp, 1965).

Pectin Decomposers. Pectin-decomposing CLB can be obtained by plating samples, e.g., lake water, on pectin overlay agar like PEK1 agar or on PEK7 agar. Pectolytic organisms cause shallow depressions in the pectin gel or liquefaction; on PEK7 agar, the colonies are surrounded by clearing zones in a background of finely divided, dispersed pectin flakes or granules, which may be clearly seen, however, only under a dissecting microscope. If such plates are flooded with iodine solution, colonies of pectolytic organisms are surrounded by light yellow halos in a brown plate. The preparation of pectin media is somewhat tricky, and the results depend decisively on the type of pectin used. We found pectin from apple, 38% esterified (from Roth, Karlsruhe, Germany), useful.

PEK1 Agar (Güde, 1973)

Base agar:

Part A:

NH_4Cl	1 g
$CaCl_2 \cdot 2H_2O$	3 g

In 100 ml water.

Part B:

K_2HPO_4	0.5 g

In 50 ml water.

Part C:

$MgSO_4 \cdot 7H_2O$	0.2 g
Agar	15 g
Tris HCl buffer, 1 M, pH 8.0	100 ml

In 750 ml of water.

After autoclaving the solutions are combined, standard trace elements (see The Genus *Pelobacter* in this Volume) are added, and plates are poured.

Top layer:

12 g pectin is slowly dissolved in 300 ml water of 70°C, the pH is adjusted to 7.2, and the medium is autoclaved for exactly 10 min at 110°C. The solution is poured as a thin layer on base agar plates and will form a gel within 5 h. During autoclaving, the medium may become dark, but this does not interfere with its usefulness.

PEK7 Agar

Top agar:

Casitone (Difco)	0.2%
Pectin	0.5%
$MgSO_4 \cdot 7H_2O$	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.2%

The pH is adjusted to 7.2. The medium is autoclaved and poured on CY agar plates.

Pectolytic CLB are very common in freshwater (Güde, 1973). They have also been isolated from soil samples after enrichment by percolation of a 1% pectin solution through the samples (Dorey, 1959; his *Fv. pectinovorum* is actually a CLB: Christensen, 1977b). The pectolytic CLB do not depend on pectin but may be stimulated by the presence of a carbohydrate like glucose. They can easily be purified by plating a diluted cell suspension on, e.g., CY agar + 0.3% glucose (= CYG2 agar) or on MYX agar.

MYX Agar

Na glutamate	0.5%
Yeast extract (Difco)	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%

Glucose (autoclaved separately)	0.2%
Agar	1.5%

The pH is adjusted to 7.2. The medium is autoclaved.

Fish Pathogens. CLB pathogenic for fish can usually be isolated and cultivated on relatively simple media. For freshwater strains, AO agar has been used with good results (e.g., Anacker and Ordal, 1955; Bullock, 1972; Pacha and Ordal, 1967; Snieszko and Bullock, 1976).

AO Agar (Anacker and Ordal, 1955)

Tryptone (Difco)	0.05%
Yeast extract (Difco)	0.05%
Beef extract	0.02%
Na acetate	0.02%
Agar	1.2%

The pH is adjusted to 7.2. The medium is autoclaved.

The agar concentration may be varied depending on the purpose for which the medium is used; a reduction to 0.9% is recommended for the first isolation from fish (for AOW agar) and to 0.4% for stock cultures (for AOS agar).

Infested material from gills, skin lesions, or internal organs, such as kidney or spleen, is streaked on agar plates and incubated at 12 to 18°C or at 30°C depending on the origin of the fish. Initially, media containing diluted fish infusion were used for isolation (Ordal and Rucker, 1944), but this seems not to be required, at least not in all cases, because similar results have been obtained with beef extract (Bernardet, 1989). However, sometimes, the fish pathogens grow very slowly at first and need an adaptation period before they can conveniently be handled (Ordal and Rucker, 194). This is not totally unusual, for it has been observed that a CLB that had been growing for a long time in a chemostat lost its ability to grow on plates, so that the plate count dropped to 20% of the viable cell count (Höfle, 1983). The medium used for isolation is, of course, not exclusive for pathogenic CLB, or CLB at all, and many kinds of saprophytic bacteria will grow on it as well. This may become a problem especially if the fish has already been dead for some time, because it is quickly colonized by putrefying organisms. A study of the drug resistance of a collection of *Cy. columnaris* strains revealed that the organism is resistant to a number of antibiotics (Fijan and Voorhees, 1969), and by the addition of neomycin (5 μg/ml) + polymyxin B (10 units/ml) to AO agar, the isolation yields could be improved (Fijan, 1969). The pathogenic CLB can be recognized on the plate by their yellow to orange colonies, which give a positive flexirubin reaction, and, in the case of *Cy. columnaris*, by a very typical, rhizoid-colony morphology. Spreading of the colonies is not always well pronounced, especially with *Cy. psychrophila*, but this organism can easily be recognized, because it does not grow at 30°C and, in contrast to environmental CLB, does not utilize sugars.

For isolating CLB pathogenic for marine fish AOW agar prepared with artificial seawater (= MAOW agar) or, for estuarine sources, with one-third-strength artificial seawater (= BAOW agar) is useful. Also, a richer medium, Marine Agar (Difco), which is a peptone (0.5%)-yeast extract (0.1%) agar (1.5%) in artificial seawater, has been used with good results (Hikida et al., 1979; Campbell and Buswell, 1982). In another case, the pathogen proved more fastidious and could only be isolated and cultivated on an agar medium containing 5% of an enzymatic digest of fish muscle in addition to peptone (0.1%) and yeast extract (0.1%) in seawater (Anderson and Conroy, 1969). In this case it was also shown that the organism depended on a high salt concentration and that a NaCl solution could not replace seawater. In fact, the fish—rainbow trout with severe lesions on the jaws and snout ("erroded mouth disease")—could be healed simply by transfer to fresh water. *Cy. columnaris*, on the other hand, is completely inhibited by 1% NaCl (Fijan and Voorhees, 1969; Pacha and Ordal, 1970).

The Genus *Capnocytophaga*. The capnocytophagas are normal inhabitants of the human oral cavity, where they can be found in tooth pockets and in the dental plaque. They may also occur in large numbers in periodontal disorders and infected lesions in the mouth. The genus was established by Leadbetter et al. 1979, but the organisms are very probably identical with the fast-gliding, anaerobic, slender rods discovered 20 years earlier during a study of fusospirilloses of the human oral cavity, which were described under the name *Sphaerocytophaga* (Gräf, 1961). In fact, they were known even before that time, but as *Bacteroides* species. Although the capnocytophagas have a strictly fermentative metabolism, they are not particularly sensitive to oxygen and grow both in the presence and in the absence of oxygen, provided an elevated level of CO_2 is supplied. The capnocytophagas are not fastidious at all and can easily be isolated and cultivated (Forlenza and Newman, 1983; Gräf, 1961; Leadbetter et al., 1979). Originally they were isolated on 10% sheep's blood agar. The samples were streaked onto the agar surface, and the cultures were incubated for 7 to 8 days at 37°C in Fortner plates, i.e., a co-culture with a facultative anaerobe. Spreading colonies were obtained, which showed a reddish hue and consisted of cells that were more or less yellow when scraped together (Gräf, 1961).

Leadbetter et al. 1979 recommended the following procedure: The sample material is quickly transferred to a screw-cap tube containing a modified Ringer's solution (NaCl, 0.9%; KCl, 0.042%; $CaCl_2 \cdot 2H_2$, 0.025%). Plaque material may be homogenized by a short burst of ultrasound (5 to 10 sec). Within 30 min after sampling, streaks are made on trypticase soy broth agar (TSB agar), which may be supplemented with 5% sheep's blood; the blood is, however, not essential. The cultures are incubated at 37°C in an atmosphere of $H_2 + CO_2 + N_2$ (GasPak, Baltimore Biological Laboratories = BBL). After 2 to 3 days, spreading colonies may be observed, but higher yields are obtained after 5 days (100% rather than 45%; Forlenza and Newman, 1983). There are three types of colonies—grey, pink, and yellow—but the cell masses of all three are yellow when scraped together. The shapes of the colonies depend critically on the ingredients used for preparing the medium: With stocks from other suppliers than BBL, nonspreading, small, compact colonies were obtained on the same type of medium (variations of this kind could, however, also be a result of using different batches of a medium component from the same supplier). The strains are purified by repeatedly streaking out material from swarm edges.

TSB Agar (Leadbetter et al., 1979)

Trypticase soy broth (BBL)	3%
Agar	3%

The pH is 7.3 (unadjusted). The medium is autoclaved.

Trypticase soy broth consists of:

Trypticase peptone	1.7%
Phytone peptone	0.3%
NaCl	0.5%
K_2HPO_4	0.25%
Glucose	0.25%

TSB agar may be supplemented with 5% sheep's blood (for TS-blood agar).

The capnocytophagas may also be first enriched by suspending sample material in trypticase soy broth without glucose in screw-cap tubes. After tightening the lids, the tubes are incubated at 37°C for 3 days. After that, large numbers of slender fusiform rods may be found in the liquid. Isolates are obtained by streaking the cell suspension on the media mentioned above.

It may be mentioned here that free-living CLB are also known that require CO_2 for fermentative growth on glucose—for example, the facultative anaerobe *Cy. succinicans* (Anderson and Ordal, 1961a, 1961b).

In addition to capnocytophagas, CLB that are not obligatory fermentative and that do not require CO_2 have also been found in dental plaque (London et al., 1982). The organisms could be isolated on blood agar media similar to those mentioned above, e.g., Columbia blood agar (BBL) or modified Schaedler agar with 5% defibrinated horse blood. The plates were incubated at 37°C in a GasPak atmosphere, but the bacteria can grow equally well in a normal atmosphere. The organisms were recognized by their spreading swarm colonies.

Purification

Purification of most members of the Cytophagales is relatively uncomplicated. It is usually sufficient to suspend, dilute, and plate samples from enrichment cultures by conventional methods. In some instances, e.g., with *Cy. columnaris*, the preparation of homogeneous suspensions may be difficult because the cells are embedded in a tenacious, sticky slime, but use of a small tissue homogenizer normally solves this problem. The plating medium should contain somewhat higher nutrient concentrations than the enrichment medium contains because high plating efficiency is desirable, and spreading growth is not required and should in fact be repressed. Some suitable media are CY agar, sometimes better with glucose (e.g., CYG2 agar); MYX agar; and, for marine strains, SAP2 agar. Often, pure strains are quickly obtained by repeatedly transferring material from the edges of spreading swarm colonies. These transfers should be made carefully, preferably by cutting out a little block of agar with the swarm edge using the sharp needle of a 1-ml disposable syringe. For this technique, media that favor gliding motility are, of course, preferable. The specific problems encountered with certain groups of the Cytophagales have already been discussed.

Cultivation

Most bacteria of the order Cytophagales can be cultivated on relatively simple media. VY/2 agar and CY agar have proved useful for all terrestrial and freshwater organisms except the cellulose decomposers. Cultures usually remain viable longer on VY/2 agar because growth is lighter and the pH is not changed as much as on CY agar. VY/2 agar is also useful for deciding whether an organism is a glider because it stimulates spreading growth. If heavier growth is desirable, CY agar may be enriched with glucose as, e.g., in CYG2 agar. Growth of some CLB is much stimulated by the presence of a carbohydrate. The cellulose decomposers grow very well on filter paper placed on CY agar. Other media suitable for their cultivation are listed above. If these gliding bacteria are cultivated on mineral

salts-glucose media (CA2 and CA13 agar) they often grow only if a heavy inoculum is applied.

VY/2 Agar

Bakers' yeast (by fresh weight of commercial yeast cake) 0.5%
$CaCl_2 \cdot 2H_2O$ 0.1%
Cyanocobalamin 0.5 µg/ml
Agar 1.5%

The cyanocobalamin is usually not required.

The pH is adjusted to 7.2. The medium is autoclaved. The yeast may be stored as an autoclaved stock suspension for several weeks. A uniform distribution of the yeast is obtained if the medium is melted before the yeast is added.

Many bacteria of the *Cytophaga* group produce heavy growth on HP6 agar, MYX agar, and similar media. Many will also grow well on mineral salts-glucose media like CA2 and CA13 agar. Special media that allow the detection of the ability of a strain to decompose certain macromolecules have already been mentioned. A medium useful for the demonstration of starch decomposition is STK2 agar.

HP6 Agar

Na glutamate	1%
Yeast extract (Difco)	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
Cyanocobalamin	0.5 µg/ml
Glucose (autoclaved separately)	0.5%
Agar	1.5%

The cyanocobalamin is usually not required.

The pH is adjusted to 7.2. The medium is autoclaved.

STK2 Agar

Base agar:

Casitone (Difco)	0.1%
Yeast extract (Difco)	0.05%
$MgSO_4 \cdot 7H_2O$	0.1%
Agar	1.2%

The pH is adjusted to 7.2. The medium is autoclaved.

Top agar:
As base agar, but agar concentration increased to 1.5%. The medium is prepared with only about 85% of the total water volume. In the remaining water are dissolved, referring to the total volume of the top agar, the following:

Soluble starch	0.2%
K_2HPO_4	0.02%

The starch solution should be heated on a water bath before autoclaving in order to prevent clumping of the starch. The two solutions are combined after autoclaving, and the mixture is poured as a thin layer on plates of the base agar. Starch degradation can be discovered if, after a suitable incubation time, the culture plates are flooded with an iodine solution, e.g., Lugol's solution diluted 1 : 5.

The fish pathogens can be cultivated on AO agar but usually also grow well on CY, MYX, and HP6 agar. Most strains do not utilize carbohydrates at all. The capnocytophagas grow on TSB agar with or without sheep's blood.

The same types of media can be used for the marine Cytophagales, only prepared with natural or artificial seawater instead of distilled water. No case is known in which the latter would not do, but a simple NaCl solution is often not suitable (e.g., Anderson and Conroy, 1969). The tolerated salinity range differs depending on the organism and often depends on the origin of the strain (e.g., Lewin and Lounsbery, 1969; Reichardt et al., 1983). Marine cellulose decomposers may be cultivated on filter paper placed, e.g., on SW5 agar. Alternatively, the mineral salts-glucose media as described for the terrestrial organisms may be used, when prepared with seawater.

The type of peptone added to a medium is not as crucial in the case of the Cytophagales as it is for myxobacteria; nevertheless, very different results may be obtained with different preparations and even with different batches. An example has already been given for the capnocytophagas. In our experience, enzymatic digests of casein, e.g., casitone (Difco) or peptone from casein (Marcor, Merck, and Oxoid), are the most universally useful peptones. Soy peptones also often give excellent results. Addition of yeast extract may stimulate growth appreciably. Again, not all preparations are equally satisfactory; good results are obtained, e.g., with Difco yeast extract. Further, it should be kept in mind that quite a few bacteria of this group grow on inorganic nitrogen sources, and it is always worthwhile to test an isolate for that possibility.

As a rule, members of the Cytophagales immediately produce homogeneous cell suspensions when inoculated into liquid media and shaken. Typically, the liquid shows a silky shimmer when rotated. Rarely, the bacteria grow in tiny granules or flakes, and then they probably always are surrounded by thick capsules or slime layers. The medium sometimes becomes more or less viscous, especially when sugars are included, and harvesting the cells by centrifugation may then make difficulties. In one such case, the addition of 10% $(NH_4)_2SO_4$ (w/v) resulted in satisfactory separation (Verma and Martin, 1967).

Most Cytophagales grow well in liquid media that contain peptone, e.g., CAS liquid medium or FXA liquid medium and, for marine organisms, SP5 liquid medium. Addition of a sugar, such as in FXAG liquid medium, may stimulate growth considerably and may even be a prerequisite for cultivation. Glucose, galactose, mannose, saccharose, and maltose are often readily utilized. The sugar has two effects: it is a convenient carbon and energy source, and it is partly transformed into acids that delay the rise of pH of the medium that results from ammonia production. Shifts of pH may cause problems in

shake cultures. Concentrations of buffers high enough to stabilize the pH reliably are often inhibitory to growth. With *Fx. filiformis* (former *Fx. elegans* Fx el), appreciable inhibition was observed at the following buffer concentrations (at a pH of about 7.0): citrate above 15 mM; phosphate, Tris HCl, and Tris-maleic acid-KOH above 20 mM; 3-morpholino-propane sulfonic acid (= MOPS) above 50 mM (E. Fautz, personal communication).

CAS Liquid Medium

Casitone (Difco)	1%
$MgSO_4 \cdot 7H_2O$	0.1%

The pH is 6.8 and needs no adjustment if Difco casitone is used. The medium is autoclaved.

FXA Liquid Medium

Casitone (Difco)	1%
Yeast extract (Difco)	0.2%
$MgSO_4 \cdot 7H_2O$	0.1%

Other similar, enzymatically digested, casein peptones may also be used, e.g., Peptone from casein, tryptically digested (Merck, Darmstadt, Germany).

The pH is adjusted to 7.0. After autoclaving, 0.2% of separately autoclaved glucose may be added, for FXAG liquid medium.

SP5 Liquid Medium

Casitone (Difco)	0.9%
Yeast extract (Difco)	0.1%

In artificial seawater.

The pH is adjusted to 7.2. The medium is autoclaved.

The fish pathogens can be cultivated in the same media mentioned for their isolation, e.g., AO broth (AO agar without agar) (e.g., Bernardet and Kerouault, 1989; Bullock, 1972; reference is not an exact match Pacha, 1968). A supplement to AO broth of 5% fish peptone, prepared from brook trout, has been recommended and gave superior results with some isolates, although it was not strictly required; it may sometimes be useful with fresh isolates (Kincheloe, 1962). For marine fish pathogens, AO broth in seawater or TCY liquid medium may be used (e.g., Hikida et al., 1979).

TCY Liquid Medium (Hikida et al., 1979)

Tryptone (Difco)	0.1%
Casamino acids, technical (Difco)	0.1%
Yeast extract (Difco)	0.02%
NaCl	3.13%
KCl	0.07%
$MgCl_2 \cdot 2H_2O$	1.08%
$CaCl_2 \cdot 2H_2O$	0.1%

In distilled water.

The pH is adjusted to 7.0 to 7.2. The medium is autoclaved.

One study showed that growth of *Cy. columnaris* can be substantially improved by a fine-tuning of the medium formulation (Song et al., 1988). The optimal medium was SO broth, which was a modification of a medium used by Shieh (1980), from which glucose, pyruvate, and citrate have been omitted, because these compounds had no effect on growth. The medium was much superior to tryptone-yeast extract and tryptone-yeast infusion broth. The generation time was reduced to 150 min compared to 210 min in the next-best medium.

SO Liquid Medium (Song et al., 1988)

Peptone	0.5%
Yeast extract	0.05%
Na acetate	0.001%
$BaCl_2 \cdot H_2O$	0.001%
K_2HPO_4	0.01%
KH_2PO_4	0.005%
$MgSO_4 \cdot 7H_2O$	0.03%
$NaHCO_3$	0.005%
$CaCl_2 \cdot 2H_2O$	6.7 mg/liter
$FeSO_4 \cdot 7H_2O$	1.0 mg/liter

The capnocytophagas are obligatory fermenters and grow luxuriantly in trypticase soy broth (TSB agar without agar) supplemented with 1% glucose; the cultures are incubated under the exclusion of oxygen (Forlenza et al., 1981).

Many Cytophagales can also be cultivated in simple synthetic media, e.g., SY liquid medium. Stabilization of the pH is especially difficult in this case and is effectively achieved only in bioreactors.

SY Liquid Medium

KH_2PO_4	0.07%
$Na_2HPO_4 \cdot 2H_2O$	0.14%
$(NH_4)_2SO_4$	0.2%
$MgSO_4 \cdot 7H_2O$	0.02%
$FeSO_4$	5 mg/l
$MnSO_4$	5 mg/l

After autoclaving, 1% glucose is added from a separately autoclaved stock solution.

Cellulose decomposers grow well in liquid media with powdered cellulose, e.g., in CEL1 liquid medium or in Dubos liquid medium with 0.5% cellulose. We use, with good success, a peptone-mineral salts medium with cellulose powder (= M9 liquid medium). Also, CAS liquid medium with cellulose powder (0.5%) often allows good growth. Further, many strains of cellulose degraders may be cultivated in glucose-containing liquid media such as GLU liquid medium (Kath, 1990; Verma and Martin, 1967).

CEL1 Liquid Medium

ST5 liquid medium is supplemented with 0.1% powdered cellulose (e.g., MN300 for chromatographic purposes from Macherey and Nagel, Düren, Germany), autoclaved separately as a stock suspension.

M9 Liquid Medium

Base medium:

NH_4Cl	0.1%
K_2HPO_4	0.075%
Casitone (Difco)	0.3%
Yeast extract (Difco)	0.09%

Supplement A:

$MgSO_4 \cdot 7H_2O$	4.5%

Supplement B:

$CaCl_2 \cdot 2H_2O$	0.2%
$FeCl_3 \cdot 6H_2O$	0.15%
$MnSO_4 \cdot H_2O$	0.15%

Supplement C:

Cellulose powder	10%

After autoclaving all solutions, 1% (v/v) of supplements A and B, 5% (v/v) of supplement C, and filter-sterilized standard trace elements (see, e.g., The Genus *Pelobacter* in this Volume) are added to the base medium.

GLU Liquid Medium (Kath, 1990)

The percentages indicated for each ingredient are with respect to the final volume of the medium.

Solution A:

NH_4Cl	0.1%
K_2HPO_4	0.075%
Casamino acids (Difco)	0.3%
Na-Fe^{III}-EDTA	0.002%
HEPES	1.19%

In 90% of the total volume.

The pH is adjusted to 7.2.

Solution B:

$MgSO_4 \cdot 7H_2O$	0.045%
$CaCl_2 \cdot 2H_2O$	0.01%
Glucose	0.5%

In 10% of the total volume.

After autoclaving, solutions A and B are combined, and standard trace elements are added from a filter-sterilized stock solution (see, e.g., The Genus *Pelobacter* in this Volume).

Most members of the Cytophagales can be grown without difficulties on a large scale in bioreactors. In ordinary batch cultures, typical yields obtained are between 10 and 15 g wet weight, or 2 and 4 g dry weight, per liter on peptone media with sugar; the ratio of dry to wet weight is 0.20 to 0.25. Generation times are in the range of one to several hours. Thus, e.g., *Fx. filiformis* Fx el grows in peptone media at 30°C with a doubling time of 2 h and 15 min; in mineral salts-glucose medium (SY liquid medium) it grows with a doubling time of 6 h and 20 min (H. Fink, personal communication). *Tx. ocellatus* grows at 30°C with generation times of 1 h and 50 min in peptone liquid medium, 2 h in dilute peptone medium, and 3 h and 30 min in casamino acids-glucose medium.

Little is known about specific mineral and vitamin requirements by members of the Cytophagales. At least, they appear not to be particularly fastidious in that respect. In a study on trace element effects on cellulose-degrading *Sporocytophaga* growing in a mineral salts-cellulose medium, a stimulation of cellulose decomposition by Ca^{2+}, Fe^{2+}, Mn^{2+}, and sometimes Cu^{2+} was demonstrated (Bortels, 1956). Certain Zn salts (ZnO, ZnS, 0.1%) were found to encourage growth of a series of cytophagas and CLB; $ZnSO_4$ (0.1%) suppressed swarming and slime production on plates, and its use in isolation media was suggested (Mehra et al., 1967). In a comparative study of a large number of different marine Cytophagales, a dependence on thiamine and cyanocobalamin was found in a few instances (Lewin and Lounsbery, 1969).

Except for the capnocytophagas, the Cytophagales are strict aerobes. Those that are known to grow anaerobically are usually facultative anaerobes. Some are able to respire on nitrate. Not one of the tested strains could use fumarate as an electron acceptor (Callies and Mannheim, 1978). Several Cytophagales require carbon dioxide for fermentative metabolism, so that care should be taken that enough CO_2 is present in the atmosphere when strains are being tested for anaerobic growth (GasPak, candle jar, Fortner plates, carbonate in the medium). The only strictly fermentative Cytophagales known so far are the capnocytophagas, which are, however, not oxygen sensitive but do require an increased level of CO_2; if that is provided, they can grow even under aerobic conditions (Leadbetter et al., 1979). They need CO_2 because they depend on the activity of phosphoenolpyruvate carboxykinase for growth (Kapke et al., 1980). There seem also to be *Capnocytophaga* strains that grow at CO_2 levels found in air. The existence of strictly anaerobic Cytophagales is not yet excluded.

All known Cytophagales are restricted to pH values between 6 and 8. Their temperature range is wide, from about 0°C to more than 40°C. On plates, most strains will grow more or less at 30°C. In contrast, liquid cultures of many strains, particularly those isolated from aquatic environments, grow only, or grow much better, at lower temperatures (usually between 18 and 26°C).

Preservation

Agar cultures of many Cytophagales stay viable for months when stored at low temperatures (2 to 8°C). At room temperature or higher, plate cultures can usually be kept for 2 to 6 weeks. In general, organisms from aquatic environments are more sensitive than those from soil. Media that are rich in peptone or contain higher concentrations of a sugar are always less suitable for

stock cultures, because they produce heavier growth and lead to unfavorable pH shifts. Good media for stock cultures are often VY/2, CYT, and SAP2 agar. For the fish pathogens, a reduction of the agar concentration in AO medium to 0.4% is advantageous. Cellulose decomposers are kept best on filter paper on top of CY or ST6 agar.

For long-term storage, essentially all standard methods for the preservation of bacteria can be applied to the Cytophagales. In our experience, the following methods give excellent results.

Drying in Skim Milk

From a young plate culture, a heavy cell suspension is prepared in about 0.7 ml of sterile skim milk. Three to five drops of this suspension are placed on a plug of skim milk predried by lyophilization in an ampule. The plug must not become solubilized but only wetted by the amount of added cell suspension. The loaded ampules are stored at 4 to 6°C until the whole set has been finished (not longer than one h) and are then dried in a desiccator over silica gel at a good vacuum for about 4 h at room temperature. The desiccator is kept overnight under vacuum, then pumped again for 15 min, and filled with nitrogen gas (preferably by attaching it to a nitrogen-filled balloon). Then the ampules are sealed and stored at room temperature or at 4 to 6°C (we do not yet know whether there is a difference in survival times). So far, organisms have been revived by us after up to 10 years of storage at room temperature. Successful lyophilization is also reported for *Cy. columnaris* (Anacker and Ordal, 1959). Lyophilized cultures of *Cy. columnaris* and *Cy. psychrophila* which had been kindly supplied to us by Dr. R.E. Pacha (Ellensburg, Washington) could be reactivated without problems after 26 and 20 years, respectively.

Skim Milk Preparation (for Preservation)

> 40 g of powdered skim milk (e.g., Oxoid L31; must be free of antibiotics) are dissolved in 200 ml water and filtered through gauze. The solution is distributed into tubes (5 ml per tube) or ampules (1 ml per 5-ml ampule) and autoclaved at exactly 115°C for 15 min. May be stored in the cold for several weeks.

Storage at Ultra-Low Temperatures

From a dense liquid culture, e.g., in FXA or CAS liquid medium, 2-ml amounts are pipetted into screw-cap tubes and placed directly into a deep freeze at –70 to –80°C. Samples of marine organisms, e.g., in SP5 liquid medium, should be kept in an inclined position during freezing to prevent cracking of the tubes. Cellulose decomposers have been successfully frozen in CEL1 liquid medium.

Alternatively, a heavy loopful of cells from a plate culture is deposited in 1 ml of a peptone-containing liquid medium, like the ones mentioned above, and frozen in the same way. The peptone medium can also be used for cellulose decomposers. Fish pathogens can be frozen at –70°C as agar cultures in AO agar with 0.9% agar; they were found to remain viable for at least one year (Fijan and Voorhees, 1969). Of course, all cultures for preservation should be young, well-growing cultures in an optimal state.

Alternatively, cell suspensions in peptone liquid media may be stored frozen in liquid nitrogen. A special freezing program is not required. We routinely add 5% dimethyl sulfoxide (DMSO) to the suspension medium, although our experience suggests that this is not necessary. In a systematic study on cryopreservation of Cytophagales, mainly marine ones, good survival in liquid nitrogen was demonstrated when the cells were suspended in a medium containing 10% glycerol, while 10% DMSO proved less suitable. Also without additives, at least one cycle of freezing and thawing was usually well tolerated. Many strains remained viable even for some weeks at –22°C without additives, but eventually, after varying storage times, most strains died (Sanfilippo and Lewin, 1970).

For revival, the frozen tube or ampule is quickly thawed, best by placing it into cold water. Immediately after the medium has become liquid, the bacteria are transferred to a suitable growth medium. For reviving a culture, it is advisable to use several different agar and liquid media in parallel, because one or the other may be more appropriate depending on the organism. Also, we found that facultative anaerobes are occasionally better reactivated under anaerobic conditions; they sometimes refuse to start to grow in an aerobic atmosphere, even when they thrive ordinarily very well under such conditions. Thus far we have tested survival at –80°C for up to 15 years and in liquid nitrogen for up to 10 years and have not yet observed any losses.

Storage in 50% Glycerol at –25°C

Using a young plate culture, a heavy cell suspension is prepared in 50 mM phosphate buffer, pH 7.2. Of this suspension, 1 ml is transferred to a screw-cap tube that contains 2 ml of 87% glycerol precooled to –25°C. The suspension is thoroughly mixed and stored at –25°C in an ordinary household deep freeze. Marine organisms may be suspended in SP5 liquid medium; 1 ml of the suspension is then transferred to 2 ml of precooled 87% glycerol. Alternatively, a heavy

loopful of cells is suspended directly in precooled SP5 liquid medium + 87% glycerol (1:2, vol/vol).

To start an active culture, a loopful of the suspension is streaked on a suitable agar medium. The remaining suspension may be returned to the deep freeze, provided the temperature was kept low during handling. CLB preserved by this method may survive for at least several years (I. Hirsch, personal communication), but we have in some cases observed losses, particularly with marine strains, so this technique is advisable for long-term storage, especially when better alternatives are available.

Storage in 0.9% Saline or in Distilled Water

Starting from young plate cultures, cell suspensions are prepared in 0.9% saline or in distilled water. The cell density should not be very high; we usually prepare a suspension of about 10^7 to 10^8 cells/ml (a suspension just faintly turbid), and this stock suspension is then diluted fivefold. Then, 0.5-ml amounts of both suspensions are transferred in parallel to 1-ml ampules, which are sealed under air and stored at room temperature (18 to 22°C) in the dark. Marine organisms may be suspended in artificial seawater. In our laboratory, a number of *Flexibacter* and CLB strains stored in saline for 7 years could be reactivated with ease and produced heavy growth within 2 days; it is likely that they would have survived for even longer periods. However, the organisms probably are not completely at rest in such suspensions and continue to grow, even if extremely slowly, so that over a long period variant strains may arise.

Characterization

Cell Morphology and Cytology

The cells of all Cytophagales without exception are rod-shaped (Fig. 1 and 2). The proportions and dimensions of the rods vary, however, over a wide range. The CLB have very short to moderately long rod cells; the short ones are often relatively fat, and the longer ones may be rather slender; they typically measure 0.5 to 1.0 × 2 to 8 μm; the cell ends are usually slightly tapered. The cells of *Capnocytophaga* resemble those of CLB with moderately long rods. *Taxeobacter* has relatively plump cylindrical rods with rounded ends. The rod cells of the cellulose decomposers (*Sporocytophaga*, *Cytophaga*) tend to be moderately long and very delicate, measuring 0.3 to 0.7 × 3 to 8 μm; *Cytophaga* cells are often spindle-shaped. *Flexibacter* and (*Microscilla*) have long thread cells which contain either very few or no cross-walls and typically measure 0.4 to 0.8 × 10 to 50 μm. Many species readily form spheroplasts. Especially in cultures of cellulose-decomposing true cytophagas, large numbers of lemon-shaped intermediary forms can often be seen (Fig. 1f). This was a source of much confusion for the early investigators (who had no phase contrast microscopes), and fancy developmental cycles have been constructed on such observations (for a review, see Imschenezki, 1959). Inflated, spindle-shaped rods may still be motile and viable and may recover, but it is doubtful whether such stages are of any advantage for the organism, e.g., for a rearrangement of the genetic apparatus, as has been suggested. Lemon-shaped degeneration forms are also typical for *Taxeobacter.* Under starvation, a *Cy. johnsonae* strain was observed to produce coccoid cells which looked like spheroplasts, did not show a peptidoglycan layer, often had lost most of their cytoplasm, and were osmotically stable (Reichardt and Morita 1982). Nevertheless, they were found to be viable, and at least some of them could grow out again into rods. It was suggested that they represent survival forms. Spheroplastlike cells have also been interpreted as resting stages in several other Cytophagales (e.g., Gräf, 1961, 1962; Bauer, 1962), and generic names have even been proposed based on such observations ("*Sphaerocytophaga,*" "*Sphaeromyxa*"). But the evidence for a function as resting forms is not convincing in any of those cases.

Several Cytophagales change their cell morphology in response to environmental conditions. Marine *Flexithrix dorotheae* is described to grow quasi-mycelially with long, sheathed filaments that release short, gliding fragments, which may again grow into filaments (Lewin, 1970). Unfortunately, the type strain, which is the only known strain of that organism, does not show this behavior any more; at least I have not been able to verify it in spite of many efforts.

The vegetative cells of *Sporocytophaga* may convert by shortening and fattening into small (diameter 1.2 to 1.4 μm), spherical, optically refractile microcysts. The microcysts are desiccation resistant; we have isolated strains from dry soil samples that had been stored for up to 9 years at room temperature. Their high heat tolerance, which allows them to survive at 60°C, in some cases even at 100°C for a short time, has already been mentioned.

Fx. filiformis (Fx el) and related species go through a spectacular shape change in the course of culture development (Fig. 2c to 2e). When inoculated into a liquid medium, e.g., CAS or MD1 liquid medium, the organism grows into long (15 to 50 μm and more), slender, flexible cells. These cells are not the usual filaments or

trichomes, for they contain no cross-walls or septa, or only very few widely separated ones (10 to 30 μm); I therefore call them thread cells. The thread cells are extremely agile and can glide, bend, twist, and wriggle like little worms. When the culture ages, the cells become shorter and shorter and may finally end up as very short coccobacilli (this stage is not always reached). The shorter rods also become slightly fatter and darker, which clearly indicates that the shortening is not simply a fragmentation of the thread cells. Under certain conditions the organism may grow exclusively in the long or in the short form. One factor that determines the length of the cells is obviously population-density connected. The same cycle can also be observed in plate cultures, e.g., on VY/2 agar, on which fast-spreading swarms are produced. Here, agile thread cells are found at the edge, while shorter and shorter rods are found moving towards the center of the colony. Below a certain length, the rods become nonmotile. The same developmental cycle has been described for *Chitinophaga*, only there the coccobacilli were interpreted as microcysts (Sangkhobol and Skerman, 1981); this appears, however, not to be correct. A study on the length control of *Flexibacter* strain FS-1 revealed that the long thread cells grew exponentially with a generation time of 90 min at 30°C. When the culture was shifted to 35°C they divided into three or four cells within the following 120 min. The culture continued to grow exponentially with about the same growth rate, but with shorter cells. When the culture was shifted back to 30°C, the shorter cells stopped dividing immediately but continued to grow, and the thread cells reappeared. When the thread cells divided, the constriction always occurred in the middle of the cell. The biochemical and genetic mechanisms that control all these fascinating morphogenetic processes are not at all understood and have not been thoroughly studied. In particular, *Fx. filiformis* should provide a beautiful model system for such investigations (Humphrey and Marshall, 1980; Poos et al., 1972; Reichenbach et al., 1974; Simon and White, 1971).

The fine structure of the cells of the Cytophagales is essentially that of typical Gram-negative bacteria, with a few remarkable peculiarities. The outer surface of the cells is usually corrugated or undulated and densely covered with cushionlike knobs and folds (e.g., Bovallius, 1979; Follett and Webley, 1965; Humphrey et al., 1979; Oyaizu et al., 1982; Strohl, 1979; van der Meulen et al., 1974). Often, vesicular and long, uneven, tubular extensions of the outer membrane and LPS can be seen (e.g., Follett and Webley, 1965; Holt et al., 1979a; Kuhrt and Pate, 1973; London et al., 1982; Martin et al., 1968; Pate and Ordal, 1967a; Pate et al., 1967; Simon and White, 1971). These strands may become as long as 0.5 μm and have been shown to contain LPS components, like 2-keto-3-deoxyoctonate (Humphrey et al., 1979). All Cytophagales excrete slime, sometimes in substantial quantities, and this slime can usually be distinguished as a dense network of fine fibril surrounding the cells (e.g., Bovallius, 1979; Humphrey et al., 1969; Martin et al., 1969; Pate and Ordal, 1967b; Ridgway and Lewin, 1973; Ridgway et al, 1975; Strohl and Tait, 1978; Verma and Martin, 1967). The microcysts of *Sp. myxococcoides* are encased in a massive, fibrillar capsule, about 180 nm thick (Holt and Leadbetter, 1967; the diameter of the microcysts as determined in this study was an astonishing 2 to 2.5 μm, which is much greater than is usually measured in the light microscope [1.2 to 1.4 μm]).

The cell envelope of the Cytophagales consists of a thin peptidoglycan layer and the usual outer membrane. In marine, filamentous *Fx. polymorphus* (clearly different from *Flexibacter* as defined here and of uncertain taxonomic position), an unusual S-layer was discovered on top of the outer membrane (Ridgway, 1977; Ridgway and Lewin, 1973, 1983; Ridgway et al., 1975). The layer consists of densely packed, goblet-shaped subunits, which have a complicated structure and are perhaps rooted in the cytoplasmic membrane. Another very interesting structural detail has been detected in the cell envelope of certain CLB (Pate and Chang, 1979). Here, tiny rings were seen and could be isolated from cell lysates. The rings were about 20 nm wide and 10 nm thick and were arranged in long chains that seem to form a large, regular, netlike superstructure. It was hypothesized that these rings might be homologous with the flagellar rotors and that their rotation would somehow propel the cell. It may be added here that the mechanism of gliding motility has been studied for many years with several Cytophagales, but so far without a clear answer (e.g., Burchard, 1984; Chang et al., 1984; Duxbury et al., 1980; Glaser and Pate, 1973; Godwin et al., 1989; Lapidus and Berg, 1982; Pate, 1985; Speyer, 1953; Wolkin and Pate, 1984). What can be said is that there is some motility apparatus in the cell surface, because latex beads move along the surface of immobilized cells (Lapidus and Berg, 1982); that the excreted slime is essential for gliding because it acts as a Stefan adhesive allowing easy translocation but preventing efficient detachment of the cells from the substrate (Humphrey et al., 1979); that the cells rotate around their long axis during gliding (Godwin et al., 1989); and that the energy for gliding may not be provided by ATP, at least not exclusively (Pate and Chang, 1979; Ridgway, 1977). The speed of gliding may be on the order of 100 to 150 μm/min, with large deviations in

both directions, depending both on the organism and on environmental conditions (e.g., Duxbury et al., 1980; Garnjobst, 1945; Lapidus and Berg, 1982; Speyer, 1953; Stanier, 1942).

Fine longitudinal fibrils, which were arranged in wide bands just below the outer membrane, have been discovered in *Cy. columnaris* (Pate and Ordal, 1967b). They were suggested to represent the machinery of gliding motility. The fibrils could indeed not be seen in nonmotile mutants (Glaser and Pate, 1973). In freeze-etched preparations, tiny beads of about the same diameter as the fibrils can be seen on the inner surface of the outer membrane (Burchard and Brown, 1973). When the cells are fixed with glutaraldehyde before being fractured, those beads arrange themselves in long lines which resemble fibrils. Thus it seems possible that the fibrils seen in ultrathin sections are merely fixation artifacts.

Lysing cells of *Cy. columnaris* release unusual tubular structures that appear to derive by invagination from the cytoplasmic and mesosomal membranes (Pate et al., 1967). They are composed of lipid and protein, although the number of protein bands is much reduced in comparison with the cytoplasmic membrane (Kuhrt and Pate, 1973). Similar tubules are also known from other Gram-negative bacteria including *Sporocytophaga* (e.g., Holt and Leadbetter, 1967; Martin et al., 1968; Yamamoto, 1967); they are not identical with rhapidosomes as has occasionally been suggested. The latter were originally observed in *Saprospira* (see The Order Cytophagales in this Volume for a detailed discussion); they were later found also in other bacteria, e.g., *Sp. myxococcoides* (Pate et al., 1967), and may be phage tails.

The cells of the Cytophagales divide in the usual way by formation of a septum, which is normally connected with a mesosome (e.g., Holt and Leadbetter, 1967; Poos et al., 1972; Ridgway et al., 1975).

Appendages like fimbriae and pili seem never to have been observed with Cytophagales. Pili were, however, isolated from *Fv. branchiophilum*, where they supposedly are important for the attachment of this fish pathogen to gill filaments (Heo et al., 1990).

In several *Capnocytophaga* strains, acid and alkaline phosphatases could be localized by cytochemical and immunochemical reactions in the periplasmic space; in fact the enzymes were associated with the outer membrane, probably the LPS, and about 12% of the activity was exposed on the outer cell surface. Most of the enzyme molecules were released from the cells when the cultures became older, a good part of them in a membrane-bound form (reference is not an exact match Poirer and Holt, 1983b, reference is not an exact match 1983c). While the bacteria normally cannot penetrate the periodontal tissue it seems conceivable that enzymes like the phosphatases may do so and thus are responsible for damage inflicted to the host by *Capnocytophaga*.

Chemical Composition

The peptidoglycan appears as a clearly distinguishable layer in ultrathin sections of all Cytophagales studied in this respect; it is between 2 and 5 nm thick, but increases to 9 nm in *Sporocytophaga* microcysts (e.g., Pate and Ordal, 1967a; reference is not an exact match Verma and Martin, 1967a). The murein sacculus has been isolated from *Cy. hutchinsonii* and *Sp. myxococcoides* and chemically analyzed (Verma, 1970; Verma and Martin, 1967a, 1967b). It had the composition of a typical Gram-negative peptidoglycan with L-Ala, D-Glu, *m*-DAP, D-Ala, and 70% direct cross-linking between *m*-DAP and D-Ala. Diaminopimelic acid (0.2 to 0.3% of the dry weight) was also observed in an early study on two flexibacters; the diamino acid came most probably from the cell wall (Holm-Hansen et al., 1965). The same organisms appear also to contain bound ornithine (0.08 to 0.7% of the dry weight); the origin of that amino acid is obscure (Holm-Hansen and Lewin, 1965; it could come from an ornithine-containing lipid, see below). Also, the lipopolysaccharide (LPS) of the Cytophagales seems to closely resemble that of the enterobacteria. The LPS of *Fx. filiformis* Fx el (= Fx $^{1}/_{2}$) was found to contain mannose, galactose, glucose, rhamnose, ribose, glucosamine, galactosamine, and small quantities of xylose, arabinose, 2-keto-3-deoxyoctonate (KDO), and heptose (Rosenfelder et al., 1974). From 17 strains of CLB and one strain each of *Cy. aurantiaca* and *Sp. myxococcoides*, the LPS was extracted with phenol, and its sugar composition was subsequently analyzed (Sutherland and Smith, 1973). LPS yields were between 0.4 and 3.4% of the dry weight (in addition, substantial amounts of polysaccharide could be harvested from the culture supernatant by ultracentrifugation). The LPS of all strains contained a high proportion of ribose (in the absence of an appreciable ultraviolet [UV] absorption at 260 nm), mannose, and glucosamine; further, most LPS preparations yielded glucose, galactose, and galactosamine, and 7 out of 19 also rhamnose. The hexosamines remained after removal of lipid A by mild hydrolysis. Because of analytical problems, KDO and heptose could not be determined reliably; but KDO always appeared to be present, whereas heptose remained questionable and was

there in very small amounts, if at all. The LPS of *Flexibacter* BH3 contained galactose, glucose, arabinose, rhamnose, glucosamine, deoxysugar, and KDO, but heptose could not be detected (Humphrey et al., 1979). An endotoxin, which was highly active immunologically as well as in the *Limulus* test, was isolated from a CLB; its main carbohydrate constituents (above 0.5% of the total) were rhamnose, fucose, mannose, galactose, glucose, glucosamine, galactosamine, and an astonishing 34% of (tentatively identified) heptose; KDO is not mentioned (Flaherty et al., 1984). The inner and outer membrane of *Cy. johnsonae* were separated and showed densities of 1.18 and 1.14 g/cm^3, respectively; the outer membrane contained the sulfonolipids that will be discussed later, but little or no KDO (Godchaux and Leadbetter, 1988). Occasionally, members of the Cytophagales produce copious amounts of extracellular slime. In the case of *Cy. hutchinsonii* and *Sp. myxococcoides*, that slime was found to be an acidic heteropolysaccharide consisting of xylose, arabinose, mannose, glucose, and glucuronic acid; the slime of *Sporocytophaga* also contains galactose (Martin et al., 1968; Verma and Martin, 1967b). This slime seems to be broken down enzymatically in the later stages of the culture. *Flexibacter* BH3 produces an extracellular, water-soluble, glycoprotein slime with glucose, fucose, galactose, and some uronic acid in the carbohydrate part (Humphrey et al., 1979). The extracellular slime of *Cy. columnaris* was determined to consist of a homopolymer of α-(1-4)-linked D-galactosamine (Johnson and Chilton, 1966).

The fatty acid patterns of the Cytophagales consistently show two peculiarities (Collins et al., 1982; Fautz et al., 1979; Godchaux and Leadbetter, 1984; Kath, 1990; Liebert et al., 1984; Oyaizu and Komagata, 1981; Walker, 1969):

1. The dominant species are branched fatty acids, mainly 15:0 (15 to 45% of the total; in *Capnocytophaga* up to 77%; Collins et al., 1982). Branching is mostly of the iso type, but small amounts of anteiso fatty acids are usually also present. Only *Cy. hutchinsonii* contains larger quantities of anteiso fatty acids, almost exclusively 17:0 (26% of the total), but as the identification was tentative, this should be reinvestigated (Walker, 1969). It was also stated that *Capnocytophaga* and *Sporocytophaga* exclusively contain anteiso fatty acids (Holt et al., 1979a), but at least for *Capnocytophaga* this could not be corroborated (Collins et al., 1982). In one study, 1 to 3% of the total fatty acids from CLB have been identified with (delta 17)-cyclopropane fatty acids (Oyaizu and Komagata, 1981). Other major fatty acids are straight chain 16:1, 16:0, and 15:0, usually in that order. The double bond has been found to be in the *cis* configuration and in the C-11 to C-12 position (Kath, 1990; Oyaizu et al., 1982; Walker, 1969).

2. There are always substantial quantities (15 to 55% of the total fatty acids) of 2- and 3-hydroxy fatty acids (Collins et al., 1982; Fautz et al., 1979, 1981; Kath, 1990; Liebert et al., 1984; Oyaizu and Komagata, 1981; Oyaizu et al., 1982). In *Capnocytophaga*, only 3-hydroxy fatty acids could be identified (Collins et al., 1982). Only a small part of these hydroxy fatty acids seem to rise from LPS, where, in addition, exclusively 3-hydroxy fatty acids are found (Rosenfelder et al., 1974).

Most of them rather appear to have their origin in novel sulfonolipids, the capnoids, which were originally discovered in *Capnocytophaga* but have since been isolated from many other Cytophagales and may indeed be a chemosystematic marker for that group (Godchaux and Leadbetter, 1980, 1983, 1984). The capnoids are essentially sulfonated sphingolipids, in which the hydroxyl on C-1 of the sphinganine base, capnine, is replaced by a sulfonic acid group. Free capnine was found only in *Capnocytophaga*; normally the amino group on C-2 bears an amide-bonded, long-chain, fatty acid as in true ceramides. The sulfonilipids may comprise as much as 20% of the cell's total lipids and are localized in the outer membrane (Godchaux and Leadbetter, 1988). It appears that there is a connection between sulfonolipids and gliding motility, for capnoid-negative mutants of *Cy. johnsonae* are no longer able to glide, but gliding may recover when sulfonolipid synthesis is restored (Abbanat et al., 1986). The biosynthesis of the capnines is presumably due to the reaction of cysteine or, more likely, of cysteic acid with a matching fatty acyl-CoA compound (Abbanat et al., 1985; White, 1984).

The fatty acid pattern of marine *Fx. polymorphus* is unique among the Cytophagales in that it consists of relatively low amounts of (mainly iso-) branched species and large quantities of polyunsaturated fatty acids, viz. 18% 20:5 (Johns and Perry, 1977). This, together with the ultrastructural details discussed above, suggests that this organism may not be correctly classified among the Cytophagales.

It is interesting to note that very similar lipid patterns to those just discussed have been demonstrated in nonmotile but phylogenetically related flavobacteria, i.e., a high proportion of branched and of 2- and 3-hydroxy fatty acids (e.g., Dees et al., 1979; Fautz et al., 1981; Moss and Dees, 1978; Oyaizu and Komagata, 1981; Yabuuchi et al., 1983; Yano et al., 1976) and the occurrence of capnoids (Godchaux and

Leadbetter, 1983) and typical sphingophospholipids and ceramides (e.g., Asselineau and Pichinoty, 1983; Yabuuchi et al., 1983; Yano et al., 1983).

The complex lipids of the Cytophagales are still incompletely known. In all organisms that have been investigated (*Capnocytophaga*, *Cy. hutchinsonii*, *Sp. myxococcoides*) phosphatidylethanolamine was by far the major phospholipid (Collins et al., 1982; Holt et al., 1979c; Iizuka et al., 1987; Walker, 1969). The phospholipid content of the capnocytophagas was between 30 and 36% of the total cellular lipids when estimated as acetone-soluble lipid. The organisms also contained an ornithine lipid, some phosphatidylserine, substantial amounts of a polyprenol, and 5% squalene; two laboratories report the absence of phosphatidylglycerol (Collins et al., 1982; Holt et al. 1979a) while in a third laboratory (Iizuka et al., 1987), this compound was detected along with phosphatidylinositol and cardiolipin. In *Cy. hutchinsonii*, 45% of the dominating 16:1 fatty acid was recovered from phosphatidylethanolamine; no other phospholipid could be demonstrated in this organism, but it contained substantial quantities of two unidentified glycolipids (Walker, 1969).

The respiratory quinones of the Cytophagales are exclusively menaquinones (Callies and Mannheim, 1978; Collins et al., 1982; Kleinig et al., 1974; Oyaizu and Komagata, 1981). Different species contain either MK-6 or MK-7, and the distribution of the two types may be of taxonomic significance. Thus, all cellulose decomposers contain MK-7 and differ in that respect neatly from most terrestrial CLB, which are presently classified with them in the same genus but contain MK-6 (M.D. Collins, personal communication). The respiratory quinone of *Capnocytophaga* is MK-6 with traces of MK-5 (Collins et al., 1982), and that of *Flexibacter* is MK-7 (Kleinig et al., 1974). Again, the true flavobacteria are also menaquinone organisms (Callies and Mannheim, 1978; Oyaizu and Komagata, 1981).

Most Cytophagales synthesize pigments. Apart from an occasional strain that produces a dark, diffusing, probably melaninlike substance (e.g., "*Cy. krzemieniewskae*": Stanier, 1941; *Fv.* (*Cy.*) *uliginosum*: ZoBell and Upham, 1944), the pigments are always cell-bound. Chemically they are of two kinds, either carotenoids or flexirubin-type pigments. The two pigment types may occur in the same organism but then are typically localized in different places: the carotenoids in the cytoplasmic and the flexirubins in the outer membrane (Irschik and Reichenbach, 1978).

It had been suggested for many years that the yellow and orange pigments that can be extracted from many Cytophagales might be carotenoids (e.g., Anderson and Ordal, 1961a; Fox and Lewin, 1963; Lewin and Lounsbery, 1969; Verma and Martin, 1967a). Unequivocal proof of zeaxanthin (4,4′-dihydroxy-β,β-carotene) could be provided for the yellow pigment of marine *Ft. dorotheae* (Aasen and Liaaen-Jensen, 1966c); the same pigment was demonstrated by chromatographic comparison in marine *Cy. lytica* (Lewin and Lounsbery, 1969), and it was later chemically identified also in a terrestrial CLB (*Cy. johnsonae*: Achenbach et al., 1978b). Two monocyclic ketocarotenoids, flexixanthin and deoxyflexixanthin, have been isolated from a flexibacter (probably *Fx. roseolus*: Lewin and Lounsbery, 1969); their chemical structures have been elucidated (Aasen and Liaaen-Jensen, 1966b). *Cy. diffluens* appears to contain the monocyclic dihydroxy carotenoid saproxanthin (Lewin and Lounsbery, 1969), which was originally isolated from *Saprospira grandis* and identified by chemical methods (Aasen and Liaaen Jensen, 1966a).

A novel pigment type, the flexirubins, which later turned out to be of considerable chemosystematic relevance, has been discovered in *Fx. filiformis* (formerly *Fx. elegans* Fx el: Achenbach et al., 1974, 1976; Reichenbach et al., 1974). The chromophore is an omega-phenyloctaenic acid which is connected via an ester bond to a resorcinol bearing two hydrocarbon chains (Fig. 4). This basic chemical structure may be modified by variation of the length and branching of the hydrocarbon chains on the resorcinol and by the introduction of additional substituents on the omega-phenyl ring, specifically methyl and chlorine; actually, for all pigment species, chlorinated counterparts are found in every flexirubin-producing organism (for reviews of their chemistry and biosynthesis, see: Achenbach, 1987; Achenbach et al., 1978). In this way, a large variety of different flexirubin-type pigments arise, and one single strain may synthesize more than 25 different compounds (Achenbach et al., 1979). Still, certain structural types seem to be characteristic for certain taxa, e.g., a methyl in the *meta* position on the omega-phenyl ring seems to occur only in *Flexibacter*. Biosynthetically, the omega-phenyl ring with the first three carbon atoms of the chain derives from tyrosine, the rest of the chain from acetate, and the resorcinol ring with its hydrocarbon substituents derives from acetate and various starter molecules, like propionate or isovalerate, via an orsellinic acid homologue; only the methyl on the omega-phenyl comes from methionine; the linking of the two halves of the molecule is the last biosynthetic step (Achenbach et al., 1972, 1982, 1983). A total synthesis of flexirubin has also been performed (Achenbach and Witzke, 1977). Flexirubin-type pigments have been found so far only in the Cytophagales and in the

true flavobacteria (Achenbach et al., 1981; Reichenbach et al., 1981; Weeks, 1981). However, not all Cytophagales contain flexirubin-type pigments, and even closely related species, and perhaps even strains of the same species, may differ in that respect. Still, the presence of these pigments in a strain appears to be a reliable criterion that the isolate belongs to the Cytophagales and flavobacteria. The existence of flexirubin-type pigments in a strain can be demonstrated in three ways: 1) As already mentioned, the colonies show a reversible color shift when covered with a 20% KOH solution (see color plate I in *The Prokaryotes*, 1st edition). 2) This preliminary test can be confirmed by a simple thin-layer chromatography of an acetone extract of the bacteria, with an extract of a known flexirubin-producer as a reference; this test can be made even more reliable by recording the absorption spectra of the eluted spots and by performing the alkali reaction in the cuvette (Reichenbach et al., 1974). 3) Finally, as the only other bacterial pigments that give a similar color reaction appear to be aromatic carotenoids (Kohl et al., 1983), the pigments may be specifically labeled by feeding radioactive tyrosine (flexirubins) or mevalonic acid (carotenoids); the result is analyzed by autoradiography of the chromatograms (Fautz and Reichenbach, 1980). Incidentally, the color reaction of cellulose-decomposing cytophagas or sporocytophagas with alkali was already observed long ago, although its meaning was not understood at the time (Fåhraeus, 1947; Walker and Warren, 1938).

Occasionally, strains of CLB with a thin, film-like growth show a striking greenish-reddish iridescence (see color plate I in *The Prokaryotes*, 1st edition). This is probably a physical color due to interference effects. Also, it has been suggested that the greenish color that is sometimes observed in cultures of Cytophagales may arise from the combination of a yellow cell mass and dark melaninlike pigments (Lewin and Lounsbery, 1969).

Pigment production by Cytophagales may be influenced by environmental conditions. Thus, *Cy. succinicans* is colorless when grown anaerobically, but yellow-orange under aerobic conditions (Anderson and Ordal, 1961a). Illumination may stimulate carotenoid synthesis; but the synthesis of the flexirubins is light-independent. In the latter case, however, the specific pigment content of the cells varied substantially with some other culture parameters; in particular, a high phosphate content and a low pH appear to reduce pigment synthesis (Reichenbach et al., 1974).

Little else is known about the chemical composition of the Cytophagales. The amino acid composition of the protein of a cellulose-decomposing cytophaga was analyzed and found to be rich in nutritionally valuable, essential amino acids (Chang and Thayer, 1975). The GC content of the DNA of the Cytophagales varies over a wide range from 28 to 65 mol% (e.g., Behrens, 1978; Kath, 1990; Mandel and Leadbetter, 1965; Mandel and Lewin, 1969; Oyaizu and Komagata, 1981). The base sequences of large chunks of the 16S rRNA of a series of *Cytophaga*, *Sporocytophaga*, and CLB strains have been determined (Kath, 1990). An unusual multicopy, single-stranded DNA-RNA species (msDNA) discovered in myxobacteria (see The Genus *Pelobacter* in this Volume) was also found in *Fx. filiformis* (= *Fx. elegans* Fx el), but it was ruled out for *Cy. johnsonae* and *Tx. ocellatus* (Dhundale et al., 1985); it should be interesting to reinvestigate the isolated occurrence of msDNA in *Flexibacter* with the inclusion of other strains.

Phages and Bacteriocins

Phages have been reported for several CLB and appear not to be difficult to obtain (Anacker and Ordal, 1955; Kingsbury and Ordal, 1966; Pate et al., 1979; Richter and Pate, 1988; Stürzenhofecker, 1966; Valentine and Chapman, 1966). There is considerable morphological variability. Most of the phages bear a tail, which usually seems to be contractile. However, two temperate phages have been isolated from *Cy. johnsonae* strains that appear to have no tails; these phages also were sensitive to chloroform, and their plaque formation was inhibited by agar (Richter and Pate, 1988). In all cases that have been investigated, the phages contained double-stranded DNA. A phage of *Cy. columnaris* could not be propagated in the presence of streptomycin (70 μg/ml), probably because the antibiotic prevented the injection of the phage DNA into the host, which for its part was resistant to streptomycin (Kingsbury and Ordal, 1966). Much higher phage yields were often observed when $CaCl_2$ (1 to 4 mM) was present during infection. Interestingly, nonmotile mutants of *Cy. johnsonae* were completely resistant to all phages tested on them (Pate et al., 1988; Wolkin and Pate, 1986).

A bacteriocinlike activity was discovered in a strain of *Sp. myxococcoides* (Tchan and Giuntini, 1950). The strain inhibited other *Sp. myxococcoides* strains, but not *Cy. aurantiaca*; the activity could not be propagated, and no phage particles could be demonstrated under the electron microscope. Bacteriocin activity was found in many strains of the fish pathogen *Cy. columnaris* (Anacker and Ordal, 1959b). The target specificity of the bacteriocins produced by different *Cy. columnaris* strains varied, so that with the aid of seven selected bacteriocin preparations, 134 strains could be grouped into nine sensitivity

classes. No connection could be seen between a certain bacteriocin class and its distribution in nature. The same results were observed for serological classes of *Cy. columnaris* (Anacker and Ordal, 1959a). Bacteriocin activities were recorded in 20 out of 30 tested strains of *Cy. johnsonae* (Richter and Pate, 1988). The activities were destroyed by protease treatment and acted bactericidally on sensitive strains. With respect to their specificity, two types could be distinguished: 1) bacteriocins that acted on motile and nonmotile strains and 2) ones that, like the phages discussed above, attacked only motile strains. With the latter, nonmotile mutants could be selected.

The occurrence in various members of the Cytophagales of rhapidosomes, which appear to be tails of defective phages, has already been mentioned.

Colony Morphology

The colonies of the Cytophagales typically are spreading swarms (Fig. 3). Spreading growth depends, however, on several environmental factors, so that organisms that are well able to move by gliding still may not produce swarms (e.g., Chang et al., 1984; Perry, 1973; Wolkin and Pate, 1984). Thus, e.g., 70% of yellow bacteria isolated from sewage plants showed gliding motility under the microscope, but only 25% also formed spreading colonies (Güde, 1980). It has been found in many cases that low nutrient concentrations favor swarming (e.g., Agbo and Moss, 1979; Bauer, 1962; Garnjobst, 1945; Wolkin and Pate, 1984). Therefore, if spreading is to be observed, agar media with a low peptone content (0.1% or less) should be used; also, VY/2 agar stimulates swarming in many cases. Other factors that may influence the spreading behavior are the kind of nutrients supplied, temperature, and humidity. No general rules can be given; however, often a relatively low temperature and a high humidity favor spreading.

The morphology of the swarm colonies varies very much, as is to be expected with such a large assembly of widely differing organisms. Often the swarms are almost unstructured, thin sheets; sometimes their surface is covered with flat mounds, tiny warts, or veins or it appears fibrous and feltlike; or the swarms form a network which may become confluent towards the center of the colony. While the swarm sheet is always somewhat slimy, the production of copious amounts of slime is relatively rare; it is observed, e.g., when the cellulose decomposers are grown on a glucose-containing agar (see also Strohl and Tait, 1978). The slime is usually soft, so that the cells can easily be scraped off the plate; but sometimes it becomes very tough, and the cell mass can hardly be removed from the agar surface, e.g., with *Cy. columnaris* (Garnjobst, 1945) and *Cy. uliginosa.* The organisms also often penetrate the agar, and some species even grow and spread preferentially within the plate, e.g., microaerophilic and agar-attacking organisms (e.g., Veldkamp, 1961). Colony variations have been reported for several species (Anderson and Ordal, 1961a; Bachmann, 1955; Oyaizu et al., 1982; Pacha, 1968; Stanier, 1942, 1947; Veldkamp, 1961). In one case, migrating microcolonies were observed (Strohl and Tail, 1978). The colonies of the cellulose decomposers on filter paper are slowly expanding, glassy, unstructured, slimy patches that are usually bright yellow to orange. Grey areas in the colonies of *Sporocytophaga* usually signal generous microcyst formation.

A sickening odor has been reported for *Cy. columnaris* on nutrient agar (Garnjobst, 1945). On peptone-containing media, and particularly with liquid cultures, a strong, cheesy smell is produced by many CLB. The cellulose degraders produce a pleasant sour-fruity odor when growing on glucose agar.

Physiology and Enzymology

With a few exceptions, the physiology and enzymology of the various members of the Cytophagales have not been thoroughly investigated, and virtually no information is available about most basic biochemical pathways. Further, many of the studies were published a long time ago, and a reevaluation of the data in the light of modern biochemical insights would be desirable. On the other hand, nothing discovered so far suggests that the metabolism of these bacteria would differ in some basic manner from the metabolism of other comparable organisms.

Most Cytophagales are strict aerobes, but there also are microaerophilic species, facultative anaerobes, and organisms with a strictly fermentative metabolism (Anderson and Ordal, 1961a, 1961b; Bachmann, 1955; Hirsch, 1980; Leadbetter et al., 1979; Stanier, 1947; Veldkamp, 1961). It appears that all Cytophagales that are capable of fermentative growth require elevated CO_2 levels (optimum 0.3 to 1% $NaHCO_3$: *Cy. fermentans, Cy. succinicans, Cy. salmonicolor, Capnocytophaga*). For strictly fermentative *Capnocytophaga* it has been shown that CO_2 is necessary because the organism depends on phosphoenolpyruvate carboxykinase (PEPCK) in order to synthesize ATP and the terminal electron acceptor, oxalacetate; the latter is reduced under regeneration of NAD to the level of succinate, which is then excreted as the major fer-

mentation product. Obviously PEPCK replaces in that organism the more usual pyruvate kinase. The enzyme is regulated by the energy charge of the cell: low ATP levels stimulate, high ones inhibit PEPCK activity (Kapke et al., 1980). A condensation of CO_2 with phosphoenolpyruvate was also suggested for fermentatively growing *Cy. succinicans* (Anderson and Ordal, 1961b). The reaction was stimulated by GDP. A CO_2-independent fermentation of fructose-1,6-diphosphate could be performed in cell extracts supplemented with lactate dehydrogenase. The data could be well reconciled with the mechanism outlined for *Capnocytophaga*. As *Cy. succinicans* is a facultative anaerobe, it is likely to have alternative pathways starting from PEP. The fermenting organisms may utilize various sugars, mannitol, and polysaccharides such as agar or starch; however, as a rule, organic acids are not suitable fermentation substrates; one exception is *Cy. succinicans*, which grows anaerobically on pyruvate. Fermentation products are mainly succinate and acetate, usually also propionate and formate, and sometimes small quantities of lactate and ethanol. *Cy. salmonicolor* produces CO_2 and H_2 during fermentative growth (Veldkamp, 1961).

Nitrate respiration allows vigorous growth of *Cy. johnsonae* var. *denitrificans* (Stanier, 1947). More recently, a CLB was isolated that grew anaerobically with NO_3^-, NO_2^-, and N_2O as terminal electron acceptors (Adkins and Knowles, 1984, 1986). If small quantities of sulfide (0.4 μM) were available, this organism was able to reduce N_2O even in the presence of acetylene in concentrations (4 kPa) that normally inhibit N_2O reduction. This is also of interest because acetylene-inhibited N_2O reduction is generally accepted as a measure of denitrification in soils. However, other denitrifying CLB (*Cy. johnsonae* strains) did not show acetylene-insensitive N_2O reduction (Adkins and Knowles, 1986). The strains differed in their ability to reduce various nitrogen compounds. In one case, N_2O reduction turned out to be inducible by nitrite, but not by nitrate. Anaerobic growth under reduction of nitrite or N_2O has also been found among flavobacteria (Pichinoty et al., 1976). Growth with fumarate as the terminal electron acceptor was not possible with any of the Cytophagales and flavobacteria tested, but several of the organisms, e.g., *Cy. hutchinsonii* and *Cy. johnsonae*, could use fumarate for limited NAD regeneration under anaerobic conditions (Callies and Mannheim, 1978). As already mentioned, no strict anaerobes are known among the Cytophagales.

It appears that all Cytophagales, including the cellulose decomposers, grow on organic nitrogen compounds, e.g., peptones, as the sole nitrogen source. Many also use the same compounds for carbon and energy and grow vigorously on peptone media, even if, as a rule, carbohydrates are the preferred carbon and energy sources. Some organisms, however, require the addition of a carbohydrate, e.g., apparently all cellulose decomposers. On the other hand, there are also organisms that cannot utilize carbohydrates at all, e.g., *Cy. psychrophila* (Pacha, 1968) and many *Cy. columnaris* strains (e.g., Song et al., 1988a).

Many Cytophagales can be cultivated on inorganic nitrogen compounds. NH_4^+ is usually preferred to NO_3^-. The latter can sometimes not be utilized at all, thus by *Cy. fermentans* (Bachmann, 1955). The spectrum of utilizable carbon compounds is wide and varies with the individual organisms. Glucose is almost always accepted. One exception is the *Cy. diffluens* group: These bacteria require some other sugar like galactose or sucrose (Lewin and Lounsbery, 1969). Acid is often produced from carbohydrates even under aerobic conditions (e.g., Oyaizu and Komagata, 1981; van der Meulen et al., 1974). *Sp. myxococcoides* metabolizes glucose via the Embden-Meyerhof-Parnas pathway (Hanstveit and Goksøyr, 1974), as does *Cy. johnsonae* (Reichardt and Morita, 1982a).

Many of the Cytophagales are able to degrade biomacromolecules, particularly all kinds of polysaccharides, like agar, starch, cellulose, yeast cell-wall β-glucan, succinoglycan (Oyaizu et al., 1982), pectin, alginate, and heparin. As many of the responsible exoenzymes potentially are of some practical interest, these activities will be discussed under "Practical Aspects," this chapter.

DNA and RNA are efficiently degraded by many Cytophagales (e.g., Greaves et al., 1970; Mitchell et al., 1967). A particularly strong DNAse activity is observed with *Cy. columnaris*. It seems, however, that no organism is able to grow solely on nucleic acids. Phosphatases are very common among the Cytophagales (e.g., Christensen, 1977a; Hirsch, 1980; Reichardt et al., 1983). An alkaline and an acid phosphatase of *Cp. ochracea* have been isolated and studied in detail (Poirier and Holt, 1983b). The enzymes are able to remove phosphate from phosphoseryl residues of phosvitin and may play a role in the dephosphorylation of phosphoproteins. CLB are able to grow on washing-powder polyphosphates as phosphate sources; the polyphosphates are well tolerated up to 0.0025% and are rapidly hydrolyzed (Ruschke and Köhn, 1970). Lipolytic activities are also known from Cytophagales, e.g., cleavage of tributyrin and of Tweens (e.g., Christensen, 1977a; Hirsch, 1980; Jooste et al., 1985; Oyaizu et al., 1982; Reichardt et al., 1983), but the responsible enzymes seem never to have been characterized.

The patterns of antibiotic sensitivities have been determined for many members of the Cytophagales, although the data often are not really comparable because of differences in methods (e.g., Agbo and Moss, 1979; Arlet et al., 1987; Christensen, 1977a; Forlenza et al., 1981; Gräf and Morhard, 1966; Reichardt et al., 1983; van der Meulen et al., 1974; Warke and Dhala, 1968). It appears that many organisms are relatively resistant to penicillins, polymyxin B, aminoglycosides, and chloramphenicol. Kanamycin resistance has been used in the isolation of cellulose-decomposing *Cytophaga* and *Sporocytophaga* strains (2.5 to 10 mg kanamycin sulfate per liter); but it is not known whether these organisms are always resistant to that antibiotic (Rivière, 1961a). Capnocytophagas respond to several conventional antibiotics and are thus easy to control.

A genetics of the Cytophagales is still nonexistent.

Classification and Identification

As mentioned in the introduction, 16S rRNA studies have shown that the Cytophagales are a main branch, or phylum, of their own in the bacterial phylogenetic system (Paster et al., 1985). They are found there together with some other, phenotypically rather different bacteria; although our current knowledge suggests that some common characteristics may indicate connections between those groups, on this basis, probably, nobody would have dared earlier to propose the existence of a natural relationship among all those bacteria. Thus, e.g., *Capnocytophaga* resembles *Bacteroides* morphologically and metabolically to such a degree that it was formerly classified in this genus. Also, sphingolipids, in general rare in bacteria, are found in *Bacteroides* and in certain flavobacteria (but also in nonrelated myxobacteria). Further, the 16S rRNA tree of descent demonstrates unequivocally that the present genera of the Cytophagales are heterogeneous and that we may even need new definitions for families and perhaps orders.

The order Cytophagales is defined here using conventional phenotypic characteristics so that new isolates can be classified relatively easily and reliably. As discussed in the introduction, only part of the group of phylogenetically related bacteria is included in the phenotypically defined order. As defined here, the order Cytophagales contains unicellular, Gram-negative, rod-shaped, gliding bacteria that often exhibit a pronounced cellular shape change or at least pleomorphism. Most species produce more or less brightly colored, yellow, orange, pink, or red colonies. The yellow and orange ones often give a positive flexirubin reaction. The respiratory quinones are exclusively menaquinones.

This definition presents us with two problems: First, a practical one—how to distinguish cytophagas from *Lysobacter*, which shares many of the above-mentioned characteristics. Differences in cell shape and pigmentation, as mentioned in the introduction, would quickly differentiate most, but unfortunately not all, Cytophagales from *Lysobacter*. In case of doubt, more subtle characteristics have to be examined. It seems that all lysobacters degrade chitin, while many Cytophagales do not. The GC content of *Lysobacter* DNA is high, between 65 and 70 mol%, values that are only reached by red *Taxeobacter* among the Cytophagales. The respiratory quinones of *Lysobacter* are ubiquinones (Q-8), while all Cytophagales appear to contain menaquinones exclusively (M.D. Collins, personal communication). Finally, no marine and no parasitic lysobacters have been found so far.

The second problem is how to integrate the flavobacteria into the order Cytophagales. The old genus *Flavobacterium* was plainly heterogeneous, but after exclusion of the high-GC-content species, the remaining, low-GC-content (30–45 mol%) species appear to be naturally related among themselves and with the Cytophagales. Those species of flavobacteria for which 16S rRNA catalogs exist are connected to the phylogenetic tree of the Cytophagales at various levels and are often found close to the gliding species. This suggests that the genus *Flavobacterium* is still heterogeneous and that a gliding species may be more closely related to a nonglider than to another glider. The situation became even more confusing after several organisms that were originally described as nonmotile and consequently classified as *Flavobacterium* species were later discovered to glide; they would therefore have to be classified among the cytophagas. This is the case for the type species of *Flavobacterium*, *Fv. aquatile*, and for *Fv. pectinovorum*, *Fv. uliginosum*, and *Fv. heparinum*. The (nonmotile) flavobacteria also have, in common with the Cytophagales, menaquinones as the only respiratory quinones and often have flexirubin-type pigments. Therefore, most probably, gliding motility will lose its determining character in this taxonomic group, and eventually there may be order(s), families, and perhaps even genera that comprise motile and nonmotile species, just as there are in groups of flagellated bacteria. But before the new boundaries can be defined, more data, especially molecular and chemosystematic ones, are needed.

As set out above, the Cytophagales are restricted here to gliding organisms which can be differentiated into genera according to the following key:

Key to the Genera of the Cytophagales

1. Cellulose decomposers that grow on filter paper as the only carbon source; may utilize inorganic or organic N sources; cannot grow on peptone alone but require in addition a carbohydrate, like cellulose (filter paper) or a suitable sugar (e.g., glucose) 2

1′. Do not attack filter paper cellulose; usually grow well on peptones, but some are much stimulated by carbohydrate or depend on it for fermentative growth 3

2. Produce microcysts . . . . . *Sporocytophaga*

2′. Do not produce microcysts, terrestrial . . . . . *Cytophaga*

2″. Do not produce microcysts, marine . . . . . *Cytophaga* (?)

3. Strictly fermentative; require CO_2 and carbohydrate for growth; inhabitants (mainly) of the oral cavity of man and mammals . . . . . *Capnocytophaga*

3′. Strict aerobes or facultative anaerobes . . . 4

4. Vegetative cells in young cultures are long (20 to 100 μm) thread cells 5

4′. Vegetative cells in young cultures are short to moderately long (2 to 15 μm) rods 6

5. Soil and freshwater bacteria; several species show a very conspicuous (cyclic) cellular shape change: in young cultures, long (20–30 μm), extremely agile thread cells; in older cultures, short immotile rods or coccobacilli; many strains contain flexirubins; GC content, 40 to 50 mol% . . . . . *Flexibacter*

5′. Marine organisms; cellular shape change not observed . . . . . *Microscilla*

5″. Marine organisms; very long (sometimes multicellular and nonmotile) filaments which may or may not have a sheath and release shorter, but often still rather long, gliding thread cells; colonies yellow due to zeaxanthin, no flexirubins; GC content, 37 mol% . . . . . *Flexithrix dorotheae*

6. Short, stout rod cells with rounded ends, often arranged at the edges of the colonies in a palisadelike fashion; colonies more or less brick red; GC content, 55 to 65 mol% . . . *Taxeobacter*

6′. Cell shape very variable: short to very short, but sometimes also moderately long (5 to 15 μm) rods, delicate or plump, with rounded or tapering ends; colonies often fast spreading, filmlike swarms, yellow to orange, marine organisms sometimes pink; many terrestrial isolates contain flexirubin-type pigments, marine isolates usually do not; in soil, freshwater, and marine habitats; some are fish pathogens; GC content, 30 to 40 mol% . . . . . *Cytophaga*-like bacteria (CLB)

(6″.) Cells are relatively short, cylindrical rods with rounded ends, but often long thread cells or cell chains are also found in cultures; colonies (slowly) spreading, often rather slimy, pale grey to greenish yellow, brick red, or brown due to diffusing pigments; respiratory quinone is Q-8; GC content, 65 to 70 mol%: *these organisms do not belong to the Cytophagales* . . . (*Lysobacter*)

The cellulose decomposers were historically the first cytophagas described, and thus taxonomically they represent the type of the whole group. For many years it has been suggested that these cellulose decomposers are rather specialized organisms that are not closely enough related to the other organisms of the group to be united with them in one genus. Experimental evidence for this hypothesis has been provided recently: Surveys of the respiratory quinones of gliding bacteria showed that many CLB contain MK-6, but all cellulose decomposers contain MK-7 (Oyaizu and Komagata, 1981; M.D. Collins, personal communication). Quantitative fatty acid analyses, DNA-DNA hybridization data, and especially comparisons of 16S rRNA base sequences suggest that the two type strains of the named species of the cellulose-decomposing cytophagas and of several new isolates are relatively closely related among themselves. Further, it could be shown that *Sporocytophaga* strains are equally closely related among themselves and, finally, that the latter are relatively closely related to the cellulose-decomposing cytophagas, but that other CLB, like *Cy. johnsonae* and *Cy. heparina*, are much farther off from both (Kath, 1990). The conclusion is that the genus *Cytophaga* should indeed be restricted to the cellulose decomposers. New genera will have to be defined for the other organisms hitherto classified in the genus *Cytophaga*, but this should not be done until after the boundaries between the various groups have been clearly established by molecular taxonomy. In the present situation it may be best to follow the practice used in the present review and to group these other organisms under the label *Cytophaga*-like bacteria (CLB). *Sporocytophaga* appears to be a separate and equally natural genus, at least as far as cellulose decomposers are concerned. Sporocytophaga isolates can easily be recognized by the presence of microcysts; these are usually pro-

duced in enormous numbers in enrichment cultures, and their formation can also be reliably induced with pure strains that no longer produce them in ordinary plate cultures when the strain is grown in M9 liquid medium (Kath, 1990). *Sp. cauliformis* appears to be a misnomer (Gräf, 1962a): It seems neither to decompose cellulose nor to form microcysts and probably belongs to the large assembly of CLB.

Another source of confusion is the distinction between *Flexibacter* and *Cytophaga.* The definition of the genus *Flexibacter* given here is based on the original description by Soriano (1945, 1947), but it is in disagreement with that used in *Bergey's Manual*, eighth edition (Leadbetter, 1974). The definition of *Flexibacter* has recently been changed again in *Bergey's Manual of Systematic Bacteriology* (Reichenbach, 1990), where it is now placed in accordance with the ideas presented here. (A detailed explanation of the rather complex taxonomic situation may be found in the latter reference, but it will not be repeated here.) The organisms united in the present genus *Flexibacter* have long, highly flexible cells. The organisms also have in common a somewhat higher GC content of 40 to 50 mol% (Behrens, 1978; Mandel and Lewin, 1969) and they all seem to contain MK-7 as respiratory quinone (M.D. Collins, personal communication). The type species is *Fx. flexilis*, which shows rather sluggish movements and is flexirubin negative. Much more fascinating is *Fx. elegans* Soriano (1945), which for many years was regarded as the typical *Flexibacter* and about which many details have been published, especially about strain Fx el (e.g., Achenbach et al., 1978; Behrens, 1978; Fautz et al., 1979, 1981; Hirsch, 1979; Paster et al., 1985; Poos et al., 1972; Rosenfelder et al., 1974; Reichenbach et al., 1974, 1981; Simon and White, 1971). In young cultures and at the edges of spreading swarms, the organism has long and extremely agile thread cells. These cells change their shape in the later stages of the culture, becoming shorter and shorter and finally becoming nonmotile (Fig. 2; Poos et al., 1972; Simon and White, 1971). Under certain conditions, the organism may grow exclusively in the short form, but the cells readily grow out into thread cells again if conditions are changed. Soriano (1945) did not mention any shape change, perhaps because he did not observe it, perhaps because he thought his cultures were contaminated, or perhaps because he regarded his organisms to consist of multicellular filaments and therefore judged fragmentation to be commonplace. But his description of *Fx. elegans*, and especially of the movements of the thread cells, leaves no doubt that his and our organisms are at least closely related. It was later discovered that there existed in the literature an even earlier description that exactly fitted *Fx. elegans* as outlined above, shape change, color, and physiology included—that of *Myxococcus filiformis* by Solntseva (1940), whose only mistake was to misinterpret the short cells as myxospores. Unfortunately, the situation was further confounded by the description under the name of *Fx. elegans* of an organism that quite obviously was not identical with Soriano's species (Lewin, 1969; Lewin and Lounsbery, 1969). As the name *Fx. elegans* has been conserved with Lewin's stain as the type species, it has been proposed to change the name of the organisms discussed above into *Fx. filiformis* (Reichenbach, 1990). To complicate things even more, an organism has been described more recently under the name of *Chitinophaga*, without any reference to the publications cited above, which is clearly closely related or identical with *Fx. filiformis* (Sangkhobol and Skerman, 1981). In this case, microcysts have again been postulated, but the published pictures are not at all convincing, and when studying the type strain the present author has never observed anything even remotely resembling a microcyst (H. Reichenbach, unpublished observations). In the 16S rRNA phylogenetic tree, *Fx. filiformis* Fx el is found in a side branch far away from *Sporocytophaga* and the CLB (Paster et al., 1985). It remains to be established whether the genus *Flexibacter* in its present state is really homogeneous. The genus is restricted so far to soil and freshwater organisms; many, but not all, contain flexirubin pigments, which have in fact been discovered in strain Fx el. The marine counterpart of *Flexibacter* may be *Microscilla*; no cellular shape change is known for this genus (Lewin, 1969; Lewin and Lounsbery, 1969; Pringsheim, 1951). It has not been well studied, and the status of the genus and its relation to the other Cytophagales are unknown.

The CLB are unquestionably a very large and heterogeneous assembly of terrestrial and marine bacteria. They will probably have to be distributed over several new genera, as discussed above. In the absence of a sound taxonomy, it is not even remotely possible at the moment to estimate the number of existing species. From DNA-DNA hybridization data and other studies it may be deduced that there are many more genera than are named so far. Some efforts have been made to establish a reliable taxonomy for the fish pathogens, which would obviously be of great practical importance. DNA-DNA hybridization data showed that the fish pathogens *Cy. columnaris*, *Cy. psychrophila*, and *Cy. maritima* are all well-separated, homogeneous, genomic species, which show little relatedness among themselves or to the many environmental species included in the studies (Baxa et al., 1987; Bernar-

det and Grimont, 1989; Bernardet and Kerouault, 1989; Bernardet et al., 1990). There are, however, strong indications that these are not the only fish pathogens among the CLB and that there are still other, hitherto undescribed species (Kent et al., 1988; Pyle and Shotts, 1981). Further, the data seem to suggest that the fish pathogens are restricted to fish and cannot be isolated as free-living, environmental strains, although the latter possibility is difficult to rule out definitely. Information about an environmental reservoir of the fish pathogens would, of course, be of central importance for epidemiological considerations and control measures. Serological data speak also against a close relationship between fish-pathogenic and environmental CLB (Pacha and Ordal, 1970; Pacha and Porter, 1968). Serological tests have therefore been very useful for a quick diagnosis of the fish pathogens, which is important for practical reasons. Early studies showed that species-specific antigens exist in *Cy. columnaris* and *Cy. psychrophila* (Anacker and Ordal, 1959; Bullock, 1972; Pacha and Porter, 1968). *Cy. columnaris* has in addition at least seven type-specific antigens, which can be used to subdivide a collection of 325 strains into four serological groups; no link could be seen between those serological groups and levels of virulence (Anacker and Ordal, 1959; Pacha and Ordal, 1970). Fluorescence-labeled specific antibodies against *Cy. maritima* could be used to demonstrate the presence of the pathogen in various tissues (Baxa et al., 1988). Incidentally, the fish themselves develop antibodies against *Cy. columnaris,* and these seem to limit natural infection and can be applied to protection by vaccination (e.g., Becker and Fujihara, 1978; Liewes et al., 1982).

In another study, the isoenzyme patterns of 106 strains of spreading and nonspreading, yellow-pigmented bacteria from fish and other habitats were used to classify CLB. While one group coincided neatly with isolates that had been identified with *Cy. columnaris*, the other three groups were clearly heterogeneous, one of them even containing other *Cy. columnaris* isolates (Starliper et al., 1988). Thus, the results were inconclusive, and isoenzyme studies do not appear promising for taxonomic purposes.

The recently discovered genus *Taxeobacter* (H. Reichenbach, unpublished observations) has a special position among the soil Cytophagales. It is distinguished from the CLB by its cell morphology, the arrangement of the cells in spreading colonies (Figs. 1 and 3), and the brick red color of the latter. It is a common organism that can be cultivated without difficulties and has probably only been overlooked because it is not easily isolated by conventional techniques. By 16S rRNA cataloging, *Taxeobacter* (= Myx 2105) has been located in the *Fx. filiformis* side branch close to *Cy. heparina* (Paster et al., 1985).

As already mentioned, *Capnocytophaga* has been known for many years under different names. It is a fermenter requiring an atmosphere with 5% CO_2, at least for isolation, and produces mainly acetate and succinate from glucose. Initially only known from the human oral cavity, related species have since been found also in animals, e.g., in dogs (Brenner et al., 1989) and in the rice rat (Shklair and Ralls, 1988). The genetic homogeneity of the three human species and the identity of one of them with *Bacteroides ochraceus* (type strain) has been established by DNA-DNA hybridization (Williams and Hammond, 1979; Williams et al., 1979).

Practical Aspects

The Cytophagales are of considerable practical interest. Their importance becomes even more impressive if we take also into consideration the many isolates classified as *Flavobacterium* in the literature, which would be justified because those strains may often have been CLB with unrecognized gliding motility, and even if they were true flavobacteria, they would be closely related organisms. A general review of the practical aspects of gliding bacteria including the Cytophagales has been published (Reichenbach, 1988).

Bacteria belonging to this large complex of species are found virtually everywhere in nature, at least in aerobic and microaerobic environments. They definitely play a major role in the turnover of matter. Their activities are, of course, not always beneficial for humans. Some undesired effects with respect to the dairy industry (see also Cousin, 1982; Guamis et al., 1987) and the rotting of cellulose fabrics have already been mentioned. Other examples of this less pleasant side are the participation of pectolytic CLB in the spoilage of vegetables (Liao and Wells, 1986; Lund, 1969) and of proteolytic CLB in the putrefaction of fish, although in the latter case, gliding bacteria may be associated with freshly caught fish and are gradually replaced by other bacteria during deterioration (Cho et al., 1984; Gennari and Tomaselli, 1988; Liston, 1960; Shewan, 1971). Also in nature the actions of CLB are not always beneficial. Thus, e.g., CLB have been found tunneling in the cell walls of seagrass leaves, apparently killing the leaves in the process (Porter et al., 1989). A case of symbiosis was discovered in calcareous sponges of the genus *Clathrina;* the bacterium may be sponge-specific and has been associated with the sponge for a long time (Burlando et al., 1988). There may yet be many more examples of similar symbioses to be discov-

ered. One such case may be the yellowish-brown bacterium living in the mycetomes of the granary weevil *Sitophilus granarius* (Bhatnager and Musgrave, 1970).

In a different context, the destruction of organic molecules may become a positive asset. The occurrence of large populations of CLB in sewage plants has already been discussed (see also Bauer, 1962). Cellulolytic *Cytophaga* and *Sporocytophaga* seem to play a role during composting of cattle manure (Godden and Penninckx, 1984). Many articles describe the decomposition by flavobacteria of recalcitrant chemicals such as pesticides, and, as just explained, some of these could really have been CLB. Thus, flavobacteria have been found to attack chlorobenzoic acid (Baggik, 1985), pentachlorophenol (Brown et al., 1986; Crawford and Mohn, 1985), parathion (Mulbry et al., 1986), nylon oligomers (Negoro and Okada, 1982), biphenyl and phenanthrene (Stucki and Alexander, 1987), and aliphatic diols (Willetts, 1983), to give just a few examples. The digestion of hardwood mesquite (*Prosopis* sp.) with a cellulose-decomposing *Cytophaga* strain has been studied with the aim of improving the feed quality of the plant material for cattle (Chang and Thayer, 1975). It was shown that the *Cytophaga* protein is particularly rich in essential amino acids, but feeding experiments with mice gave unsatisfactory results.

The production by members of the Cytophagales of special enzymes for technical application could be another field of practical interest. Not too much is known about such enzymes, but a number of potentially useful hydrolases and lyases has indeed been discovered and more or less characterized. The following examples may illustrate this. The *Cytophaga* and *Sporocytophaga* species are very active cellulose decomposers and have been studied since the beginning of the century (e.g., Berg et al., 1972; Fåhraeus, 1947; Hutchinson and Clayton, 1919; Imshenetski and Solntseva, 1936; Krzemieniewska, 1930, 1933; Rivière, 1961; Sijpesteijn and Fåhraeus, 1949; Stanier, 1942; Winogradsky, 1929). Initially it was believed that enzymatic attack required close contact between cell and fiber, but later it was found that in the culture supernatant of *Sp. myxococcoides* there were also enzymes that could solubilize cellulose, at least to a certain degree (Kauri and Kushner, 1985; Osmundsvåg and Goksøyr, 1975; Vance et al., 1980). With other organisms, however, cell-free cellulases could not be demonstrated (Chang and Thayer, 1977). Also, transglycosylations to β-1-3 and β-1-6 oligosaccharides (Charpentier, 1965) and a conversion of β-D-glucose into α-D-glucose by the activity of an exoglucanase (Charpentier and Robic, 1974) have been demonstrated. Relatively little is known about enzymes themselves, and a reinvestigation using modern methods would be desirable.

Another group of enzyme producers known for some time are the pectolytic CLB (e.g., Donderski, 1982; Dorey, 1959; Güde, 1973). The responsible enzyme is a randomly cleaving polygalacturonate lyase, which catalyzes a trans-elimination reaction; a pectin methyl esterase could not be found (Kurowski and Dunleavy, 1976; Sundarraj and Bhat, 1971). Only one single enzyme was found in *Cy. johnsonae*, and this enzyme was the smallest one (35 kDa) of all pectate lyases characterized (Liao, 1989). The pectolytic activity of *Cy. johnsonae* made water-stored spruce logs more permeable for preservatives and was therefore considered to be of practical relevance (Kurowski and Dunleavy, 1976; Ward and Fogarty, 1974).

Although chitin degradation is often observed among CLB and flexibacters, relatively little is known about the enzymes (Donderski, 1984; Reichardt et al., 1983; Stanier, 1947; Sundarraj and Bhat, 1972). The enzymes of *Cy. johnsonae*, a chitinase and a chitobiase, may be excreted or cell-bound. Deacetylation seems to be the first degradation step. The chitinolytic strain of *Cy. johnsonae* studied by Veldkamp (1955) was either a *Lysobacter*, judging from the strain deposited at NCIB (no. 8501), or a *Flexibacter*, as may be concluded from the published description of the organism.

Several enzymes from CLB that degrade polysaccharides derived from marine algae have been characterized, mainly agarases (Duckworth and Turvey, 1969a, 1969b, 1969c; van der Meulen and Harder, 1975, 1976), but also a porphyranase (Turvey and Christison, 1967) and a large (100 kDa) extracellular carrageenase (Sarwar et al., 1987).

Many Cytophagales efficiently hydrolyze starch (e.g., Christensen, 1977a), but the enzymes never seem to have been studied. In two cases, special enzymes have been found in CLB because they had been screened for them for an application in structural studies. A strain was found and identified with *Cy. johnsonae* that produced endo-β-glucanases, which could then be used to study the composition of yeast cell walls (Bacon et al., 1970; Webley et al., 1967). The discovery of a strain that attacked heparin (Payza and Korn, 1956a, 1956b) proved a decisive contribution to the elucidation of the chemical structure of this complex heteropolysaccharide (e.g., Dietrich, 1969). The organism was first classified as *Flavobacterium heparinum* but later recognized to be a CLB (Christensen, 1980). Enzyme production has to be induced by the addition of heparin, and yields are not particularly good. Later, another

(tentatively classified) *Flavobacterium* strain was isolated that produced heparinase constitutively and at a 10-fold higher level (Joubert and Pitout, 1985). The enzyme may be of considerable practical interest for the deheparinization of blood.

Many Cytophagales excrete strong proteinases. A few of these enzymes have been further characterized because of their unusual specificities. In a screening, strains were discovered that solubilized autoclaved feathers and wool and could grow on these substrates as the only C and N sources; they did not attack the native material (Christison and Martin, 1971; Martin and So, 1969). The organisms resembled *Cy. johnsonae* in their characteristics but were never clearly classified. The keratinolytic enzyme appears to be associated with acidic polysaccharides on the surface of the cells. *Capnocytophagas* show strong aminopeptidase activities (e.g., Nakamura and Slots, 1982). In a screening for arylaminopeptidases among oral bacteria, several different activities at high levels were discovered in capnocytophagas, including monopeptide-, dipeptide-, and tripeptide-cleaving enzymes, some of which were only seen in capnocytophagas, e.g., enzymes attacking ala-, leu-, his-, lys-, and met-β-naphthylamines (Suido et al., 1986). Such proteolytic enzymes could contribute to the occasional pathogenicity of the organisms. Also the fish-pathogenic CLB show very strong proteolytic activities, which may be responsible for at least part of their infectivity. In an effort to prepare compounds with immunoadjuvant activity from cell walls of *Staphylococcus epidermis*, an interesting endopeptidase was isolated from the culture supernatant of the gliding bacterium *Cytophaga* B-30 (Kawata et al., 1984). The identity of the organism is not unequivocally established, and the description does not rule out the possibility that it was really a lysobacter. The enzyme released long-chain polysaccharide peptides from *Staphylococcus*, but little or nothing from *Streptococcus* and *Micrococcus* peptidoglycan. In cells and in the culture supernatant of *Cytophaga* NCMB 1314, an agent was discovered, presumably an enzyme, that solubilized active cholinesterase from fish muscle (plaice, *Pleuronectes platessa*). Again, the identity of the organism is not completely clear. Release of this "S-factor" into the medium was stimulated by cultivation under magnesium limitation (Bovallius, 1978, 1979; Lundin, 1968; Lundin and Bovallius, 1966).

Substantial phospholipase A_2 activity has been seen in *Capnocytophaga (Cp.) ochracea* strains; it has been suggested that this enzyme is a factor contributing to the pathogenic potential of the organism (Sandholm et al., 1988).

Interesting immunological effects may be produced with certain cell components of the Cytophagales. The allergenic activity of *Cytophaga allerginae* endotoxin, leading to humidifier fever, a lung disorder, has already been mentioned; the chemical composition of the endotoxin has been determined (Flaherty et al., 1984; Liebert et al., 1984). The purified endotoxin proved to be a novel mitogen that induced peripheral blood lymphocytes in vitro to synthesize IgG and IgA; as the immunoglobin induction is not inhibited by cyclosporin A, it seems to be T-cell independent (Alevy and Compas, 1987). Another potent mitogen called gliding bacteria adjuvant (GBA) was recovered in relatively large quantities (140 to 400 mg/l) from the culture supernatant of the *Cytophaga* GB-2. The psychrotrophic bacterium was first isolated from contaminated fetal calf serum. GBA was free of endotoxin and protein and perhaps consisted of a complex slime polysaccharide. It stimulated mouse B lymphocyte proliferation in vitro, the production of immunoglobulins, and the release of colony-stimulating factor and interleukin 1 by macrophages (Shiigi et al., 1977; Usinger et al., 1985). Purified exopolysaccharide from *Capnocytophaga* cultures activated the human complement system in vitro (maximum effect at 28 μg/ml plasma); this activation may be a factor leading to tissue destruction in periodontal lesions (Bolton and Dyer, 1986). A large (155-kDa), lectinlike protein was isolated from the outer membrane of *Cp. gingivalis* (Kagermeier and London, 1986). The lectin appears to be specific for neuraminic acid, *N*-acetylgalactosamine, and *N*-acetylglucosamine, and it is probably responsible for coaggregation with other bacteria, like *Actinomyces israelii*, in the formation of dental plaque. Similar adhesins with specificities for rhamnose and fucose were found on *Cp. ochracea* (Weiss et al., 1987). A lactose-specific, lectinlike receptor was demonstrated on *Cp. sputigena* and *Cp. gingivalis* (Saito et al., 1988).

Some members of the Cytophagales have been found to produce very interesting secondary metabolites. In a screening for antibiotic activities, about 20% of the 270 tested strains showed inhibitory effects (Reichenbach et al., 1984). Several β-lactams were isolated from *Flexibacter* strains, including new monobactams with oligopeptide side chains (Cooper et al., 1983; Sing et al., 1983) and monobactams with formylamino- and glucuronic acid substituents, the formacidins (Hida et al., 1985; Katayama et al., 1985). Monobactams with a dehydroasparagine residue were obtained from *Cy. johnsonae* (Kato et al., 1987a, 1987b), and deacetoxycephalosporin C and 7-formylamidocephalosporins were obtained from a *Flavobacterium* (Shoji et

al., 1984; Singh et al., 1982, 1984). *Cy. uliginosa* (formerly *Fv. uliginosum*) contributed marinactan, a heteropolysaccharide with marked antitumor activity in mice (Umezwawa et al., 1983).

Finally, there are some important medical and veterinary aspects of the Cytophagales: Flexibacteria are able to pass through membrane filters of 0.1-μm pore size used for sterilizing seawater; the contaminants could be eliminated only by pasteurization (Little et al., 1987). Immunological effects of cell components and products of diverse Cytophagales and resulting health problems have already been reviewed. A further example are the mitogenic and immunomodulatory effects exerted by cell wall fractions from a *Capnocytophaga* strain which had been isolated from a patient with juvenile periodontitis; the effects were also given by fractions that were free of LPS and peptidoglycan (Murayama et al., 1982). It is not known to what extent or whether *Capnocytophaga* contributes to periodontal disorders. Cytopathogenic effects were observed for "*Sphaerocytophaga*" (Gräf, 1962b, 1962c). These bacteria were present in large numbers in acute and chronic inflammations in the human oral cavity. In cell cultures (calf kidney cells), they caused cell lysis, and they destroyed human leukocytes in vitro. When inoculated subcutaneously into guinea pigs, they produced inflammations and ulcerations, but were not pathogenic for mice and rabbits when applied intraperitoneally and intravenously, respectively. It was found that neutrophils from patients with large *Capnocytophaga* populations in dental infections showed abnormal behavior and that such behavior could be induced in normal neutrophils when they were exposed to sonic extracts of *Capnocytophaga* (Shurin et al., 1979). Sonic extracts of *Cp. sputigena* showed moderate toxicity for human gingival fibrablasts as indicated by inhibition of cell proliferation and thymidine incorporation (Stevens and Hammond, 1988). Heat-killed *Cp. gingivalis* cells elicited strong endotoxin reactions in the *Limulus* and the Shwartzman test (e.g., Fumarola et al., 1981). As capnocytophagas are usually found in large numbers in cases of severe destructive periodontitis, especially in juvenile periodontitis, and as they produce similar symptoms when inoculated into healthy gnotobiotic rats, they may indeed not be completely harmless, even if they are always present in the normal buccal flora (Forlenza and Newman, 1983). Perhaps they also play a certain role in the "piggyback" transport of nonmotile bacteria into periodontal pockets. While the situation appears complex and somewhat controversial with periodontal pathogenicity, the case is clear when general infections occur (see also Hawkey et al., 1984). Newly described *Cp. canimorsus* and *Cp. cynodegmi* have been considered to be responsible for septicemia and localized wound infections in humans after dog bites; the organisms seem occasionally also to be transmitted by cats (Brenner et al., 1989). Capnocytophagas respond well to antibiotics, like various β-lactams, quinolones, chloramphenicol, and tetracycline (see also Arlet et al., 1987).

As already discussed in the section on "Habitats," the fish-pathogenic CLB are showing a steadily increasing economic impact on fish cultivation and, to a lesser extent, also on wildlife, in fresh water as well as in seawater. *Cy. columnaris*, *Cy. psychrophila*, *Cy. maritima*, and *Fv. branchiophilum* appear to be the major pathogens and have been reported worldwide, but other species are probably also involved (see also Amin et al., 1988; Farkas and Oláh, 1984; Kuo et al., 1980, 1981; Kusuda and Kimura, 1982; Ostland et al., 1989; Spangenberg, 1975). The outbreaks of disease may follow a seasonal pattern (e.g., Kuo et al., 1981). This is easily understandable, for the fish, as well as the pathogen, usually have well-defined temperature optima; thus, for example, *Cy. columnaris* is a typical warm-water, *Cy. psychrophila* a cold-water pathogen (e.g., Snieszko, 1974). Losses due to infections by CLB can be severe and sometimes approach 100% within a few days. Individual strains of the same species may differ substantially in their virulence. It seems that the pathogens become a problem mainly when the fish are kept under suboptimal conditions. The bacteria are sensitive to certain antibiotics, e.g., chloramphenicol, tetracyclines, and erythromycin, but better suited for practical application are chemotherapeutics like nifurpirinol or Mefarol. As pointed out already, control of the disease may also be effected by vaccination or transfer of the fish to water with a higher or lower temperature or salinity, depending on the specific case. Numerous studies on the various aspects of fish diseases caused by CLB have been published in the last 40 years, and some references to specific topics are *preconditioning by environmental factors:* Chen et al., 1982; Chowdhury and Wakabayashi, 1989a, 1989b; Hanson and Grizzle, 1985; Snieszko, 1974; *experimental infection:* Baxa et al., 1987b, Chowdury and Wakabayashi, 1989a; Kuo et al., 1987b; *histopathology:* Pacha and Ordal, 1967; and *control measures:* Amin et al., 1988; Deufel, 1974; Liewes et al., 1982; Ostland et al., 1989; Snieszko, 1953, summarizes the older literature.

Finally, it seems safe to predict that, beyond the examples given earlier, more diseases and disorders of aquatic animals other than fish will be discovered that are connected to CLB.

Literature Cited

Aasen, A. J., S. Liaaen Jensen. 1966a. The carotenoids of flexibacteria II. A new xanthophyll from *Saprospira grandis*. Acta Chem. Scand. 20:811–819.

Aasen, A. J., S. Liaaen Jensen. 1966b. Carotenoids of flexibacteria III. The structures of flexixanthin and deoxyflexixanthin. Acta Chem. Scand. 20:1970–1988.

Aasen, A. J., S. Liaaen Jensen. 1966c. Carotenoids of flexibacteria IV. The carotenoids of two further pigment types. Acta Chem. Scand. 20:2322–2324.

Abbanat, D. R., W. Godchaux, G. Polychroniou, E. R. Leadbetter. 1985. Biosynthesis of a sulfonolipid in gliding bacteria. Biochem. Biophys. Res. Commun. 130:873–878.

Abbanat, D. R., E. R. Leadbetter, W. Godchaux, A. Escher. 1986. Sulphonolipids are molecular determinants of gliding motility. Nature 342:367–369.

Achenbach, H. 1987. The pigments of the flexirubin-type. A novel class of natural products. Fortschr. Chem. Org. Naturst. 52:73–111.

Achenbach, H., A. Böttger-Vetter, E. Fautz, H. Reichenbach. 1982. On the origin of the branched alkyl substituents on ring B of flexirubin-type pigments. Arch. Microbiol. 132:241–244.

Achenbach, H., A. Böttger-Vetter, D. Hunkler, E. Fautz, H. Reichenbach. 1983. Investigations on the biosynthesis of flexirubin—The origin of benzene ring B and its substituents. Tetrahedr. 39:175–185.

Achenbach, H., A. Böttger, W. Kohl, E. Fautz, H. Reichenbach. 1979. Untersuchungen zur Biogenese des Flexirubins—Herkunft des Benzolringes A und der aromatischen C-Methylgruppen. Phytochem. 18:961–963.

Achenbach, H., W. Kohl, A. Böttger-Vetter, H. Reichenbach. 1981. Untersuchungen der Pigmente aus *Flavobacterium* spec. Stamm C1/2. Tetrahedr. 37:559–563.

Achenbach, H., W. Kohl, H. Reichenbach. 1976. Flexirubin, ein neuartiges Pigment aus *Flexibacter elegans*. Chem. Ber. 109:2490–2502.

Achenbach, H., W. Kohl, H. Reichenbach. 1978. The flexirubin-type pigments—a novel class of natural pigments from gliding bacteria. Revista Latinoamericana de Quimica 9:111–124.

Achenbach, H., W. Kohl, H. Reichenbach. 1979. Die Konstitutionen der Pigmente vom Flexirubin-Typ aus *Cytophaga johnsonae Cy jl*. Chem. Ber. 112:1999–2011.

Achenbach, H., W. Kohl, H. Reichenbach, H. Kleinig. 1974. Zur Struktur des Flexirubins. Tetrahedr. Lett. 1974:2555–2556.

Achenbach, H., W. Kohl, W. Wachter, H. Reichenbach. 1978b. Investigations of the pigments from *Cytophaga johnsonae Cy jl*. Arch. Microbiol. 117:253–257.

Achenbach, H., J. Witzke. 1977. Totalsynthese des Flexirubin-dimethylethers. Angew. Chem. 89:198–199.

Adkins, A. M., R. Knowles. 1984. Reduction of nitrous oxide by a soil *Cytophaga* in the presence of acetylene and sulfide. FEMS Microbiol. Lett. 23:171–174.

Adkins, A. M., R. Knowles. 1986. Denitrification by *Cytophaga johnsonae* strains and by a gliding bacterium able to reduce nitrous oxide in the presence of acetylene and sulfide. Can. J. Microbiol. 32:421–424.

Agbo, J. A. C., M. O. Moss. 1979. The isolation and characterization of agarolytic bacteria from a lowland river. J. Gen. Microbiol. 115:355–368.

Ajmal, M., B. C. Hobbs. 1967. Causes and effect of columnaris-type diseases in fish. Nature 215:141–142.

Alevy, Y. G., M. B. Compas. 1987. Induction of human immunoglobulin synthesis (IgG, IgA) by the novel, T cell independent mitogen *Cytophaga allerginae* endotoxin. Int. Arch. Allergy Appl. Immunol. 84:79–84.

Amin, N. E., I. S. Abdallah, M. Faisal, M. El-S. Easa, T. Alaway, S. A. Alyan. 1988. Columnaris infection among cultured Nile tilapia *Oreochromis niloticus*. Antonie van Leeuwenhoek 54:509–520.

Anacker, R. L., E. J. Ordal. 1955. Study of a bacteriophage infecting the myxobacterium *Chondrococcus columnaris*. J. Bacteriol. 70:738–741.

Anacker, R. L., E. J. Ordal. 1959. Studies on the myxobacterium *Chondrococcus columnaris* I. Serological typing. J. Bacteriol. 78:25–32.

Anacker, R. L., E. J. Ordal. 1959b. Studies on the myxobacterium *Chondrococcus columnaris* II. Bacteriocins. J. Bacteriol. 78:33–40.

Anderson, J. I. W., D. A. Conroy. 1969. The pathogenic myxobacteria with special reference to fish diseases. J. Appl. Bacteriol. 32:30–39.

Anderson, R. L., E. J. Ordal. 1961a. *Cytophaga succinicans* sp. n., a facultatively anaerobic aquatic myxobacterium. J. Bacteriol. 81:130–138.

Anderson, R. L., E. J. Ordal. 1961b. CO_2-dependent fermentation of glucose of *Cytophaga succinicans*. J. Bacteriol. 81:139–146.

Arlet, G., M. J. Sanson-Le Pors, I. M. Casin, M. Ortenberg, Y. Perol. 1987. In vitro susceptibility of 96 *Capnocytophaga* strains, including a β-lactamase producer, to new β-lactam antibiotics and six quinolones. Antimicrob. Agents Chemother. 31:1283–1284.

Asselinearu, J., F. Pichinoty. 1983. Lipid composition of strains of *Flavobacterium* and *Sphingobacterium*. FEMS Microbiol. Lett. 20:375–378.

Bachmann, B. J. 1955. Studies on *Cytophaga fermentans*, n. sp., a facultatively anaerobic lower myxobacterium. J. Gen. Microbiol. 13:541–551.

Bacon, J. S. D., A. H. Gordon, D. Jones, I. F. Taylor, D. M. Webley. 1970. The separation of β-glucanases produced by *Cytophaga johnsonii* and their role in the lysis of yeast cell walls. Biochem. J. 120:67–78.

Baechler, C. A., R. S. Berk. 1972. Ultrastructural observations of *Pseudomonas aeruginosa*: Rhapidosomes. Microstructures 3:24–31.

Baggi, G. 1985. Ricerche sulla degradazione di acidi clorobenzoici. Ann. Microbiol. 35:71–78.

Bauer, L. 1962. Untersuchungen an *Sphaeromyxa xanthochlora*, n. sp., einer auf Tropfkörpern vorkommenden Myxobakterienart. Arch. Hyg. Bakteriol. 146:392–400.

Baxa, D. V., K. Kawai, R. Kusuda. 1987a. Molecular taxonomic classification of gliding bacteria isolated from diseased cultured flounder. Fish Pathol. 22:11–14.

Baxa, D. V., K. Kawai, R. Kusuda. 1987b. Experimental infection of *Flexibacter maritimus* in black sea bream (*Acanthopagrus schlegeli*) fry. Fish Pathol. 22:105–109.

Baxa, D. V., K. Kawai, R. Kududa. 1988. Detection of *Flexibacter maritimus* by fluorescent antibody technique in experimentally infected black sea bream fry. Fish Pathol. 23:29–32.

Becker, C. D., M. P. Fujihara. 1978. The bacterial pathogen *Flexibacter columnaris* and its epizootiology among Columbia River fish, monograph no. 2. American Fish. Society Washington, DC.

Behrens, H. 1978. Charakterisierung der DNA gleitender Bakterien der Ordnung Cytophagales. Doctoral thesis, Technical University Braunschweig. Germany.

Berg, B., B. V. Hofsten, G. Pettersson. 1972. Electron-microscopic observations on the degradation of cellulose fibres by *Cellvibrio fulvus* and *Sporocytophaga myxococcoides*. J. Appl. Bacteriol. 35:215–219.

Bernardet, J. F. 1989. Étude phénotypique et génomique des bactéries appartenant aux genres *Cytophaga* et *Flexibacter* (ordre des Cytophagales) et comparaison avec le genre *Flavobacterium*; application à l'identification et à la taxonomie des espèces ichthyopathogènes. Doctoral thesis, University of Paris VII.

Bernardet, J. F., A. C. Campbell, J. A. Buswell. 1990. *Flexibacter maritimus* is the agent of "black patch necrosis" in Dover sole in Scotland. Dis. Aquat. Org. 8: 233–237.

Bernardet, J. F., P. A. D. Grimont. 1989. Deoxyribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris* sp. nov., nom. rev., *Flexibacter psychrophilus* sp. nov., nom. rev., and *Flexibacter maritimus* Wakabayashi, Hikida, and Masumura 1986. Int. J. Syst. Bacteriol. 39:346–354.

Bernardet, J. F., B. Kerouault. 1989. Phenotypic and genomic studies of "*Cytophaga psychrophila*" isolated from diseased rainbow trout (*Oncorhynchus mykiss*) in France. Appl. Environm. Microbiol. 55:1796–1800.

Bhatnagar, R. D. S., A. J. Musgrave. 1970. Cytochemistry, morphogenesis, and tentative identification of mycetomal microorganisms of *Sitophilus granarius* L. (Coleoptera). Can. J. Microbiol. 16:1357–1362.

Bolton, R. W., J. K. Dyer. 1986. Human complement activation by purified *Capnocytophaga* exopolysaccharide. Measurement by radioimmunoassay. J. Period. Res. 21:634–639.

Bootsma, R., J. P. M. Clerx. 1976. Columnaris disease of cultured carp *Cyprinus carpio* L. Characterization of the causative agent. Aquacult. 7:371–384.

Bortels, H. 1956. Die Bedeutung einiger Spurenelemente für *Cellvibrio-* und *Cytophaga*-Arten. Arch. Mikrobiol. 25:226–245.

Bovallius, Å. 1978. Increased extracellular production of a cholinesterase-solubilising factor by *Cytophaga* NCMB 1314 during magnesium starvation. Can. J. Microbiol. 24:381–385.

Bovallius, Å. 1979. Morphological and chemical characteristics of a *Cytophaga* sp. grown under conditions of magnesium excess and magnesium limitation. J. Gen. Microbiol. 113:137–145.

Brenner, D. J., D. G. Hollis, G. R. Fanning, R. E. Weaver. 1989. *Capnocytophaga canimorsus* sp. nov. (formerly CDC group DF-2), a cause of septicemia following dog bite, and *C. cynodegmi* sp. nov., a cause of localized wound infection following dog bite. J. Clin. Microbiol. 27:231–235.

Brockman, E. R. 1967. Fruiting myxobacteria from the South Carolina coast. J. Bacteriol. 94:1253–1254.

Brown, E. J., J. J. Pignatello, M. M. Martinson, R. L. Crawford. 1986. Pentachlorophenol degradation: a pure bacterial culture and an epilithic microbial consortium. Appl. Environm. Microbiol. 52:92–97.

Bullock, G. L. 1972. Studies on selected myxobacteria pathogenic for fishes and on bacterial gill disease in hatchery-reared salmon. 1–30. Technical papers, Bureau of Sport Fisheries and Wildlife. U.S. Dept. Interior. Washington, DC.

Burchard, R. P. 1984. Inhibition of *Cytophaga* U67 gliding motility by inhibitors of polypeptide synthesis. Arch. Microbiol. 139:248–254.

Burchard, R. P., D. T. Brown. 1973. Surface structure of gliding bacteria after freeze-etching. J. Bacteriol. 114:1351–1355.

Burlando, B., M. A. Sabatini, E. Gaino. 1988. Association between calcareous *Clathrina cerebum* (Haeckel) and bacteria: electron microscope study. J. Exp. Mar. Biol. Ecol. 116:35–42.

Callies, E., W. Mannheim. 1978. Classification of the *Flavobacterium-Cytophaga* complex on the basis of respiratory quinones and fumarate respiration. Int. J. Syst. Bacteriol. 28:14–19.

Campbell, A. C., J. A. Buswell. 1982. An Investigation into the bacterial aetiology of "black patch necrosis" in Dover sole, *Solea solea* L. J. Fish Dis. 5:495–508.

Chang, L. Y. E., J. L. Pate, R. J. Betzig. 1984. Isolation and characterization of nonspreading mutants of the gliding bacterium *Cytophaga johnsonae*. J. Bacteriol. 159:26–35.

Chang, W. T. H., D. W. Thayer. 1975. The growth of *Cytophaga* on mesquite. Developm. Industr. Microbiol. 16:456–464.

Chang, W. T. H., D. W. Thayer. 1977. The cellulase system of a *Cytophaga* species. Can. J. Microbiol. 23:1285–1292.

Charpentier, M. 1965. Étude de l'activité cellulolytique de *Sporocytophaga myxococcoides*. Ann. Inst. Pasteur 109:771–797.

Charpentier, M., D. Robio. 1974. Dégradation de la cellulose par un microorganisme du sol: *Sporocytophaga myxococcoides*: Caractérisation d'une exoglucanase. C. R. Acad. Sc. Paris. 279:863–866.

Chen, C. R. L., H. Y. Chung, G. H. Kou. 1982. Studies on the pathogenicity of *Flexibacter columnaris*—I. Effect of dissolved oxygen and ammonia on the pathogenicity, of *Flexibacter columnaris* to eel (*Anguilla japonica*). CAPD Fish. Series 8:1–7.Counc. Agricult. Plann. Devel. Rep. China. Taipei, Taiwan.

Cho, Y., H. Shinano, M. Akiba. 1984. Studies on the microbiological ecology of mackerel stored by the method of partial freezing—I. Changes in microflora and chemical compounds in mackerel stored by partial freezing. Bull. Faculty Fish. (Hokkaido Univ.) 35:271–285.

Chowdhury, B. R., H. Wakabayashi. 1988. Effects of sodium, potassium, calcium and magnesium ions on *Flexibacter columnaris* infection in fish. Fish Pathol. 23:237–241.

Chowdhury, B. R., H. Wakabayashi. 1989. A study on the mechanism of the bacterial competitive effects on *Flexibacter columnaris* infection: Effects of the time-lag between the exposures of fish to *F. columnaris* and its competitor. Fish Pathol. 24:105–110.

Christensen, P. J. 1973. Studies on soil and freshwater cytophagas. Ph. D. thesis, University of Alberta. Edmonton, Canada.

Christensen, P. J. 1977a. The history biology, and taxonomy of the *Cytophaga* group. Can. J. Microbiol. 23:1599–1653.

Christensen, P. J. 1977b. Synonomy of *Flavobacterium pectinovorum* Dorey with *Cytophaga johnsonae* Stanier. Int. J. Syst. Bacteriol. 27:122–132.

Christensen, P. J. 1980. Description and taxonomic status of *Cytophaga heparina* (Payza and Korn) comb. nov. (Basionym: *Flavobacterium heparinum* Payza and Korn 1956). Int. J. Syst. Bacteriol. 30:473–475.

Christensen, P. J., F. D. Cook. 1978. *Lysobacter*, a new genus of nonfruiting gliding bacteria with a high base ratio. Int. J. Syst. Bacteriol. 28:367–393.

Christison, J., S. M. Martin. 1971. Isolation and preliminary characteristics of an extracellular protease of *Cytophaga* sp. Can. J. Microbiol. 17:1207–1216.

Colgrove, D. J., J. W. Wood. 1966. Occurrence and control of *Chondrococcus columnaris* as related to Fraser River sockeye salmon. Progress Report No. 15 of the International Pacific Salmon Fisheries Commission. New Westminster, British Columbia. Canada.

Collins, M. D., H. N. Shah, A. S. McKee, R. M. Kroppenstedt. 1982. Chemotaxonomy of the genus *Capnocytophaga* (Leadbetter, Holt & Socransky). J. Appl. Bacteriol. 52:409–415.

Collins, V. G. 1970. Recent studies of bacterial pathogens of freshwater fish. Water Treatm. Exam. 19:3–31.

Cooper, R., K. Bush, P. A. Principe, W. H. Trejo, J. S. Wells, R. B. Sykes. 1983. Two new antibiotics produced by a *Flexibacter* sp. I. Taxonomy, fermentation, isolation and biological properties. J. Antibiot. 36:1252–1257.

Couke, P., J. P. Voets. 1967. The mineral requirements of *Polyangium cellulosum*. Zschr. Allg. Mikrobiol. 7:175–182.

Cousin, M. A. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products: a review. J. Food Protect. 45:172–207.

Crawford, R. L., W. W. Mohn. 1985. Microbiological removal of pentachlorophenol from soil using a *Flavobacterium*. Enzyme Microb. Technol. 7:617–620.

Davis, H. S. 1921/1922. A new bacterial disease of fresh-water fishes. Bull. U. S. Bur. Fish. 38:261–280.

Davis, H. S. 1949. *Cytophaga columnaris* as a cause of fish epidemics. Transact. Am. Fish. Soc. 77:102–104.

Dawson, R. M. C., W. H. Elliott, K. M. Jones (ed.). 1969. Data for biochemical research 2nd ed. Clarendon Press. Oxford.

Dees, S. B., C. W. Moss, R. E. Weaver, D. Hollis. 1979. Cellular fatty acid composition of *Pseudomonas paucimobilis* and groups IIk-2, Ve-1, and Ve-2. J. Clin. Microbiol. 10:206–209.

Deufel, J. 1974. Wirkung von Mefarol auf Fische und den Erreger der bakteriellen Kiemenschwellung. Der Fischwirt. 24:27–29.

Dhundale, A. R., T. Furuichi, S. Inouye, M. Inouye. 1985. Distribution of multicopy single-stranded DNA among myxobacteria and related species. J. Bacteriol. 164:914–917.

Dietrich, C. P. 1969. Enzymatic degradation of heparin. Biochem. J. 111:91–95.

Donderski, W. 1982. Studies on pectolytic bacteria in water and bottom sediments of two lakes of different trophy. Acta Microbiol. Pol. 31:293–299.

Donderski, W. 1983. Chitinolytic bacteria in water and bottom sediments of two lakes of different trophy. Acta Microbiol. Pol. 33:163–170.

Dorey, M. J. 1959. Some properties of a pectolytic soil flavobacterium. J. Gen. Microbiol. 20:91–104.

Dubos, R. 1928. The decomposition of cellulose by aerobic bacteria. J. Bacteriol. 15:223–234.

Duckworth, M., J. R. Turvey. 1969a. An extracellular agarase from a *Cytophaga* species. Biochem. J. 113:139–142.

Dungan, C. F., R. A. Elston, M. H. Schiewe. 1989. Evidence for colonization and destruction of hinge ligaments in cultured juvenile Pacific oysters (*Crassostrea gigas*) by *Cytophaga*-like bacteria. Appl. Environm. Microbiol. 55:1128–1135.

Duxbury, T., B. A. Humphrey, K. C. Marshall. 1980. Continuous observations of bacterial gliding motility in a dialysis microchamber: The effects of inhibitors. Arch. Microbiol. 124:169–175.

Fåhraeus, G. 1947. Studies on the cellulose decomposition by *Cytophaga*. Symbolae Botanicae Upsalienses 9(2):1–128.

Farkas, J., J. Oláh. 1984. Occurrence, experimental infection and treatment of myxobacterial gill disease of carp. Symp. Biol. Hungarica (Budapest) 23:55–61.

Fautz, E., L. Grotjahn, H. Reichenbach. 1981. Hydroxy fatty acids as valuable chemosystematic markers in gliding bacteria and flavobacteria. 127–133. H. Reichenbach and O. B. Weeks (ed.) The *Flavobacterium-Cytophaga* group. Verlag Chemie. Weinheim, Germany.

Fautz, E., H. Reichenbach. 1980. A simple test for flexirubin-type pigments. FEMS Microbiol. Lett. 8:87–91.

Fautz, E., G. Rosenfelder, L. Grotjahn. 1979. Iso-branched 2- and 3-hydroxy fatty acids as characteristic lipid constituents of some gliding bacteria. J. Bacteriol. 140:852–858.

Fijan, N. N. 1969. Antibiotic additives for the isolation of *Chondrococcus columnaris* from fish. Appl. Microbiol. 17:333–334.

Fijan, N. N., P. R. Voorhees. 1969. Drug sensitivity of *Chondrococcus columnaris*. Veterinarski Arhiv (Zagreb) 39:259–267.

Flaherty, D. K., F. H. Deck, M. A. Hood, C. Liebert, F. Singleton, P. Winzenburger, K. Bishop, L. R. Smith, L. M. Bynum, W. B. Witmer. 1984. A *Cytophaga* species endotoxin as a putative agent of occupation-related lung disease. Infect. Immun. 43:213–216.

Follett, E. A. C., D. M. Webley. 1965. An electron microscope study of the cell surface of *Cytophaga johnsonii* and some observations on related organisms. Antonie van Leeuwenhoek 31:361–382.

Forlenza, S., M. G. Newman. 1983. *Capnocytophaga*. 45–66. E. J. Bottone (ed.) Unusual microorganisms. Marcel Dekker. New York.

Forlenza, S. W., M. G. Newman, A. L. Horikoshi, U. Blachman. 1981. Antimicrobial susceptibility of *Capnocytophaga*. Antimicrob. Agents Chemother. 19:144–146.

Forlenza, S. W., M. G. Newman, A. I. Lipsey, S. E. Siegel, U. Blachman. 1980. *Capnocytophaga* sepsis: a newly recognized clinical entity in granulocytopenic patients. Lancet 1980:567–568.

Fox, D. L., R. A. Lewin. 1963. A preliminary study of the carotenoids of some flexibacteria. Can. J. Microbiol. 9:753–768.

Fujihara, M. P., F. P. Hungate. 1972. Seasonal distribution of *Chondrococcus columnaris* infection in river fishes as determined by specific agglutinins. J. Fish. Res. Bd. Can. 29:173–178.

Fujihara, M. P., P. A. Olson, R. E. Nakatani. 1971. Some factors in susceptibility of juvenile rainbow trout and chinook salmon to *Chondrococcus columnaris*. J. Fish. Res. Bd. Can. 28:1739–1743.

Fumarola, D., P. D. Laforgia, R. Monno, G. Miragliotta, F. Mangini. 1981. Endotoxin-like activity with *Capnocytophaga gingivalis*. IRCS Medical Sci. 9:720.

Garnjobst, L. 1945. *Cytophaga columnaris* (Davis) in pure culture: a myxobacterium pathogenic to fish. J. Bacteriol. 49:113–128.

Gennari, M., S. Tomaselli. 1988. Changes in aerobic microflora of skin and gills of Mediterranean sardines (Sar-

dinia pilchardus) during storage in ice. Int. J. Food Microbiol. 6:341–347.

Ghittion, P. 1972. Mortalità massiva per Malattia Branchiale Batterica (MBB) in cieche di anguilla europea (*Anguilla anguilla*). Riv. Ital. Piscic. Ittiop. 7:83–85.

Glaser, J., J. L. Pate. 1973. Isolation and characterization of gliding motility mutants of *Cytophaga columnaris*. Arch. Mikrobiol. 93:295–309.

Godchaux, W., E. R. Leadbetter. 1980. *Capnocytophaga* spp. contain sulfonolipids that are novel in prokaryotes. J. Bacteriol. 144:592–602.

Godchaux, W., E. R. Leadbetter. 1983. Unusual sulfonolipids are characteristic of the *Cytophaga-Flexibacter* group. J. Bacteriol. 153:1238–1246.

Godchaux, W., E. R. Leadbetter. 1984. Sulfonolipids of gliding bacteria. Structure of the *N*-acylaminosulfonates. J. Biol. Chem. 259:2982–2990.

Godchaux, W., E. R. Leadbetter. 1988. Sulfonolipids are localized in the outer membrane of the gliding bacterium *Cytophaga johnsonae*. Arch. Microbiol. 150:42–47.

Godden, G., M. J. Penninckx. 1984. Identification and evolution of the cellulolytic microflora present during composting of cattle manure: On the role of actinomycetes sp. Ann. Microbiol. (Inst. Pasteur) 135B:69–78.

Godwin, S. L., M. Fletcher, R. P. Burchard. 1989. Interference reflection microscopic study of sites of association between gliding bacteria and glass substrata. J. Bacteriol. 171:4589–4595.

Gräf, W. 1961. Anaerobe Myxobakterien, neue Mikroben in der menschlichen Mundhöhle. Arch. Hyg. Bakteriol. 145:405–459.

Gräf, W. 1962a. Über Wassermyxobakterien. Arch. Hyg. Bakteriol. 146:114–125.

Gräf, W. 1962b. Die zytopahtogenen Eigenschaften der anaeroben Myxobakterien. Arch. Hyg. Bakteriol. 146:481–491.

Gräf, W. 1962c. Die Pathogenität anaerober Myxobakterien (*Sphaerocytophaga*) im Tierversuch. Arch. Hyg. Bakteriol. 146:492–500.

Gräf, W., H. Morhard. 1966. Antibiotikaempfindlichkeit von anaeroben Myxobakterien. Arch. Hyg. Bakteriol. 150:135–139.

Greaves, M. P., D. Vaughan, D. M. Webley. 1970. The degradation of nucleic acids by *Cytophaga johnsonii*. J. Appl. Bacteriol. 33:380–389.

Guamis, B., T. Huerta, E. Garay. 1987. SDS-PAGE study of milk proteolysis by selected psychrotrophs from raw milk. Milchwissensch. 42:89–91.

Güde, H. 1973. Untersuchungen über aerobe pektinzersetzende Bakterien in einem eutrophen See. Arch. Hydrobiol. Suppl. 42:483–496.

Güde, H. 1980. Occurrence of cytophagas in sewage plants. Appl. Environm. Microbiol. 39:756–763.

Hansen, G. H., J. A. Olafsen. 1989. Bacterial colonization of cod (*Gadus morhua* L.) and halibut (*Hippoglossus hippoglossus*) eggs in marine aquaculture. Appl. Environm. Microbiol. 55:1435–1446.

Hanson, L. A., J. M. Grizzle. 1985. Nitrite-induced predisposition of channel catfish to bacterial diseases. Progr. Fish-Cult. 47:98–101.

Hanstveit, A. O., J. Goksøyr. 1974. The pathway of glucose catabolism in *Sporocytophaga myxococcoides*. J. Gen. Microbiol. 81:27–35.

Hawkey, P. M., H. Malnick, S. Glover, N. Cook, J. A. Watts. 1984. *Capnocytophaga ochracea* infection: two cases and a review of the published work. J. Clin. Pathol. 37:1066–1070.

Hayes, P. R. 1977. A taxonomic study of flavobacteria and related Gram-negative yellow pigmented rods. J. Appl. Bacteriol. 3:345–367.

Heo, G. J., H. Wakabayashi, S. Watabe. 1990. Purification and characterization of pili from *Flavobacterium branchiophila*. Fish Pathol. 25:21–27.

Heyn, A. N. J. 1957. Bacteriological studies on cotton. Textile Res. J. 27:591–603.

Hida, T., S. Tsubotani, N. Katayama, H. Okazaki, S. Harada. 1985. Formadicins, new monocyclic β-lactam antibiotics of bacterial origin II. Isolation, characterization and structures. J. Antibiot. 38:1128–1140.

Hikida, M., H. Wakabayashi, S. Egusa, K. Masumura. 1979. *Flexibacter* sp., a gliding bacterium pathogenic to some marine fishes in Japan. Bull. Jap. Soc. Sci. Fish. 45:421–428.

Hirsch, I. 1979. Beiträge zur Taxonomie der Cytophagales. Ph. D. thesis, Technical University. Braunschweig, Germany.

Hoeniger, J. F. M. 1985. Microbial decomposition of cellulose in acidifying lakes of south-central Ontario. Appl. Environm. Microbiol. 50:315–322.

Höfle, M. G. 1982. Glucose uptake of *Cytophaga johnsonae* studied in batch and chemostat culture. Arch. Microbiol. 133:289–294.

Höfle, M. G. 1983. Long-term changes in chemostat cultures of *Cytophaga johnsonae*. Appl. Environm. Microbiol. 46:1045–1053.

Höfle, M. G. 1984. Transient responses of glucose-limited cultures of *Cytophaga johnsonae* to nutrient excess and starvation. Appl. Environm. Microbiol. 47:356–362.

Holm-Hansen, O., R. A. Lewin. 1965. Bound ornithine in certain flexibacteria and algae. Physiol. Plant. 18:418–423.

Holm-Hansen, O., R. Prasad, R. A. Lewin. 1965. Occurrence of α-, ε-diaminopimelic acid in algae and flexibacteria. Phycologia 5:1–14.

Holt, S. C., J. Doundowlakis, B. J. Takas. 1979a. Phospholipid composition of gliding bacteria: Oral isolates of *Capnocytophaga* compared with *Sporocytophaga*. Infect. Immun. 26:305–310.

Holt, S. C., G. Forcier, B. J. Takacs. 1979b. Fatty acid composition of gliding bacteria: Oral isolates of *Capnocytophaga* compared with *Sporocytophaga*. Infect. Immunity 26:298–304.

Holt, S. C., E. R. Leadbetter. 1967. Fine structure of *Sporocytophaga myxococcoides*. Arch. Mikrobiol. 57:199–213.

Holt, S. C., E. R. Leadbetter, S. S. Socransky. 1979c. *Capnocytophaga*: new genus of Gram-negative gliding bacteria. II. Morphology and ultrastructure. Arch. Microbiol. 120:231–238.

Humphrey, B. A., K. C. Marshall. 1980. Fragmentation of some gliding bacteria during the growth cycle. J. Appl. Bacteriol. 49:281–289.

Hutchinson, H. B., J. Clayton. 1919. On the decomposition of cellulose by an aerobic organism (*Spirochaeta cytophaga* nov. sp.). J. Agricult. Sci. 9:143–173.

Iizuka, T., K. Fujikawa, K. Ito, S. Murai. 1987. The phospholipid components of bacteria related to periodontitis. J. Nihon Univ. Sch. Dent. 29:189–195.

Imschenezki, A. A. (also: Imshenetski). 1959. Mikrobiologie der Cellulose. "Translated from the Russian edition of 1953." Akademie Verlag. Berlin.

Imshenetski, A. A., L. Solntseva. 1936. On aerobic cellulose-decomposing bacteria. [In Russian, with English summary.] Izvestiia Akademii Nauk SSSR. Bulletin de l'Académie des Sciences de l'URSS. Classe des Sciences Mathématique et Naturelles. Série Biologie 1115–1172.

Irschik, H., H. Reichenbach. 1978. Intracellular location of flexirubins in *Flexibacter elegans* (Cytophagales). Biochim. Biophys. Acta 510:1–10.

Johns, R. B., G. J. Perry. 1977. Lipids of the marine bacterium *Flexibacter polymorphus*. Arch. Microbiol. 114:267–271.

Johnson, J. L., W. S. Chilton. 1966. Galactosamine glycan of *Chondrococcus columnaris*. Science 152:1247–1248.

Jooste, P. J. 1985. The taxonomy and significance of *Flavobacterium-Cytophaga* strains from dairy sources. Doctoral thesis, University of Bloemfontein, RSA.

Jooste, P. J., T. J. Britz, J. de Haast. 1985. A numerical taxonomic study of *Flavobacterium-Cytophaga* strains from dairy sources. J. Appl. Bacteriol. 59:311–323.

Joubert, J. J., M. J. Pitout. 1985. A constitutive heparinase in a *Flavobacterium* sp. Experientia 41:1541.

Kadota, H. 1956. A study of the marine aerobic cellulose-decomposing bacteria. Memoirs of the College of Agriculture, Kyoto University, no. 74:1–128.

Kagermeier, A., J. London. 1986. Identification and preliminary characterization of a lectinlike protein from *Capnocytophaga gingivalis* (emended). Infect. Immun. 51:490–494.

Kapke, P. A., A. T. Brown, T. T. Lillich. 1980. Carbon dioxide metabolism by *Capnocytophaga ochracea*: Identification, characterization, and regulation of phosphoenolpyruvate carboxykinase. Infect. Immun. 27:756–766.

Katayama, N., Y. Nozaki, K. Okonogi, H. Ono, S. Harada, H. Okazaki. 1985. Formadicins, new monocyclic β-lactam antibiotics of bacterial origin I. Taxonomy, fermentation and biological activities. J. Antibiot. 38:1117–1127.

Kath, T. K. L. 1990. Untersuchungen zur natürlichen Verwandtschaft cellulosezersetzender *Cytophaga*-artiger Bakterien. Doctoral thesis, Technical University. Braunschweig, Germany.

Kato, T., H. Hinoo, J. Shoji, K. Matsumoto, T. Tanimoto, T. Hattori, K. Hirooka, E. Kondo. 1987. PB-5266 A, B and C, new monobactams. I. Taxonomy, fermentation and isolation. J. Antibiot. 40:135–138.

Kato, T., H. Hinoo, Y. Terui, J. Kikuchi, J. Shoji. 1988. The structures of katanosins A and B. J. Antibiot. 41:719–725.

Kato, T., H. Hinoo, Y. Terui, J. Nishikawa, Y. Nakagawa, Y. Ikenishi, J. Shoji. 1987. PB-5266 A, B and C, new monobactams II. Physico-chemical properties and chemical structures. J. Antibiot. 40:139–144.

Kauri, T., D. J. Kushner. 1985. Role of contact in bacterial degradation of cellulose. FEMS Microbiol. Ecol. 31: 301–306.

Kawata, S., T. Takemura, K. Yokogawa, S. Kotani. 1984. Isolation of bacteriolytic endopeptidase from a strain of *Cytophaga* and its application to preparation of hydrosoluble polysaccharide peptide from *Staphylococcus epidermidis* peptidoglycan. Agric. Biol. Chem. 48:2253–2263.

Kent, M. L., C. F. Dungan, R. A. Elston, R. A. Holt. 1988. *Cytophaga* sp. (Cytophagales) infection in seawater penreared Atlantic salmon *Salmo salar*. Dis. Aquat. Org. 4:173–179.

Kincheloe, J. W. 1962. The cultivation and drug sensitivity of myxobacteria isolated from diseased fish. The Progressive Fish-Culturist 24:119–126.

Kingsbury, D. T., E. J. Ordal. 1966. Bacteriophage infecting the myxobacterium *Chondrococcus columnaris*. J. Bacteriol. 91:1327–1332.

Kleinig, H., H. Reichenbach, N. Theobald, H. Achenbach. 1974. *Flexibacter elegans* and *Myxococcus fulvus:* aerobic Gram-negative bacteria containing menaquinones as the only isoprenoid quinones. Arch. Microbiol. 101:91–93.

Kohl, W., H. Achenbach, H. Reichenbach. 1983. The pigments of *Brevibacterium linens*: Aromatic carotenoids. Phytochem. 22:207–210.

Krzemieniewska, H. 1930. Le cycle évolutif de *Spirochaeta cytophaga* Hutchinson et Clayton. Acta Soc. Botan. Polon. 7:507–519.

Krzemienieqska, H. 1933. Contribution à l'étude du genre *Cytophaga* (Winogradsky). Arch. Mikrobiol. 4:394–408.

Kuhrt, M., J. L. Pate. 1973. Isolation and characterization of tubules and plasma membranes from *Cytophaga columnaris*. J. Bacteriol. 114:1309–1318.

Kuo, S. C., H. Y. Chung, G. H. Kou. 1980. Studies on identification and pathogenicity of the gliding bacteria in cultured fishes. CAPD Fish. Series 3:53–66. Counc. Agricult. Plann. Devel. Rep. China. Taipei, Taiwan.

Kuo, S. C., H. Y. Chung, G. H. Kou. 1981. Studies on artificial infection of the gliding bacteria in cultured fishes. Fish Pathol. 15:309–314.

Kurowski, W. M., J. A. Dunleavy. 1976. Pectinase production by bacteria associated with improved preservative permeability in sitka spruce: synthesis and secretion of polygalacturonate lyase by *Cytophaga johnsonii*. J. Appl. Bacteriol. 41:119–128.

Kusuda, R., H. Kimura. 1982. Characteristics of gliding bacterium isolated from cultured yellowtail *Seriola quinqueradiata*. Bull. Japan. Soc. Sci. Fish. 48:1107–1112.

Lapidus, I. R., H. C. Berg. 1982. Gliding motility of *Cytophaga* sp. strain U67. J. Bacteriol. 151:384–398.

Leadbetter, E. R. 1974. Cytophagales. 99–112. R. E. Buchanan and N. E. Gibbons (ed.) Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins. Baltimore.

Leadbetter, E. R., S. C. Holt, S. S. Socransky. 1979. *Capnocytophaga*: New genus of Gram-negative gliding bacteria. I. General characteristics, taxonomic considerations and significance. Arch. Microbiol. 122:9–16.

Lewin, R. A. 1969. A classification of flexibacteria. J. Gen. Microbiol. 58:189–206.

Lewin, R. A. 1970. *Flexithrix dorotheae* gen. et sp. nov. (Flexibacterales); and suggestions for reclassifying sheathed bacteria. Can. J. Microbiol. 16:5411–515.

Lewin, R. A., D. M. Lounsbery. 1969. Isolation, cultivation and characterization of flexibacteria. J. Gen. Microbiol. 58:145–170.

Liao, C. H. 1989. Analysis of pectate lyases produced by soft rot bacteria associated with spoilage of vegetables. Appl. Environm. Microbiol. 55:1677–1683.

Liao, C. H., J. M. Wells. 1986. Properties of *Cytophaga johnsonae* strains causing spoilage of fresh produce at food markets. Appl. Environm. Microbiol. 52:1261–1265.

Liebert, C., M. A. Hood, F. H. Deck, K. Bishop, D. K. Flaherty. 1984. Isolation and characterization of a new *Cytophaga* species implicated in work-related lung disease. Appl. Environm. Microbiol. 48:936–943.

Liewes, E. W., R. H. van Dam, M. G. Vos-Maas, R. Bootsma. 1982. Presence of antigen sensitized leukocytes in carp

(*Cyprinus carpio* L.) following bath immunization against *Flexibacter columnaris*. Vet. Immunol. Immunopathol. 3:603–609.

Liston, J. 1960. The bacterial flora of fish caught in the Pacific. J. Appl. Bacteriol. 23:469–470.

London, J., R. Celesk, P. Kolenbrander. 1982. Physiological and ecological properties of the oral Gram-negative gliding bacteria capable of attaching to hydroxyapatite. 76–85. R. F. Genco and S. E. Mergenhagen (ed.) Host-parasite interactions in periodontal disease. Am. Soc. Microbiol. Washington, DC.

Lund, B. M. 1969. Properties of some pectolytic, yellow pigmented, Gram-negative bacteria isolated from fresh cauliflowers. J. Appl. Bacteriol. 32:60–67.

Lundin, S. J. 1968. A bacterial factor capable of solubilizing cholinesterase from plaice body muscle. Acta Chem. Scand. 22:2519–2528.

Lundin, S. J., Å. Bovallius. 1966. The solubilization of a cholinesterase from plaice muscle by bacteria. Acta Chem. Scand. 20:395–402.

Mandel, M., E. R. Leadbetter. 1965. Deoxyribonucleic acid base composition of myxobacteria. J. Bacteriol. 90:1795–1796.

Mandel, M., R. A. Lewin. 1969. Deoxyribonucleic acid base composition of flexibacteria. J. Gen. Microbiol. 58:171–178.

Martin, H. H., H. J. Preusser, J. P. Verma. 1968. Über die Oberflächenstruktur von Myxobakterien II. Anionische Heteropolysaccharide als Baustoffe der Schleimhülle von *Cytophaga hutchinsonii* und *Sporocytophaga myxococcoides*. Arch. Mikrobiol. 62:72–84.

Martin, S. M., V. So. 1969. Solubilization of autoclaved feathers and wool by myxobacteria. Can. J. Microbiol. 15: 1393–1397.

Mayer, H., H. Reichenbach. 1978. Restriction endonucleases: General survey procedure and survey of gliding bacteria. J. Bacteriol. 136:708–713.

Mehra, I. J., G. M. Warke, S. A. Dhala. 1967. Effect of zinc salts on *Cytophaga* spp. Ind. J. Microbiol. 7:75–78.

Moss, C. W., S. B. Dees. 1978. Cellular fatty acids of *Flavobacterium meningosepticum* and *Flavobacterium* species group IIb. J. Clin. Microbiol. 8:772–774.

Mulbry, W. W., J. S. Karns, P. C. Kearney, J. O. Nelson, C. S. McDaniel, J. R. Wild. 1986. Identification of a plasmid-borne parathion hydrolase gene from *Flavobacterium* sp. by Southern hybridization with *opd* from *Pseudomonas diminuta*. Appl. Environm. Microbiol. 51:926–930.

Murayama, Y., K. Muranishi, H. Okada, K. Kato, S. Kotani, H. Takada, M. Tsujimoto, A. Kawasaki, T. Ogawa. 1982. Immunological activities of *Capnocytophaga* cellular components. Infect. Immun. 36:876–884.

Nakamura, M., J. Slots. 1982. Aminopeptidase activity of *Capnocytophaga*. J. Periodont. Res. 17:597–603.

Negoro, S., H. Okada. 1982. Physical map of nylon oligomer degradative plasmid pOAD2 harbored in *Flavobacterium* sp. KI72. Agric. Biol. Chem. 46:745–750.

Newman, M. G., S. S. Socransky, E. D. Savitt, D. A. Propas, A. Crawford. 1976. Studies of the microbiology of periodontosis. J. Periodontol. 47:373–379.

Ordal, E. J., R. R. Rucker. 1944. Pathogenic myxobacteria. Proc. Soc. Exptl. Biol. Med. 56:15–18.

Osmundsvåg, K., J. Goksøyr. 1975. Cellulases from *Sporocytophaga myxococcoides*. Eur. J. Biochem. 57:405–409.

Ostland, V. E., H. W. Ferguson, R. M. W. Stevenson. 1989. Case report: bacterial gill disease in goldfish *Carassias auratus*. Dis. Aquat. Org. 6:179–184.

Owen, R. J., J. J. S. Snell. 1976. Deoxyribonucleic acid reassociation in the classification of flavobacteria. J. Gen. Microbiol. 93:89–102.

Oyaizu, H., K. Komagata. 1981. Chemotaxonomic and phenotypic characterization of the strains of species in the *Flavobacterium-Cytophaga* complex. J. Gen. Appl. Microbiol. 27:57–107.

Oyaizu, H., K. Komagata, A. Amemura, T. Harada. 1982. A succinoglycan-decomposing bacterium, *Cytophaga arvensicola* sp. nov. J. Gen. Appl. Microbiol. 28:369–388.

Pacha, R. E. 168. Characteristics of *Cytophaga psychrophila* (Borg) isolated during outbreaks of bacterial cold-water disease. Appl. Microbiol. 16:97–101.

Pacha, R. E., E. J. Ordal. 1967. Histopathology of experimental columnaris disease in young salmon. J. Comparative Pathol. 77:419–423.

Pacha, R. E., E. J. Ordal. 1970. Myxobacterial diseases of salmonids. 243–257. S. F. Snieszko (ed.) A symposium on diseases of fishes and shellfishes. Am. Fish. Soc., Special Publ. no. 5. Washington, DC.

Pacha, R. E., S. Porter. 1968. Characteristics of myxobacteria isolated from the surface of freshwater fish. Appl. Microbiol. 16:1901–1906.

Paerregaard, A., E. Gutschick. 1987. *Capnocytophaga* bacteremia complicating premature delivery by cesarean section. Eur. J. Clin. Microbiol. 6:580–581.

Paster, B. J., W. Ludwig, W. G. Weisburg, E. Stackebrandt, R. B. Hespell, C. M. Hahn, H. Reichenbach, K. O. Stetter, C. R. Woese. 1985. A phylogenetic grouping of the bacteroides, cytophagas, and certain flavobacteria. System. Appl. Microbiol. 6:34–42.

Pate, J. L. 1985. Gliding motility in *Cytophaga*. Microbiol. Sciences 2:289–295.

Pate, J. L., L. Y. E. Chang. 1979. Evidence that gliding motility in prokaryotic cells is driven by rotary assemblies in the cell envelopes. Current Microbiol. 2:59–64.

Pate, J. L., J. L. Johnson, E. J. Ordal. 1967. The fine structure of *Chondrococcus columnaris* II. Structure and formation of rhapidosomes. J. Cell Biol. 35:15–35.

Pate, J. L., E. J. Ordal. 1967a. The fine structure of *Chondrococcus columnaris* I. Structure and formation of mesosomes. J. Cell Biol. 35:1–13.

Pate, J. L., E. J. Ordal. 1967b. The fine structure of *Chondrococcus columnaris* III. The surface layers of *Chondrococcus columnaris*. J. Cell Biol. 35:37–51.

Pate, J. L., S. J. Petzold, L. Y. E. Chang. 1979. Phages for the gliding bacterium *Cytophaga johnsonae* that infect only motile cells. Current Microbiol. 2:257–262.

Payza, A. N., E. D. Korn. 1956a. The degradation of heparin by bacterial enzymes. J. Biol. Chem. 223:853–864.

Payza, A. N., E. D. Korn. 1956b. Bacterial degradation of heparin. Nature 177:227–232.

Perry, L. B. 1973. Gliding motility in some nonspreading flexibacteria. J. Appl. Bacteriol. 36:227–232.

Pichinoty, F., J. Bigliardi-Rouvier, M. Mandel, B. Greenway, G. Méténier, J. L. Garcia. 1976. The isolation and properties of a denitrifying bacterium of the genus *Flavobacterium*. Antonie van Leeuwenhoek 42:349–354.

Poirier, T. P., S. C. Holt. 1983a. Acid and alkaline phosphatases of *Capnocytophaga* species. I. Production and cytological localization of the enzymes. Can. J. Microbiol. 29:1350–1360.

Poirier, T. P., S. C. Holt. 1983b. Acid and alkaline phosphatases of *Capnocytophaga* species. II. Isolation, purification, and characterization of the enzymes from

Capnocytophaga ochracea. Can. J. Microbiol. 29:1361–1368.

Poirier, T. P., S. C. Holt. 1983c. Acid and alkaline phosphatases of *Capnocytophaga* species. III. The relationship of the enzymes to the cell wall. Can. J. Microbiol. 29:1369–1381.

Poos, J. C., F. R. Turner, D. White, G. D. Simon, K. Bacon, C. T. Russell. 1972. Growth, cell division, and fragmentation in a species of *Flexibacter*. J. Bacteriol. 112:1387–1395.

Porter, D., S. Y. Newell, W. L. Lingle. 1989. Tunneling bacteria in decaying leaves of a seagrass. Aquat. Bot. 35:395–401.

Pringsheim, E. G. 1951. The Vitreoscillaceae: a family of colorless, gliding, filamentous organisms. J. Gen. Microbiol. 5:124–149.

Pyle, S. W., E. B. Shotts. 1981. DNA homology studies of selected flexibacteria associated with fish disease. Can. J. Fish. Aquat. Sci. 38:146–151.

Reichardt, W. 1974. Ecophysiology of some aquatic bacteria from the *Flavobacterium-Cytophaga* group. Zbl. Bakteriol. 1. Abt. Orig., Reihe A 227:85–93.

Reichardt, W., B. Gunn, R. R. Colwell. 1983. Ecology and taxonomy of chitinoclastic *Cytophaga* and related chitin-degrading bacteria isolated from an estuary. Microb. Ecol. 9:273–294.

Reichardt, W., R. Y. Morita. 1982a. Influence of temperature adaptation on glucose metabolism in a psychrotrophic strain of *Cytophaga johnsonae*. Appl. Environm. Microbiol. 44:1282–1288.

Reichardt, W., R. Y. Morita. 1982b. Survival stages of a psychrotrophic *Cytophaga johnsonae* strain. Can. J. Microbiol. 28:841–850.

Reichenbach, H. 1988. Gliding bacteria in biotechnology. 673–696. H. J. Rehm and G. Reed (ed.) Biotechnology, vol. 6b. VCH Verlagsges. Weinheim, Germany.

Reichenbach, H. 1989. *Flexibacter*. 2061–2071. J. Staley (ed.) Bergey's manual of systematic bacteriology, vol. 3. Williams and Wilkins. Baltimore.

Reichenbach, H., K. Gerth, H. Irschik, B. Kunze, G. Höfle, H. Augustiniak, R. Jansen, T. Kemmer, W. Kohl, H. Steinmetz, W. Trowitzsch. 1984. Results of a screening for antibiotics with gliding bacteria. Third Eur. Congr. Biotechnol., München 1:15–21.Verlag Chemie. Weinheim, Germany.

Reichenbach, H., H. Kleinig, H. Achenbach. 1974. The pigments of *Flexibacter elegans*: novel and chemosystematically useful compounds. Arch. Microbiol. 101:131–144.

Reichenbach, H., W. Kohl, H. Achenbach. 1981. The flexirubin-type pigments, chemosystematically useful compounds. 101–108. H. Reichenbach and O. B. Weeks (ed.) The *Flavobacterium-Cytophaga* group. Verlag Chemie. Weinheim, Germany.

Reichenbach, H., W. Kohl, A. Böttger-Vetter, H. Achenbach. 1980. Flexirubin-type pigments in *Flavobacterium*. Arch. Microbiol. 126:291–293.

Reichenbach, H., W. Ludwig, E. Stackebrandt. 1986. Lack of relationship between gliding cyanobacteria and filamentous gliding heterotrophic eubacteria: Comparison of 16S rRNA catalogues of *Spirulina, Saprospira*, *Vitreoscilla, Leucothrix* and *Herpetosiphon*. Arch. Microbiol. 145:391–395.

Reichenbach, H., O. B. Weeks (ed.). 1981. The *Flavobacterium-Cytophaga* group. Verlag Chemie. Weinheim, Germany.

Richter, C. A., J. L. Pate. 1988. Temperate phages and bacteriocins of the gliding bacterium *Cytophaga johnsonae*. J. Gen. Microbiol. 134:253–262.

Ridgway, H. F. 1977a. Source of energy for gliding motility in *Flexibacter polymorphus*: Effects of metabolic and respiratory inhibitors on gliding movement. J. Bacteriol. 131:544–556.

Ridgway, H. F. 1977b. Ultrastructural characterization of goblet-shaped particles from the cell wall of *Flexibacter polymorphus*. Can. J. Microbiol. 23:1201–1213.

Ridgway, H. F., R. A. Lewin. 1973. Goblet shaped sub-units from the wall of a marine gliding microbe. J. Gen. Microbiol. 79:119–128.

Ridgway, H. F., R. A. Lewin. 1983. Subunit composition of goblet-shaped particles from the cell wall of *Flexibacter polymorphus*. Can. J. Microbiol. 29:1689–1693.

Ridgway, H. F., R. M. Wagner, W. T. Dawsey, R. A. Lewin. 1975. Fine structure of the cell envelope layers of *Flexibacter polymorphus*. Can. J. Microbiol. 21:1733–1750.

Rivière, J. 1961. Activité cellulolytique des bactéries aérobies du sol. I. Isolement et purification. Ann. Agron. 12:385–398.

Rivière, J. 1961b. Activité cellulolytique des bactéries aérobies due sol. II. Action des facteurs due milieu sur la production de cellulases bactériennes. Ann. Agron. 12:399–424.

Rosenfelder, G., O. Lüderitz, O. Westphal. 1974. Composition of lipopolysaccharides from *Myxococcus fulvus* and other fruiting and non-fruiting myxobacteria. Eur. J. Biochem. 44:411–420.

Rucker, R. R., B. J. Earp, E. J. Ordal. 1953. Infectious diseases of Pacific salmon. Transact. Am. Fish. Soc. 83:297–312.

Ruschke, R. 1968. Die Bedeutung von Wassermyxobakterien für den Abbau organischen Materials. Mitt. Internat. Verein. Limnol. 14:164–167.

Ruschke, R., K. Köhn. 170. Untersuchungen zum Abbau kondensierter Phosphate aus Waschmiteln durch *Sporocytophaga cauliformis* un *Pseudomonas fluorescens*. Zbl. Bakteriol., 2. Abt. 124:81–90.

Ruschke, R., M. Rath. 1966. *Sporocytophaga cauliformis* Knorr and Gräf, eine Myxobakterienart mit groβer Bedeutung für den Abbau organischen Materials. Arch. Hydrobiol., Suppl. 28:377–402.

Saito, A., I. Takazoe, K. Okuda. 1988. Comparison of hemagglutinating activity, adsorption to saliva-treated hydroxyapatite, and cell surface hydrophobicity of Gram-negative periodontopathic bacteria. Bull. Tokyo Dent. Coll. 29:51–57.

Sandholm, L., K. Mahlberg, H. Jousimies-Somer. 1988. Phospholipase A_2: a possible virulence factor of *Capnocytophaga ochracea*. Oral Microbiol. Immunol. 3:18–21.

Sanfilippo, A., R. A. Lewin. 1970. Preservation of viable flexibacteria at low temperatures. Can. J. Microbiol. 16:441–444.

Sangkhobol, V., V. B. D. Skerman. 1981. *Chitinophaga,* a new genus of chitinolytic myxobacteria. Int. J. System. Bacteriol. 31:285–293.

Sarwar, G., S. Matayoshi, H. Oda. 1987. Purification of a κ-carrageenase from marine *Cytophaga* species. Microbiol. Immunol. 31:869–877.

Shewan, J. M. 1971. The microbiology of fish and fishery products—a progress report. J. Appl. Bacteriol. 34:299–315.

Shewan, J. M., T. A. McMeekin. 1983. Taxonomy (and ecology) of *Flavobacterium* and related genera. Annu. Rev. Microbiol. 37:233–252.

Shieh, H. S. 1980. Studies on the nutrition of a fish pathogen, *Flexibacter columnaris*. Microbios Lett. 13:129–133.

Shiigi, S. M., R. R. Capwell, K. H. Grabstein, R. I. Mishell. 1977. Sera and the in vivo induction of immune responses III. Adjuvant obtained from gliding bacteria with properties distinct from gliding bacteria with properties distinct from enteric bacterial lipopolysaccharide. J. Immunol. 119:679–684.

Shklair, I. L., S. A. Ralls. 1988. Periodontopathic microorganisms in the rice rat (*Oryzomys palustris*). Microbios 55:25–31.

Shlaes, D. M., M. J. Dul, P. I. Lerner. 1982. *Capnocytophaga* bacteremia in the compromised host. Am. J. Clin. Pathol. 77:359–361.

Shoji, J., H. Hinoo, K. Matsumoto, T. Hattori, T. Yoshida, S. Matsuura, E. Kondo. 1988. Isolation and characterization of katanosins A and B. J. Antibiot. 41:713–718.

Shoji, J., T. Kato, R. Sakazaki, W. Nagata, Y. Terui, Y. Nakagawa, M. Shiro, K. Matsumoto, T. Hattori, T. Yoshida, E. Kondo. 1984. Chitinovorins A, B and C, novel β-lactam antibiotics of bacterial origin. J. Antibiot. 37:1486–1490.

Shurin, S. B., S. S. Socransky, E. Sweeney, T. P. Stossel. 1979. A neutrophil disorder induced by *Capnocytophaga*, a dental microorganism. New Engl. J. Med. 301:849–854.

Sijpesteijn, A. K., G. Fåhraeus. 1949. Adaptation of *Sporocytophaga myxococcoides* to sugars. J. Gen. Microbiol. 3:224–235.

Simon, G. D., D. White. 1971. Growth and morphological characteristics of a species of *Flexibacter*. Arch. Mikrobiol. 78:1–16.

Singh, P. D., J. H. Johnson, P. C. Ward, J. S. Wells, W. H. Trejo, R. B. Stykes. 1983. SQ 28,332, a new monobactam produced by a *Flexibacter* sp. Taxonomy, fermentation, isolation, structure determination and biological properties. J. Antibiot. 36:1245–1251.

Singh, P. D., P. C. Ward, J. S. Wells, C. M. Ricca, W. H. Trejo, P. A. Principe, R. B. Sykes. 1982. Bacterial production of deacetoxycephalosporin. C. J. Antibiot. 35:1397–1399.

Singh, P. D., M. G. Young, J. H. Johnson, C. M. Cimarusti, R. B. Sykes. 1984. Bacterial production of 7-formamidocephalosporins. Isolation and structure determination. J. Antibiot. 37:773–780.

Snieszko, S. F. 1953. Therapy of bacterial fish diseases. Transact. Americ. Fish. Soc. 83:313–330.

Snieszko, S. F. 1974. The effects of environmental stress on outbreaks of infectious diseases of fishes. J. Fish Biol. 6:197–208.

Snieszko, S. F., G. L. Bullock. 1976. Columnaris disease of fishes, Fish disease leaflet no. 45. U. S. Department of the Interior, Fish and Wildlife Service. Washington, DC.

Solntseva, L. I. 1940. Biology of myxobacteria. I. *Myxococcus*. Mikrobiologiya 9:217–232 (In Russian, with English summary).

Song, Y. L., J. L. Fryer, J. S. Rohovec. 1988a. Comparison of six media for the cultivation of *Flexibacter columnaris*. Fish Pathol. 23:91–94.

Song, Y. L., J. L. Fryer, J. S. Rohovec. 1988b. Comparison of gliding bacteria isolated from fish in North America and other areas of the Pacific rim. Fish Pathol. 23:197–202.

Soriano, S. 1945. El nuevo orden Flexibacterales y la clasificación de los órdenes de las bacterias. Revistaa Argentina de Agronomia (Buenos Aires) 12:120–140.

Soriano, S. 1947. The Flexibacterales and their systematic position. Antonie van Leeuwenhoek 12:215–222.

Spangenberg, R. 1975. Orientierende Untersuchungen über das Vorkommen von Myxobakterien bei der Kiemennekrose des Karpfens. Ztschr. Binnenfisch. DDR 22: 121–127.

Speyer, E. 1953. Untersuchungen an *Sporocytophaga myxococcoides* (Stanier 1940). Arch. Mikrobiol. 18:245–272.

Stanier, R. Y. 1941. Studies on marine agar digesting bacteria. J. Bacteriol. 42:527–558.

Stanier, R. Y. 1942. The *Cytophaga* group: A contribution to the biology of myxobacteria. Bacteriol. Rev. 6:143–196.

Stanier, R. Y. 1947. Studies on nonfruiting myxobacteria. I. *Cytophaga johnsonae* n. sp., a chitin-decomposing myxobacterium. J. Bacteriol. 53:297–315.

Stapp, C., H. Bortels. 1934. Mikrobiologische Untersuchungen über die Zersetzung von Waldstreu. Zbl. Bakteriol. 2. Abt. 90:28–66.

Starliper, C. E., E. B. Shotts, T. C. Hsu, W. B. Schill. 1988. Genetic relatedness of some Gram-negative yellow pigmented bacteria from fishes and aquatic environments. Microbios 56:181–198.

Stevens, R. H., B. F. Hammond. 1988. The comparative cytotoxicity of a periodontal bacteria. J. Periodontol. 59:741–749.

Strohl, W. R. 1979. Ultrastructure of *Cytophaga johnsonae* and *C. aquatilis* by freeze-etching. J. Gen. Microbiol. 112:261–268.

Strohl, W. R., L. R. Tait. 1978. *Cytophaga aquatilis* sp. nov., a facultative anaerobe isolated from the gills of freshwater fish. Int. J. Syst. Bacteriol. 28:293–303.

Stucki, G., M. Alexander. 1987. Role of dissolution rate and solubility in biodegradation of aromatic compounds. Appl. Environm. Microbiol. 53:292–297.

Stürzenhofecker, P. 1966. Bakteriophagen bei Wassermyxobakterien. Arch. Hyg. Bakteriol. 150:153–157.

Suido, H., M. Nakamura, P. A. Mashimo, J. J. Zambon, R. J. Genco. 1986. Arylaminopeptidase activities of oral bacteria. J. Dent. Res. 5:1335–1340.

Sundarraj, N., J. V. Bhat. 1971. Endo-polygalacturonate lyase of *Cytophaga johnsonii*. Arch. Mikrobiol. 77:155–164.

Sundarraj, N., J. V. Bhat. 1972. Breakdown of chitin by *Cytophaga johnsonii*. Arch. Mikrobiol. 85:159–167.

Sutherland, I. W., M. L. Smith. 1973. The lipopolysaccharides of fruiting and non-fruiting myxobacteria. J. Gen. Microbiol. 74:259–266.

Tchan, Y. T., J. Giuntini. 1950. Action antagoniste chez les Cytophagaceae. Ann. Inst. Pasteur 50:415–416.

Turvey, J. R., J. Christison. 1967. The hydrolysis of algal galactans by enzymes from a *Cytophaga* species. Biochem. J. 105:311–316.

Umezawa, H., Y. Okami, S. Kurasawa, T. Ohnuki, M. Ishizuka, T. Takeuchi, T. Shiio, Y. Yugari. 1983. Marinactan, antitumor polysaccharide produced by marine bacteria. J. Antibiot. 36:471–477.

Usinger, W. R., G. C. Clark, E. Gottschalk, S. Holt, R. I. Mishell. 1985. Characteristics of bacterium GB-2, a presumptive *Cytophaga* species with novel immunoregulatory properties. Current Microbiol. 12:203–208.

Valentine, A. F., G. B. Chapman. 1966. Fine structure and host-virus relationship of a marine bacterium and its bacteriophage. J. Bacteriol. 92:1535–1554.

Vance, I., C. M. Topham, S. L. Blayden, J. Tampion. 1980. Extracellular cellulase production by *Sporocytophaga myxococcoides* NCIB 8639. J. Gen. Microbiol. 117:235–241.

van der Meulen, H. J., W. Harder. 1975. Production and characterization of the agarase of *Cytophaga flevensis*. Antonie van Leeuwenhoek 41:431–447.

van der Meulen, H. J., W. Harder. 1976. Characterization of the neoagarotetra-ase and neoagarobiase of *Cytophaga flevensis*. Antonie van Leeuwenhoek 42:81–94.

van der Meulen, H. J., W. Harder., H Veldkamp. 1974. Isolation and characterization of *Cytophaga flevensis* sp. nov., a new agarolytic flexibacterium. Antonie van Leeuwenhoek 40:329–346.

Veldkamp, H. 1955. A study of the aerobic decomposition of chitin by microorganisms. Mededelingen van de Landbouwhogeschool te Wageningen 55:127–174.

Veldkamp, H. 1961. A study of two marine agar-decomposing, facultatively anaerobic myxobacteria. J. Gen. Microbiol. 26:331–342.

Veldkamp, H. 1965. Isolation of *Cytophaga* and *Sporocytophaga*. Zbl. Bakteriol. 1. Abt., Suppl. 1:81–90.

Verma, J. P. 1970. The amino acid sequence of mureins of *Cytophaga hutchinsonii* and *Sporocytophaga myxococcoides*. Proc. Ind. Nat. Sci. Acad. 36B:364–368.

Verma, J. P., H. H. Martin. 1967b. Chemistry and ultrastructure of surface layers in primitive myxobacteria: *Cytophaga hutchinsonii* and *Sporocytophaga myxococcoides*. Folia Microbiol. 12:248–254.

Verma, J. P., H. H. Martin. 1967. Über die Oberflächenstruktur von Myxobakterien I. Chemie und Morphologie der Zellwände von *Cytophaga hutchinsonii* und *Sporocytophaga myxococcoides*. Arch. Mikrobiol. 59:355–380.

Wakabayashi, H., S. Egusa. 1974. Characteristics of myxobacteria associated with some freshwater fish diseases in Japan. Bull. Jap. Soc. Sci. Fish. 40:751–757.

Wakabayashi, H., M. Hikida, K. Masumura. 1986. *Flexibacter maritimus* sp. nov., a pathogen of marine fishes. Int. J. Syst. Bacteriol. 36:396–398.

Wakabayashi, H., G. J. Huh, N. Kimura. 1989. *Flavobacterium branchiophila* sp. nov., a causative agent of bacterial gill disease of freshwater fishes. Int. J. Syst. Bacteriol. 39:213–216.

Wakabayashi, H., K. Kira, S. Egusa. 1970a. Studies on columnaris disease of pond-cultured eels. I. Characteristics and pathogenicity of *Chondrococcus columnaris* isolated from pond-cultured eels [In Japanese, with English summary.]. Bull. Jap. Soc. Scientif. Fish. 36:147–154.

Wakabayashi, H., K. Kira, S. Egusa. 1970b. Studies on columnaris disease of pond-cultured eels. II. The relation between gill disease and *Chondrococcus columnaris* [In Japanese, with English summary.]. Bull. Jap. Soc. Scientif. Fish. 36:678–685.

Walker, E., F. L. Warren. 1938. Decomposition of cellulose by *Cytophaga*. I. Biochem. J. 32:31–43.

Walker, R. W. 1969. Cis-11-hexadecenoic acid from *Cytophaga hutchinsonii* lipids. Lipids 4:15–18.

Ward, O. P., W. M. Fogarty. 1974. Polygalacturonate lyase production by *Bacillus subtilis* and *Flavobacterium pectionvorum*. Appl. Microbiol. 27:346–350.

Warke, G. M., S. A. Dhala. 1968. Use of inhibitors for selective isolation and enumeration of cytophagas from natural substrates. J. Gen. Microbiol. 51:43–48.

Webley, D. M., E. A. C. Follett, I. F. Taylor. 1967. A comparison of the lytic action of *Cytophaga johnsonii* on a eubacterium and a yeast. Antonie van Leeuwenhoek 33:159–165.

Weeks, O. B. 1974. Flavobacterium. 357–364. R. E. Buchanan and N. E. Gibbons (ed.) Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins. Baltimore.

Weeks, O. B. 1981. Preliminary studies of the pigments of *Flavobacterium breve* NCTC 11099 and *Flavobacterium odoratum* NCTC 11036. 109–114. H. Reichenbach and O. B. Weeks (ed.) The *Flavobacterium-Cytophaga* group. Verlag Chemie. Weinheim, Germany.

Weiss, E. I., J. London, P. E. Kolenbrander, A. S. Kagermeier, R. N. Andersen. 1987. Characterization of lectinlike surface components on *Capnocytophaga ochracea* ATCC 33596 that mediate coaggregation with Gram-positive oral bacteria. Infect. Immun. 55:1198–1202.

White, R. H. 1984. Biosynthesis of the sulfonolipid 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid in the gliding bacterium *Cytophaga johnsonae*. J. Bacteriol. 159:42–46.

Willets, A. 1983. Bacterial metabolism of aliphatic diols. Function of alcohol oxidases and catalase in *Flavobacterium* sp. NCIB 11171. J. Gen. Microbiol. 129:997–1004.

Williams, B. L., B. F. Hammond. 1979. *Capnocytophaga*: new genus of Gram-negative gliding bacteria. IV. DNA base composition and sequence homology. Arch. Microbiol. 122:35–39.

Williams, B. L., D. Hollis, L. V. Holdeman. 1979. Synonymy of strains of Center for Disease Control group DF-1 with species of *Capnocytophaga*. J. Clin. Microbiol. 10:550–556.

Winogradsky, S. N. 1929. Études sur la microbiologie du sol. Sur la dégradation de la cellulose dans le sol. Ann. Inst. Pasteur 43:549–633.

Woese, C. R., E. Stackebrandt, T. J. Macke, G. Fox. 1985. A phylogenetic definition of the major eubacterial taxa. System. Appl. Microbiol. 6:143–151.

Wolkin, R. H., J. L. Pate. 1984. Translocation of motile cells of the gliding bacterium *Cytophaga johnsonii* depends on a surface component that may be modified by sugars. J. Gen. Microbiol. 130:2651–2669.

Wolkin, R. H., J. L. Pate. 1986. Phage adsorption and cell adherence are motile-dependent characteristics of the gliding bacterium *Cytophaga johnsonii*. J. Gen. Microbiol. 132:355–367.

Yabuuchi, E., T. Kaneko, I. Yano, C. W. Moss, N. Miyoshi. 1983. *Sphingobacterium* gen. nov., *Sphingobacterium spiritivorum* comb. nov., *Sphingobacterium multivorum* comb. nov., *Sphingobacterium mizutae* sp. nov., and *Flavobacterium indologenes* sp. nov.: glucose-nonfermenting Gram-negative rods in CDC groups IIK2- and IIb. Int. J. Syst. Bacteriol. 33:580–598.

Yamamoto, T. 1967. Presence of rhapidosomes in various species of bacteria and their morphological characteristics. J. Bacteriol. 94:1746–1756.

Yano, I., S. Imaizumi, I. Tomiyasu, E. Yabuuchi. 1983. Separation and analysis of free ceramides containing 2-hydroxy fatty acids in *Sphingobacterium* species. FEMS Microbiol. Lett. 20:449–453.

Yano, I., Y. Ohno, M. Masui, K. Kato, E. Yabuuchi, A. Ohyama. 1976. Occurrence of 2-and 3-hydroxy fatty acids in high concentrations in the extractable and bound lipids of *Flavobacterium meningosepticum* and *Flavobacterium* IIb. Lipids 11:685–688.

ZoBell, C. E., H. C. Upham. 1944. A list of marine bacteria including descriptions of sixty new species. Bull. Scripps Inst. Oceanogr. Univ. Calif. (Technical Series) 5:239–292.

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CHAPTER 6.8

The Genus *Saprospira*

HANS REICHENBACH

Gram-negative gliding bacteria that form helical, multicellular filaments are grouped in the genus *Saprospira* (Fig. 1). All known species live in aquatic environments. They are moderately common but relatively difficult to isolate and therefore have not been studied very well, although some of them can be cultivated without problems. Relatively little has been added to our knowledge of *Saprospira* since the first edition of *The Prokaryotes* (Reichenbach and Dworkin, 1981).

The genus *Saprospira* was defined by Gross (1911). He described two marine species, *S. grandis* and *S. nana*, of which only the former has since been isolated by other investigators. The two organisms differed considerably in their dimensions, as shown in Table 1, which lists some characteristics of the known strains of the genus.

It seems, however, that at least three other investigators had observed saprospiras before Gross. Kolkwitz (1909) described a freshwater saprospira under the name of *Spirulina albida.* He obviously believed his organism to be an apochlorotic blue-green "alga" (cyanobacterium).

In 1875, van Tieghem (1880) observed a delicate white scum that resembled *Beggiatoa* covering the mud in the water course of an old mill. Under the microscope, he discovered that the organism consisted of long, fine, helical filaments that were so tightly wound up that the coils touched. The helices moved by rotation around their long axis. The long filaments were also actively bending. He called this organism *Spirulina alba,* thereby deliberately emphasizing its close affinity to the blue-green algae of the same genus. Although his description is rather scant, it seems almost certain that he was dealing with a *Saprospira* species.

In the same year, Warming (1875) observed a very large helical organism in marine debris from the Danish coast and named it *Spirochaeta gigantea*. From his careful description, one can deduce that this organism, too, may have been a *Saprospira* species.

Not much was published in the five decades following the definition of the genus. In addition, until the middle of the 20th century, *Saprospira* was regularly regarded as belonging to the spirochetes, which caused considerable confusion. Gross (1912) himself stressed the similarity between *Cristispira*, which was also first defined by him, and *Saprospira*. To him and most other investigators at the time, the latter was essentially a spirochete without a crista or an axial filament. Unfortunately, in stained preparations, both organisms also showed a multichambered aspect, which was in fact a staining artifact but obscured the difference in organization of the two bacteria. Dobell (1912) described a new freshwater species, *Saprospira flexuosa*. From the careful description given by Dobell, one may deduce that his organism really was a *Saprospira*, perhaps *S. albida* as suggested by Lewin (1962), although this point cannot be decided because we do not know how many different freshwater saprospiras exist. During a study on helical bacteria in the digestive tract of oysters, Dimitroff (1926) observed several types of organisms which he regarded as saprospiras. One he identified with *S. grandis*, the others he described as new species, *S. lepta* and *S. puncta*. His account of the movements of his isolate of *S. grandis* leaves considerable doubt whether the bacterium was really *Saprospira*. On the other hand, he observed true cristispiras under the microscope and distinguished their very fast movements quite clearly from those of *Saprospira*. If one takes into account the fact that before the days of the phase contrast microscope, it was not so easy to observe living bacteria, it is easier to understand this uncertainty. It might be worthwhile to investigate again for the presence of saprospiras in shellfish. The two new species could never be observed by Dimitroff in the living state so their motility behavior is not known. They both had long, tapering ends with a sharp point, which is not known for any *Saprospira* species. The giant, gliding "spirochete" observed by Soriano in an enrichment culture and very briefly mentioned by him in the legend to a rather suggestive drawing (Soriano, 1945), was probably a (freshwater) saprospira. Dyar (1947) described the isolation and cultivation of a freshwater "spirochete." This organism resembled *Saprospira* in many respects and indeed was later identified with *S. albida* by Pringsheim (1963). Skuja (1948) described the new species, *Achroonema spiroideum,* from Swedish lakes. The organism was later equated with *S. albida* (Lewin, 1962), but the very lax coils typical for *Achroonema spiroideum* leave some doubts whether this assignment is correct.

Many details about the morphology and physiology of the saprospiras were elucidated between 1960 and 1970 through the research of Lewin and his collaborators, who also defined several new species and emended the taxonomic position of the genus (Lewin, 1962, 1965a, 1965b, 1969, 1972; Lewin and Lounsbery, 1969; Lewin and Mandel, 1970). The phylogenetic relationship of *Saprospira grandis* was recently established by 16S rRNA oligonucleotide cataloging: The organism belongs to the *Bacteroides-Flavobacterium-Cytophaga* phylum (Paster et al., 1985). A relationship with the cyanobacterium *Spirulina* has specifically been ruled out (Reichenbach et al., 1986).

A short review of the genus *Saprospira* has recently been published (Reichenbach, 1989). A movie showing the movements and the development of the swarm colonies of *Saprospira* is also available (Reichenbach et al., 1975/1976; Reichenbach, 1980).

This chapter was taken unchanged from the second edition.

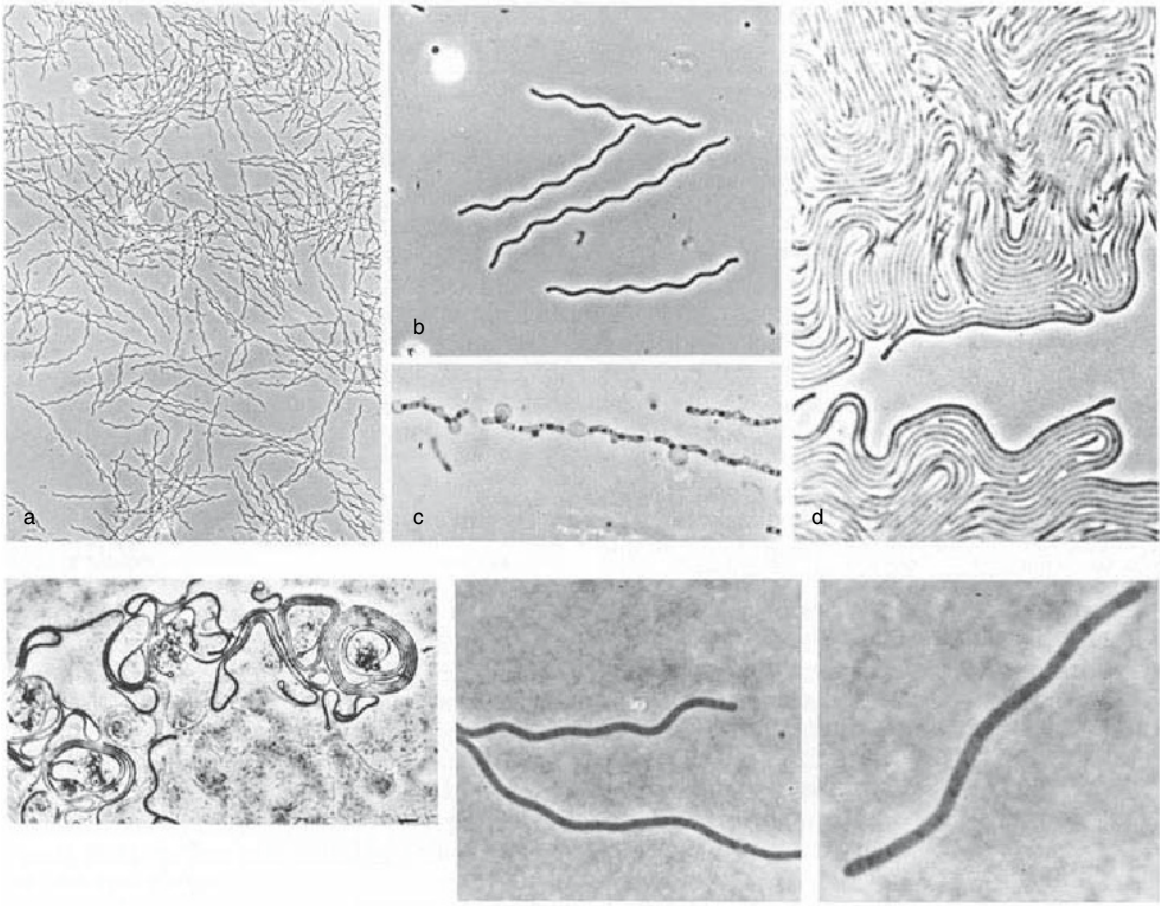

Fig. 1. Phase contrast photomicrographs of *Saprospira grandis*. (a) Helical filaments from a 1-day-old liquid culture, survey picture; ×180. (b) Filaments at higher magnification; ×500. (c) Decaying filament showing the individual cells; ×900. (d) Growing in the narrow space between agar and cover glass in a chamber culture, the filaments have lost most of their helical shape and concomitantly their ability to glide; ×920. (Lower left) Nonhelical filaments in a chamber culture with large, optically refractile, terminal bulbs; ×470. (Lower center and lower right) At very high magnification (×1000 and × 1200; Zeiss Axiomat) the cross-walls in the filaments are visible. The lower filament shows a constriction near one end, perhaps one possible way of filament fragmentation.

Habitats

The saprospiras appear to live exclusively in aquatic environments. *S. grandis* and other marine species have been isolated from sand and mud and collected at the sea coast at many different locations all over the world (Gross, 1911; Lewin and Lounsbery, 1969; Lewin and Mandel, 1970). One strain of *S. grandis* has been obtained from a rotting crab carapace (Reichenbach, 1980). As discussed above, several species may possibly be found within oysters (Dimitroff, 1926). Saprospiras are common also in fresh water (Lewin, 1965b). They seem to prefer eutrophic conditions and are often associated with algae. Saprospiras have been obtained from the mud in rivers and lakes (Kolkwitz, 1909; Pringsheim, 1963), from planktonic algae and cyanobacteria, as well as from the upper layers of surface waters, in which the filaments may be free-floating (Dobell, 1912; Skuja, 1948; Brunel, 1949; Ashton and Robarts, 1987), from decaying organic matter in a sulfur spring (Dyar, 1947), from a bog (Jarosch, 1967), and from sewage plants, including activated sludge (Kolkwitz, 1909; Cyrus and Sladká, 1970; Sladká and Ottová, 1973). *S. thermalis* was first isolated from a hot spring in Iceland, but the organism is not thermophilic and in fact not even thermotolerant, for its maximum temperature is 35–37°C (Lewin, 1965a). Only one strain of *S. albida* was ever isolated from soil. The sample came from Canada, but unfortunately nothing was said about the water conditions of that soil (Lewin, 1965b).

Interesting details about the interaction of a saprospira species with the cyanobacterium

Table 1. Characteristics of the *Saprospira* strains.

Strain	Morphological characteristic (μm)[a]					GC content (mol%)	Habitat
	Length of filament	Diameter of filament	Width of helix	Pitch of helix	Length of cell		
S. grandis, Gross 1911	6–100	0.8		6–6.5	1.5–2.2		Marine
S. grandis, Lewin 1962	10–500	0.8–1.2	1.5–2	4–10	1–2.5	46–48	Marine
S. grandis, Dimitroff 1926[b]	50–90	1.2–1.4[c]		20–28			Oyster, marine
S. grandis, Reichenbach 1980	15–450	0.8–0.9	1.4–1.8	5–6.5	2.7–5.5	47	Marine
S. nana, Gross 1911	36	0.5		2.3–3	1.5–3		Marine
S. gigantea, Warming 1875	About 400	1.5–3	5–9	25–40			Marine
S. toviformis, Lewin and Mandel 1970	10–500	0.8	1.5	4–9	1–2.5	38	Marine
S. lepta, Dimitroff 1926[b,d]	54–92	0.5	1.6–4.8	5–13			Oyster, marine
S. puncta, Dimitroff 1926[b,d]	60–100	0.9–1.2		4–8			Oyster, marine
S. albida, Kolkwitz 1909		1	4–5				Fresh water
S. albida, Dyar 1947	About 400		2	3–6.5			Fresh water
S. albida, Lewin 1965b	10–500	0.8–1.2	1.5–2	3–7	2–3	40–43	Fresh water
S. albida, Ashton and Roberts 1987	10–450	0.8–0.9	1.4–1.6	2.9–4.9	2.1–2.7		Fresh water
S. flexuosa, Dobell 1912[e]	3–50	0.8	2	3			Fresh water
S. spiroidea, Skuja 1948[f]	10–250	0.3–0.5	2.8–5.7	9–25	3–15		Fresh water
S. flammula, Lewin 1965b	10–500	1.0	1.5	3–4	2–3	48	Fresh water
S. thermalis, Lewin 1965a	10–500	1.0	1.5–2.5	7–17	2–5	35–37	Fresh water

In the older literature, measurements were often taken from fixed and stained specimens which may have been considerably distorted.
From the description by Dimitroff it cannot be decided with confidence whether his organisms were true saprospiras.
It is not clear whether Dimitroff was talking about the width of the filament or the width of the helix.
With tapering ends.
Probably actually *S. albida*.
Achroonema spiroideum was equated with *S. albida*, but it forms a very lax helix, and, in this respect, it resembles *S. thermalis* more closely than *S. albida*.

Microcystis aeruginosa in a South African reservoir have been published (Ashton and Robarts, 1987). Usually *Saprospira* is found in the surface layers of the lake at a very low density of less than 10 filaments per liter, but during *Microcystis* blooms, its density increases to more than 10,000 filaments per liter, and the saprospiras colonize and lyse the floating colonies of the cyanobacterium. Curiously, the *Saprospira* seems selectively to attack a toxic variant of *M. aeruginosa,* which is dominant at the site for most of the year. It has been suggested that saprospiras could be useful to control this undesired organism.

Isolation

No specific enrichment methods are known for saprospiras. Gross (1911) described a collection technique that makes use of the tendency of *Saprospira* to attach itself to a substrate surface. He filled a large petri dish with a sample of marine sand, covered it with sea water, placed coverslips on the water surface, and left them floating overnight. Most of the saprospiras stuck to the glass surface and could be removed from the crude culture.

To isolate saprospiras, samples are taken from appropriate habitats (see above) and placed as well-separated streaks or spots onto the surface of dry agar plates. To prevent excessive growth of contaminants, the media should contain nutrients in low concentrations only. For marine samples, MS1, SW2, or SP6 agar can be used. It appears that for saprospiras, natural and artificial seawater are equivalent, so that the following media could be prepared with either one.

MS1 Agar

Agar 1.5%

Natural seawater

The pH is not adjusted. The medium is autoclaved.

SW2 Agar

Agar	1.5%
NH_4Cl	0.1%
Na acetate	0.002%

Artificial seawater (see below)

The pH is adjusted to 7.2. The medium is autoclaved.

Artificial Seawater (Dawson et al., 1972)

The following recipe has been used by us with good results: NaCl, 24.7 g; KCl, 0.7 g; $MgSO_4 \cdot 7H_2O$, 6.3 g; $MgCl_2 \cdot 6H_2O$, 4.6 g; $CaCl_2$ (anhydrous), 1.0 g; $NaHCO_3$, 0.2 g. Bring to 1 liter with water. To avoid precipitation, $CaCl_2$ and $NaHCO_3$ are autoclaved as separate stock solutions.

SP6 Agar

Casitone (Difco)	0.03%
Yeast extract (Difco)	0.01%
Agar	1.5%

Artificial seawater (see above)
The pH is adjusted to 7.2. The medium is autoclaved.

The freshwater saprospiras are often more fastidious, and most investigators were only able to maintain their isolates for longer periods in mixed cultures, e.g., in fouling soil suspensions such as soil enriched with proteinaceous material in water (Pringsheim, 1963). Repeatedly, it has been found that small quantities of H_2S are stimulatory, although the organisms do not really depend on it (Dobell, 1912; Dyar, 1947). Perhaps the good results with Pringsheim's technique were also due to the production of H_2S from the added protein. Dyar (1947) seems to have been the first to succeed in isolating and cultivating a strain of *S. albida*. She used a leaf-infusion agar with a few drops of blood for isolation.

Several freshwater saprospiras were isolated by Lewin using LEW1 agar (Lewin 1965a, 1965b).

LEW1 Agar (Lewin, 1965b)

$Ca(NO_3)_2 \cdot 4H_2O$	0.05%
$MgSO_4 \cdot 7H_2O$	0.01%
K_2HPO_4	0.01%
Tryptone (Difco)	0.01%
Agar	1%

Yeast extract (Difco) can also be used in place of tryptone.

The crude cultures are incubated at 30°C, at room temperature, or in the refrigerator, depending on the starting material. For freshwater strains, 23–27°C is recommended (Lewin, 1965a). Although marine *S. grandis* has a temperature optimum around 30°C, it is useful to keep crude and enrichment cultures at 6°C, since the organism still grows and spreads reasonably well at that temperature, while the growth of contaminants is considerably reduced (H. Reichenbach, unpublished observations). The crude cultures are examined from time to time under a dissecting microscope. Oblique illumination should be applied to make delicate, spreading colonies also visible. Already after 3 to 4 days or, at lower temperatures, after a longer interval of up to 2 to 3 weeks, spreading colonies with long, rootlike tongues (Fig. 2) or with irregular, flamelike edges may be found. This is the typical appearance of colonies of gliding bacteria. *Saprospira* colonies often also show a very regular striped pattern (Dyar, 1947; Reichenbach et al., 1975/76; Reichenbach, 1980), which is seen with other screw-shaped bacteria, e.g., *Methanospirillum hungatii* (Ferry et al., 1974). A microscopic control should show the typical *Saprospira* filaments (Fig. 1) and their characteristic movements (Reichenbach et al., 1975/76). Further purification is achieved by making transfers from the spreading edges of the swarm colonies; it is best to cut out a piece of the colony using the sharp point of a 1-ml disposable syringe. The material is streaked on the same medium as before or, as soon as the colonies appear sufficiently pure, also on a richer medium, e.g., SAP2 agar for marine and LEW2 and LEW3 agar for freshwater strains.

SAP2 Agar

Tryptone (Difco)	0.1%
Yeast extract (Difco)	0.1%
Agar	1.5%

Artificial seawater (see above)
The pH is adjusted to 7.2. The medium is autoclaved.

LEW2 and LEW3 Agar (Lewin, 1965a)

The recipe is the same as for LEW1 agar, but the tryptone concentration is raised to 0.03% and 0.3%, respectively.

Finally, diluted filament suspensions may be plated on the richer versions of the above-mentioned media. The most troublesome contaminants are other gliding bacteria and, in marine samples, agar decomposers. Both groups should be eliminated at the very beginning of the enrichment process.

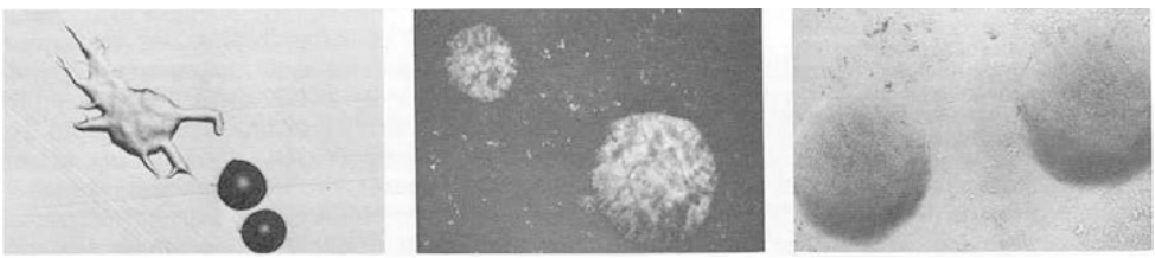

Fig. 2. Colonial morphology of *Saprospira grandis*. (Left) Colony on peptone-seawater agar together with two colonies of a nongliding organism. The *Saprospira* colony is rather tough and remains relatively compact; it spreads mainly along the grooves scratched into the agar surface by the inoculation loop. Leitz Aristophot; ×7.2. (Middle) Compact colonies on SP2 agar, in dark field; ×33. (Right) Colonies under oblique illumination; ×33. In both cases, the appearance of the colonies suggests some orderly arrangement of the filaments; in bright field, a pattern of fine, parallel, concentric stripes can be seen; Zeiss Axiomat.

Cultivation

All *Saprospira* strains that have been studied so far are strictly aerobic organotrophs and seem to prefer amino acids as carbon, nitrogen, and energy sources, often without an alternative. Many marine strains grow well on relatively simple media, such as SAP1 or SAP2 agar.

SAP1 Agar (Lewin, 1962)

Tryptone (Difco)	0.5%
Yeast extract (Difco)	0.5%
Agar	1.5%

Artificial seawater (see above)
The pH is adjusted to 7.2. The medium is autoclaved.

The marine saprospiras can be cultivated without difficulties and with good yields in liquid media, such as SP5 liquid medium or SAP1 liquid medium (as for SAP1 agar, but without agar). They usually form homogeneous suspensions in shake cultures.

SP5 Liquid Medium

Casitone (Difco)	0.9%
Yeast extract (Difco)	0.1%

Artificial seawater (see above)
The pH is adjusted to 7.2. The medium is autoclaved.

An almost fully defined medium is reported for *S. grandis* (Lewin, 1972). It is composed of nine essential amino acids, mineral salts, and an unknown growth factor present in yeast nucleic acid hydrolysate. The marine saprospiras almost always require at least half-strength sea water, but *S. toviformis* and a few *S. grandis* strains also grow at freshwater salt concentrations (Lewin and Lounsbery, 1969).

Among the freshwater saprospiras, *S. thermalis* is the least fastidious one and can be grown on relatively simple media, e.g., LEW3 agar (see above), and in LEW4 liquid medium. A synthetic medium also has been developed (Lewin, 1965a).

LEW4 Liquid Medium (Lewin, 1965a)

Casamino acids (Difco)	0.1%
Sodium glutamate	0.1%
Glucose (sterilized separately)	0.1%
Tris (hydroxymethyl) aminomethane	0.1%
$MgSO_4 \cdot 7H_2O$	0.01%
KNO_3	0.01%
$CaCl_2 \cdot 2H_2O$	0.01%
Sodium glycerophosphate	0.01%
Thiamine	1 mg/liter
Cobalamine	1 µg/liter
Fe	0.5 mg/liter
Zn	0.3 mg/liter
B, Co, Cu, Mn, Mo each	0.1 mg/liter

The pH is adjusted to 7.5. The medium is autoclaved.

Good growth was also obtained in a medium containing the mineral salts (and glycerophosphate) of LEW4 liquid medium plus tryptone (Difco) 0.02% and glucose 0.02%. If the tryptone is replaced by casamino acids, the addition of the vitamins became necessary (as in LEW4 liquid medium). The casamino acids in turn can be replaced by 0.2% sodium glutamate and three essential amino acids, i.e., leucine (50 mg/liter), isoleucine (50 mg/l), and valine (100 mg/liter). Growth was substantially faster when tyrosine (100 mg/liter) or phenylalanine (250 mg/liter) was also added.

The freshwater saprospiras of the *S. albida* type are more difficult to cultivate. Dyar (1947) could grow pure cultures on blood agar containing 5–10% erythrocytes and 1–1.5% agar. The organism did not grow if blood serum or heat-sterilized blood was used, which seems to indicate that it required either a heat-labile growth factor or enzyme in erythrocytes. Lewin (1965b) recommended a semisolid medium, LEW5 agar, for those strains, but even then they grow rather slowly.

LEW5 Agar (Lewin, 1965b)

Tryptone (Difco)	0.1%
Yeast extract (Difco)	0.1%
Gelatin (Difco)	0.1%
Glucose (sterilized separately)	0.1%
Tris buffer	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
KNO_3	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
NaCl	0.1%
Sodium glycerophosphate	0.01%
Fe	0.5 mg/liter
Zn	0.3 mg/liter
B, Co, Cu, Mn, Mo	0.1 mg/liter each
Agar	0.25%

The pH is adjusted to 7.5. The medium is autoclaved.

Unlike the other freshwater saprospiras, *S. flammula* is stimulated by 0.3% NaCl. It does not grow on casamino acids alone, but can be cultivated on a medium containing 0.5% tryptone, 0.3% NaCl and 0.25% agar (Lewin, 1965b).

Most saprospiras grow well at 30°C. Many strains even grow at 40°C (Lewin and Lounsbery, 1969). The temperature optimum is near 25°C for (marine) *S. toviformis* (Lewin and Mandel, 1970), 26°C for (freshwater) *S. albida* (the range is still 15–34°C; Dyar, 1947), and about 37°C for (marine) *S. grandis* (Lewin, 1962). *S. grandis* survives 46°C for 30 min (Lewin, 1972). This high temperature optimum of *S. grandis* is somewhat surprising, for the organism usually is found in a relatively cold environment. Thus, strain Sa gl was isolated at Roscoff, Brittany, where the Atlantic Ocean rarely reaches 17–18°C and is much colder most of the year. This strain also

grows rather well at 6°C and thus has a very wide temperature range.

The optimal pH is around neutral, and the range in which growth is possible is 6.5 to 7.5 and occasionally slightly beyond. The doubling time of *S. grandis* is 2 to 3 h at 30°C in SAP1 liquid medium (Lewin, 1962, 1972), and that of *S. thermalis* is 6 to 7 h at 30°C (Lewin, 1965a). A yield of about 250 mg dry cell mass per liter has been found with *S. thermalis* (Lewin, 1965a). With *S. grandis* in SP5 liquid medium at 30°, after 24 h of cultivation we obtained 12.5 g bacteria per liter by wet weight, corresponding to 2.5 to 3 g dry weight. Aasen and Liaaen-Jensen (1966a) harvested 400 g wet cells, giving 51.5 g freeze-dried bacteria, from 200 liters of culture broth after 20 h at 30°C.

Preservation

Cultures of *S. grandis* strain Sa gl on SAP2 agar remain viable for 2 to 3 weeks at room temperature (21°C). Lysis within 3 days at 30°C and within a few weeks at 25°C, on all tested liquid and agar media, has been reported for other strains (Lewin, 1962). Dyar (1947) found that her *S. albida* survived on blood agar for about one month but died after a short time in the refrigerator.

Filaments of *S. grandis* strain Sa gl can be suspended in SP5 liquid medium and frozen at –80°C or in liquid nitrogen without any further precautions. We were able to revive both cultures without difficulty after 2.5 years, the longest period tested so far. They will certainly survive for a much longer time. We had no success with drying in skim milk (H. Reichenbach, unpublished observations).

Sanfilippo and Lewin (1970) tested several freezing techniques with 27 strains of five *Saprospira* species. The longest survival times they found for cultures frozen at –22°C was 2 to 3 weeks (only marine species). In media containing 10% glycerol, all marine strains survived in liquid nitrogen for 1 year, the longest period tested. Of the freshwater species, only some strains of *S. thermalis* could be preserved in this way. Dimethyl sulfoxide (DMSO, 10%) proved toxic for all saprospiras: all strains were killed within 24 h at room temperature.

Characterization

The saprospiras form helical, multicellular, unbranched filaments (see Fig. 1) that move by gliding when in contact with a substrate surface. The length of the filaments is not fixed and varies considerably, even within one culture. The filaments tend to be longer in liquid media than on agar plates. *S. grandis* strain Sa gl, e.g., measures 15–130 μm when grown on a plate and 20–450 μm when grown in a liquid medium of the same composition. Similar figures have been reported for other strains and species (see Table 1). The diameter of the filaments is relatively constant and usually around 1 μm.

The filaments are composed of cylindrical cells that are 1–5 μm long and closely attached to one another. No constrictions are visible at the surface of the filaments at the sites of the septa. The cells at the ends of the filaments are rounded, but otherwise do not seem different from the rest. (As mentioned above, two dubious species with pointed filaments have been described from oysters; Dimitroff, 1926.) The length of the cells within one filament is variable, which suggests that the cells divide along the whole filament and independently from one another. With a good microscope and applying phase or interference contrast, the cross-walls can be seen in the living filament. Also, dark-field microscopy shows the septa clearly (Dyar, 1947; Jarosch, 1967). The cell boundaries become more apparent when the filaments begin to disintegrate (see Fig. 1c). Drying of the filaments to the slide may be sufficient to induce fragmentation (Dyar, 1947). Further, the multicellular nature of the screws can be seen after staining, e.g., with an I-KI solution (Skuja, 1948) or with Heidenhain's hematoxylin (Gross, 1911). Other staining methods often used in the older literature, e.g., with safranine, borax carmine, or methylene blue, also produced chambered filaments in *Saprospira*, but this may sometimes have been an artifact due to shrinkage of the protoplast, for true spirochetes also showed such a chambered appearance (Dobell, 1912; Dimitroff, 1926). Another identified artifact was an "axial filament" in heavily stained mounts of *S. albida* (Dyar, 1947). Of course, the septa are easily recognizable in thin sections under the electron microscope (Lewin, 1962). Also, in the election microscope, a typical Gram-negative cell wall with a thin peptidoglycan layer and an outer membrane can be seen. As usual, the septa consist only of the two cytoplasmic membranes and the peptidoglycan layer. The filaments multiply by breaking in two, apparently sometimes at the sites of necridia, i.e., dead cells (Gross, 1911; see also The Genus *Herpetosiphon* in this Volume). It seems that reproduction by simultaneous fragmentation into many short, one- or few-celled pieces can also take place (Gross, 1911, 1912; Dobell, 1912; Lewin, 1962, 1965b). The short pieces move only very slowly if at all, but with growing length their movements speed up again (Dobell, 1912; Skuja, 1948).

The helix of *Saprospira* usually is relatively loose (Fig. 1). Its pitch is 3–10 μm, and its width

is 1.5–2.5 μm. Some species show especially lax helices, e.g., *S. thermalis* and *S. (Achroonema) spiroideum*. But pitch and width are not constant features of a strain and can vary somewhat even within one culture. With the freshwater species, the helices appear usually to be coiled sinistrally (Lewin, 1965b), while the marine strains are coiled dextrally (Lewin and Mandel, 1970). The screw shape of the filaments may completely disappear during cultivation, in which case the organism would be difficult to recognize as a *Saprospira* (Dyar, 1947; Lewin, 1965b). Thus, the very long filaments seen in liquid cultures generally form only rudimentary and irregular helices. Also after improper fixation (e.g., with picric acid) the filaments tend to straighten out (Gross, 1911). In chamber cultures when restricted to the narrow space between the agar surface and the cover glass, *S. grandis* grows in the shape of very long, meandering filaments that are completely straight and, interestingly, also nonmotile (Fig. 1d; Reichenbach et al., 1975/1976; Reichenbach, 1980).

In such chamber cultures of *S. grandis*, filaments can sometimes be seen with the end cells inflated into spherical, optically refractile bulbs of 1.4–3 μm diameter (Fig. 2; Reichenbach, 1980). Those bulbs are probably a degeneration phenomenon but should not be confused with the spheroplasts that are found in old cultures all along the filament (Dyar, 1947) or can be produced by adding a 5 to 10% NaCl solution ("blisters"; Dobell, 1912). Filaments of *S. grandis* from liquid cultures appear to be very sensitive to changes in environmental conditions. Simply mounting them on a slide is sufficient to induce a decay of the filament and the formation of spherical protrusions at the surface of the cells (Fig. 1c).

Inside the cells of *S. albida* many highly refractive granules can be seen, sometimes extending from one side of the cell to the other (Dobell, 1912; Dyar, 1947). Dyar distinguishes two types: 1) volutin granules, i.e., polyphosphate, which show metachromatic staining with methylene blue and dissolve in (hot) water or in 0.02% $NaHCO_3$ solution, and 2) lipid globules, which stain with Sudan black B. The latter could well consist of poly-β-hydroxybutyrate. The inclusions are also seen under the electron microscope (Dyar, 1947). They appear to be relatively rare in *S. grandis* (Lewin, 1962; Lewin and Kiethe, 1965).

Gross (1911) mentioned that, in aging cultures of *S. grandis*, series of cells with increased stainability could occasionally be seen within filaments. Such cells later became spherical and surrounded themselves with a heavy wall. He suggested that those cells were spores, although he could not observe any further development. This observation has not yet been corroborated by other investigators.

A DNA phage of *S. grandis* with an icosahedral head, a contractile tail sheath, and rigid tail spines has been isolated and characterized (Lewin et al., 1964). Peculiar rod-shaped particles with long, wicklike tails, found in lysing cultures of *S. grandis* (Lewin, 1963; Lewin and Kiethe, 1965; Reichle and Lewin, 1968) and of the freshwater species *S. albida* and *S. flammula* (Lewin, 1965b), have been described as "rhapsidosomes." The preparations showed no infectivity whatsoever. Initially it was thought that the particles consisted of a ribonucleoprotein containing a highly 2′-*O*-methylated RNA (Correll and Lewin, 1964; Correll, 1968). This, however, turned out to be an analytical error, for the particles are composed exclusively of protein (Delk and Dekker, 1969, 1972; Price and Rottman, 1970). Very similar particles were later found in a myxobacterium and identified as the tails of a defective phage (Reichenbach, 1967). The *Saprospira* particles very probably have the same origin (Delk and Dekker, 1972). In fact, defective phage tails are rather common in bacteria, and many examples are known today. Unfortunately, the term rhapidosome was later also used for rod-shaped particles of a totally different morphology and nature, leading to unnecessary confusion (e.g., Pate et al., 1967; Yamamoto, 1967; Baechler and Berk, 1972).

In autolysed cultures of *S. thermalis*, but not of any other *Saprospira*, cell-wall fragments with a periodic hexagonal substructure are seen under the electron microscope, obviously remnants of a surface layer (Lewin, 1965b).

The helical filaments of *Saprospira* move by gliding when in contact with a suitable substrate surface, e.g., an agar or glass surface. The speed of *S. grandis* at room temperature (25°C) is 50–120 μm/min, and the movement is thus very obvious (Lewin, 1962). A speed of 120–180 μm/min has been determined for *S. thermalis* (Lewin, 1965b), one of 600 μm/min for *S. spiroidea* (Skuja, 1948), and one of only 14 μm/min for *S. flexuosa* (Dobell, 1912). The gliding screws rotate around their long axis and are also able to bend actively. Bending is particularly conspicuous when the filaments are suspended in a liquid. The helices move equally well in both directions and may quickly and frequently reverse the sense of their movement. As already mentioned, loss of the helical arrangement of the cells leads to a loss of translocation. The locomotory machinery still seems to be operative, however, since tiny particles stuck to the surface of the filaments can be seen to migrate back and forth over a limited distance, presumably the length of a cell (Reichenbach et al., 1975/1976). Obviously, the proper spatial arrangement of the cells in the

helix is a prerequisite for their efficient cooperation during movement (Reichenbach, 1980). As with all other gliding bacteria, the mechanism of locomotion is not understood in *Saprospira*. A movement of Indian ink particles on a helical path along the surface of filaments of *S. albida* has been explained by the movement of a helical slime belt that could also be responsible for the translocation of the filaments (Jarosch, 1967), but what moves the slime is still an open question. A sticky surface is, no doubt, essential for gliding motility, and the surface of *Saprospira* is indeed very sticky (Gross, 1911). Slimy material has been isolated from viscous, nonlysing liquid cultures of *S. thermalis* (27 mg/liter), and various sugars have been provisionally identified in the hydrolysate (glucose, xylose, rhamnose, traces of galactose and mannose; Lewin, 1965a).

The colonies of *S. grandis* on rich agar media are compact with entire edges and of a rather tough consistency (Fig. 2). On dilute media, however, the colonies spread, albeit often slowly and to a much lesser extent than that sun with the swarms of many other gliding bacteria. Long, unbranched, rhizoidal tongues may radiate from the edges of those colonies (Fig. 2). In other cases, the swarms are thin and filmlike (e.g., *S. grandis* on SP6 agar). The spreading colonies and, less so, also the compact ones show a surprising and very regular pattern of narrow stripes (Fig. 3). The striations are particularly impressive under dark-field illumination. As already mentioned, the phenomenon was first described for *Saprospira* by Dyar (1947) and is also known for other, unrelated, helical bacteria. It may be explained by an exact alignment of the screws (Fig. 3d). The optical pattern seems to arise from differences in light scattering in the rising and falling gyres of the helices, perhaps in combination with an interference effect. Colonies of *S. grandis* Sa gl show a periodicity of the stripes of 7.6 to 8.6 μm, while the pitch of the helices in suspension is 4.9 to 6.5 μm. Apparently, the helices are slightly distorted on the plate.

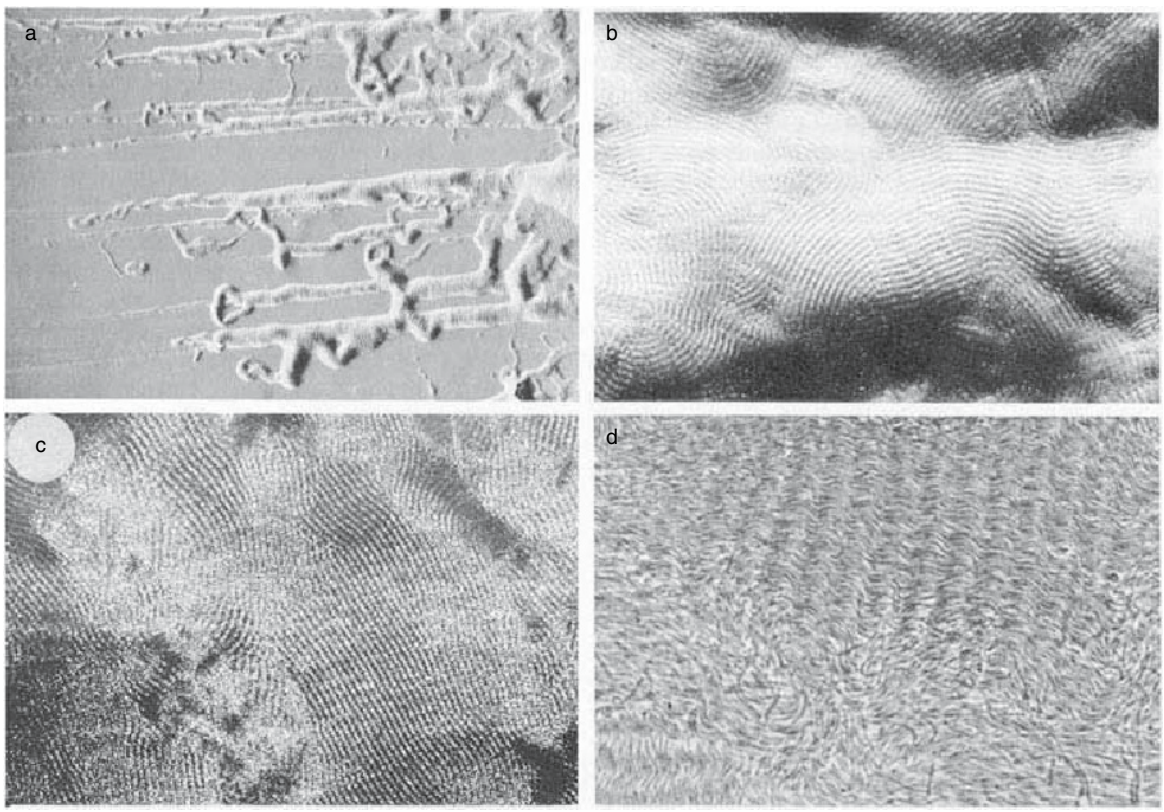

Fig. 3. Striated colonies of *Saprospira grandis*. (a) Colony spreading along grooves in the agar surface; the stripes run almost perpendicular to the direction of the spreading movement; Zeiss Standard Microscope, with oblique illumination; ×60. (b) Surface of a colony under oblique illumination; the filaments are just recognizable as a very delicate striation perpendicular to the stripes; Zeiss Axiomat; ×120. (c) Under dark-field illumination, the pattern of stripes becomes even more distinct; Zeiss Axiomat; ×120. (d) At high magnification it can be seen that in the striated areas the filaments lie strictly parallel, with the screws "in phase"; Zeiss Axiomat; ×580.

Relatively little is known about the chemical composition of *Saprospira*. The respiratory chain contains menaquinone MK-7 exclusively (M. D. Collins, personal communication). While initial studies seemed to indicate an *o*-type cytochrome as the terminal oxidase (Webster and Hackett, 1966), it was later found that *S. grandis* has four functional cytochromes, one of which has an unusual maximum at 603 nm (77°K) in the difference spectrum, and that an a,a_3-type cytochrome is most probably the oxidase (Dietrich and Biggins, 1971). NADH-oxidation by *Saprospira* particles is insensitive to antimycin A, rotenone, and amytal, but it is inhibited by cyanide and HOQNO.

The GC content of the DNA of the various species is 35–48 mol% (see Table 1).

The saprospiras are colored in shades of yellow, orange, pink, and red. The pigments are cell-bound. Initial studies provided a preliminary characterization of the pigment patterns by thin-layer chromatography and of the pigment type by absorption spectra of chromatographic fractions (Fox and Lewin, 1963; Lewin and Lounsbery, 1969). It soon became apparent that the pigments were probably carotenoids, but so far only one, the new xanthophyll saproxanthin (a modified 3,1′-dihydroxy-γ-carotene) from *S. grandis*, has been chemically characterized (Aasen and Liaaen-Jensen, 1966a). The pigment content was 0.02% of the freeze-dried cells. From *S. thermalis*, a single carotenoid, apparently a keto-carotenoid, was obtained, but because of very low yields (0.002% of the dry weight) its chemical structure could not be determined (Aasen and Liaaen-Jensen, 1966b). The early investigators described *S. albida* as completely unpigmented (however, they may have thought mainly of chlorophylls), but when a sufficiently high cell density is obtained, the organism shows a pale yellow tint (Fox and Lewin, 1963). Carotenoid synthesis by *S. thermalis* is stimulated by light, by suboptimal concentrations of cobalamine or tyrosine, and by subinhibitory concentrations of thiamine or sodium thiosulfate (Lewin, 1965a).

As already mentioned, *S. grandis* depends entirely on amino acids for growth (Lewin, 1972). It can be cultivated on various peptones, such as tryptone, casitone, phytone, or yeast extract. It can also be grown on casamino acids, but only if the medium is supplemented with tryptophan, asparagine, and a small quantity of yeast nucleic acid hydrolyzate (the growth factor supplied by the latter could not be identified). In that case, the casamino acids could be replaced by a mixture of essential amino acids, i.e., arginine, histidine, isoleucine, leucine, valine, methionine, phenylalanine, threonine, and perhaps lysine. Curiously, asparagine cannot be substituted by aspartate or glutamine. In nature, *S. grandis* may provide itself with the required amino acids by the degradation of proteinaceous matter. Gelatin liquefaction, the clotting and digestion of milk casein, and the decomposition of bacterial cells by *S. grandis* have been reported. In accordance with the metabolic restrictions outlined above, polysaccharides like starch, alginate, carboxymethyl cellulose, and agar are not hydrolyzed (Lewin and Lounsbery, 1969). Strain Sa gl also does not attack chitin and yeast cells. Marine *S. toviformis* differs from *S. grandis* in that it grows on casamino acids alone and does not require asparagine. Also, it is stimulated by lactate and acetate and depolymerizes carboxymethyl cellulose. It, too, does not utilize any of the tested sugars (Lewin and Lounsbery, 1969).

S. thermalis, the only freshwater species studied in some detail, utilizes glucose but none of the other tested sugars, sugar alcohols, ethanol, or acetate (Lewin, 1965a; Lewin and Lounsbery, 1969). This organism also hydrolyzes starch. *S. thermalis* grows well in a mineral salts/glucose medium with tryptone. The latter can be replaced by 1) yeast extract; 2) casamino acids plus the vitamins cobalamine and thiamine; or 3) glutamate plus the essential amino acids valine, leucine, and isoleucine and the vitamins. Much better growth is obtained if phenylalanine or tyrosine is also added to the synthetic medium. The main nitrogen source in the latter medium, glutamate, can be substituted by asparagine, methionine, or threonine, to a certain extent also by NH_4^+ or urea, but not by NO_3. *S. thermalis* too is proteolytic. The organism appears to operate a citric acid cycle (Lewin, 1965a). A fructose-bisphosphate aldolase has been purified 240-fold from *S. thermalis* (Willard and Gibbs, 1968). It is a type II aldolase, as is characteristic for bacteria, requiring cysteine, Zn^{2+}, and K^+ for maximum activity.

All saprospiras are catalase negative and produce ammonia from amino acids; none reduces nitrate to nitrite. Only *S. thermalis* produces H_2S and minor amounts of indole.

Taxonomy

An extensive study on the taxonomy of flexibacteria, including several types of saprospiras, was performed in the late 1960s (Lewin, 1969; Lewin and Lounsbery, 1969). The study was based mainly on a set of about 70 morphological and physiological features, but the GC content of the strains was also determined (Mandel and Lewin, 1969; in case of a discrepancy between data in the literature, the GC values given in Table 1 were taken from this study). The data were pro-

cessed in two different ways: by an Adansonian analysis (Colwell, 1969) and by a recurrent group analysis (Fager, 1969). While in both schemes, the strains of *S. grandis*, *S. thermalis*, and *S. toviformis* turn out as coherent and clearly separated groups, the connections between those groups, and between them and other flexibacteria, differ substantially. Considering the limitations of such a phenotypic classification it may, however, be too early to conclude that the various saprospiras are not closely enough related to be included in one genus.

The phylogenetic relationship of *S. grandis* with the cytophagas and flavobacteria has already been mentioned. The branching point is, however, relatively low, and the connection to the other members of the group is consequently weak.

At present, only one species of *Saprospira* is accepted, *S. grandis* (Reichenbach, 1989). Although there are no conclusive arguments to prove it, at least some of the various organisms classified as saprospiras in the past (Table 1) may indeed represent other *Saprospira* species. Apparently, a similar situation exists with *Saprospira* as with *Beggiatoa*, for which strains or species of widely different dimensions have also been reported. Unfortunately, with the exception of *S. grandis*, no other *Saprospira* seems to be currently available for comparison in a public culture collection, so that all planned studies will have to start with new isolates.

Applications

Apart from the remote possibility discussed earlier for using saprospiras for the control of freshwater cyanobacteria, no other practical interest in the saprospiras is obvious at the moment.

Literature Cited

Aasen, A. J., S. Liaaen-Jensen. 1966a. The carotenoids of flexibacteria II. A new xanthophyll from *Saprospira grandis*. Acta Chem. Scand. 20:811–819.

Aasen, A. J., S. Liaaen-Jensen. 1966b. Carotenoids of flexibacteria IV. The carotenoids of two further pigment types. Acta Chem. Scand. 20:2322–2324.

Ashton, P. J., R. D. Robarts. 1987. Apparent predation of *Microcystis aeruginosa* Kütz. emend. Elenkin by a *Saprospira*-like bacterium in a hypertrophic lake (Hartbeespoort Dam, South Africa). J. Limnol. Soc. South Afr. 13:44–47.

Baechler, C. A., R. S. Berk. 1972. Ultrastructural observations of *Pseudomonas aeruginosa*: Rhapsidosomes. Microstructures 3:24–31.

Brunel, J. 1949. *Achroonema spiroideum* Skuja 1948, of the Trichobacteriales, discovered simultaneously in Sweden and in Canada. Contrib. Inst. Bot. Univ. Montreal 64:21–27.

Colwell, R. R. 1969. Numerical taxonomy of the flexibacteria. J. Gen. Microbiol. 58:207–215.

Correll, D. L. 1968. Rhapidosomes: 2′-O-methylated ribonucleoproteins. Science 161:372–373.

Correll, D. L., R. A. Lewin. 1964. Rod-shaped ribonucleoprotein particles from *Saprospira*. Can. J. Microbiol. 10:63–74.

Cyrus, Z., A. Sladká. 1970. Several interesting organisms present in activated sludge. Hydrobiologia 35:383–396.

Dawson, R. M. C., D. C. Elliott, W. H. Elliott, K. M. Jones (ed.) 1972. Data for biochemical research, p. 508. Clarendon Press. Oxford.

Delk, A. S., C. A. Dekker. 1969. Rhapidosomes: Absence of a highly 2′-O-methylated RNA component. Science 166:1646–1647.

Delk, A. S., C. A. Dekker. 1972. Characterization of rhapidosomes of *Saprospira grandis*. J. Mol. Biol. 64:287–295.

Dietrich, W. E., J. Biggins. 1971. Respiratory mechanisms in the Flexibacteriaceae: Terminal oxidase systems of *Saprospira grandis* and *Vitreoscilla* species. J. Bacteriol. 105:1083–1089.

Dimitroff, V. T. 1926. Spirochetes in Baltimore market oysters. J. Bacteriol. 12:135–177.

Dobell, C. 1912. Researches on the spirochaets and related organisms. Arch. Protistenk. 26:117–240.

Dyar, M. T. 1947. Isolation and cytological study of a free-living spirochete. J. Bacteriol. 54:483–493.

Fager, E. W. 1969. Recurrent group analysis in the classification of flexibacteria. J. Gen. Microbiol. 58:179–187.

Ferry, J. G., P. H. Smith, R. S. Wolfe. 1974. *Methanospirillum,* a new genus of methanogenic bacteria, and characterization of *Methanospirillum hungatii* sp. nov. Internat. J. Syst. Bacteriol. 24:465–469.

Fox, D. L., R. A. Lewin. 1963. A preliminary study of the carotenoids of some flexibacteria. Can. J. Microbiol. 9:753–768.

Gross, J. 1911. Über freilebende Spironemaceen. Mitt. Zool. Stat. Neapel 20:188–203.

Gross, J. 1912. Über Systematik, Struktur und Fortpflanzung der Spironemacea. Zbl. Bakteriol. 1. Abt. Orig. 65:83–98.

Jarosch, R. 1967. Studien zur Bewegungsmechanik der Bakterien und Spirochäten des Hochmoors. Österr. Bot. Zschr. 114:255–306.

Kolkwitz, R. 1909. Kryptogamenflora der Mark Brandenburg, Band V , p. 137. Gebrüder Borntraeger. Leipzig,

Lewin, R. A. 1962. *Saprospira grandis* Gross; and suggestions for reclassifying helical, apochlorotic, gliding organisms. Can. J. Microbiol. 8:555–563.

Lewin, R. A. 1963. Rod-shaped particles in *Saprospïra*. Nature 198:103–104.

Lewin, R. A. 1965a. Isolation and some physical features of *Saprospira thermalis*. Can. J. Microbiol. 11:77–86.

Lewin, R. A. 1965b. Freshwater species of *Saprospira*. Can. J. Microbiol. 11:135–139.

Lewin, R. A. 1969. A classification of flexibacteria. J. Gen. Microbiol. 58:189–206.

Lewin, R. A. 1972. Growth and nutrition of *Saprospira grandis* Gross (Flexibacterales). Can. J. Microbiol. 18:361–365.

Lewin, R. A., J. Kiethe. 1965. Formation of rhapidosomes in *Saprospira*. Can. J. Microbiol. 11:935–938.

Lewin, R. A., D. M. Lounsbery. 1969. Isolation, cultivation and characterization of flexibacteria. J. Gen. Microbiol. 58:145–170.

Lewin, R. A., M. Mandel. 1970. *Saprospira toviformis* nov. spec. (Flexibacterales) from a New Zealand seashore. Can. J. Microbiol. 16:507–510.

Lewin, R. A., D. M. Crothers, D. L. Correll, B. E. Reimann. 1964. A phage infecting *Saprospira grandis*. Can. J. Microbiol. 10:75–85.

Mandel, M., R. A. Lewin. 1969. Deoxyribonucleic acid base composition of flexibacteria. J. Gen. Microbiol. 58:171–178.

Pate, J. L., J. L. Johnson, E. J. Ordal. 1967. The fine structure of *Chondrococcus columnaris.* II. Structure and formation of rhapidosomes. J. Cell. Biol. 35:15–35.

Paster, B. J., W. G. Weisburg, E. Stackebrandt, R. B. Hespell, C. M. Hahn, H. Reichenbach, K. O. Stetter, C. R. Woese. 1985. A phylogenetic grouping of the bacteroides, cytophagas and certain flavobacteria. Syst. Appl. Microbiol. 6:34–42.

Price, A. R., F. Rottman. 1970. Nucleic acids from *Saprospira grandis*: The absence of 2′-O-methylated RNA. Biochim. Biophys. Acta 199:288–291.

Pringsheim, E. G. 1963. Farblose Algen. Gustav Fischer Verlag. Stuttgart, 111.

Reichenbach, H. 1967. Die wahre Natur der Myxobakterien-"Rhapidosomen." Arch. Mikrobiol. 56:371–383.

Reichenbach, H. 1980. *Saprospira grandis* (Leucotrichales): Growth and movement. Publik. Wiss. Film, Sekt. Biol., Ser. 13, Nr. 26:1–21.

Reichenbach, H. 1989. Genus *Saprospira* Gross 1911, 202. *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.) Bergey's manual of systematic bacteriology. Williams and Wilkins. Baltimore, 3:2077–2082.

Reichenbach, H., M. Dworkin. 1981. The order Cytophagales (with addenda on the genera *Herpetosiphon*, *Saprospira*, and *Flexithrix*). *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.) The prokaryotes. Springer-Verlag. Berlin, 356–379.

Reichenbach, H., H. K. Galle, H. H. Heunert. 1975/1976. *Saprospira grandis* (Leucotrichales). Enzyclopaedia Cinematographica E 2424, film* of the Institut für den wissenschaftlichen Film, Göttingen. Wachstum und Bewegung. Federal Republic of Germany.

Reichenbach, H., W. Ludwig, E. Stackebrandt. 1986. Lack of relationship between gliding cyanobacteria and filamentous gliding heterotrophic eubacteria: Comparison of 16S rRNA catalogues of *Spirulina, Saprospira*, *Vitreoscilla, Leucothrix*, and *Herpetosiphon*. Arch. Microbiol. 145:391–395.

Reichle, R. E., R. A. Lewin. 1968. Purification and structure of rhapidosomes. Can. J. Microbiol. 14:211–213.

Sanfilippo, A., R. A. Lewin. 1970. Preservation of viable flexibacteria at low temperatures. Can. J. Microbiol. 16:441–444.

Skuja, H. 1948. Taxonomie des Phytoplanktons einiger Seen in Uppland, Schweden. Symbolae Botanicae Upsalienses 9:1–399.

Sladká, A., V. Ottová. 1973. Filamentous organisms in activated sludge. Hydrobiologia 43:285–299.

Soriano, S. 1945. Un nuovo orden de bacterias: Flexibacteriales. Ciencia e Investigacion 1:92–93.

van Tieghem, P. 1880. Observations sur les Bacteriacées vertes, sur des Phycochromacées blanches, et sur les affinités de ces deux familles. Bull. Soc. Bot. France 27:174–179.

Warming, E. 1875. Om nogle ved Danmarks Kyster levende Bakterier. Videnskabelige Meddelelser fra den naturhistoriske Forening i Kjöbenhaven for Aaret 1875:307–420.

Webster, D. A., D. P. Hackett. 1966. Respiratory chain of colorless algae II. Cyanophyta. Plant Physiol. 41:599–605.

Willard, J. M., M. Gibbs. 1968. Purification and characterization of the fructose diphosphate aldolases from *Anacystis nidulans* and *Saprospira thermalis*. Biochim. Biophys. Acta 151:438–448.

Yamamoto, T. 1967. Presence of rhapidosomes in various species of bacteria and their morphological characteristics. J. Bacteriol. 94:1746–1756.

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CHAPTER 6.9

The Genus *Haliscomenobacter*

EPPE GERKE MULDER AND MARIA H. DEINEMA

In the 1st edition of *The Prokaryotes,* the genus *Haliscomenobacter* was considered together with bacteria of the genera *Sphaerotilus* and *Leptothrix*, but *Haliscomenobacter* is not closely related to these other Gram-negative sheathed bactera.

Habitats

Strains of *Haliscomenobacter hydrossis*, the only species of the genus *Haliscomenobacter* isolated so far, are nearly always present in activated sludge flocs, sometimes in large amounts. The conditions for such an abundant development, which gives rise to bulking sludge, are not understood.

Straight, thin, needle-shaped, sheath-forming chains of cells protruding from the sludge flocs (Fig. 1) may interfere with clumping and compacting of the solids.

Isolation

The following procedure (van Veen, 1973) can successfully be used to isolate *Haliscomenobacter* sp. from bulking activated sludge.

I Medium (g/liter of distilled water)

Glucose	0.15
$(NH_4)_2SO_4$	0.5
$Ca(NO_3)_2$	0.01
K_2HPO_4	0.05
$MgSO_4 \cdot 7H_2O$	0.05
KCl	0.05
$CaCO_3$	0.1
Vitamin B_{12}	10^{-5}
Thiamine	4×10^{-4}
Agar (Oxoid)	10

S.C.Y. Medium (g/liter of distilled water)

Sucrose	1.0
Casitone (Difco)	0.75
Yeast extract (Difco)	0.25
Trypticase soy broth without dextrose (BBL)	0.25
Vitamin B_{12}	10^{-5}
Thiamine	4×10^{-4}
Agar (Oxoid)	10

The vitamins are sterilized separately by filtration.

A sample of 0.10–0.50 ml of sludge with a relatively large amount of *Haliscomenobacter* threads is pipetted into tubes containing 10 ml sterile tap water. The tubes are stirred for several minutes with a tube mixer and the flocs allowed to settle. The settling time depends on the characteristics of the sludge and especially on the degree of bulking. This treatment of the sludge is repeated until sufficient filamentous organisms are observed microscopically in the upper layers of the supernatant. During moderate agitation and rotating movements of the dilute floc suspensions, fragments of threads are severed from the protruding filaments. The low total numbers of organisms and the favorable ratio between filamentous and other bacteria in the upper liquid layers permit a direct inoculation of the plates without further dilution of the suspension. The time of agitation of the flocs in the tubes appears to be related to the mechanical properties of the stirrer and to the nature and number of filamentous organisms present in activated sludge.

Very small droplets containing sufficient numbers of filamentous bacteria are transferred to the previously dried surfaces of 30 to 40 agar plates containing I medium. The cells are regularly spread over the whole surface of the agar plates by rubbing firmly with a sterile glass rod. The plates are incubated for 3 to 4 weeks at a temperature between 17 and 20°C.

The small colonies that develop from filamentous bacteria are often difficult to detect with a stereomicroscope, but recognition of the poorly contrasting, filamentous microcolonies on the agar is facilitated by low-magnification (150×) phase-contrast microscopy. Isolation is performed by transferring bacterial cells to S.C.Y. medium using sterile capillary tubes.

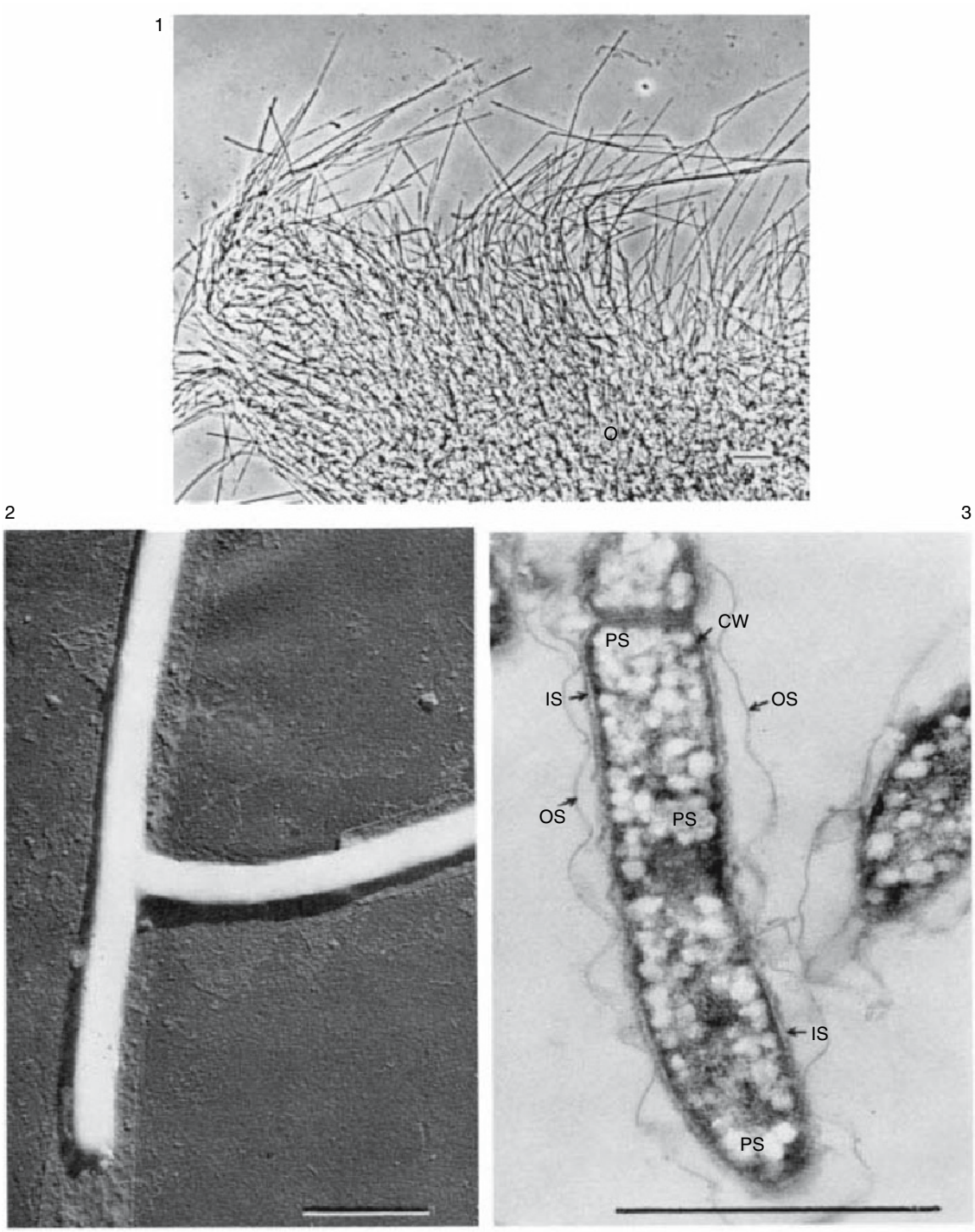
Preservation of Cultures

Stock cultures of *Haliscomenobacter hydrossis* can be maintained in the following way. Three milliliters of sterile tap water are pipetted onto the surface of the S.C.Y. agar slopes. The inoculated tubes are incubated at 20–25°C until turbid growth has developed in the liquid layer on the agar. The cells remain viable during 3 months' storage at 4°C.

Identification

The main characteristics of *Haliscomenobacter* spp. are presented in Table 1. Pinkish, smooth, or slightly filamentous colonies about 1–3 mm in diameter are formed on S.C.Y. medium. The cells

This chapter was taken unchanged from the second edition.



Figs. 1–3. *Haliscomenobacter hydrossis*. (1) Activated sludge flocs with many filaments. Bar = 10 µm. (2) Branched filament with thin hyaline sheath. Bar = 1 µm. (3) Fine structure: OS, outer layer of the sheath; IS, inner layer of the sheath; CW, cellwall; PS, polysaccharide globules. Bar = 1 µm.

Table 1. Main characteristics of *Haliscomenobacter*.

Cells	
Width (μm)	0.35–0.45
Length (μm)	3–5
Branchings	Real
Structure of sheath surface[a]	Smooth
C source:	
Glucose	+
Sucrose	+
Glycerol	–
Lactate	–
N source:	
NH_4^+	+
NO_3^-	+
Aspartic and glutamic acids	+
Casamino acids	+
Vitamin B_{12}	+
Thiamine	+
Optimum pH	7.0–8.0
$Fe(OH)_3$ accumulation	–
Mn^{2+} oxidation	–
Carotenoid pigments	+
Reserve material:	
PHB	–
Polysaccharide	+
GC content (mol%)	48.3–49.7

[a]Electron microscope observations.

are nearly always enclosed by a narrow, hardly visible, hyaline sheath (Fig. 2). A holdfast, as may be present in *Sphaerotilus natans*, has never been detected. The sheaths do not attach to glass walls of culture flasks; occasionally single cells are liberated from the sheaths in fast-growing, aerated cultures. Flagella have not been detected by electron microscope examinations, and motility in liquid or hanging-drop cultures has never been observed. Glucose, glucosamine, lactose, sucrose, and starch have been found to be excellent carbon and energy sources. The cells grow very well with $(NH_4)_2SO_4$, KNO_3, glutamate, or casamino acids as the nitrogen source. Both vitamin B_{12} and thiamine are required for growth. Maximum growth was generally obtained after an incubation period of 4–6 days on a rotary shaker at 25°C (Krul, 1977; van Veen et al., 1973).

Instead of poly-β-hydroxybutyrate (present in *Sphaerotilus* and *Leptothrix* spp.), polysaccharide globules are present in cells from media with a high C/N ratio (Fig. 3) (Deinema et al., 1977). Strains of *Haliscomenobacter* isolated so far belong to one species, *H. hydrossis* (Crombach et al., 1974; Eikelboom, 1975; van Veen et al., 1973).

The GC content of the DNA of *H. hydrossis* strains was found to be 49.0 ± 0.7 mol% (Crombach et al., 1974). This value, together with the other properties of this sheathed bacterium (Table 1), confirms the theory that except for the presence of a sheath, bacteria of the genus *Haliscomenobacter* are entirely different from those belonging to the genera *Sphaerotilus* and *Leptothrix*.

Three strains of *Haliscomenobacter hydrossis* have been deposited with the American Type Culture Collection (ATCC 27775–7) in Rockville, Maryland, and with the Deutsche Sammlung für Mikroorganismen (DSM 1100) in Göttingen.

Literature Cited

Crombach, W. H. J., van Veen, W. L., van der Vlies, A. W., Bots, W. C. P. M. 1974. DNA base composition of some sheathed bacteria. Antonie van Leeuwenhoek Journal of Microbiology and Serology 40:217–220.

Deinema, M. H., Henstra, S., Werdmüller von Elgg, E. 1977. Structural and physiological characteristics of some sheathed bacteria. Antonie van Leeuwenhoek Journal of Microbiology and Serology 43:19–29.

Eikelboom, D. H. 1975. Filamentous organisms observed in activated sludge. Water Research 9:65–388.

Krul, J. M. 1977. Experiments with *Haliscomenobacter hydrossis* in continuous culture without and with *Zoogloea ramigera*. Water Research 11:197–204.

van Veen, W. L. 1973. Bacteriology of activated sludge, in particular the filamentous bacteria. Antonie van Leeuwenhoek Journal of Microbiology and Serology 39:189–205.

van Veen, W. L., van der Kooij, D., Geuze, E. C. W. A., van der Vlies, A. W. 1973. Investigations on the sheathed bacterium *Haliscomenobacter hydrossis* gen. n., sp. n., isolated from activated sludge. Antonie van Leeuwenhoek Journal of Microbiology and Serology 39:207–216.

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CHAPTER 6.10

Sphingomonas and Related Genera

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Introduction

The genus *Sphingomonas* was defined by Yabuuchi et al. (1990) as a group of Gram-negative, rod-shaped, chemoheterotrophic, strictly aerobic bacteria that possess ubiquinone 10 as the major respiratory quinone, contain glycosphingolipids (GSLs) instead of lipopolysaccharide in their cell envelopes, and typically produce yellow-pigmented colonies. By 2001, the genus included more than 20 species that were quite diverse in terms of their phylogenetic, ecological, and physiological properties. As a result, Takeuchi et al. (2001) subdivided *Sphingomonas* into four genera: *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*. These genera are referred to collectively as "sphingomonads" in this chapter. The sphingomonads are widely distributed in nature, having been isolated from many different aqueous and terrestrial habitats, as well as from plant root systems, clinical specimens, and other sources. Sphingomonads are metabolically versatile and, thus, are able to utilize a wide range of naturally occurring organic compounds as well as many types of refractory environmental contaminants. Because of this, there has been much interest in the metabolic pathways of these organisms, the properties of the enzymes involved with those pathways, and the genetics of their catabolic processes. Some of the sphingomonads (especially *Sphingomonas paucimobilis*) also play a role in human disease, primarily by causing a range of mostly nosocomial, non-life-threatening infections that typically are easily treated by antibiotic therapy. Owing to their remarkable biodegradative and biosynthetic capabilities, sphingomonads have been utilized for a wide range of biotechnological applications, from bioremediation of environmental contaminants to production of extracellular polymers such as sphingans used extensively in the food and other industries.

Taxonomy and Phylogeny

The genus *Sphingomonas* was defined by Yabuuchi et al. (1990) as a group of Gram-negative, rod-shaped, chemoheterotrophic, strictly aerobic bacteria that possess ubiquinone 10 as the major respiratory quinone, contain glycosphingolipids (GSLs) in their cell envelopes, and typically produce yellow-pigmented colonies. Other characteristics, such as presence of certain lipids and the utilization of various substrates, were also included in the definition of the genus. However, it was the presence of GSLs that most clearly distinguished *Sphingomonas* from other members of the α-subclass of the Proteobacteria (Woese, 1987; Stackebrandt et al., 1988) at that time. Yabuuchi et al. (1990) characterized 16 strains of bacteria in the Japan Collection of Microorganisms (JCM) that had been received as *Pseudomonas paucimobilis* (including the type strain, JCM 7516^T; Holmes et al., 1977), *Xanthomonas* sp., *Flavobacterium* sp., *Flavobacterium devorans*, *Flavobacterium capsulatum* (Leifson, 1962), or *Sphingobacterium* sp. On the basis of physiological, biochemical, and phylogenetic evidence, 14 of these strains were classified into five species of the new genus: *Sphingomonas paucimobilis* (the type species), *Sphingomonas parapaucimobilis*, *Sphingomonas adhaesiva*, *Sphingomonas capsulata* and *Sphingomonas yanoikuyae*.

Between 1990 and 1999, various research groups added 15 more novel species to the genus. Takeuchi et al. (1993) defined *Sphingomonas macrogoltabidus*, *Sphingomonas sanguis* and *Sphingomonas terrae* after examining several strains known to degrade polyethylene glycol (PEG), as well as two *Sphingomonas* strains that were included in the Yabuuchi et al. (1990) study but not assigned to any species. Two years later, Takeuchi et al. (1995) defined four more species that consisted of strains isolated from plant roots: *Sphingomonas asaccharolytica* (from apple tree roots), *Sphingomonas mali* (also from apple tree roots), *Sphingomonas pruni* (peach tree roots), and *Sphingomonas rosa* (rose roots). Nohynek et al. (1995) proposed *Sphingomonas chlorophenolica*, based on a reexamination of four strains (originally described as *Arthrobacter, Flavobacterium* or *Pseudomonas* species; Edgehill and Finn, 1982; Saber and Crawford, 1985; Radehaus and Schmidt, 1992; Resnick and Chapman, 1994) known to degrade

polychlorinated biphenyls (PCBs). A *Flavobacterium* strain (strain MH; Horvath et al., 1990) that degrades the herbicide 2-(2,4-dichlorophenoxy)-propionic acid (Mecoprop™) was reclassified as *Sphingomonas herbicidovorans* (Zipper et al., 1996). Similarly, Nohynek et al. (1996) reclassified three strains of *Pseudomonas saccharophila* that can degrade polychlorinated biphenyls (PCBs; Puhakka et al., 1995) as *Sphingomonas subarctica*. Strains from deep terrestrial subsurface environments (Atlantic coastal plain aquifer sediments) were added to the genus by Balkwill et al. (1997a), who described *Sphingomonas aromaticivorans*, *Sphingomonas stygia* and *Sphingomonas subterranea*. Kämpfer et al. (1997) then reclassified "*Pseudomonas azotocoligens*" (Anderson, 1955; Hill and Postgate, 1969) as *Sphingomonas trueperi*, and Denner et al. (1999) renamed *Pseudomonas echinoides* (Heumann, 1962) *Sphingomonas echinoides*. Yrjälä et al. (1998) reclassified *Pseudomonas* strain HV3 (which degrades aromatic and chloroaromatic compounds; Kilpi et al., 1980) as *Sphingomonas* HV3, but this strain was never recognized as a distinct species.

By 1999, the genus *Sphingomonas* included no fewer than 20 recognized species. It was obvious from phylogenetic analyses of 16S rRNA gene sequences that this group of bacteria was genetically rather diverse, forming several distinct clusters of strains within the α-4 subclass of the Proteobacteria in phylogenetic trees. As a result, several authors speculated that the genus was too diverse and might best be separated into two or more distinct genera at some point in the future (e.g., Van Bruggen et al., 1993; Segers et al., 1994; Takeuchi et al., 1994; Takeuchi et al., 1995; Balkwill et al., 1997a). Moreover, Kämpfer et al. (1997) reported that *Sphingomonas* species with *sym*-homospermidine as the major polyamine formed a relatively homogeneous subgroup within the genus. Nevertheless, Yabuuchi et al. (1999) expanded the genus even further by transferring in three more species from other genera because their cell envelopes contain GSLs. *Blastomonas natatoria* (Sly and Cahill, 1997), *Erythromonas ursincola* (Yurkov et al., 1997), and *Rhizomonas suberifaciens* (Van Bruggen et al., 1990) were renamed *Sphingomonas natatoria*, *Sphingomonas ursincola* and *Sphingomonas suberifaciens*, respectively. This change added phytopathogenic and photosynthetic organisms to *Sphingomonas*, thereby creating an unusually diverse genus in terms of its physiological and ecological characteristics. Shortly after the Yabuuchi et al. (1999) proposal, Hiraishi et al. (2000) emended the description of the genus *Blastomonas*, redefining it as a photosynthetic genus and proposing that *Erythromonas ursincola* (*Sphingomonas ursincola* in Yabuuchi et al., 1999) be transferred to it as *Blastomonas ursincola*. This proposal has been recognized, removing one species from *Sphingomonas*, but the other two species transferred by Yabuuchi et al. (1999) remain in the genus at this time (see Table 1).

Takeuchi et al. (2001) carried out a thorough reevaluation of the then-recognized species of *Sphingomonas* to better define the genus and, if necessary, subdivide it into additional genera. An important conclusion of this study was that, though the presence of GSLs in the cell envelope is significant, species should not be assigned to the genus *Sphingomonas* solely on the basis of this characteristic. *Rhizomonas* and *Blastomonas* (including the former *Erythromonas ursincola*) nested in the overall *Sphingomonas* cluster during phylogenetic analyses of 16S rRNA gene sequences. However, they were clearly separated, along a distinct line of descent, from any of the four monophyletic clusters of *Sphingomonas* species seen in phylogenetic trees (see below and Fig. 1 in Takeuchi et al., 2001). This was also true for *Erythrobacter* and *Porphyrobacter*, two other genera in the α-4 subclass of the Proteobacteria that possess GSLs. *Rhizomonas* and *Blastomonas* also differed from the *Sphingomonas* clusters in possessing phytopathogenic or photosynthetic properties, which are generally considered to be taxonomically very significant. Therefore, it was suggested that *Blastomonas* and *Rhizomonas* be left as they were prior to the Yabuuchi et al. (1999) revision and not be included in the genus *Sphingomonas*, a proposal that has not yet been recognized in the case of *Rhizomonas suberifaciens* and *Blastomonas natatoria* (see Table 1).

Takeuchi et al. (2001) found that phylogenetic analyses separated the remaining species of *Sphingomonas* into four monophyletic and genetically distinct clusters (in phylogenetic trees) that were supported by high levels of confidence (>86%) in bootstrap analyses. These clusters could also be distinguished on the basis of certain physiological and biochemical characteristics. Therefore, it was proposed that the four monophyletic clusters of species represent four distinct genera, including *Sphingomonas* and three novel genera: *Sphingobium*, *Novosphingobium* and *Sphingopyxis*. The principal traits of these four genera, as reported by Takeuchi et al. (2001), are summarized in Table 2. *Sphingomonas* differs from the other three genera primarily in its lipid composition (major fatty acids and major 2-OH fatty acids), the presence of homospermidine as the major polyamine, and signature nucleotide bases at certain positions within the 16S rRNA gene. *Sphingobium* and *Novosphingobium* are

Table 1. Reorganization and subdivision of the genus *Sphingomonas* by Takeuchi et al. (2001).

Original *Sphingomonas* species name	Taxonomy reference	Proposal by Takeuchi et al. (2001)	Current taxonomic status
S. adhaesiva	Yabuuchi et al., 1990	Retain as *Sphingomonas adhaesiva*	*Sphingomonas adhaesiva*
S. aromaticivo rans	Balkwill et al., 1997a	Reclassify: *Novosphingobium aromaticivorum*	*Novosphingobium aromaticivorans*
S. asaccharolytica	Takeuchi et al., 1995	Retain as *Sphingomonas asaccharolytica*	*Sphingomonas asaccharolytica*
S. capsulata	Yabuuchi et al., 1990	Reclassify: *Novosphingobium capsulatum*	*Novosphingobium capsulatum*[a]
S. chlorophenolica	Nohynek et al., 1995	Reclassify: *Sphingobium chlorophenolicum*	*Sphingobium chlorophenolicum*
S. echinoides	Denner et al., 1999	Retain as *Sphingomonas echinoides*	*Sphingomonas echinoides*
S. herbicidovorans	Zipper et al., 1996	Reclassify: *Sphingobium herbicidovorans*	*Sphingobium herbicidovorans*
S. macrogoltabidus	Takeuchi et al., 1993	Reclassify: *Sphingopyxis macrogoltabida*	*Sphingopyxis macrogoltabida*[a]
S. mali	Takeuchi et al., 1995	Retain as *Sphingomonas mali*	*Sphingomonas mali*
S. natatoria	Yabuuchi et al., 1999	Remove from genus; return to *Blastomonas*	*Sphingomonas natatoria*
S. parapaucimobilis	Yabuuchi et al., 1990	Retain as *Sphingomonas parapaucimobilis*	*Sphingomonas parapaucimobilis*
S. paucimobilis	Yabuuchi et al., 1990	Retain as *Sphingomonas paucimobilis*	*Sphingomonas paucimobilis*[a]
S. pruni	Takeuchi et al., 1995	Retain as *Sphingomonas pruni*	*Sphingomonas pruni*
S. rosa	Takeuchi et al., 1995	Reclassify: *Novosphingobium roseae*	*Novosphingobium rosa*
S. sanguis	Takeuchi et al., 1993	Retain as *Sphingomonas sanguinis*	*Sphingomonas sanguinis*
S. stygia	Balkwill et al., 1997a	Reclassify: *Novosphingobium stygiae*	*Novosphingobium stygium*
S. subarctica	Nohynek et al., 1996	Reclassify: *Novosphingobium subarcticum*	*Novosphingobium subarcticum*
S. suberifaciens	Yabuuchi et al., 1999	Remove from genus; return to *Rhizomonas*	*Sphingomonas suberifaciens*
S. subterranea	Balkwill et al., 1997a	Reclassify: *Novosphingobium subterraneae*	*Novosphingobium subterraneum*
S. terrae	Takeuchi et al., 1993	Reclassify: *Sphingopyxis terrae*	*Sphingopyxis terrae*
S. trueperi	Kämpfer et al., 1997	Retain as *Sphingomonas trueperi*	*Sphingomonas trueperi*
S. ursincola	Yabuuchi et al., 1999	Remove from genus; return to *Blastomonas*[b]	*Blastomonas ursincola*
S. yanoikuyae	Yabuuchi et al., 1990	Reclassify: *Sphingobium yanoikuyae*	*Sphingobium yanoikuyae*[a]
Sphingomonas HV3	Yrjälä et al., 1998	Not examined	Not recognized as species

[a]Type species.
[b]Reclassified as *Blastomonas ursincola* by Hiraishi et al. (2000).

virtually identical in terms of lipid composition, signature nucleotide bases, and various other traits, but can be distinguished by the presence or absence of nitrate reductase activity (in addition to the clustering patterns in phylogenetic trees). *Sphingopyxis* differs from the other three genera primarily in its lipid composition and signature nucleotide bases (see Table 2). The Takeuchi et al. (2001) proposal to redefine and subdivide the genus *Sphingomonas* was accepted and the current taxonomic positions of the species examined in this study are summarized in Table 1.

At present, the genus *Sphingomonas* officially includes ten species (Table 3) that were not examined by Takeuchi et al. (2001) because the papers describing them were incomplete at the time of that study or have appeared since it was published. On the basis of the phylogenetic analyses presented in the respective publications, it appears that five of these recently described species are members of the genus *Sphingomonas* as redefined by Takeuchi et al. (2001): *Sphingomonas pituitosa* (an extracellular slime producer; Denner et al., 2001), *Sphingomonas roseiflava* (from plant roots; Yun et al., 2000), *Sphingomonas cloacae* (a nonylphenol-degrading strain; Fujii et al., 2001), and *Sphingomonas aquatilis* and *Sphingomonas koreensis* (isolated from natural mineral water sources in Korea; Lee et al., 2001). Three of the recently described species appear to be members of the genus *Sphingobium* on the basis of their positions in phylogenetic trees. These are: *Sphingomonas chungbukensis* (an aromatic degrader; Kim et al., 2000), *Sphingomonas wittichii* (which degrades dibenzo-*p*-dioxin; Yabuuchi et al., 2001), and *Sphingomonas xenophaga* (two strains that degrade xenobiotics; Stolz et al., 2000). *Sphingomonas alaskensis*, an oligotrophic organism isolated from seawater (Vancanneyt et al., 2001), clusters with *Sphingopyxis* species in phylogenetic trees and thus is most likely to be a member of that genus. The remaining species, *Sphin-*

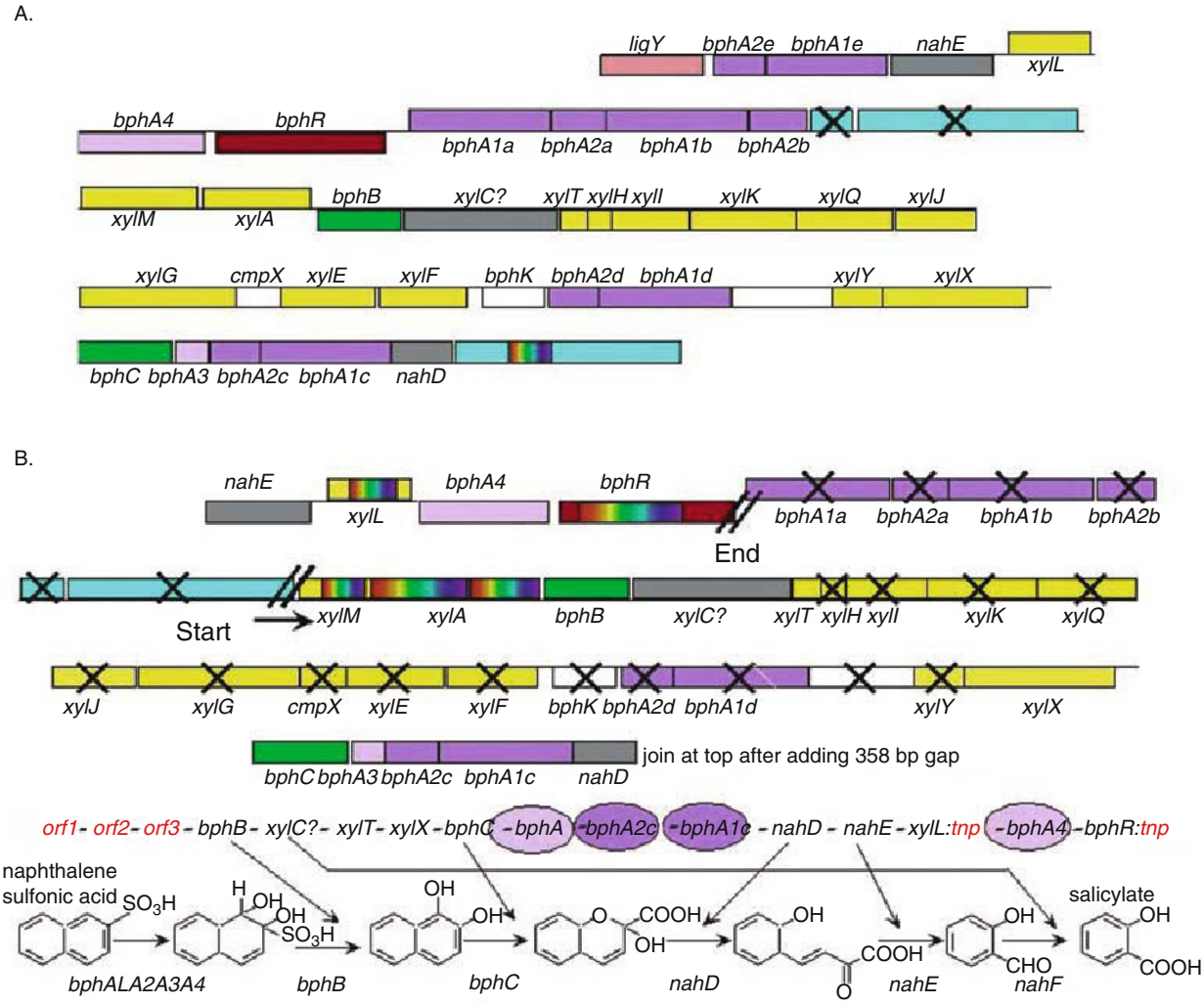

Fig. 1.

gomonas taejonensis, was described by Lee et al. (2001) and could be a member of either *Sphingopyxis* or *Sphingomonas*. The clusters in the phylogenetic tree published by Lee et al. (2001) are not in complete agreement with those described by Takeuchi et al. (2001) and this makes it difficult to speculate about the correct taxonomic placement of this species without additional information. At some point, the official taxonomic status of the 10 species listed in Table 3 will need to be clarified. Moreover, new *Sphingomonas* species proposed in the future should be characterized specifically in regard to their placement within the genera described by Takeuchi et al. (2001).

The subdivision of the genus *Sphingomonas* into four genera (Takeuchi et al., 2001) is a rather recent development, and very few reports since then have focused on the redefined genus or on any of the three new genera as separate entities. Moreover, many strains of *Sphingomonas* described in the literature were never assigned to a species and, therefore, are referred to simply as "*Sphingomonas* sp." At this time, it is not known whether these strains are members of the redefined genus *Sphingomonas* or one of the three new genera. Consequently, this chapter treats all members of the four genera proposed by Takeuchi et al. (2001) as a single group of organisms (i.e., as they were prior to reorganization of the genus). The new names proposed by Takeuchi et al. (2001) are used when the actual taxonomic position of an organism is reasonably certain. The phrase "*Sphingomonas* and related genera" refers to the four genera proposed by Takeuchi et al. (2001), and the term "sphingomonads" refers in general to the members of all of those genera.

Table 2. Principal characteristics of *Sphingomonas* and related genera.

Characteristic	*Sphingomonas*	*Sphingobium*	*Novosphingobium*	*Sphingopyxis*
Cell morphology				
Gram reaction	–	–	–	–
Shape	Rods	Rods	Rods	Rods
Size	0.3–0.8 × 1.0–1.9μm	0.3–0.7 × 1.0–3.5μm	0.3–0.5 × 1.0–3.0μm	0.3–0.5 × 0.9μm
Spore-formation	–	–	–	–
Motility	+/–	+/–	+/–	+/–
Colony morphology				
Color	Yellow or off-white	Yellow or whitish-brown	Yellow or whitish-brown	Yellow or whitish-brown
Physiological characteristics				
Strictly aerobic	+	+	+	+
Strictly chemoorganotrophic	+	+	+	+
Catalase activity	+	+	+	+
Nitrate reduction	(+/–)	–	+	–
Biochemical characteristics				
Major respiratory quinone	Q-10	Q-10	Q-10	Q-10
Lipid composition				
Major fatty acids	18:1, 16:0, and/or 17:1	18:1 (16:0 minor)	18:1 (16:0 minor)	18:1, 16:0 and 16:1
Major 2-hydroxy fatty acids	2-OH 14:0 or 2-OH 15:0	2-OH 14:0	2-OH 14:0	2-OH 14:0, 2-OH 15:0, and/or 2-OH 16:0
Presence of GSLs in envelope	+	+	+	+
Major polyamine	Homospermidine	Spermidine	Spermidine	Spermidine
Genetic characteristics:				
G+C content (mol%)	62–68	62–67	62–67	63–65
Signature nucleotide bases at these positions in 16S rRNA gene:				
52:359	C:G	U:A	U:A	C:G
134	G	G	G	G
593	G	U	U	U
987:1218	G:C	A:U	A:U	G:C
990:1215	U:G	U:G	U:G	U:G

Abbreviation and symbols: GSLs, glycosphingolipids; +, always present; –, always absent; +/–, present sometimes. From Takeuchi et al. (2001).

Table 3. *Sphingomonas* species not included in the Takeuchi et al. (2001) revision of the genus.

Species	Taxonomy reference	Probable genus assignment based on 16S rRNA phylogeny
S. alaskensis	Vancanneyt et al., 2001	*Sphingopyxis*
S. aquatilis	Lee et al., 2001	*Sphingomonas*
S. chungbukensis	Kim et al., 2000	*Sphingobium*
S. cloacae	Fujii et al., 2001	*Sphingomonas*
S. koreensis	Lee et al., 2001	*Sphingomonas*
S. pituitosa	Denner et al., 2001	*Sphingomonas*
S. roseiflava	Yun et al., 2000	*Sphingomonas*
S. taejonensis	Kim et al., 2000	*Sphingomonas* or *Sphingopyxis*
S. wittichii	Yabuuchi et al., 2001	*Sphingobium*
S. xenophaga	Stolz et al., 2000	*Sphingobium*

Habitat

The habitats for the type strains of the 32 currently recognized species of *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* are listed in Table 4. Habitats for selected additional strains of these genera are listed in Table 5. (Table 5 is not meant to be an exhaustive list; rather, strains were selected to provide a reasonably representative variety of habitats.) It is quite apparent from these tables that sphingomonads

Table 4. Habitats of type strains for *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* species.

Species and strain designation	Culture accession nos.	Habitat	References
Sphingomonas species			
S. adhaesiva GIFU 11458^T	JCM 7370^T = ATCC 51229^T = DSM 7418^T	"Sterile" (UV-irradiated) water used before surgery	Yabuuchi et al., 1990
S. alaskensis RB2256^T	DSM 13593^T = LMG 18877^T	Seawater, Resurrection Bay, Alaska	Vancanneyt et al., 2001
S. aquatilis JSS7^T	KCTC 2881^T = KCCM 41067^T	Natural mineral water source, Korea	Lee et al., 2001
S. asaccharolytica Y-345^T	IFO 15499^T = ATCC 51839^T = DSM 10564^T	Roots of apple tree, Japan	Takeuchi et al., 1995
S. chungbukensis DJ77^T	KCTC 2955^T = IMSNU 11152^T	Chemically contaminated freshwater sediment near Taejon, Republic of Korea	Kim et al., 1986, 2000
S. cloacae S-3^T	JCM 10874^T = IAM 14885^T = DSM 14926^T	Water in sewage treatment plant	Fujii et al., 2001
S. echinoides DSM 1805^T	ATCC 14820^T = NCIB 9420^T	Laboratory contaminant on nutrient agar plate	Heumann, 1962; Denner et al., 1999
S. koreensis JSS26^T	KCTC 2882^T = KCCM 41069^T	Natural mineral water source, Korea	Lee et al., 2001
S. mali Y-347^T	IFO 15500^T = ATCC 51840^T = DSM 10565^T	Roots of apple tree, Japan	Takeuchi et al., 1995
S. natatoria ATCC 35951^T	JCM 10396^T, DSM 3183^T	Water in swimming pool	Sly, 1985 Yabuuchi et al., 1999
S. parapaucimobilis GIFU 11387^T	JCM 7510^T = ATCC 51231^T = DSM 7463^T	Urine specimen	Yabuuchi et al., 1990
S. paucimobilis GIFU 2395^T	JCM 7516^T = ATCC 29837^T = DSM 1098^T	Hospital respirator	Holmes et al., 1977 Yabuuchi et al., 1990
S. pituitosa EDIV^T	CIP 106154^T = DSM 13101^T	Water in eutrophic fountain (Vienna) during algal bloom	Denner et al., 2001
S. pruni Y-250^T	IFO 15498^T = ATCC 51838^T = DSM 10566^T	Roots of *Prunus persica* (peach tree), Japan	Takeuchi et al., 1995
S. roseiflava MK341^T	IAM 14823^T	Ears of plants in family Gramineae	Kawahara et al., 1994 Yun et al., 2000
S. sanguinis GIFU 2397^T	JCM 7514^T = ATCC 51382^T = DSM 13885^T = IFO 13937^T	Blood specimen	Yabuuchi et al., 1990 Takeuchi et al., 1993, 2001
S. suberifaciens ATCC 49355^T	JCM 7516^T = NCPPB 3629^T = DSM 7465^T	Corky root of lettuce	van Bruggen et al., 1990 Yabuuchi et al., 1999
S taejonensis JSS54^T	KCTC 2884^T = KCCM 41068^T	Natural mineral water source, Korea	Lee et al., 2001
S. trueperi ATCC 12417^T	DSM 7225^T = NCIMB 9391^T	Soil	Anderson, 1955 Kämpfer et al., 1997
S. wittichii RW1^T	DSM 6014^T = JCM 10273^T	Elbe River water	Wittich et al., 1992 Yabuuchi et al., 2001
S. xenophaga BN6^T	DSM 6383^T	Elbe River water	Nörtemann et al., 1986 Stolz et al., 2000
Sphingobium species			
S. chlorophenolicum ATCC 33790^T	DSM 7098^T = JCM 10275^T	Pentachlorophenol-contaminated soil	Edgehill and Finn, 1982 Nohynek et al., 1995 Takeuchi et al., 2001
S. herbicidovorans MH	DSM 11019^T = IFO 16415^T = ATCC 700291^T	Soil	Horvath et al., 1990 Zipper et al., 1996 Takeuchi et al., 2001
S. yanoikuyae GIFU 9882^T	JCM 7371^T = ATCC 51230^T = DSM 7462^T	Clinical specimen	Yabuuchi et al., 1990 Takeuchi et al., 2001
Novosphingobium species			
N. aromaticivorans F199^T	SMCC F199^T = ATCC 700278^T = DSM 12444^T	Deep Atlantic coastal plain aquifer sediments	Balkwill et al., 1997a Takeuchi et al., 2001

Table 4. *Continued*

Species and strain designation	Culture accession nos.	Habitat	References
N. capsulatum GIFU 11526^T	JCM 7508^T = ATCC 14666^T = DSM 30196^T	Stored distilled water	Leifson, 1962 Yabuuchi et al., 1990 Takeuchi et al., 2001
N. rosa IFO 15208^T	ATCC 51837^T = DSM 7285^T = NCPPB 2661^T	Hairy root of *Rosa* (rose) sp., United States	Holmes and Roberts, 1981 Takeuchi et al., 1995, 2001
N. stygium B0712	SMCC B0712^T = ATCC 700280^T = DSM 12445^T	Deep Atlantic coastal plain aquifer sediments	Balkwill et al., 1997a Takeuchi et al., 2001
N. subarcticum KF1^T	DSM 10700^T = HAMBI 2110^T = IFO 16058^T	Activated sludge used in fluidized-bed reactor	Puhakka et al., 1995 Nohynek et al., 1996 Takeuchi et al., 2001
N. subterraneum B0478^T	SMCC B0478^T = ATCC 700279^T = DSM 12447^T	Deep Atlantic coastal plain aquifer sediments	Balkwill et al., 1997a Takeuchi et al., 2001
Sphingopyxis species			
S. macrogoltabida 203^T	IFO 15033^T = ATCC 51380^T = DSM 8826^T	Soil	Kawai et al., 1984 Takeuchi et al., 1993, 2001
S. terrae E-1-A^T	IFO 15098^T = ATCC51381^T = DSM 8831^T	Activated sludge	Kawai et al., 1984 Takeuchi et al., 1993, 2001

Abbreviations: GIFU, Dept. of Microbiology, Gifu Univ., Gifu, Japan; JCM, Japan Collection of Microorganisms; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; LMG, Universiteit Gent, Laboratorium voor Mikrobiologie, Gent, Belgium; KCTC, Korean Collection For Type Cultures; IFO, Institute For Fermentation, Osaka, Japan; IMSNU, Institute of Microbiology, Seoul National University, Seoul, Korea; IAM, Institute of Applied Microbiology, University of Tokyo, Institute of Molecular and Cellular Bioscience, Tokyo, Japan; NCIB, National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland, United Kingdom; CIP, Institut Pasteur; NCPBB, National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, United Kingdom; and HAMBI, Cult. Coll. Fac. of Agric. and Forestry, Univ. of Helsinki, Finland.

Table 5. Habitats of selected additional strains of *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*.

Genus and strain(s)[a]	Habitat from which strain was isolated	References
Sphingomonas strains		
S. alaskensis AF01	Seawater, Cape Muroto, Japan	Eguchi et al., 2001
S. alaskensis RB255, RB2515, RB2109, RB2108, RB2510, and RB2519	Seawater, Resurrection Bay, Alaska	Vancanneyt et al., 2001
S. mali Y-348 and Y-351	Roots of apple tree, Japan	Takeuchi et al., 1995
S. parapaucimobilis GIFU 2135	Vaginal swab	Yabuuchi et al., 1990
S. parapaucimobilis GIFU 2266 and 11693	Sputum specimen	Yabuuchi et al., 1990
S. paucimobilis 2322	Creosote-contaminated soil	Dutta et al., 1998
S. paucimobilis BPSI-3	PCB-contaminated soil	Davison and Veal, 1993
S. paucimobilis EPA505	Creosote-contaminated soil	Mueller et al., 1990
S. paucimobilis GIFU 2137	Vaginal swab	Yabuuchi et al., 1990
S. paucimobilis GIFU 2396 and 2406	Spinal fluid	Yabuuchi et al., 1990
S. paucimobilis GIFU 11385	Urine specimen	Yabuuchi et al., 1990
S. paucimobilis GIFU 11386	Sputum specimen	Yabuuchi et al., 1990
S. paucimobilis GS1	Soil near Baroda, India	Ashtaputre and Shah, 1995
S. paucimobilis P4	Derived from culture of NCIMB 11942	Lobas et al., 1994
S. paucimobilis Q1	Soil	Furukawa et al., 1983
S. paucimobilis SS86	Field exposed to gamma-hexachlorocyclohexane	Senoo and Wada, 1989
S. paucimobilis SYK-6	Pulp-bleaching wastewater, Japan	Sonoki et al., 2000
S. paucimobilis TA-2	Soil	Ohe et al., 1990
S. paucimobilis UT26	Mutant of *S. paucimobilis* SS86	Imai et al., 1989
Sphingomonas str. 1CX	Waste liquor from sewage treatment plant	Coughlin et al., 1999
Sphingomonas str. 2.10, 2.11, 2.12, 2.15, and 2.18	Biofilm on glass coupon exposed to groundwater from borehole in England	Buswell et al., 1997 Rickard et al., 2002
Sphingomonas str. 72MPII	Contaminated marine sediment near petroleum refinery, Lavera, France	Gilewicz et al., 1997

Table 5. *Continued*

Genus and strain(s)[a]	Habitat from which strain was isolated	References
Sphingomonas str. A1	Soil	Momma et al., 1999
Sphingomonas str. ANT-17 and ANT-20	JP8 fuel-contaminated soil, Antarctica	Lloyd-Jones and Lau, 1997
Sphingomonas str. CF06	Soil that had been exposed to carbufuran	Feng et al., 1997
Sphingomonas str. DhA-33	Batch reactor used to treat synthetic pulp mill effluent	Mohn, 1995 Mohn et al., 1999
Sphingomonas str. DhA-95	Hydrocarbon-contaminated Arctic Soil, Ellesmere Island	Mohn et al., 1999
Sphingomonas str. HV3	Soil, Finland	Kilpi et al., 1980 Yrjälä et al., 1998
Sphingomonas JS5	Creosote-contaminated soil	Sabaté et al., 1999
Sphingomonas str. K6, K16, K39, K74, and K101	Chlorophenol-contaminated groundwater near adjacent to sawmill, Finland	Männistö et al., 1999
Sphingomonas str. KT-1	Arakawa River water, Japan	Tabata et al., 1999
Sphingomonas str. RP001, RP003, RP006, and WP01	PAH-contaminated soil, New Zealand	Lloyd-Jones and Lau, 1997
Sphingomonas str. S88	Pond water	Yamazaki et al., 1996
Sphingomonas str. SRS2	Agricultural soil treated with herbicide isoproturon, Demark	Sørensen et al., 2001
Sphingomonas str. SS3	Soil contaminated with industrial waste	Schmidt et al., 2001
Sphingomonas str. TZS-7	Crude oil	Lu et al., 1999
Sphingomonas str. UG30	Pentachlorophenol-contaminated soil	Leung et al., 1997
Sphingomonas str. V21	Deep glacial ice core, Lake Vostok, Antarctica	Christner et al., 2001
Sphingobium strains		
S. chlorophenolicum ATCC 39723	Pentachlorophenol-contaminated soil	Saber and Crawford, 1985 Nohynek et al., 1995
S. chlorophenolicum RA2	Pentachlorophenol-contaminated soil	Radehaus and Schmidt, 1992 Nohynek et al., 1995
S. chlorophenolicum SR3	Pentachlorophenol-contaminated soil from wood preserving facility	Resnick and Chapman, 1994 Nohynek et al., 1995
S. yanoikuyae B1	Polluted stream	Gibson et al., 1973 Gibson, 1999
S. yanoikuyae IFO 15163	Roots of *Psychotria nairobiensis*	Takeuchi et al., 1995
S. yanoikuyae IFO 15164	Roots of *Ardisia crispa*	Takeuchi et al., 1995
Novosphingobium strains		
N. aromaticivorans B0522 and B0695	Deep coastal plain aquifer sediments	Balkwill et al., 1997a
N. rosa IFO 15209 and IFO 15210	Roots of *Rosa* (rose) sp., United States	Takeuchi et al., 1995
N. subarcticum KF3 and NKF1	Activated sludge used as inoculum in fluidized-bed reactor	Puhakka et al., 1995 Nohynek et al., 1996
N. subarcticum PR-P12	Rhizosphere of salt marsh plants	Daane, et al., 2001
Sphingopyxis strains		
S. macrogoltabida TFA	Rhine River mud	Hemáez et al., 1999

Abbreviations: PCB, polychlorobiphenyl; JP-8, a jet fuel grade; and PAH, polycyclic aromatic hydrocarbon.
[a]In many cases, it has not been confirmed that the strain is a member of the indicated genus and/or species.

can occupy a broad range of different types of environments. Aqueous habitats in which they have been shown to occur include freshwater environments such as river water, pond water, and groundwater, as well as marine environments such as seawater near the coasts of Alaska and Japan. Sphingomonads also have been isolated from soils and freshwater, marine, or deep terrestrial subsurface sediments.

Many strains of *Sphingomonas* and related genera have been isolated from comparatively pristine environments, but a considerable proportion of them (e.g., one third of those in Tables 4 and 5) were discovered in contaminated (sometimes rather badly contaminated) environments. The contaminants in these environments include a variety of potentially toxic and/or recalcitrant compounds, such as PCBs, creosote, pentachlorophenol, herbicides, etc. Many of the strains isolated from contaminated habitats were able to utilize one or more of the contaminants as sole carbon sources, a reflection of the exten-

sive biodegradative versatility of these organisms (see the section Physiology in this Chapter). Given their versatile degradative abilities, it seems likely that natural enrichments have occurred for species of *Sphingomonas* and related genera in some of the contaminated habitats cited in Tables 4 and 5.

Many of the sphingomonads described to date were isolated from low-nutrient habitats (e.g., soils and sediments), some of which may be very oligotrophic (e.g., seawater and deep subsurface environments). In fact, the type strain of *Novosphingobium capsulatum* (ATCC 14666^T) was isolated from stored distilled water (Leifson, 1962). In contrast, various strains of *Sphingomonas*, *Sphingobium* and *Novosphingobium* have been isolated from roots or other parts of plants, where one would expect the concentrations of organic carbon to be much higher than in soils, sediments, or seawater. *Sphingomonas* and *Sphingobium* also have been isolated from clinical specimens with relatively high concentrations of organic nutrients, such as blood and sputum. Some of these isolates, especially strains of *Sphingomonas paucimobilis*, have been implicated in certain types of (mostly nosocomial) infections in humans (see the section Disease in this Chapter). These findings indicate that sphingomonads can survive and function over a rather wide range of nutrient concentrations.

In all likelihood, the complete range of habitats that can be occupied by *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* has yet to be determined. In any case, it is obvious from the research reported to date that these organisms are exceedingly widespread in nature and that this is due primarily to their physiological and metabolic versatility, especially their extensive catabolic capabilities.

Isolation

To the best of our knowledge, no one has published a protocol designed specifically for the isolation of *Sphingomonas*, *Sphingobium*, *Novosphingobium* and/or *Sphingopyxis* species. Virtually all strains of these genera reported to date were isolated more or less fortuitously, in studies that were carried out for various reasons but with no intent to concentrate specifically on *Sphingomonas*-like organisms. The primary objective of some of these studies was to examine the composition of culturable aerobic microbial communities in certain types of environments, in which case environmental samples typically were diluted (if necessary) and plated on commonly available microbiological media. Comparatively dilute media have been used in most studies in which sphingomonads were isolated. Examples include R2A agar (described by Reasoner and Geldreich [1985] and used by Christner et al. [2001]; see also Rickard et al., 2002); a dilute peptone-tryptone-yeast extract-glucose (1% PTYG) medium (Balkwill, 1989; Fredrickson et al., 1995) and PYGV, a dilute peptone-yeast extract-glucose medium with vitamins described by Staley (1968) and used by Männistö et al. (1999). However, sphingomonads have also been isolated using media that have higher concentrations of nutrients, such as Czapek-Dox agar with sucrose (30 g/liter; Denner et al., 2001) and blood agar (Morrison and Shulman, 1986). In all of these studies, sphingomonads just happened to be among the bacteria detected and isolated. Several researchers have mentioned the growth of yellow-pigmented colonies, and this could serve as an initial indication that these types of bacteria may be part of the community being examined. Interestingly, Lee et al. (2001) focused on yellow-pigmented bacteria in natural mineral water sources in Korea because earlier studies had focused on nonpigmented forms. As a result, they isolated several new strains of *Sphingomonas* that were shown to represent three novel species (*S. aquatilis*, *S. koreensis* and *S. taejonensis*).

Many strains of *Sphingomonas* and related genera have been isolated in studies in which enrichment cultures were used to investigate bacteria that degrade specific organic compounds, most often compounds that are important as environmental contaminants. The inoculum for the enrichment in these studies usually is water, soil, or sediment from a contaminated environment. The enrichment itself typically is carried out in some type of mineral-salts medium (which may or may not include vitamins), with the target compound serving as the sole source of carbon and energy. Examples of target compounds used in enrichments that have resulted in the isolation of sphingomonads include 6-aminonaphthalene-2-sulfonic acid (Nörtemann et al., 1986), biphenyl (Furukawa et al., 1983), dibenzo-*p*-dioxin (Wittich et al., 1992), *N*,*N*-dimethylaniline (Schmidt, 1994), fluoranthene (Mueller et al., 1990), 4-fluorodiphenyl ether (Schmidt et al., 1992), Orange II (an azo dye; Coughlin et al., 1999), 2-methylphenanthrene (Gilewicz et al., 1997; Sabaté et al., 1999), nonylphenol (Fujii et al., 2001), pentachlorophenol (Saber and Crawford, 1985; Radehaus and Schmidt, 1992), phenanthrene (Dutta et al., 1998), the herbicide Isoproturon™ (Sørensen et al., 2001), tetralin (Hernáez et al., 1999), and several others.

To isolate organisms that degrade compounds like those listed above, it usually has been nec-

essary to run the initial enrichment culture for an extended period of time and to subculture at least twice in fresh batches of the enrichment medium. While isolating a 2-methylphenanthrene-degrading *Sphingomonas* strain (2MPII), for example, Gilewicz et al. (1997) incubated an initial enrichment for one month and then made two successive transfers into fresh medium, incubating each of the transfers for another month. Sørensen et al. (2001) used 15 successive subcultures in fresh medium during the isolation of a *Sphingomonas* strain (SRS2) that degrades the phenylurea herbicide Isoproturon™. Variations on the enrichment approach have included the use of multiple substrates instead of a single target compound (Puhakka et al., 1995), a soil column (Horvath et al., 1990), and/or a continuous-flow fluidized-bed bioreactor (Puhakka et al., 1995). Regardless of the details, enrichment and subculturing may yield a mixed culture, in which case one still has to isolate the strain that actually degrades the compound of interest, assuming the reactions are carried out by a single strain as opposed to a consortium. This is most often done by plating the mixed culture on a general growth medium, isolating the different colony types by re-streaking on fresh medium, and then testing each of the pure cultures for their ability to degrade the target compound. However, some authors have developed indicator media on which the colonies that degrade the target compound are recognized by a change in the appearance of the surrounding medium (e.g., Saber and Crawford, 1985; Sabaté et al., 1999). This approach greatly lessens the time required to detect and isolate the desired strain(s).

A few strains of *Sphingomonas* have been obtained by using very specialized procedures for isolation of organisms with certain types of physiological traits other than the ability to use a specific compound as a carbon source. For example, Eguchi et al. (2001) and Vancanneyt et al. (2001) used extinction dilution techniques and enrichments in filter-sterilized seawater to isolate oligotrophic strains of *Sphingomonas alaskensis* from seawater near Cape Muroto in Japan and Resurrection Bay in Alaska, respectively. Ashtaputre and Shah (1995) isolated a *Sphingomonas paucimobilis* strain (GS1) that produces a viscous, gel-forming exopolysaccharide by plating soil from Baroda, India, on a nitrogen-free sucrose agar medium and looking for viscous colonies.

Cultivation

The types of media that have been used to cultivate sphingomonads are nearly as varied as the habitats from which they have been isolated (see the section Habitat in this Chapter). A variety of complex media can be utilized for routine cultivation of most species. For example, many of them have been cultured for phylogenetic and biochemical studies on various combinations of peptone, yeast extract, and a source of carbon and energy (e.g., glucose; Takeuchi et al., 1993; Takeuchi et al., 1995; Balkwill et al., 1997a). However, other complex media have also been used. Examples include trypticase soy broth (TSB) or agar (TSA; Zipper et al., 1996; Denner et al., 2001; Lee et al., 2001; Yabuuchi et al., 2001), Luria broth (Kim et al., 2000), nutrient agar (Lee et al., 2001), and brain-heart infusion agar (for pathogens; Takeuchi et al., 1993).

As a group, sphingomonads are metabolically very diverse and able to degrade both a broad range of naturally occurring organic compounds and many different types of refractory environmental pollutants. As a result, a variety of chemically defined media have been used to cultivate these organisms, typically for enrichment cultures using a single organic compound as the sole source of carbon and energy (e.g., Saber and Crawford, 1985; Katayama et al., 1988; Mueller et al., 1990; Schmidt et al., 1992; Wittich et al., 1992; Takeuchi et al., 1993; Parsons et al., 1998; Wittmann et al., 1998; Fukuda et al., 2002). Our laboratory utilized a defined mineral salts medium designed for cultivation of aerobic pseudomonads to grow aromatic-degrading isolates from subsurface sediments (Fredrickson et al., 1995). Similar media have been used by others (Fortnagel et al., 1990) and often contain phosphates (which also serve to buffer pH), $(NH_4)_2SO_4$, magnesium and calcium salts, and a trace element solution (for examples, see Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) media 457 and 465; http://www.dsmz.de/media). Mineral media are sometimes supplemented with peptone and/or vitamins to enhance growth (Zipper et al., 1996).

Most strains of sphingomonads are mesophiles and, therefore, are routinely cultured at 30°C. Although some strains have been isolated from low-temperature environments and some are even psychrotrophic (Eguchi et al., 1996), they also grow well at mesophilic temperatures (Christner et al., 2001). No thermophilic sphingomonads have been isolated to our knowledge. All known species are obligate aerobes; to date, no facultative strains been described. Some of the marine-derived strains have been grown in media containing NaCl at concentrations similar to those in seawater, but it is not clear whether these strains actually require salt (Eguchi et al., 1996).

Identification

There is no currently available rapid-kit-based or automated method for identification of all of the various species of *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* described to date. One might suspect that a new strain (depending on the conditions under which it was isolated) is a member of one of these genera if it is a Gram-negative, rod-shaped organism that forms yellow, off-white, or whitish brown colonies on general media such as nutrient agar or trypticase soy agar. Perhaps the easiest way to establish whether the isolate is very likely to be a member of one of these genera is to determine its 16S ribosomal RNA gene sequence (or partial sequence) and then analyze that sequence phylogenetically to determine the strain's evolutionary relatedness to previously described species. Standard approaches for doing this have been detailed by many of the authors who have proposed new *Sphingomonas* species in recent years (e.g., Takeuchi et al., 1995; Takeuchi et al., 2001; Balkwill et al., 1997a). The clustering patterns in the resulting phylogenetic trees and the signature nucleotide bases at certain positions in the 16S rRNA gene (see Table 2) will indicate whether the isolate is highly likely to be a member of one of the four *Sphingomonas*-related genera described by Takeuchi et al. (2001).

Following phylogenetic analysis, several additional analyses should be done to confirm that an isolate is a member of *Sphingomonas*, *Sphingobium*, *Novosphingobium* or *Sphingopyxis* on the basis of the distinguishing characteristics of these genera (Table 2) described by Takeuchi et al. (2001). At a minimum, one should show that glycosphingolipids (GSLs) are present in the cell envelope and that ubiquinone Q10 is the principal respiratory quinone. GSLs can be detected and identified by extraction with chloroform:methanol, followed by thin layer chromatography (TLC; Kawahara et al., 1991). Isoprenoid quinones can be extracted with chloroform:methanol, separated by TLC, and identified by high-performance liquid chromatography (HPLC) analysis (Takeuchi et al., 1993). Lipid composition, especially the major fatty acids and 2-hydroxy fatty acids, should also be determined and various methods can be used to do this (for examples, see Balkwill et al. [1997a], Yabuuchi et al. [1999], and Takeuchi et al. [2001]). Some authors have utilized the Microbial Identification System (MIDI Inc., Newark, DE, USA) for lipid analysis (for examples, see Nohynek et al. [1995] and Lee et al. [2001]).

To identify an isolate to the species level within one of the *Sphingomonas*-related genera, it generally would be necessary to perform substrate-utilization and other physiological analyses. For details, the reader is referred to the species descriptions and techniques cited in the taxonomy papers for each recognized species in Tables 1 and 3. It may appear from these analyses that an isolate is a new species. If so, one would then have to establish this by showing either that 16S rRNA gene sequence similarities are below 97% or that chromosomal DNA-DNA reassociation values are below 70% when the isolate is compared to the phylogenetically most closely related established species (see Stackebrandt and Goebel, 1994).

Preservation

Strains of *Sphingomonas* and related genera are readily preserved by lyophilization, and culture collections routinely supply these organisms in the form of freeze-dried stocks. To provide an extra measure of insurance for long-term preservation, however, they should also be frozen in a cryoprotectant and stored at very low temperature. Cultures typically are frozen in their usual growth medium containing either 20–25% glycerol or 7% dimethylsulfoxide (DMSO) as the cryoprotectant. In our laboratory, we grow broth cultures to the late exponential phase, concentrate the cells by centrifugation, and resuspend them in a relatively small volume of fresh medium containing 7% DMSO prior to freezing. After freezing by placement in a –80°C freezer or dry ice-methanol bath, the frozen stocks can be stored at –80°C and/or under liquid nitrogen.

Physiology

Catabolism of Organic Compounds

Sphingomonas, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* species are aerobic (nonfermentative), yellow-pigmented, nonsporeforming Gram-negative bacteria that either are nonmotile or contain a single polar flagellum. Glucose is assimilated by most strains (Balkwill et al., 1997a) and is frequently used as a growth substrate in both complex and defined media. A wide variety of other sugars including arabinose, fucose, galactose, lactose, mannose, melibiose, sucrose, trehalose, and xylose also are commonly assimilated (Balkwill et al., 1997a; Denner et al., 2001; Fujii et al., 2001). Many sphingomonads can degrade polysaccharides in addition to mono- and disaccharides. For example, *Sphingomonas* strain A1 can use high-molecular-weight alginate for growth by depolymerizing the polymer with an intracellular alginate lyase

(Momma et al., 1999). This organism has developed a novel pit-dependent endocytosic uptake system for the metabolism of macromolecules (Hashimoto et al., 2001).

Sphingomonads are well recognized for their metabolic versatility and ability to degrade a broad range of environmental contaminants in addition to various natural organic compounds primarily of plant origin. Some strains have even been isolated from arsenic-contaminated mine tailings and can reduce arsenate to arsenite under aerobic conditions (Macur et al., 2001). They are particularly adept at degrading aromatic compounds including single and multi-ring structures with various substitutions and functionality. Examples of the classes and types of organic compounds that can be degraded by sphingomonads are provided in Table 6. Because of their metabolic versatility, the metabolic pathways of sphingomonads have been studied in detail (e.g., Schmidt et al., 1992; Wittich et al., 1992; Zylstra and Kim, 1997), as have the properties of their associated catabolic enzymes (Bünz and Cook, 1993a; Bünz et al., 1993b; Bertini et al., 1995; Armengaud and Timmis, 1998; Hynkova et al., 1999; Xun et al., 1999; Byun et al., 2001; Vuilleumier et al., 2001; Oakley et al., 2002).

Extracellular Biopolymers

Sphingomonas strains are known to produce extracellular biopolymers, including certain gellan-related exopolysaccharides (Lobas et al., 1992; Martins et al., 1996; Hashimoto and Murata, 1998). For example, some strains of *S. paucimobilis* produce a gellan-related polysaccharide that forms a gel if deacetylated (Anson et al., 1987). Gellan-related polysaccharides synthesized by strains of *Sphingomonas* are collectively referred to as "sphingans" (Pollock, 1993). They share the same carbohydrate backbone structure (–X-glucose-glucuronic acid-glucose-X–) in which X is either L-rhamnose or L-mannose. The yield, molecular structure, and properties of sphingans are significantly affected by growth medium composition (Fialho et al., 1999). *Sphingomonas paucimobilis* GS1 produces an anionic heteropolysaccharide with a viscosity that is 5.5-fold greater than that of xanthan gum. This compound is stable over a pH range of 2–10 and in the presence of NaCl con-

Table 6. Organic compounds metabolized by members of the genus *Sphingomonas*.

Compound class or compound	Representative strain(s) (reference)
Nonylphenol	*Sphingomonas cloacae* (Fujii et al., 2001)
Dibenzo-*p*-dioxin, dibenxofuran	*Sphingomonas wittichii* strain RW1 (Wittich et al., 1992)
Diphenyl ethers	*Sphingomonas* sp. strain SS3 (Schmidt et al., 1992)
Polychlorinated hydrocarbons:	
Pentachlorophenol	*Sphingomonas* sp. strain ATCC 39723 (Crawford and Ederer, 1999);
Polychlorinated	*S. chlorophenolica* RA2 (Witmann et al., 1998)
biphenyls	*S. paucimobilis* BPSI-3 (Davison et al., 1996)
Monoaromatics:	
toluene, xylene, cresol	*S. aromaticivorans* F199 (Balkwill et al., 1997a);
	S. yanoikuyae B1 (Zylstra and Kim, 1997)
Polyaromatic hydrocarbons:	
naphthalene, anthracene,	*S. yanoikuyae* B1 (Gibson, 1999)
phenanthrene	*S. paucimobilis* EPA 505 (Mueller et al., 1990)
fluoranthene	*S. paucimobilis* 2322 (Dutta et al., 1998);
substituted naphthalenes	*Sphingomonas* sp. strain BN6 (Riegert et al., 1999)
Pesticides:	
lindane	*S. paucimobilis* UT26 (Nagata et al., 1999)
diclofop-methyl	*S. paucimobilis* (Adkins, 1999)
2,4-D, mecoprop	*S. herbicidovorans* MH (Kohler, 1999)
carbofuran	*Sphingomonas* sp. strain CF05 (Feng et al., 1997)
isoproturon	*Sphingomonas* sp. strain SRS2 (Sørensen et al., 2001)
Lignin monomers & dimers:	*S. paucimobilis* SYK-6 (Masai et al., 1999)
Triterpenoids (resin acids): dehydroabietic acid, abietic acid, palustric acid, dichlorodehydroabietic acid	*S.* spp. DhA-33 (Mohn and Stewart, 1997; Mohn et al., 1999)
Polymers:	
Polyethers (polyethylene glycol)	*S. macrogoltabidus* strain 203,
	S. terrae E-1-A (Kawai, 1999; Takeuchi et al., 1993)

centrations up to 50 g · liter^{-1}, has a gel strength four times that of agar, and is able to withstand autoclaving (Ashtaputre and Shah, 1995). Functions of extracellular polysaccharides produced by sphingomonads include the attachment of cells to surfaces (Azeredo and Oliveira, 2000), the formation of biofilms (Johnsen et al., 2000; Kuehn et al., 2001), and the probable enhancement of pathogenicity (Pollock, 1993). In addition to sphingans, some sphingomonads can produce enzymes that are involved in the depolymerization of polysaccharides (Hashimoto et al., 1996; Sutherland and Kennedy, 1996; Hashimoto and Murata, 1998). Some strains of *Sphingomonas* exhibit a planktonic/sessile dimorphism, with the sessile state being marked by production of a viscous exopolysaccharide capsule (Pollock and Armentrout, 1999). The factors that control these behaviors in *Sphingomonas* are complex and poorly understood, but it is clear that culture conditions, such as the level of aeration, can influence shifts between the planktonic and sessile states and, hence, exopolysaccharide production.

In an effort to increase gellan yield in "*Sphingomonas elodea*," Vartak et al. (1995) noted that mutants deficient in 6-phosphogluconate dehydrogenase were unaffected in CO_2 production or gellan yield from glucose. This result combined with other enzyme analyses indicated that "*S. elodea*" utilizes the Entner-Doudoroff and pentose-phosphate pathways, rather than the Embden-Meyerhof glycolytic pathway, for glucose metabolism.

Membrane Structure

Sphingomonads are nearly unique among Gram-negative bacteria in that they are devoid of lipopolysaccharides (LPS) that can carry endotoxins. Instead, the cell envelope structure of *S. paucimobilis* includes a cell membrane that consists of proteins, phospholipids, and respiratory quinones and an outer membrane that contains glycosphingolipids (GSLs) with the carbohydrate moiety facing away from the cell surface (Kawasaki et al., 1994). The GSLs therefore occupy a position similar to that of LPS in other Gram-negative bacteria and likely provide many of the same functions (White et al., 1996), such as a barrier to bactericidal substances. *Sphinogomonas paucimobilis* GSLs include a glycosyl portion consisting of D-mannose-*p*-(1→2)-α-D-galactose-*p*-(1→6)-α-D-glucosamine-*p*-(1→4)-α-D-glucuronic acid-1-α-(18:0)/(18:1) ω5 dihydrosphingosine with an amide-linked 2-hydroxy (14:0) at the 2 position of the long-chain base (Kawahara et al., 1991). The structure of GSLs can vary from species to species (Kawahara et al., 1999; Kawahara et al., 2001). Because the carbohydrate part of a GSL is far shorter than that of LPS, the cell surface of sphingomonads is more hydrophobic that that of other Gram-negative bacteria (Kawahara et al., 2001). This hydrophobicity likely contributes to the ability of sphingomonads to degrade hydrophobic polycyclic aromatic hydrocarbons (PAHs; Mueller et al., 1990; Ye et al., 1996; Ryeom et al., 2000; Daane et al., 2001) and susceptibility to hydrophobic antibiotics (Smalley et al., 1983).

Oligotrophy

Sphingomonas species such as *S. alaskensis* appear to be the dominant members of the picoplankton and important contributors to the total biomass and nutrient recycling in some low-nutrient marine environments (Eguchi et al., 2001). The type strain of *S. alaskensis* (RB2256) was isolated from Resurrection Bay in Alaska using a dilution-to-extinction method (Schut et al., 1997) and produces ultramicrocells (<0.08 μm^3) in dilute or rich medium (Eguchi et al., 2001). *Sphingomonas alaskensis* RB2256 appears to be well-adapted for survival under oligotrophic conditions as it can grow in liquid sea water medium containing less than 1 mg · liter^{-1} of dissolved organic carbon (Schut et al., 1997) and has an intermediate sized genome (~3.25 Mbp; Eguchi et al., 2001) with a single copy of the rRNA operon (Fegatella et al., 1998). Moreover, gene expression in this strain is highly regulated in response to starvation, including considerable co- and post-translational modification of proteins (Fegatella and Cavicchioli, 2000). Both growing and starved cells were remarkably resistant to stresses such as elevated temperature and high concentrations of ethanol and hydrogen peroxide, indicating that strain RB2256 has stress-resistance mechanisms that are distinct from those of other non-differentiating bacteria (Eguchi et al., 1996). Maximum growth rates were reached almost immediately when carbon-limited or starved cells of strain RB2256 were exposed to excess glucose, indicating that protein synthesis was constitutively regulated and that this organism is well adapted to life in seawater.

Genetics

Genetic systems have been used primarily to study aromatic catabolic metabolism and sphingan biosynthetic pathways in sphingomonads. Conjugation of broad host range plasmids

pUFR042 (IncW; De Feyter and Gabriel, 1991) into *N. aromaticivorans* F199 (M. F. Romine, unpublished results) and pSEB24 (IncQ; Yamazaki et al., 1996) or pRK311 (IncP; Ditta et al., 1985) into *Sphingomonas* S88 (Yamazaki et al., 1996) have been successful. Sphingomonads are inherently resistant to polymyxin B, which inhibits proper cytoplasmic membrane formation in most other Gram-negative bacteria, including *Escherichia coli*, and therefore makes an excellent selectable marker to discriminate against donor cells in conjugation experiments. The majority of the genetic studies on sphingomonads have focused on the construction of transposon mutants generated either with *Tn10* (Pollock et al., 1998) or with *Tn5* (Kim and Zylstra, 1995; Feng et al., 1997; Zylstra and Kim, 1997; Bünz et al., 1999; Romine et al., 1999; Kaneko et al., 2000; Bastiaens et al., 2001; Story et al., 2001).

Deletion mutants in *Sphingomonas* strain S88 have been constructed by standard double crossover exchange, utilizing a narrow host range plasmid, pSEB26, which contains the pMB1 (pUC12/13) replication origin. The ability of many sphingomonad strains to metabolize sucrose suggests that use of the sucrose sensitivity phenotype that is conferred by levansucrase (*sacB*) as a selectable marker for the second crossover event will not be suitable for most wildtype strains of these organisms. Indeed, a *sacB* homolog was found in the genome sequence of the sucrose-metabolizing *Novosphingobium aromaticivorans* strain F199.

The aromatic catabolic properties of many sphingomonads reside on large plasmids (>50 Kb). Plasmids have been cured from *Sphingomonas* sp. strain CF06 by growing cells at elevated temperature (42°C) for 2 days (Feng et al., 1997). In *Tn5* mutants selected for their inability to metabolize carbofuran, these plasmids were found to have undergone rearrangements, deletions, or loss. Because of their large size, specialized techniques are required to isolate sphingomonad plasmids (Furukawa and Chakrabarty, 1982; Stillwell et al., 1995; Feng et al., 1997). Transfer of the *N. aromaticivorans* pNL1 conjugative plasmid to *Sphingomonas* strain S88m260 has been demonstrated. The *sspB*$^{-}$ phenotype of the recipient strain makes it resistant to the peptidoglycan synthesis inhibitory antibiotic, bacitracin (Pollock et al., 1998), to which other sphingomonads are sensitive. Stringent starvation protein B (SspB) is required for biosynthesis of capsular polysaccharide (Pollock et al., 1998). Similar spontaneous bacitracin-resistant recipient strains for conjugal transfer can be isolated by selection in the presence of bacitracin (T. J. Pollock, personal communication).

Genomics

Novosphingobium aromaticivorans F199 harbors a 185,457-bp conjugative, aromatic catabolic plasmid, pNL1, that has been completely sequenced (Romine et al., 1999). The entire genome of this organism was sequenced to near completion by the United States Department of Energys Joint Genome Institute, and the data are available at (the Joint Genome Institute website). Draft annotations of the genome sequence are available at (Oak Ridge National Laboratory Computational Biology website (DB1)) and at PEDANT (Protein Extraction, Description, and Analysis Tool) website). To date, this is the only publicly available genomic sequence information for a sphingomonad. Strain F199, which was isolated from deep subsurface sediments Fredrickson et al., 1995), has an additional plasmid, designated "pNL2" (~480 kbp) and an estimated genome size of 2.86 Mb (R. Schneider, unpublished data), but genome sequence analyses suggested that the genome size is closer to 3.8 Mb. The average G+C content of pNL1 is 62 mol% and that of the genome is 65.1 mol%. Genome sizes of other *Novosphingobium* strains from terrestrial subsurface environments were estimated to be approximately 2.9 Mb (*N. subterraneum* B0478), 3.76 Mb (*N. aromaticivorans* B0522), 3.82 Mb (*N. aromaticivorans* B0695), and 3.81 Mb (*N. stygium* B0712), whereas those for the type strains of *N. capsulatum* (ATCC 14666) and *S. paucimobilis* (ATCC 29837) were estimated to be 4.18 Mb and 2.6 Mb, respectively (Fredrickson et al., 1999). Extrachromosomal elements hosted by these strains ranged between 48 Kb and 750 Kb (150, 230, 420, and 630 Kb for strain B0478; 50, 130, and 270 Kb for strain B0712; 180 and 650 Kb for strain B0522; 48, 150, and 750 Kb for strain B0695; 230 and 600 Kb for ATCC 14666; and 360 Kb for ATCC 29837). These genome sizes are comparable to those of *S. alaskensis* strains RB2256, AF01, and KT-1, whose genome sizes have been estimated to range between 3.11 and 3.25 Mb, with a G+C content between 64.8 and 65.3 mol% (Eguchi et al., 2001).

Over one-third of *N. aromaticivorans* plasmid pNL1 (83 open reading frames) is devoted to aromatic catabolic genes. The remainder of the plasmid encodes for genes that are necessary for conjugation and replication. The replication region includes two transposons and two group II intron-associated maturases. Group II introns commonly have been found in the genomes of eukaryotic organelles in fungi and plants and, more recently, in bacteria. In bacteria, they are most often associated with insertion sequence elements (Ferat et al., 1994; Knoop and Brennicke, 1994; Kulaeva et al., 1998; Martínez-

Abarca et al., 1998), conjugative transposons (Mullany et al., 1996), conjugative plasmids (Mills et al., 1996), and chromosomal sex factors (Shearman et al., 1996), suggesting that these elements can be disseminated via horizontal transfer. Homing by mobile group II introns results in unidirectional, lateral transposition of the intron to a specific site within an intron-less copy of the intron's normal host gene. Group II homing is unique in that it does not require recombinase activity, is not critically dependent on exon homology, and occurs without marker conversion in flanking exons (Cousineau et al., 1998). These properties are conducive to interspecies spread of the introns, which may account for the wide distribution in prokaryotes and eukaryotes. A possible role for group II introns in the transfer and evolution of biodegradative functions is suggested by studies by Yeo et al. (1997), who demonstrated that when a conjugative plasmid was utilized to capture genes responsible for 2,5-xylenol catabolism, a group II intron was concurrently trapped on the plasmid. Mobile group II introns can be re-targeted and used for highly specific chromosomal gene disruption. Consequently, these introns have been proposed as a useful tool for genetic engineering and functional genomics (Karberg et al., 2001).

The aromatic catabolic cluster includes genes responsible for catabolism of naphthalene, biphenyl, and *m*-xylene. Seven sets of oxygenase large and small subunits are present on pNL1. Enzymes belonging to this family typically catalyze the initial ring oxidation step in aromatic catabolic pathways. The duplication of these genes, but not those that encode enzymes needed for further conversion of oxygenase catabolic products to catechol, suggests that the enzymes they encode may be responsible for the broad substrate specificity of *N. aromaticivorans* strain F199. Analysis of the genome sequence also showed duplication of genes encoding cytochrome *c* and flavocytochrome methylhydroxylase components of enzymes that likely catalyze the initial oxidation of substrates such as *p*-cresol, vanillyl alcohol, and eugenol.

Surprisingly, instead of being clustered by the catabolic pathway in which their products participate, catabolic genes in *N. aromaticivorans* F199 plasmid pNL1 are co-located with those for other types of catabolic enzymes within eleven putative operons. The DNA sequences in the catabolic gene-encoding region of pNL1 have approximately 70% identity to sequences available for *S. yanoikuyae* B1 (Kim, 1996a) and *S. xenophaga* BN6 (GenBank accession number U65001). While the gene order and orientation of the compared genes in strains B1 and F199 are identical, two genes that encode putative membrane proteins are absent in strain B1 and a third membrane-encoding gene in strain B1 has an insertion sequence (Fig. 1a). In strain BN6, gene deletions, insertions, and rearrangements relative to pNL1 result in inactivation or deletion of the catechol extradiol cleavage genes and *m*-xylene catabolic genes (Fig. 1b). Unlike strain F199, strains B1 (Gibson et al., 1973) and BN6 (Stolz et al., 2000) were isolated from waters contaminated with anthropogenic aromatic hydrocarbons. One can speculate that these strains share a common ancestor with F199 and that the selective pressures produced by the pollutants resulted in genetic recombinatorial events that improved the ability of these strains to compete and to grow on man-made aromatic hydrocarbons. The Middendorf formation sediments from which strain F199 was isolated are estimated to have been deposited about 100 million years ago, and the groundwater that flows through this formation has been dated at 4,000 years of age (Fredrickson et al., 1999; Murphy et al., 1992). The primary carbon and energy source available to microorganisms living in these sediments is recalcitrant sedimentary organic matter or lignite. The heterogeneity of lignite and the extremely slow rates of carbon exchange in these sediments may explain why strain F199 maintains so many aromatic catabolic genes and the absence of transposons or similar mobile elements in the regions that encode them.

Ecology

Although sphingomonads have been isolated from a wide range of environments and the physiological and metabolic properties of select strains have been examined in detail, relatively little is known regarding the ecology of this diverse group of microorganisms. Two aspects of their metabolism probably contribute to their widespread distribution in the environment: their ability to utilize a wide range of organic compounds and their ability to grow and survive under low-nutrient or starvation conditions. *Sphingomonas* is a dominant member of the picoplankton population in some marine environments, as evidenced by the isolation of *S. alaskensis* from a 10^5 dilution of seawater from near Cape Muroto, Japan, where the standing bacterial count was 3.1×10^5 cells $\cdot$ ml^{-1} (Eguchi et al., 2001). Similarly, seven phylogenetically related strains of *S. alaskensis* were isolated from a 10^6-fold dilution of seawater from Resurrection Bay, a deep fjord of the Gulf of Alaska (Vancanneyt et al., 2001). The physiological properties of these strains, as described ear-

lier, showed that they are well adapted to life in oligotrophic marine environments. *Sphingomonas* isolates were also among psychrotrophic bacteria cultured from Antarctic sea ice (Bowman et al., 1997) and from water collected at the mouth of Yaquina Bay, Oregon (Suzuki et al., 1997). The widespread distribution and numerical abundance of these organisms suggest that they play an important role in the cycling of carbon and other nutrients in marine environments. In addition to general ecological roles in nutrient cycling, *Sphingomonas* has also been identified as the pathogenic agent of coral plague, a disease that was first identified on coral of the Florida Reef Tract in 1995 and has spread rapidly since (Richardson, 1997; Richardson et al., 1998). A strain of *S. paucimobilis* served as a host for several temperate marine phage (Jiang et al., 1998). Although the extent of this association in nature is unknown, the interactions between marine viruses and their hosts likely have a significant impact on the genetic diversity and composition of marine microbial communities. Additional investigations will most likely reveal even more extensive ecological roles of *Sphingomonas* in marine environments.

Consistent with the idea of sphingomonads being adapted to oligotrophic environments and having the ability to assimilate a wide range of organic compounds was the isolation of three new species of *Sphingomonas* (now renamed "*Novosphingobium*" species) from deeply buried (>200 m below land surface) sediments of the Atlantic Coastal Plain (ACP) of the eastern United States (Fredrickson et al., 1995). The organic carbon content of the groundwater from ACP aquifers is generally very low, <0.5 mg · liter^{-1}, but sedimentary organic carbon can be as high as 1.7% (wt/wt; Fredrickson et al., 1999). We have previously suggested that the extensive catabolic capabilities (e.g., assimilation of aromatic compounds) of these deep subsurface strains might be a result of their adaptation to growth on a range of structurally diverse organic compounds that are only slowly released from solid phase carbon associated with buried sediments. Although the distribution of sphingomonads in the deep subsurface has not been investigated extensively, they have also been isolated from deep sediments from the United States Department of Energys Hanford Site in south-central Washington (Balkwill et al., 1997b; Chandler et al., 1998). Even less is known about the ecology of sphingomonads in the terrestrial subsurface than in marine environments, but they likely fill a similar niche as oligotrophic heterotrophs that contribute to the turnover of organic carbon and perhaps other nutrients.

Many strains of sphingomonads with novel biodegradative properties have been isolated from contaminated soils and sediments (see Tables 4 and 5). They have also been detected in contaminated sediments with molecular-based techniques (Kasai et al., 2001). These findings suggest that the sphingomonads have an important ecological role in contaminated environments, but specific information in this regard is limited. There is some evidence, however, that organic contaminant-degrading strains of sphingomonads are able to compete effectively for resources in contaminated environments and, in some cases, may be the primary organisms responsible for removal of the contaminants. A fluidized bed reactor that was utilized to treat polychlorophenol-contaminated groundwater in Finland for over six years was dominated by *Novosphingobium* sp. strain M1, which could biodegrade the three main contaminants at the in situ temperature of 8°C (Tirola et al., 2002). A strain of *S. paucimobilis* that degrades 2,4-dichlorophenoxyacetic (2,4-D) acid (strain 745) was the second most dominant population (after a genetically constructed strain of *Pseudomonas cepacia*) in a Kansas prairie soil that was treated with 2,4-D and inoculated with four strains of 2,4-D-degrading bacteria (Ka et al., 1994; Kim et al., 1996b). Although a strain of *S. paucimobilis* that degrades hexachlorocyclohexane was observed to survive poorly after it was inoculated into an unamended control soil, it survived and grew in contaminated soil, especially inside of soil aggregates less than 0.025 mm in diameter (Nishiyama et al., 1992). Contaminant-degrading sphingomonad strains have been shown to effectively degrade organic contaminants when inoculated into subsurface sediment (Fredrickson et al., 1999) or soil (Halden et al., 1999). They also maintained higher population densities in the presence of the contaminant than in its absence. These findings indicate that sphingomonads can utilize contaminants as a growth and energy source and that they can compete successfully with the indigenous organisms in complex environments.

Disease

Although it is widespread in other environments (Table 5), *Sphingomonas paucimobilis* (formerly *Pseudomonas paucimobilis*) also has a long history of being associated with equipment in hospitals and with various types of clinical specimens. When Holmes et al. (1977) described *Pseudomonas paucimobilis*, 22 of 28 strains included in the new species had come from hospital equipment, liquid solutions used in hospitals, or clinical specimens. At least one strain of three additional *Sphingomonas* species (*S.*

adhaesiva, *S. parapaucimobilis* and *S. sanguinis*) and one strain of *Sphingobium yanoikuyae* have also been isolated from various clinical sources (Tables 4 and 5). Strains of *S. paucimobilis* are thought to be widespread and endemic members of the microbiota in hospitals (Reina et al., 1991). Equipment and other sources from which they have been isolated include various types of respiratory therapy items, humidifiers, water, air, bedside water bottles, sinks, temperature probes, etc. (Holmes et al., 1977; Crane et al., 1981; Faden et al., 1981; Reina et al., 1991; Lemaitre et al., 1996). Clinical specimens from which strains of *S. paucimobilis* have been obtained include blood cultures, vaginal swabs, urine, sputum, tracheal secretions, sinus washings, spinal fluid, etc. (Holmes et al., 1977; Crane et al., 1981; Faden et al., 1981; Morrison and Schulman, 1986; Lemaitre et al., 1996).

The occurrence of *S. paucimobilis* in a clinical specimen does not necessarily mean that the organism is causing an infection. For example, Faden et al. (1981) investigated an incident in a hospital in which *P. paucimobilis* was isolated from maxillary sinus washings four times in five weeks. There was no indication that the patients examined in this incident had active cases of sinusitis. Rather, the bacterium appeared to be present as a noninfectious contaminant. The source of the contamination was identified as a leaking sink, under which a saline solution used to cleanse sinuses was stored. Similarly, Lemaitre et al. (1996) investigated a situation in which *S. paucimobilis* was isolated from the tracheal secretions of 85 mechanically ventilated babies in a neonatal intensive care unit. Fortunately, none of the neonates developed pneumonia or sepsis. The source of the contamination was traced to the temperature probes of respiratory ventilators used in the hospital.

Although *S. paucimobilis* is sometimes present in clinical specimens as a noninfectious contaminant, it is capable of causing active infections in humans, albeit somewhat infrequently. Through 1991, at least 18 cases of infections attributed to *S. paucimobilis* were described in the literature (see reviews by Morrison and Schulman [1986] and Reina et al. [1991]). Among these infections were six cases of bacteremia (bacterial infections of the bloodstream), two leg ulcers, four cases of peritonitis (in patients undergoing chronic ambulatory dialysis), a brain abscess, a cervical adenopathy, a splenic abscess, a respiratory infection, a urinary infection, and a single case of meningitis. Sporadic reports of *S. paucimobilis* infections (mostly cases of bacteremia, but also urinary, respiratory, and wound infections) have continued to appear since the Reina et al. (1991) review (e.g., Lemaitre et al., 1996; Martino et al., 1996; Gorricho et al., 1998; Hsueh et al., 1998; de Otero et al., 1998; Paster et al., 2002; Perola et al., 2002).

Fortunately, infections by *S. paucimobilis* are seldom serious or life threatening, so long as they are treated properly. Apparently, *S. paucimobilis* has limited virulence in comparison to *Pseudomonas* and other genera that have been associated with similar infections and sources of contamination (Morrison and Shulman, 1986; Reina et al., 1991; Hsueh et al., 1998). The reason for this is not known, although it has been suggested that the relative lack of virulence is a result of the absence of lipopolysaccharide (LPS) and its associated endotoxins in *Sphingomonas* and other genera with GSLs in their envelopes (Hsueh et al., 1998). In any event, infections caused by *S. paucimobilis* can be treated effectively with antibiotics. Antibiotic susceptibility patterns have been studied in some detail (Gilardi, 1984; Reina et al., 1991), and these should be kept in mind when deciding on a specific course of treatment.

As noted elsewhere in this chapter, *Sphingomonas paucimobilis* and related species are able to survive in a rather broad range of environments. This is consistent with their appearance as contaminants in many different hospital-related reservoirs of infection (equipment, solutions, water systems, etc.). Given the near ubiquity and considerable adaptability of these organisms, it might be almost impossible to prevent occasional *S. paucimobilis*- related nosocomial infections of humans, even when treatment facilities exercise rigorous precautionary measures.

The ability of *S. paucimobilis* to infect humans is now well established, but it remains to be seen whether members of *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* can cause diseases in plants. Several strains of these genera were isolated from the root systems of various plants (see Tables 4 and 5), but there is little or no detailed information on the nature of the relationship between these bacteria and the plants from which they were isolated. To the best of our knowledge, there are no indications that the bacteria are pathogenic to the extent that they are harmful to the plants with which they are associated; they are likely to be saprophytic. *Sphingomonas suberifaciens* is the only confirmed plant pathogen in the four *Sphingomonas*-related genera described by Takeuchi et al. (2001). However, these authors recommended that this species be transferred back to its former designation (*Rhizomonas suberifaciens*) because the inclusion of phytopathogenic forms created a genus that was too broad in terms of its ecological and physiological characteristics (see the section Taxonomy and Phylogeny in this Chapter).

Given that strains isolated from root systems were left in the genus, it appears that Takeuchi et al. (2001) did not consider these organisms to be plant pathogens.

Biotechnological Applications

Their metabolic diversity and ability to produce extracellular gellan-like polysaccharide (sphingans) has generated much interest in sphingomonads for biotechnological applications, and numerous patents utilizing members of this group exist world-wide. General categories of these patents include novel catalyses, bioremediation, fossil fuel desulfurization, novel enzymes, biotin production, and polysaccharide production.

Sphingans have a wide range of applications in the food, pharmaceutical, petroleum and other industries (Sutherland, 1999). One interesting and novel application of the extracellular polysaccharide (EPS) produced by *S. paucimobilis* GS-1 is as a drilling fluid component in oil exploration because its relative stability of viscosity under extremes of pH, temperature and salinity, as well as emulsification properties (Shah and Ashtaputre, 1999). *Sphingomonas* will likely continue to be an important source of organisms for research and development of novel exopolysaccharides.

Biocatalytic transformations, including the degradation of hazardous organic compounds and the conversion of commonly occurring organics to novel or specialty chemicals, have also been among the important applications of sphingomonads. A two-phase partitioning bioreactor containing *S. aromaticivorans* achieved complete biodegradation of naphthalene, phenanthrene, acenaphthene and anthracene at volumetric consumption rates of 90 $mg \cdot liter^{-1} \cdot h^{-1}$ (Janikowski et al., 2002). A strain of *S. paucimobilis* has also been used to bioremediate hexachlorocyclohexane-contaminated soil, in which case greater than 95% removal of the contaminant occurred within two months after inoculation (Senoo et al., 1996). Sphingomonads appear to be ideally suited for bioremediation applications because of their ability to complete and proliferate in contaminated soils and sediments.

Sphingomonas sp. strain HXN-200 has proven to be an excellent biocatalyst for the regio- and stereoselective hydroxylation of pyrrolidines (Li et al., 1999; 2001), *N*-substituted azetidines and piperidines (Chang et al., 2002) and pyrrolidin-2-ones (Chang et al., 2000). Because of their extensive intrinsic catabolic capabilities and amenability to genetic manipulation, sphingomonads are amenable to genetic engineering to obtain strains with improved properties (Vartak et al., 1995; Van Kranenburg et al., 1999).

Literature Cited

Adkins, A. 1999. Degradation of the phenoxy acid herbicide diclofop-methyl by Sphingomonas paucimobilis isolated from a Canadian prairie soil. J. Ind. Microbiol. Biotechnol. 23:332–335.

Anderson, G. R. 1955. Nitrogen fixation by Pseudomonas-like soil bacteria. J. Bacteriol. 70:129–133.

Anson, A., P. J. Fisher, A. F. D. Kennedy, and I. W. Sutherland. 1987. A bacterium yielding a polysaccharide with unusual properties. J. Appl. Bacteriol. 62:147–150.

Armengaud, J., and K. N. Timmis. 1998. The reductase RedA2 of the multi-component dioxin dioxygenase system of Sphingomonas sp. RW1 is related to class-I cytochrome P450-type reductases. Eur. J. Biochem. 253:437–444.

Ashtaputre, A. A., and A. K. Shah. 1995. Studies on a viscous, gel-forming exopolysaccharide from Sphingomonas paucimobilis GS1. Appl. Environ. Microbiol. 61:1159–1162.

Azeredo, J., and R. Oliveira. 2000. The role of exopolymers in the attachment of Sphingomonas paucimobilis. Biofouling 16:59–67.

Balkwill, D. L. 1989. Numbers, diversity, and morphological characteristics of aerobic, chemoheterotrophic bacteria in deep subsurface sediments from a site in South Carolina. Geomicrobiol. J. 7:33–52.

Balkwill, D. L., G. R. Drake, R. H. Reeves, J. K. Fredrickson, D. C. White, D. B. Ringelberg, D. P. Chandler, M. F. Romine, D. W. Kennedy, and C. M. Spadoni. 1997a. Taxonomic study of aromatic-degrading bacteria from deep-terrestrial-subsurface sediments and description of Sphingomonas aromaticivorans sp. nov., Sphingomonas subterranea sp. nov., and Sphingomonas stygia sp. nov. Int. J. Syst. Bacteriol. 47:191–201.

Balkwill, D. L., R. H. Reeves, G. R. Drake, J. Y. Reeves, F. H. Crocker, M. B. King, and D. R. Boone. 1997b. Phylogenetic characterization of bacteria in the Subsurface Microbial Culture Collection. FEMS Microbiol. Rev. 20:201–216.

Bastiaens, L., D. Springael, W. Dejonghe, P. Wattiau, H. Verachtert, and L. Diels. 2001. A transcriptional luxAB reporter fusion responding to fluorine in Sphingomonas sp. LB126 and its initial characterisation for whole-cell bioreporter purposes. Res. Microbiol. 152:849–859.

Bertini, I., F. Capozzi, A. Dikiy, B. Happe, C. Luchinat, and K. N. Timmis. 1995. Evidence of histidine coordination to the catalytic ferrous ion in the ring-cleaving 2,2′,3-trihydroxybiphenyl dioxygenase from the dibenzofuran-degrading bacterium Sphingomonas sp. strain RW1. Biochem. Biophys. Res. Comm. 215:855–860.

Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin. 1997. Diversity and association of psychrophilic bacteria in Antarctic sea ice. Appl. Environ. Microbiol. 63:3068–3078.

Bünz, P. V., and A. M. Cook. 1993a. Dibenzofuran 4,4a-dioxygenase from Sphingomonas sp. strain RW1: Angular dioxygenation by a three-component enzyme system. J. Bacteriol. 175:6467–6475.

Bünz, P. V., R. Falchetto, and A. M. Cook. 1993b. Purification of two isofunctional hydrolases (EC 3.7.1.8) in the degradative pathway for dibenzofuran in Sphingomonas sp. strain RW1. Biodegradation 4:171–178.

Bünz, P. V., M. Buck, S. Hebenbrock, and P. Fortnagel. 1999. Stability of mutations in a Sphingomonas strain. Can. J. Microbiol. 45:404–407.

Buswell, C. M., Y. M. Herlihy, P. D. Marsh, C. W. Keevil, and S. A. Leach. 1997. Coaggregation amongst aquatic biofilm bacteria. J. Appl. Microbiol. 83:477–484.

Byun, T., M. Tang, A. Sloma, K. M. Brown, C. Marumoto, M Fujii, and A. M. Blinkovsky. 2001. Aminopeptidase from Sphingomonas capsulata. J. Biol. Chem. 276:17902–17907.

Chandler, D. P., F. J. Brockman, T. J. Bailey, and J. K. Fredrickson. 1998. Phylogenetic diversity of archaea and bacteria in a deep subsurface paleosol. Microb. Ecol. 36:37–50.

Chang, D., B. Witholt, and Z. Li. 2000. Preparation of (S)-N-substituted 4-hydroxy-pyrrolidin-2-ones by regio- and stereoselective hydroxylation with Sphingomonas sp. HXN-200. Org. Lett. 2:3949–3942.

Chang, D., H. J. Feiten, K. H. Engesser, J. B. van Beilen, B. Witholt, and Z. Li. 2002. Practical syntheses of N-substituted 3-hydroxyazetidines and 4-hydroxypiperidines by hydroxylation with Sphingomonas sp. HXN-200. Org. Lett. 4:1859–1862.

Christner, B. C., E. Mosley-Thompson, L. G. Thompson, and J. N. Reeve. 2001. Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice. Environ. Microbiol. 3:570–577.

Coughlin, M. F., B. K. Kinkle, and P. L. Bishop. 1999. Degradation of azo dyes containing aminonaphthol by Sphingomonas sp. strain 1CX. J. Ind. Microbiol. Biotechnol. 23:341–346.

Cousineau, B., D. Smith, S. Lawrence-Cavanagh, J. E. Mueller, J. Yang, D. Mills, D. Manias, G. Dunny, A. M. Lambowitz, and M. Belfort. 1998. Retrohoming of a bacterial group II intron: mobility via complete reverse splicing, independent of homologous DNA recombination. Cell 94:451–462.

Crane, L. R., L. C. Tagle, and W. A. Palutke. 1981. Outbreak of Pseudomonas paucimobilis in an intensive care facility. J. Am. Med. Assoc. 246:985–987.

Crawford, R. L., and M. M. Ederer. 1999. Phylogeny of Sphingomonas species that degrade pentachlorophenol. J. Ind. Microbiol. Biotechnol. 23:320–325.

Daane, L. L., I. Harjono, G. J. Zylstra, and M. M. Häggblom. 2001. Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. Appl. Environ. Microbiol. 67:2683–2691.

Davison, A. D., and D. A. Veal. 1993. Storage of a mixed microbial consortium capable of growth on biphenyl. Lett. Appl. Microbiol. 17:101–103.

Davison, A. D., P. Karuso, D. R. Jardine, and D. A. Veal. 1996. Halopicilinic acids, novel products arising through the degradation of chloro- and bromo-biphenyl by Sphingomonas paucimobilis BPSI-3. Can. J. Microbiol. 42:66–71.

De Feyter, R., and D. W. Gabriel. 1991. Use of cloned DNA methylase genes to increase the frequency of transfer of foreign genes into Xanthomonas campestris pv. malvacearum. J. Bacteriol. 173:6421–6427.

Denner, E. B. M., P. Kämpfer, H.-J. Busse, and E. R. B. Moore. 1999. Reclassification of Pseudomonas echinoides Heumann 1962, 343^{AL}, in the genus Sphingomonas as Sphingomonas echinoides comb. nov. Int. J. Syst. Bacteriol. 49:1103–1109.

Denner, E. B. M., S. Paukner, P. Kämpfer, E. R. B. Moore, W.-R. Abraham, H.-J. Busse, G. Wanner, and W. Lubitz. 2001. Sphingomonas pituitosa sp. nov., an exopolysaccharide-producing bacterium that secretes an unusual type of sphingan. Int. J. Syst. Evol. Microbiol. 51:827–841.

de Otero, J., J. Masip, S. Elia, A. Betbese, J. Paez, and L. Ferrer. 1998. Bacteremia caused by Sphingomonas (Pseudomonas) paucimobilis [in Spanish]. Enferm. Infecc. Microbiol. Clin. 16:388–389.

Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X. W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski. 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid 13:149–153.

Dutta, T. K., S. A. Selifonov, and I. C. Gunsalus. 1998. Oxidation of methyl-substituted naphthalenes: Pathways in versatile Sphingomonas paucimobilis strain. Appl. Environ. Microbiol. 64:1884–1889.

Edgehill, R. U., and R. K. Finn. 1982. Isolation, characterization and growth kinetics of bacteria metabolizing pentachlorophenol. Eur. J. Appl. Microbiol. Biotechnol. 16:179–184.

Eguchi, M., T. Nishikawa, K. MacDonald, R. Cavicchioli, J. C. Gottschal, and S. Kjelleberg. 1996. Responses to stress and nutrient availability by the marine ultramicrobacterium Sphingomonas sp. strain RB2256. Appl. Environ. Microbiol. 62:1287–1294.

Eguchi, M., M. Ostrowski, F. Fegatella, J. Bowman, D. Nichols, T. Nishino, and R. Cavicchioli. 2001. Sphingomonas alaskensis strain AFO1, an abundant oligotrophic ultramicrobacterium from the North Pacific. Appl. Environ. Microbiol. 67:4945–4954.

Faden, H., M. Britt, and B. Epstein. 1981. Sinus contamination with Pseudomonas paucimobilis: A pseudoepidemic due to contaminated irrigation fluid. Infect. Control 2:233–235.

Fegatella, F., J. Lim, S. Kjelleberg, and R. Cavicchioli. 1998. Implications of rRNA operon copy number and ribosome content in the marine oligotrophic ultramicrobacterium Sphingomonas sp. strain RB2256. Appl. Environ. Microbiol. 64:4433–4438.

Fegatella, F., and R. Cavicchioli. 2000. Physiological responses to starvation in the marine oligotrophic ultramicrobacterium Sphingomonas sp. strain RB2256. Appl. Environ. Microbiol. 66:2037–2044.

Feng, X., L.-T. Ou, and A. Ogram. 1997. Plasmid-mediated mineralization of carbofuran by Sphingomonas sp. strain CF06. Appl. Environ. Microbiol. 63:1332–1337.

Ferat, J. L., M. Le Gouar, and F. Michel. 1994. Multiple group II self-splicing introns in mobile DNA from Escherichia coli. C. R. Acad. Sci. 317:141–148.

Fialho, A. M., L. O. Martins, M.-L. Donval, J. H. Leitão, M. J. Ridout, A. J. Jay, V. J. Morris, and I. Sá-Correia. 1999. Structures and properties of gellan polymers produced by Sphingomonas paucimobilis ATCC 31461 from lactase compared with those produced from glucose and from cheese whey. Appl. Environ. Microbiol. 65:2485–2491.

Fortnagel, P., H. Harms, R.-M. Wittich, S. Krohn, H. Meyer, V. Sinnwell, H. Wilkes, and W. Francke. 1990. Metabolism of dibenzofuran by Pseudomonas sp. strain HH69

and the mixed culture HH27. Appl. Environ. Microbiol. 56:1148–1156.

Fredrickson, J. K., D. L. Balkwill, G. R. Drake, M. F. Romine, D. B. Ringelberg, and D. C. White. 1995. Aromatic-degrading Sphingomonas isolates from the deep subsurface. Appl. Environ. Microbiol. 61:1917–1922.

Fredrickson, J. K., D. L. Balkwill, M. F. Romine, and T. Shi. 1999. Ecology, physiology, and phylogeny of deep subsurface Sphingomonas sp. J. Ind. Microbiol. Biotechnol. 23:273–283.

Fujii, K., N. Urano, H. Ushio, M. Satomi, and S. Kimura.. 2001. Sphingomonas cloacae sp. nov., a nonylphenol-degrading bacterium isolated from wastewater of a sewage-treatment plant in Tokyo. Int. J. Syst. Evol. Microbiol. 51:603–610.

Fukuda, K., S. Nagata, and H. Taniguchi. 2002. Isolation and characterization of dibenzofuran-degrading bacteria. FEMS Microbiol. Lett. 208:179–185.

Furukawa, K., and A. M. Chakrabarty. 1982. Involvement of plasmids in total degradation of chlorinated biphenyls. Appl. Environ. Microbiol. 44:619–626.

Furukawa, K., J. R. Simon, and A. M. Chakrabarty. 1983. Common induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in Pseudomonas paucimobilis. J. Bacteriol. 154:1356–1362.

Gibson, D. T., R. L. Roberts, M. C. Wells, and V. M. Kobal. 1973. Oxidation of biphenyl by a Beijerinckia species. Biochem. Biophys. Res. Comm. 50:211–219.

Gibson, D. T. 1999. Beijerinckia sp. strain B1: A strain by any other name. J. Ind. Microbiol. Biotechnol. 23:284–293.

Gilardi, G. L. 1984. Antimicrobial susceptibility of glucose-nonfermenting Gram-negative bacilli (NFGNB). Clin. Microbiol. Newslett. 6:149–152.

Gilewicz, M., Ni'matuzahroh, T. Nadalig, H. Budzinski, P. Doumenq, V. Michotey, and J. C. Bertrand. 1997. Isolation and characterization of a marine bacterium capable of utilizing 2-methylphenanthrene. Appl. Microbiol. Biotechnol. 48:528–533.

Gorricho, J., L. Torres, J. Navascues, M. C. Villuendas, and M. L. Marco. 1998. Bacteremia by Sphingomonas paucimobilis [in Spanish]. Enferm. Infecc. Microbiol. Clin. 16:98–99.

Halden, R. U., B. G. Halden, and D. F. Dwyer. 1999. Removal of dibenzofuran, dibenzo-p-dioxin, and 2-chlorodibenzo-p-dioxin from soils inoculated with Sphingomonas sp. strain RW1. Appl. Environ. Microbiol. 65:2246–2249.

Hashimoto, W., T. Inose, H. Nakajima, N. Sato, S. Kimura, and K. Murata. 1996. Purification and characterization of microbial gellan lyase. Appl. Environ. Microbiol. 62:1475–1477.

Hashimoto, W., and K. Murata. 1998. Alpha-L-rhamnosidase of Sphingomonas sp. R1 producing an unusual exopolysaccharide of sphingan. Biosci. Biotechnol. Biochem. 62:1068–1074.

Hashimoto, W., K. Momma, Y. Mishima, B. Mikami, and K. Murata. 2001. Super-channel in bacteria: function and structure of a macromolecular import system mediated by a pit-dependent ABC transporter. Biosci. Biotechnol. Biochem. 65:1949–1956.

Hernáez, M. J., W. Reineke, and E. Santero. 1999. Genetic analysis of biodegradation of tetralin by a Sphingomonas strain. Appl. Environ. Microbiol. 65:1806–1810.

Heumann, W. 1962. Die Methodik der Kreuzung sternbildender Bakterien. Biol. Zentbl. 81:341–354.

Hill, S., and J. R. Postgate. 1969. Failure of putative nitrogen-fixing bacteria to fix nitrogen. J. Gen. Microbiol. 58:277–285.

Hiraishi, A., H. Kuraishi, and K. Kawahara. 2000. Emendation of the description of Blastomonas natatoria (Sly 1985) Sly and Cahill 1997 as an aerobic photosynthetic bacterium and reclassification of Erythromonas ursincola Yurkov et al. 1997 as Blastomonas ursincola comb. nov. Int. J. Syst. Evol. Microbiol. 50:1113–1118.

Holmes, B., R. J. Owen, A. Evans, H. Malnick, and W. R. Willcox. 1977. Pseudomonas paucimobilis, a new species isolated from human clinical specimens, the hospital environment, and other sources. Int. J. Syst. Bacteriol. 27:133–146.

Holmes, B., and P. Roberts. 1981. The classification, identification, and nomenclature of agrobacteria. Incorporating revised descriptions for each of Agrobacterium tumefaciens (Smith & Townsend) Conn 1942, Agrobacterium rhizogenes (Riker et al.) Conn 1942, and Agrobacterium rubi (Hildebrand) Starr & Weiss 1943. J. Appl. Bacteriol. 50:443–467.

Horvath, M., G. Ditzelmüller, M. Loidl, and F. Streichsbier. 1990. Isolation and characterization of a 2-(2,4-dichlorophenoxy)propionic acid-degrading soil bacterium. Appl. Microbiol. Biotechnol. 33:213–216.

Hsueh, P.-R., L.-J. Teng, P.-C. Yang, Y.-C. Chen, H.-J. Pan, S.-W. Ho, and K.-T. Luh. 1998. Nosocomial infections caused by Sphingomonas paucimobilis: clinical features and microbiological characteristics. Clin. Infect. Dis. 26:676–681.

Hynková, K., Y. Nagata, M. Takagi, and J. Damborsky. 1999. Identification of the catalytic triad in the haloalkane dehalogenase from Sphingomonas paucimobilis UT26. FEBS Lett. 446:177–181.

Imai, R., Y. Nagata, K. Senoo, H. Wada, M. Fukuda, M. Takagi, and K. Yano. 1989. Dehydrochlorination of γ-hexachlorocyclohexane (γ-BHC) by γ-BHC-assimilating Pseudomonas paucimobilis. Agric. Biol. Chem. 53:2015–2017.

Janikowski, T. B., D. Velicogna, M. Punt, and A. J. Daugulis. 2002. Use of a two-phase partitioning bioreactor for degrading polycyclic aromatic hydrocarbons by a Sphingomonas sp. Appl. Microbiol. Biotechnol. 59:368–376.

Jiang, S. C., C. A. Kellogg, and J. H. Paul. 1998. Characterization of marine temperate phage-host systems isolated from Mamala Bay, Oahu, Hawaii. Appl. Environ. Microbiol. 64:535–542.

Johnsen, A. R., M. Hausner, A. Schnell, and S. Wuertz. 2000. Evaluation of fluorescently labeled lectins for noninvasive localization of extracellular polymeric substances in Sphingomonas biofilms. Appl. Environ. Microbiol. 66:3487–3491.

Ka, J. O., W. E. Holben, and J. M. Tiedje. 1994. Analysis of competition in soil among 2,4-dichlorophenoxyacetic acid-degrading bacteria. Appl. Environ. Microbiol. 60:1121–1128.

Kämpfer, P., E. B. M. Denner, S. Meyer, E. R. B. Moore, and H.-J. Busse. 1997. Classification of "Pseudomonas azotocolligans" Anderson 1955, 132, in the genus Sphingomonas as Sphingomonas trueperi sp. nov. Int. J. Syst. Bacteriol. 47:577–583.

Kaneko, A., H. Miyadai, H. Danbara, and K. Kawahara. 2000. Construction of mutants of Sphingomonas pauci-

mobilis defective in terminal mannose in the glycosphingolipid. Biosci. Biotechnol. Biochem. 64:1298–1301.

Karberg, M., H. Guo, J. Zhong, R. Coon, J. Perutka, and A. M. Lambowitz. 2001. Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. Nature Biotechnol. 19:1162–1167.

Kasai, Y., H. Kishira, K. Syutsubo, and S. Harayama. 2001. Molecular detection of marine bacterial populations on beaches contaminated by the Nakhodka tanker off-spill accident. Environ. Microbiol. 3:246–255.

Katayama, Y., S. Nishikawa, A. Murayama, M. Yamasaki, N. Morohoshi, and T. Haraguchi. 1988. The metabolism of biphenyl structure in lignin of the soil bacterium (Pseudomonas paucimobilis SYK-6). FEBS Lett. 233:129–133.

Kawahara, K., U. Seydel, M. Matsuura, H. Danbara, E. T. Reitschel, and U. Zähringer. 1991. Chemical structure of glycosphingolipids isolated from Sphingomonas paucimobilis. FEBS Lett. 292:107–110.

Kawahara, K., I. Mizuta, W. Katabami, M. Koizumi, and S. Wakayama. 1994. Isolation of Sphingomonas strains from ears of rice and other plants of family Gramineae. Biosci. Biotech. Biochem. 58:600–601.

Kawahara, K., H. Kuraishi, and U. Zähringer. 1999. Chemical structure and function of glycosphingolipids of Sphingomonas spp and their distribution among members of the α-4 subclass of Proteobacteria. J. Ind. Microbiol. Biotechnol. 23:408–413.

Kawahara, K., B. Lindner, Y. Isshiki, K. Jakob, Y. A. Knirel, and U. Zähringer. 2001. Structural analysis of a new glycosphingolipid from the lipopolysaccharide-lacking bacterium Sphingomonas adhaesiva. Carbohydr. Res. 333:87–93.

Kawai, F., T. Kimura, Y. Tani, and H. Yamada. 1984. Involvement of a polyethylene glycol (PEG)-oxidizing enzyme in the bacterial metabolism of PEG. Agric. Biol. Chem. 48:1349–1351.

Kawai, F. 1999. Sphingomonads involved in the biodegradation of xenobiotic polymers. J. Ind. Microbiol. Biotechnol. 23:400–407.

Kawasaki, S., R. Moriguchi, K. Sekiya, T. Nakai, E. Ono, K. Kume, and K. Kawahara. 1994. The cell envelope structure of the lipopolysaccharide-lacking Gram-negative bacterium Sphingomonas paucimobilis. J. Bacteriol. 176:284–290.

Kilpi, S., V. Backström, and M. Korhola. 1980. Degradation of 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), benzoic acid and salicylic acid by Pseudomonas sp. HV3. FEMS Microbiol. Lett. 8:177–182.

Kim, C. K., J. W. Kim, Y. C. Kim and T. I. Mheen. 1986. Isolation of aromatic hydrocarbon-degrading bacteria and genetic characterization of their plasmid genes. Korean J. Microbiol. 24:67–72.

Kim, E., and G. J. Zylstra. 1995. Molecular and biochemical characterization of two meta-cleavage dioxygenases involved in biphenyl and m-xylene degradation by Beijerinckia sp. strain B1. J. Bacteriol. 177:3095–3103.

Kim, E. 1996a. Molecular analysis of aromatic hydrocarbon degradation by Sphingomonas yanoikuyae B1 [PhD thesis]. Rutgers State University of New Jersey. New Brunswick, NJ.

Kim, E., P. J. Aversano, M. F. Romine, R. P. Schneider, and G. J. Zylstra. 1996b. Homology between genes for aromatic hydrocarbon degradation in surface and deep-subsurface Sphingomonas strains. Appl. Environ. Microbiol. 62:1467–1470.

Kim, S.-J., J. Chun, K. S. Bae, and Y.-C. Kim. 2000. Polyphasic assignment of an aromatic-degrading Pseudomonas sp., strain DJ77, in the genus Sphingomonas, as Sphingomonas chungbukensis sp. nov. Int. J. Syst. Evol. Microbiol. 50:1641–1647.

Knoop, V., and A. Brennicke. 1994. Evidence for a group II intron in Escherichia coli inserted into a highly conserved reading frame associated with mobile DNA sequences. Nucleic Acids Res. 22:1167–1171.

Kohler, H. P. E. 1999. Sphingomonas herbicidovorans MH: A versatile phenoxyalkonic acid herbicide degrader. J. Ind. Microbiol. Biotechnol. 23:336–340.

Kuehn, M., M. Mehl, M. Hausner, H. J. Bungartz, and S. Wuertz. 2001. Time-resolved study of biofilm architecture and transport processes using experimental and simulation techniques: the role of EPS. Water Sci. Technol. 43:143–150.

Kulaeva, O. I, E. V. Koonin, J. C. Wootton, A. S. Levine, and R. Woodgate. 1998. Unusual insertion element polymorphisms in the promoter and terminator regions of the mucAB-like genes of R471a and R446b. Mutation Res. 397:247–262.

Lee, J.-S., Y. K. Shin, J.-H. Yoon, M. Takeuchi, Y.-R. Pyun, and Y.-H. Park. 2001. Sphingomonas aquatilis sp. nov., Sphingomonas koreensis sp. nov. and Sphingomonas taejonensis sp. nov., yellow-pigmented bacteria isolated from natural mineral water. Int. J. Syst. Evol. Microbiol. 51:1491–1498.

Leifson, E. 1962. The bacterial flora of distilled and stored water. III: New species of the genera Corynebacterium, Flavobacterium, Spirillum, and Pseudomonas. Int. Bull. Bacteriol. Nom. Tax. 12:161–170.

Lemaitre, D., A. Elaichouni, M. Hundhausen, G. Claeys, P. Vanhaesebrouck, M. Vaneechoutte, and G. Verschraegen. 1996. Tracheal colonization with Sphingomonas paucimobilis in mechanically ventilated neonates due to contaminated ventilator temperature probes. J. Hosp. Infect. 32:199–206.

Leung, K. T., O. Tresse, D. Errampalli, H. Lee, and J. T. Trevors. 1997. Mineralization of p-nitrophenol by pentachlorophenol-degrading Sphingomonas spp. FEMS Microbiol. Lett. 155:107–114.

Li, Z., H.-J. Feiten, J. B. van Beilen, W. Duetz, and B. Witholt. 1999. Preparation of optically active N-benzyl-3-hydroxypyrrolidine by enzymatic hydroxylation. Tetrahedron: Asymmetry 10:1323–1333.

Li, Z., H.-J. Feiten, D. Chang, W. A. Duetz, J. B. van Beilen, and B. Witholt. 2001. Preparation of (R)- and (S)-N-protected 3-hydroxypyrrolidines by hydroxylation with Sphingomonas sp. HXN-200, a highly active, regio- and stereoselective, and easy to handle biocatalyst. J. Org. Chem. 66:8424–8430.

Lloyd-Jones, G. and P. C. K. Lau. 1997. Glutathione s-transferase-encoding gene as a potential probe for environmental bacterial isolates capable of degrading polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 63:3286–3290.

Lobas, D., S. Schumpe, and W. D. Deckwer. 1992. The production of gellan exopolysaccharide with Sphingomonas paucimobilis E2 (DSM 6314). Appl. Microbiol. Biotechnol. 37:411–415.

Lobas, D., M. Nimtz, V. Wray, A. Schumpe, C. Proppe, and W.-D. Deckwer. 1994. Structure and physical properties of the extracellular polysaccharide PS-P4 produced by

Sphingomonas paucimobilis P4 (DSM 6418). Carbohydr. Res. 251:303–313.

Lu, J., T. Nakajima-Kambe, T. Shigeno, A. Ohbo, N. Nomura, and T. Nakahara. 1999. Biodegradation of dibenzothiophene and 4,6-dimethyldibenzothiophene by Sphingomonas paucimobilis strain TZS-7. Biosci. Bioengin. 88:293–299.

Macur, R. E., J. T. Wheeler, T. R. McDermott, and W. P. Inskeep. 2001. Microbial populations associated with the reduction and enhanced mobilization of arsenic in mine tailings. Environ. Sci. Technol. 35:3676–3682.

Männistö, M. K., M. A. Tirola, M. S. Salkinoja-Salonen, M. S. Kulomaa, and J. A. Puhakka. 1999. Diversity of chlorophenol-degrading bacteria isolated from contaminated boreal groundwater. Arch. Microbiol. 171:189–197.

Martínez-Abarca, F., S. Zekri, and N. Toro. 1998. Characterization and splicing in vivo of a Sinorhizobium meliloti group II intron associated with particular insertion sequences of the IS630-Tcl/IS3 transposon superfamily. Molec. Microbiol. 28:1295–1306.

Martino, R., C. Martinez, R. Pericas, R. Salazar, C. Sola, S. Brunet, A. Sureda, and A. Domingo-Albos. 1996. Bacteremia due to glucose non-fermenting Gram-negative bacilli in patients with hematological neoplasias and solid tumors. Eur. J. Clin. Microbiol. Infect. Dis. 15:610–615.

Martins, L. O., A. M. Fialho, P. L. Rodrigues, and I. Sá-Correia. 1996. Gellan gum production and activity of biosynthetic enzymes in Sphingomonas paucimobilis mucoid and non-mucoid variants. Biotechnol. Appl. Biochem. 24:47–54.

Masai, E., Y. Katayama, S. Nishikawa, and K. Fukuda. 1999. Characterization of Sphingomonas paucimobilis SYK-6 genes involved in degradation of lignin-related compounds. J. Ind. Microbiol. Biotechnol. 23:364–373.

Mills, D. A., L. L. McKay, and G. M. Dunny. 1996. Splicing of a group II intron involved in the conjugative transfer of pRS01 in lactococci. J. Bacteriol. 178:3531–3538.

Mohn, W. W. 1995. Bacteria obtained from a sequencing batch reactor that are capable of growth on dehydroabietic acid. Appl. Environ. Microbiol. 61:2145–2150.

Mohn, W. R., and G. R. Stewart. 1997. Bacterial metabolism of chlorinated dehydroabietic acids occurring in pulp and paper mill effluents. Appl. Environ. Microbiol. 63:3014–3020.

Mohn, W. W., Z. Yu, E. R. B. Moore, and A. F. Muttray. 1999. Lessons learned from Sphingomonas species that degrade abietane triterpenoids. J. Ind. Microbiol. Biotechnol. 23:374–379.

Momma, K., W. Hashimoto, O. Miyake, H.-J. Yoon, S. Kawai, Y. Mishima, B. Mikami, and K. Murata. 1999. Special cell surface structure, and novel macromolecule transport/depolymerization system of Sphingomonas sp. A1. J. Ind. Microbiol. Biotechnol. 23:425–435.

Morrison Jr., A. J., and J. A. Shulman. 1986. Community-acquired bloodstream infection caused by Pseudomonas paucimobilis: case report and review of the literature. J. Clin. Microbiol. 24:853–855.

Mueller, J. G., P. J. Chapman, B. O. Blattmann, and P. H. Pritchard. 1990. Isolation and characterization of a fluoranthene-utilizing strain of Pseudomonas paucimobilis. Appl. Environ. Microbiol. 56:1079–1086.

Mullany, P., M. Pallen, M. Wilks, J. R. Stephen, and S. Tabaqchali. 1996. A group II intron in a conjugative transposon from the Gram-positive bacterium, Clostridium difficile. Gene 174:145–150.

Murphy, E. M., J. A. Schramke, J. K. Fredrickson, H. W. Bledsoe, A. J. Francis, D. S. Sklarew, and J. C. Linehan. 1992. The influence of microbial activity and sedimentary organic carbon on the isotope geochemistry of the Middendorf Aquifer. Water Resource Res. 28:723–740.

Nagata, Y., K. Miyauchi, and M. Takagi. 1999. Complete analysis of genes and enzymes for γ-hexachlorocyclohexane degradation in Sphingomonas paucimobilis UT26. J. Ind. Microbiol. Biotechnol. 23:380–390.

Nishiyama, M., K. Senoo, H. Wada, and S. Matsumoto. 1992. Identification of soil micro-habitats for growth, death and survival of a bacterium, g-1,2,3,4,5,6-hexachlorocyclohexane-assimilating Sphingomonas paucimobilis, by fractionation of soil. FEMS Microbiol. Ecol. 101:145–150.

Nohynek, L. J., E. L. Suhonen, E.-L. Nurmiaho-Lassila, J. Hantula, and M. Salkinoja-Salonen. 1995. Description of four pentachlorophenol-degrading bacterial strains as Sphingomonas chlorophenolica sp. nov. Syst. Appl. Microbiol. 18:527–538.

Nohynek, L. J., E.-L. Nurmiaho-Lassila, E. L. Suhonen, H.-J. Busse, M. Mohammadi, J. Hantula, F. Rainey, and M. S. Salkinoja-Salonen. 1996. Description of chlorophenol-degrading Pseudomonas sp. strains KF1^T, KF3, and NKF1 as a new species of the genus Sphingomonas, Sphingomonas subarctica sp. nov. Int. J. Syst. Bacteriol. 46:1042–1055.

Nörtemann, B., J. Baumgarten, H. G. Rast, and H.-J. Knackmuss. 1986. Bacterial communities degrading amino- and hydroxynaphthalene-2-sulfonates. Appl. Environ. Microbiol. 52:1195–1202.

Oakley, A. J., Z. Prokop, M. Bohác, J. Kmunícek, T. Jedlicka, M. Monincová, I. Kutá-Smatanová, Y. Nagata, J. Damborsky, and M. C. J. Wilce. 2002. Exploring the structure and activity of haloalkane dehalogenase from Sphingomonas paucimobilis UT26: evidence for product- and water-mediated inhibition. Biochemistry 41:4847–4855.

Ohe, T., T. Ohmoto, Y. Kobayashi, A. Sato, and Y. Watanabe. 1990. Metabolism of naphthalenesulfonic acids by Pseudomonas sp. TA-2. Agric. Biol. Chem. 54:669–675.

Parsons, J. R., J. A. de Bruijne, and A. R. Weiland. 1998. Biodegradation pathway of 2-chlorodibenzo-p-dioxin and 2-chlorodibenzofuran in the biphenyl-utilising strain JB1. Chemosphere 37:1915–1922.

Paster, B. J., W. A. Falkler Jr., C. O. Enwonwu, E. O. Idigbe, K. O. Savage, V. A. Levanos, M. A. Tamer, R. L. Ericson, C. N. Lau, and F. E. Dewhirst. 2002. Prevalent bacterial species and novel phenotypes in advanced noma lesions. J. Clin. Microbiol. 40:2187–2191.

Perola, O., T. Nousiainen, S. Suomalainen, S. Aukee, U. M. Kärkkäinen, J. Kauppinen, T. Ojanen, and M.-L. Katila. 2002. Recurrent Sphingomonas paucimobilis-bacteraemia associated with a multi-bacterial waterborne epidemic among neutropenic patients. J. Hosp. Infect. 50:196–201.

Pollock, T. J. 1993. Gellan-related polysaccharides and the genus Sphingomonas. J. Gen. Microbiol. 139:1939–1945.

Pollock, T. J., W. A. T. van Workam, L. Thorne, M. J. Mikolajczak, M. Yamazaki, J. W. Kijne, and R. W. Armentrout. 1998. Assignment of biochemical functions to glycosyl transferase genes which are essential for biosynthesis of exopolysaccharides in Sphingomonas strain S88 and Rhizobium leguminosarum. J. Bacteriol. 180:586–593.

Pollock, T. J., and R. W. Armentrout. 1999. Planktonic/sessile dimorphism of polysaccharide-encapsulated sphingomonads. J. Ind. Microbiol. Biotechnol. 23:436–441.

Puhakka, J. A., R. P. Herwig, P. M. Koro, G. V. Wolfe, and J. F. Ferguson. 1995. Biodegradation of chlorophenols by mixed and pure cultures from a fluidized-bed reactor. Appl. Microbiol. Biotechnol. 42:951–957.

Radehaus, P. M., and S. K. Schmidt. 1992. Characterization of a novel Pseudomonas sp. that mineralizes high concentrations of pentachlorophenol. Appl. Environ. Microbiol. 58:2879–2885.

Reasoner, D. J., and E. E. Geldreich. 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 46:1–7.

Reina, J., A. Bassa, I. Llompart, D. Portela, and N. Borell. 1991. Infections with Pseudomonas paucimobilis: report of four cases and review. Rev. Infect. Dis. 13:1072–1076.

Resnick, S. M., and P. J. Chapman. 1994. Physiological properties and substrate specificity of a pentachlorophenol-degrading Pseudomonas species. Biodegradation 5:47–54.

Richardson, L. L. 1997. Occurrence of the black band disease cyanobacterium on healthy corals of the Florida Keys. Bull. Mar. Sci. 61:485–490.

Richardson, L. L., W. M. Goldberg, K. G. Kuta, R. B. Aronson, G. W. Smith, K. B. Ritchie, J. C. Halas, J. S. Feingold, and S. L. Miller. 1998. Florida's mystery coral-killer identified. Nature 392:557–558.

Rickard, A. H., S. A. Leach, L. S. Hall, C. M. Buswell, N. J. High, and P. S. Handley. 2002. Phylogenetic relationships and coaggregation ability of freshwater biofilm bacteria. Appl. Environ. Microbiol. 68:3644–3650.

Riegert, U., G. Heiss, A. E. Kuhm, C. Müller, M. Contzen, H.-J. Knackmuss, and A. Stolz. 1999. Catalytic properties of the 3-chlorocatechol-oxidizing 2,3-dihydroxybiphenyl 1,2-dioxygenase from Sphingomonas sp. strain BN6. J. Bacteriol. 181:4812–4817.

Romine, M. F., L. C. Stillwell, K.-K. Wong, S. J. Thurston, E. C. Sisk, C. Sensen, T. Gaasterland, J. K. Fredrickson, and J. D. Saffer. 1999. Complete sequence of a 184-kilobase catabolic plasmid from Sphingomonas aromaticivorans F199. J. Bacteriol. 181:1585–1602.

Ryeom, T. K., I. G. Lee, S. Y. Son, and T. Y. Ahn. 2000. Degradation of phenanthrene by Sphingomonas sp. 1-21 isolated from oil-contaminated soil. J. Microbiol. Biotechnol. 10:724–727.

Sabaté, J., M. Grifoll, M. Viñas, and A. M. Solanas. 1999. Isolation and characterization of a 2-methylphenanthrene utilizing bacterium: Identification of ring cleavage metabolites. Appl. Microbiol. Biotechnol. 52:704–712.

Saber, D. L., and R. L. Crawford. 1985. Isolation and characterization of Flavobacterium strains that degrade pentachlorophenol. Appl. Environ. Microbiol. 50:1512–1518.

Schmidt, S., R.-M. Wittich, D. Erdmann, H. Wilkes, W. Francke, and P. Fortnagel. 1992. Biodegradation and diphenyl ether and its monohalogenated derivatives by Sphingomonas sp. strain SS3. Appl. Environ. Microbiol. 58:2744–2750.

Schmidt, C. 1994. Isolation and growth physiology of N,N-dimethylaniline and 2,4-dimethylaniline degrading Sphingomonas sp. [PhD thesis No. 10710]. ETH Zürich. Zurich, Switzerland.

Schut, F., J. C. Gottschal, and R. A. Prins. 1997. Isolation and characterisation of the marine ultramicrobacterium Sphingomonas sp. strain RB2256. FEMS Microbiol. Rev. 20:363–369.

Segers, P., M. Vancanneyt, B. Pot, U. Torck, B. Hoste, D. Dewettinck, E. Falsen, K. Kersters, and P. De Vos. 1994. Classification of Pseudomonas diminuta Leifson and Hugh 1954 and Pseudomonas vesicularis Büsing, Döll, and Freytag 1953 in Brevundimonas diminuta comb. nov. and Brevundimonas vesicularis comb. nov., respectively. Int. J. Syst. Bacteriol. 44:499–510.

Senoo, K., and H. Wada. 1989. Isolation and identification of an aerobic γ-HCH-decomposing bacterium from soil. Soil. Sci. Plant Nutr. 35:79–87.

Senoo, K., M. Nishiyama, and S. Matsumoto. 1996. Bioremediation of gamma-HCH-polluted field soil by inoculation with an aerobic gamma-HCH-decomposing bacterium (Sphingomonas paucimobilis SS86). Soil Sci. Plant Nutr. 42:11–19.

Shah, A. K., and A. A. Ashtaputre. 1999. Evaluation of rheological properties of the exopolysaccharide of Sphingomonas paucimobilis GS-1 for application in oil exploration. J. Ind. Microbiol. Biotechnol. 23:442–445.

Shearman, C., J. J. Godon, and M. Gasson. 1996. Splicing of a group II intron in a functional transfer gene of Lactococcus lactis. Molec. Microbiol. 21:45–53.

Sly, L. I. 1985. Emendation of the genus Blastobacter Zavarzin 1961 and description of Blastobacter natatorius sp. nov. Int. J. Syst. Bacteriol. 35:40–45.

Sly, L. I., and M. M. Cahill. 1997. Transfer of Blastobacter natatorius (Sly 1985) to the genus Blastomonas gen. nov. as Blastomonas natatoria comb. nov. Int. J. Syst. Bacteriol. 47:566–568.

Smalley, D. L., V. R. Hansen, and V. S. Baselski. 1983. Susceptibility of Pseudomonas paucimobilis to 24 antimicrobial agents. Antimicrob. Agents Chemother. 23:161–162.

Sonoki, T., T. Obi, S. Kubota, M. Higashi, E. Masai, and Y. Katayama. 2000. Coexistence of two different O demethylation systems in lignin metabolism by Sphingomonas paucimobilis SKY-6: Cloning and sequencing of the lignin biphenyl-specific O-demethylase (LigX) gene. Appl. Environ. Microbiol. 66:2125–2132.

Sørensen, S. R., Z. Ronen, and J. Aamand. 2001. Isolation from agricultural soil and characterization of a Sphingomonas sp. able to mineralize the phenylurea herbicide Isoproturon. Appl. Environ. Microbiol. 67:5403–5409.

Stackebrandt, E., R. G. E. Murray, and H. G. Trüper. 1988. Proteobacteria classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives." Int. J. Syst. Bacteriol. 38:321–325.

Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44:846–849.

Staley, J. T. 1968. Prosthecomicrobium and Ancalomicrobium: new prosthecate freshwater bacteria. J. Bacteriol. 95:1921–1942.

Stillwell, L. C., S. J. Thurston, R. P. Schneider, M. F. Romine, J. K. Fredrickson, and J. D. Saffer. 1995. Physical mapping and characterization of a catabolic plasmid from the deep-subsurface bacterium Sphingomonas sp. strain F199. J. Bacteriol. 177:4537–4539.

Stolz, A., C. Schmidt-Maag, E. B. M. Denner, H.-J. Busse, T. Egli, and P. Kämpfer. 2000. Description of Sphingomonas xenophaga sp. nov. for strains BN6^T and N,N which degrade xenobiotic aromatic compounds. Int. J. Syst. Evol. Microbiol. 50:35–41.

Story, S. P., S. H. Parker, S. S. Hayasaka, M. B. Riley, and E. L. Kline. 2001. Convergent and divergent points in catabolic pathways involved in utilization of fluoranthene, naphthalene, anthracene, and phenanthrene by Sphingomonas paucimobilis var. EPA505. J. Ind. Microbiol. Biotechnol. 26:369–382.

Sutherland, I. W., and L. Kennedy. 1996. Polysaccharide lyases from gellan-producing Sphingomonas spp. Microbiology 142:867–872.

Sutherland, I. W. 1999. Microbial polysaccharide products. Biotechnol. Genet. Engin. Rev. 16:217–229.

Suzuki, M. T., M. S, Rappe, Z. W., Haimberger, H. Winfield, N. Adair, J. Ströbel, and S. Giovannoni. 1997. Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl. Environ. Microbiol. 63:983–989.

Tabata, K., K.-I. Kasuya, H. Abe, K. Masuda, and Y. Doi. 1999. Poly(aspartic acid) degradation by a Sphingomonas sp. isolated from freshwater. Appl. Environ. Microbiol. 65:4268–4270.

Takeuchi, M., F. Kawai, Y. Shimada, and A. Yokota. 1993. Taxonomic study of polyethylene glycol-utilizing bacteria: emended description of the genus Sphingomonas and new descriptions of Sphingomonas macrogoltabidus sp. nov., Sphingomonas sanguis sp. nov. and Sphingomonas terrae sp. nov. Syst. Appl. Microbiol. 16:227–238.

Takeuchi, M., H. Sawada, H. Oyaizu, and A. Yakota. 1994. Phylogenetic evidence for Sphingomonas and Rhizomonas as nonphotosynthetic members of the alpha-4 subclass of the Proteobacteria. Int. J. Syst. Bacteriol. 44:308–314.

Takeuchi, M., T. Sakane, M. Yanagi, K. Yamasato, K. Hamana, and A. Yokota. 1995. Taxonomic study of bacteria isolated from plants: proposal of Sphingomonas rosa sp. nov., Sphingomonas pruni sp. nov., Sphingomonas asaccharolytica sp. nov., and Sphingomonas mali sp. nov. Int. J. Syst. Bacteriol. 45:334–341.

Takeuchi, M., K. Hamana, and A. Hiraishi. 2001. Proposal of the genus Sphingomonas sensu stricto and three new genera, Sphingobium, Novosphingobium and Sphingopyxis on the basis of phylogenetic and chemotaxonomic analyses. Int. J. Syst. Evol. Microbiol. 51:1405–1417.

Tirola, M. A., M. K. Männistö, J. A. Puhakka, and M. S. Kulomaa. 2002. Isolation and characterization of Novosphingobium sp. strain MT1, a dominant polychlorophenol-degrading strain in a groundwater bioremediation system. Appl. Environ. Microbiol. 68:173–180.

van Bruggen, A. H. C., K. N. Jochimsen, and P. R. Brown. 1990. Rhizomonas suberifaciens gen. nov., sp. nov., the causal agent of corky root of lettuce. Int. J. Syst. Bacteriol. 40:175–188.

van Bruggen, A. H. C., K. N. Jochimsen, E. M. Steinberger, P. Segers, and M. Gillis. 1993. Classification of Rhizomonas suberifaciens, an unnamed Rhizomonas species, and Sphingomonas spp. in rRNA superfamily IV. Int. J. Syst. Bacteriol. 43:1–7.

Vancanneyt, M., F. Schut, C. Snauwaert, J. Goris, J. Swings, and J. C. Gottschal. 2001. Sphingomonas alaskensis sp. nov., a dominant bacterium from a marine oligotrophic environment. Int. J. Syst. Evol. Microbiol. 51:73–79.

van Kranenburg, R., I. C. Boels, M. Kleerebezem, and W. M. de Vos. 1999. Genetics and engineering of microbial exopolysaccharides for food: approaches for the production of existing and novel polysaccharides. Curr. Opin. Biotechnol. 10:498–504.

Vartak, N. B., C. C. Lin, J. M. Cleary, M. J. Fagan, and M. H. Saier Jr. 1995. Glucose metabolism in "Sphingomonas elodea": Pathway engineering via construction of a glucose-6-phosphate dehydrogenase insertion mutant. Microbiology 141:2339–2350.

Vuilleumier, S., Z. Ucurum, S. Oelhafen, T. Leisinger, J. Armengaud, R.-M. Wittich, and K. N. Timmis. 2001. The glutathione S-transferase OrfE3 of the dioxin-degrading bacterium Sphingomonas sp. RW1 displays maleylpyruvate isomerase activity. Chem.-Biol. Interact. 133:265–267.

White, D. C., S. D. Sutton, and D. B. Ringelberg. 1996. The genus Sphingomonas: physiology and ecology. Curr. Opin. Biotechnol. 7:301–306.

Wittich, R.-M., H. Wilkes, V. Sinnwell, W. Francke, and P. Fortnagel. 1992. Metabolism of dibenzo-p-dioxin by Sphingomonas sp. strain RW1. Appl. Environ. Microbiol. 58:1005–1010.

Wittmann C., A.-P. Zeng, and W.-D. Deckwer. 1998. Physiological characterization and cultivation strategies of the pentachlorophenol-degrading bacteria Sphingomonas chlorophenolica RA2 and Mycobacterium chlorophenolicum PCP-1. J. Ind. Microbiol. Biotechnol. 21:315–321.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–272.

Xun, L., J. Bohuslavek, and M. Cai. 1999. Characterization of 2,6-dichloro-p-hydroquinone 1,2-dioxygenase (PcpA) of Sphingomonas chlorophenolica ATCC 39723. Biochem. Biophys. Res. Commun. 266:323–325.

Yabuuchi, E., I. Yano, H. Oyaizu, Y. Hashimoto, T. Ezaki, and H. Yamamoto. 1990. Proposals of Sphingomonas paucimobilis gen. nov. and comb. nov., Sphingomonas parapaucimobilis sp. nov., Sphingomonas yanoikuyae sp. nov., Sphingomonas adhaesiva sp. nov., Sphingomonas capsulata comb. nov., and two genospecies of the genus Sphingomonas. Microbiol. Immunol. 34:99–119.

Yabuuchi, E., Y. Kosako, T. Naka, S. Suzuki, and I. Yano. 1999. Proposal of Sphingomonas suberifaciens (van Bruggen, Jochimsen and Brown 1990) comb. nov., Sphingomonas natatoria (Sly 1985) comb. nov., Sphingomonas ursincola (Yurkov et al. 1997) comb. nov., and emendation of the genus Sphingomonas. Microbiol. Immunol. 43:339–349.

Yabuuchi, E., H. Yamamoto, S. Terakubo, N. Okamura, T. Naka, N. Fujiwara, K. Kobayashi, Y. Kosako, and A. Hiraishi. 2001. Proposal of Sphingomonas wittichii sp. nov. for strain RW1^T, known as a dibenzo-p-dioxin metabolizer. Int. J. Syst. Evol. Microbiol. 51:281–292.

Yamazaki, M., L. Thorne, M. Mikolajczak, R. W. Armentrout, and T. J. Pollock. 1996. Linkage of genes essential for synthesis of a polysaccharide capsule in Sphingomonas strain S88. J. Bacteriol. 178:2676–2687.

Ye, D., M. A. Siddiqi, A. E. Maccubbin, S. Kumar, and H. C. Sikka. 1996. Degradation of polynuclear aromatic hydrocarbons by Sphingomonas paucimobilis. Environ. Sci. Technol. 30:136–142.

Yeo, C. C., J. M Tham, M. W.-C. Yap, and C. L. Poh. 1997. Group II intron from Pseudomonas alcaligenes NCIB 9867 (P25X): entrapment in plsmid RP4 and sequence analysis. Microbiology 143:2833–2840.

Yrjälä, K., S. Suomalainen, E. L. Suhonen, S. Kilpi, L. Paulin, and M. Romantschuk. 1998. Characterization and reclassification of an aromatic- and chloroaromatic-degrading Pseudomonas sp., strain HV3, as Sphingomonas sp. HV3. Int. J. Syst. Bacteriol. 48:1057–1062.

Yun, N. R., Y. K. Shin, S. Y. Hwang, H. Kuraishi, J. Sugiyama, and K. Kawahara. 2000. Chemotaxonomic and phylogenetic analyses of Sphingomonas strains isolated from ears of plants in the family Gramineae and a proposal of Sphingomonas roseoflava sp. nov. J. Gen. Appl. Microbiol. 46:9–18.

Yurkov, V., E. Stackebrandt, O. Buss, A. Vermeglio, V. Gorlenko, and J. T. Beatty. 1997. Reorganization of the genus Erythromicrobium: description of "Erythromicrobium sibiricum" as Sandaracinobacter sibiricus gen. nov., sp. nov., and of "Erythromicrobium ursincola" as Erythromonas ursincola gen. nov., sp. nov. Int. J. Syst. Bacteriol. 47:1172–1178.

Zipper, C., K. Nickel, W. Angst, and H.-P. E. Kohler. 1996. Complete microbial degradation of both enantiomers of the chiral herbicide Mecoprop [(RS)-2-(4-chloro-2-methylphenoxy)propionic acid] in an enantioselective manner by Sphingomonas herbicidovorans sp. nov. Appl. Environ. Microbiol. 62:4318–4322.

Zylstra, G. J., and E. Kim. 1997. Aromatic hydrocarbon degradation by Sphingomonas yanoikuyae B1. J. Ind. Microbiol. Biotechnol. 19:408–414.

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CHAPTER 6.11

The Genera *Empedobacter* and *Myroides*

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Introduction

Formerly belonging to the genus *Flavobacterium*, the genera *Empedobacter* and *Myroides* have now been included in the family Flavobacteriaceae as set out in the introductory chapter on Flavobacteriaceae of this edition (see An Introduction to the Family Flavobacteriaceae in this Volume by Bernardet and Nakagawa). Both these genera are known to have a clinical origin and to be resistant to a wide range of antimicrobials. The aim of this chapter is to give the reader more detail on the taxonomy, phylogeny, habitat, isolation and preservation, identification, pathogenicity and applications of these genera.

Taxonomy and Phylogeny

Members of both genera, *Empedobacter* and *Myroides*, were formerly included in the genus *Flavobacterium*, family Bacteriaceae, tribe Chromobacterieae, and were known as *Flavobacterium breve* (Bergey et al., 1923) and *Flavobacterium odoratum* (Stutzer and Kwaschnina, 1929), respectively. These genera have now been included as two of the 25 genera of the family Flavobacteriaceae (Reichenbach, 1989). The phylogeny and taxonomy of this family are discussed in detail by Bernardet and Nakagawa in Introduction to Flavobacteriaceae in this Volume.

Empedobacter

This organism was isolated for the first time in 1888 by Mori from canal water and was simply known as "the short canal bacillus" (Mori, 1888). In 1890, Lustig named it "*Bacillus brevis*," the only validly published name of this organism until 1994. This name was also used by Frankland and Frankland (1894). After that, this organism was given a variety of names which, unfortunately, were not validly published, namely "*Bacillus canalicolis brevis*" (Cornil and Babes, 1890), "*Bacillus canalis parvus*" (Eisenberg, 1891), "*Bacterium canalis parvus*" (Chester, 1897), "*Bacterium canale*" (Mez, 1898), "*Pseudobacterium brevis*" (Krasil'nikov, 1949), and "*Empedobacter breve*" (Prévot, 1961).

In 1923, Bergey et al. transferred *Bacillus brevis* to the color genus *Flavobacterium* (which they created) as *F. brevis*; this name was used by Stutzer and Kwaschnina (1929) for their isolates. In the eighth edition of *Bergey's Manual*, Weeks (1974) corrected the epithet to *Flavobacterium breve*. In conjunction with an emended description of the genus, Holmes and Owen (1979a) made a request to reject *Flavobacterium aquatile* and to replace it with *F. breve* as the type species of the genus. The Judicial Commission of the International Committee on Systematic Bacteriology, however, denied this request, stating that there was no real potential for confusion if *F. aquatile* was retained, nor were there strong arguments found in the *International Code of Nomenclature of Bacteria* for rejecting *F. aquatile* as the type species of the *Flavobacterium* genus (Wayne, 1982).

Lustig (1890) did not designate the type strain of *B. brevis*, and no neotype strain appears to have been proposed for this species until Holmes et al. (1978) proposed NCTC 11099, isolated from bronchial secretions, as the neotype strain of *F. breve*. Despite being phenotypically homogeneous, strains of *F. breve* were found to be genomically heterogeneous (Owen and Holmes, 1980; Ursing and Bruun, 1991).

In 1994, Vandamme et al. proposed new genus names for some of the species in *Flavobacterium* after extensive genotypic, chemotaxonomic and phenotypic studies. Instead of proposing a new name for *F. breve*, they proposed reviving the name *Empedobacter* (Prévot, 1961) and renamed *F. breve* "*Empedobacter brevis*."

Myroides

Isolated for the first time in 1923, members of the genus *Myroides* have historically been classified as *Bacterium faecale aromaticum* (Stutzer, 1923) and later as *Flavobacterium odoratum* (Stutzer and Kwaschnina, 1929). No further mention of this organism was made in the literature until

1977, when Holmes et al. examined nine clinical isolates and identified them as *F. odoratum*. They gave a revised description of *Flavobacterium* and proposed a neotype strain for *F. odoratum* (NCTC 11036 = ATCC 4651). In this and a subsequent study (Holmes et al., 1979b), it was found that three strains of CDC Group M-4f (Tatum et al., 1974) were identical to *F. odoratum*. These strains were originally assembled and placed into groups by the late Elizabeth O. King and her successors (King, 1959; Tatum et al., 1974). *Flavobacterium odoratum* was, however, not included in the list of species of flavobacteria in the *Bergey's Manual of Systematic Bacteriology* until 1984 (Holmes et al., 1984).

It was suggested by several authors that *F. odoratum* should be classified in a separate genus on the basis of phenotypic and phylogenetic differences (Holmes and Owen, 1981; Holmes et al., 1984; Gherna and Woese, 1992; Nakagawa and Yamasato, 1993; Bernardet et al., 1996). Heterogeneity on the basis of colonial morphology, nutritional features, fatty acid profiles, and genomic relatedness among *F. odoratum* strains, were, however, also observed (Owen and Holmes, 1978; Rasoamananjara et al., 1986; Rasoamananjara et al., 1987). It was only in 1996 that Vancanneyt et al. performed an extensive polyphasic taxonomic analysis and reclassified *F. odoratum* into a new genus as *Myroides odoratus* (type strain, ATCC 4651). They also described an additional species, namely *Myroides odoratimimus* (NCTC 11180).

Habitat

Clinical Isolates

Although originally isolated from canal water, *Empedobacter* is mainly known to have a clinical origin, although it accounts for less than 0.1% of all clinical isolates of nonfermentative Gram-negative bacteria (G. L. Gilardi, personal communication, cited in Pickett et al., 1991). Stutzer and Kwaschnina (1929) were the first investigators to report the isolation of this organism from a clinical source, namely feces from a patient with relapsing fever. Since then, *Empedobacter* strains have been isolated from human eyes (swabs), bronchial secretions, peritoneal fluid, dialysis fluid, serous cavity fluid, cervixes and vaginas (swabs), wounds, blood, and urine (Holmes et al., 1978; Bruun, 1982).

Although it is not regarded as a component of the human microflora, *Myroides* accounts for 0.2% of all clinical isolates of nonfermentative, Gram-negative bacteria (Pickett et al., 1991). The first *Myroides* strains isolated were from the human intestine (Stutzer, 1923; Stutzer and Kwaschnina, 1929). Since then this organism has been isolated from human urine, feces, wound discharge, sputum and blood (Holmes et al., 1979b; Bruun, 1982; Shewan and McMeekin, 1983; Rubin et al., 1985; Pickett et al., 1991). Although the source is uncertain, water in the hospital environment is frequently suspected of carrying this organism. It was speculated that the strain that caused necrotizing fasciitis and bacteremia in a 71-year-old female farmer in Taiwan gained access via a trivial wound contaminated with soil or water (Hsueh et al., 1995).

Environmental Isolates

Although the first *Empedobacter* and *Myroides* strains were primarily isolated from clinical sources, a number of recent studies have indicated that they are widely distributed in the environment, especially water. This accounts for their apparent presence in many habitats (e.g., Herman [1981] and Shewan and McMeekin [1983]).

Empedobacter brevis and *Flavobacterium odoratum* (*Myroides odoratus*) together with other bacteria were isolated from stable fly eggs and were found to be necessary to complete the development of stable fly larvae (Lysyk et al., 1999). These organisms were also isolated from South Atlantic fish species at the processing site and were considered potential active spoilers of the fish (i.e., producers of off-odors; Engelbrecht et al., 1996). An *Empedobacter* strain and two *Myroides odoratus* strains were also isolated from freshwater fish skin, the water of the sampling site, and the air during chill storage of the freshwater fish, but were not considered important contributors to the spoilage of these fish (González et al., 2000).

In the food environment, *Myroides* strains have been isolated from dairy sources and shown to produce thermostabile proteases and lipases which may play a role in the spoilage of dairy products (Griffiths et al., 1981; Zahran and Al-Saleh, 1997). They could, however, not be isolated from dairy sources by Jooste et al. (1985). In a study by Hebbar et al. (1991), *F. odoratum* was isolated from roots and crowns of the sunflower plant (*Helianthus annuus*), where it acted as one of the antagonistic bacteria to fungal pathogens. Chourasia (1995) isolated this organism from the geocarposphere of peanuts (*Arachis hypogaea*).

It was found that in food processing plants, welding of stainless steel surfaces has no significant influence on short-term accumulation of *Listeria monocytogenes* or a three-member bacterial consortium (including two *Myroides odoratus* strains) that was isolated from weldments in a seafood processing plant (Tide et al., 1999).

In a study by Bremer et al. (2001), it was, however, found that the attachment of *Listeria monocytogenes* to stainless steel increased significantly in the presence of *Myroides odoratus* strains as contaminating organisms of food surface areas.

Isolation and Preservation

The flavobacteria in general are not difficult to isolate, since they are chemo-organotrophic (Holmes, 1992). The general (nonselective) isolation, maintenance and preservation media used for culturing members of the Flavobacteriaceae are discussed in detail by Jooste and Hugo (1999) and Hugo and Jooste (2003).

Plate count agar containing 0.5% NaCl was used to isolate *Empedobacter* and *Myroides* strains from Cape marine fish (Engelbrecht et al., 1996) while tryptone soya agar (Oxoid) was used to isolate *Empedobacter* and *Myroides odoratus* from freshwater fish (González et al., 2000).

For the isolation of *Myroides odoratus* from the geocarposphere of peanut, 5% trypticase soy agar (Bacto tryptone, 17 g; Bacto soytone, 3 g; Bacto dextrose, 2.5 g; sodium chloride, 5 g; dipotassium phosphate, 2.5 g; Bacto agar, 20 g; and distilled water, 1 liter) and PAF (Bacto peptone, 10 g; proteose peptone, 10 g; potassium phosphate, 1.5 g; magnesium sulfate, 1.5 g; Bacto agar, 15 g; and distilled water, 1 liter) were used (Chourasia, 1995).

Cultivation of *Empedobacter* and *Myroides* strains has been reported on nutrient agar (Holmes et al., 1978; Yagci et al., 2000) and heart infusion medium with 5% rabbit blood (Dees et al., 1986). Cultivation of *Myroides* species has been reported in nutrient broth No. 2 (Oxoid; Owen and Holmes, 1978) and trypticase soy agar (BBL; Vancanneyt et al., 1996).

Although no specific medium is known for the selective isolation of *Empedobacter* and *Myroides* species, β-hydroxybutyrate agar and MacConkey agar may be used for the enumeration of *Myroides*, *E. brevis*, *Chryseobacterium* and *Weeksella* (Bernardet et al., 1996; Weyant et al., 1996; Jooste and Hugo, 1999). Selective media on which *Empedobacter* and *Myroides* species will not grow include nutrient broth containing 6% NaCl and Simmons citrate agar (Holmes et al., 1977; Jooste and Hugo, 1999). *Empedobacter* strains will also not grow on Salmonella-Shigella agar (Tatum et al., 1974; Holmes et al., 1978; Weyant et al., 1996; Jooste and Hugo, 1999).

For the preservation of *Empedobacter* and *Myroides* species, the general media used for flavobacterial maintenance may be used. These media include PMYA II (peptonized milk yeast extract agar; Christensen and Cook, 1972) and Dorset egg medium in a screw-capped bijou bottle stored at 4ºC (Cowan, 1974; Holmes et al., 1984). For longer periods of preservation, isolates may be freeze-dried (Holmes, 1992), or agar slant culture growth (18-h old) can be suspended in defibrinated rabbit blood placed in suitable containers and frozen in a mixture of dry ice and alcohol prior to storage at –50ºC (Holmes et al., 1984).

Identification

Methods for the identification of members of the Flavobacteriaceae are described by Bernardet et al. (2002). General identification methods and characteristics (chemotaxonomically significant components [respiratory quinones, fatty acids, etc.], production of enzymes, pigments, gliding motility, salt requirement, and capnophilic metabolism) of the Flavobacteriaceae are also discussed in detail in An Introduction to the Family Flavobacteriaceae in this Volume by Bernardet and Nakagawa. Only the morphological and phenotypical characteristics of *Empedobacter* and *Myroides* will be discussed in this chapter, since they play such an important role in differentiating the two genera.

Morphology

Members of both genera are Gram-negative, nonsporeforming rods that are 0.5 μm in diameter and 1–2 μm long with parallel sides and rounded ends. Longer rods of *Empedobacter* may be observed (Holmes et al., 1984; Vandamme et al., 1994). In *Myroides* species, longer rods and long chains (containing 4–10 cells) may occur in broth medium (Holmes et al., 1977).

They are nonmotile (i.e., cellular gliding and swarming of colonies are absent). Cells lack flagella (Holmes et al., 1978; Holmes et al., 1979b; Bernardet et al., 2002).

Pigmentation is light yellow (in both genera), of the flexirubin type, nonfluorescent, and insoluble in solid media (Holmes, 1992; Bernardet et al., 2002). The yellow hue of *Empedobacter* does not change with variation of medium or temperature (Holmes et al., 1984). *Empedobacter* colonies on nutrient agar at 30°C are pinpoint (to 2.0 mm), low convex, circular, smooth and shining and have an entire edge. On blood agar the colonies are pinpoint (to 2.5 mm; Holmes et al., 1984; Vandamme et al., 1994; Bernardet et al., 2002).

In *Myroides* species, four colony types may occur after incubation for 24 h on nutrient agar (Holmes et al., 1979b). Colony type 1 produces

effuse, spreading colonies (3–4 mm in diameter) with raised, shiny centers and dull, matt, spreading edges. On further incubation, the whole colony becomes smooth and shiny. Colony type 2 shows the same appearance after 24 h as colony type 1 shows, but the colonies are smaller, about 1.0–1.5 mm in diameter. Colony type 3 is smooth, shiny, and convex with no spreading edge and with a diameter of 0.5–1.0 mm after 24 h. Colony type 4 is mucoid. A characteristic fruity odor is produced by most strains (Holmes et al., 1977; Bruun, 1982; Rubin et al., 1985; Vancanneyt et al., 1996).

Phenotypic Identification and Physiology

Empedobacter

Both genera are strictly aerobic with menaquinone 6 as the major respiratory quinone. Both genera will grow at 25°C and 30°C, but not at 5°C or 42°C. All *Myroides* and most *Empedobacter* strains will grow at 37°C, but most *Empedobacter* strains will only produce acid from carbohydrates when incubated at 30°C. Prolonged incubation may then be necessary (up to 17 days; Holmes et al., 1978; Bruun, 1982; Bernardet et al., 2002).

Strong proteolytic activity occurs in both genera. Both genera will grow on MacConkey agar as well as on β-hydroxybutyrate agar. Both genera produce DNase, urease, oxidase, catalase and phosphatase. Indole is produced in *Empedobacter* but not in *Myroides*. Both genera degrade gelatin. While starch is not degraded by *Myroides*, results vary for *Empedobacter*. Neither of the genera produces β-galactosidase, reduces nitrate, or degrades agar and esculin. *Myroides* species reduce nitrite. In *Empedobacter*, several carbohydrates (not glycerol or trehalose) are oxidized. In *Myroides* there is no carbohydrate utilization (Holmes et al., 1978; Holmes et al., 1979b; Holmes et al., 1984; Bernardet et al., 2002). The differential characteristics of *Empedobacter* and *Myroides* are given in Table 1.

Table 1. Differential characteristics of *Empedobacter* and *Myroides*.

Characteristic	*Empedobacter*	*Myroides*
Growth at		
37°C	+a	+
Acid production from		
Glucose	+	–a
Maltose	+	–
Production of		
Pigment (flexirubin type)	(+)	(+)
Urease	V	+
Indole	+	–
Reduction of		
Nitrite	–	+
Degradation/hydrolysis of		
Starch		–
Tyrosine	V	V
Carbohydrate utilization	V	–
Resistance to Pen G	+	+
G+C content (mol%)	31–33	30–38

Symbols and abbreviations: a, most strains are positive or negative for this characteristic.
+, positive reaction; (+), weak positive reaction; –, negative reaction; V, varies within and/or between species: V, varies between references; and Pen G, penicillin G.
From Holmes et al. (1978, 1979, 1984) and Bernardet et al. (2002).

Both genera are known for their resistance to a wide range of antimicrobials (Holmes et al., 1984; Vandamme et al., 1994). *Myroides* in particular are resistant to the following groups of antimicrobials (although variability exists between reported cases): β-lactams, aminoglycosides, tetracyclines, quinolones and trimethoprim-sulfamethoxazole (Holmes et al., 1979b; Strandberg et al., 1983; Macfarlane et al., 1985; Hsueh et al., 1995; Yagci et al., 2000). Although an R plasmid conferring resistance to ampicillin, carbenicillin, and erythromycin has been reported in a strain of *M. odoratus* (Kono et al., 1980), plasmids have not been detected in *Myroides* species since then. Resistance in these organisms is, therefore, possibly associated with chromosomal genes (Holmes, 1992).

The dominant fatty acids in *Empedobacter* are 15:0 iso, 16:1γ7c, 16:1γ5c, 16:0, 16:0 3OH and 17:0 iso 3OH (Oyaizu and Komagata, 1981; Dees et al., 1986; Vandamme et al., 1994). The DNA contains 31–33 mol% G+C (Vandamme et al., 1994; Bernardet et al., 2002).

The dominant fatty acids of *Myroides* species are 15:0 iso, 15:0 iso 3OH, 16:0 3OH, 17:0 iso 3OH, and 17:1 iso γ9c (Oyaizu and Komagata, 1981; Dees et al., 1986; Vancanneyt et al., 1996). The DNA contains 30–38 mol% G+C (Vancanneyt et al., 1996; Bernardet et al., 2002).

The two species of *Myroides*, *M. odoratus* and *M. odoratimimus*, are difficult to differentiate phenotypically. Small differences were, however, found in carbon source assimilation (Biotype 100, bioMérieux) and oxidation (Biolog GN MicroPlate assays; Vancanneyt et al., 1996). The differential characteristics between the two species are given in Table 2.

Pathogenicity

Empedobacter

This organism was isolated for the first time in 1888 from canal water and found to be pathogenic to laboratory animals such as guinea pigs, mice and rabbits, but not pigeons (Holmes et al., 1978). Stutzer and Kwaschnina (1929) and

Table 2. Differential characteristics between *M. odoratus* and *M. odoratimimus*.

Characteristic	*M. odoratus*	*M. odoratimimus*
Growth on Biotype 100 carbon sources	Negative for all	Positive for L-aspartate, L-glutamate and L-proline Variable for L-malate, succinate and fumarate
Biolog GN MicroPlate oxidation of 95 carbon sources	Positive for all	Positive for Tween 40, Tween 80, methylpyruvate, monomethylsuccinate, acetic acid, α-hydroxybutyric acid, α-ketovaleric acid, DL-lactic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-leucine, L-ornithine, L-proline, L-serine, L-threonine, inosine, uridine and thymidine Variable for L-Fucose, formic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketoglutaric acid, L-histidine, L-phenylalanine, D-serine, urocanic acid, phenyl ethylamine, 2,3-butanediol, and glycerol
Major fatty acids	15:0 iso, 15:0 iso 3OH, 15:0 anteiso, 16:0 3OH, 17:0 iso 3OH, and 17:1 iso ω9c	13:0 iso, 15:0, 15:0 iso, 15:0 iso 3OH, 16:0 3OH, 17:0 iso 3OH, 17:1 iso ω9c, and summed feature 4

Abbreviations: 15:0, pentadecanoate; 15:0 iso, 13-methyl-tetradecanoic acid; 15:0 iso 3OH, 3-hydroxy-13-methyl-tetradecanoic acid; 13:0 iso, 11-methyl-dodecanoic acid; 16:0 3OH, methyl 3-hydroxyhexadecanoate; 17:0 iso, 15-methyl-hexadecanoic acid; 15:0 anteiso, 12-methyl-tetradecanoic acid; 17:1 iso ω9c, 15-methyl-cis-delta-7-hexadecanoic acid; summed in feature 4: 15:0 iso 2OH, 2-hydroxy-13-methyl-tetradecanoic acid; 16:1 ω7c; 16:1 ω7t and 17:0 iso 3OH, 3-hydroxy-15-methyl-hexadecanoic acid.
From Vancanneyt et al. (1996).

Holmes et al. (1978), however, found their clinical isolates did not cause disease. The resistance of this organism to a wide range of antimicrobial agents (including carbenicillin and gentamicin) suggested, however, that *Empedobacter* infections would be difficult to treat (Holmes et al., 1978).

Empedobacter brevis was easily recovered from clinical material (Weyant et al., 1996). Recently, it was shown that *E. brevis* together with strains of other bacterial species is a common contaminant of contact lens care systems. These species may facilitate *Acanthamoeba* growth and thus cause keratitis of the eyes (Bottone et al., 1992). In 1997, *E. brevis* was found to cause meningitis in a dog, but the source of the organism was not known (Haburjak and Schubert, 1997).

Myroides

Stutzer (1923) speculated that the strains he isolated played only a saprophytic role in the human intestine. He could not demonstrate any animal pathogenicity since intradermal, intraperitoneal, and intravenous injections of *Bacterium faecale aromaticum* into mice, guinea pigs and rabbits produced no pathogenic response. Stutzer and Kwaschnina (1929) isolated strains of *F. odoratum* from the feces of patients with typhoid fever, acute gastroenteritis, and relapsing fever. Although not considered to be pathogenic, this organism was isolated in a way that might reflect the pathological processes in the intestine.

Holmes et al. (1979b) first suggested a possible pathogenic role for *F. odoratum* isolated from soft tissue infections, including an amputation site and urinary tract infections. Davis et al. (1979) isolated the organism from an amputation site, but the pathogenic role of the organism in this connection was uncertain. In 1985, Macfarlane et al. reported ventriculitis caused by *F. odoratum*, which was treated with intraventricular cefotaxime. Hsueh et al. (1995) reported the first necrotizing fasciitis and bacteremia caused by *F. odoratum* (*M. odoratus*) and argued that this organism should be added to the list of etiologic agents that can cause severe skin and soft-tissue infections. Since then, a case of recurrent cellulitis with bacteremia was reported in a corticosteroid-treated patient with multiple skin lesions (Bachman et al., 1996).

In 2000, an outbreak of a *Myroides* spp. urinary tract infection was reported among urology patients with catheters in a Turkish hospital (Yagci et al., 2000). *Myroides odoratimimus* was identified as the organism causing this outbreak over a three-year period. All of the 22 affected patients, including two with bacteremia, had

pyuria, calculi and neoplasia. Yagci et al. (2000) speculated that the outbreak was maintained by contamination from an unknown source, but could not identify a single measure that ended the outbreak.

The resistance of *Myroides* to a wide range of antimicrobial agents commonly used against Gram-negative nonfermentative bacterial infections, including β-lactams (penicillins, cephalosporins, aztreonam, and carbapenems) and aminoglycosides, suggests that any infection by this organism would prove difficult to treat (Holmes et al., 1979b; Strandberg et al., 1983; Hsueh et al., 1995; Yagci et al., 2000).

Applications

No literature on applications of *E. brevis* is available. In the past 10–15 years, some applications of *Myroides* spp. have been reported. *Myroides* spp. have been used as biological control agents against bacterial and fungal pathogens of the sunflower plant (Hebbar et al., 1991). *Flavobacterium odoratum* (*M. odoratus*) has been able to colonize the geocarposphere of peanuts, resulting in the inhibition of aflatoxin production by *Aspergillus flavus* (Chourasia, 1995). In a study by Lysyk et al. (1999), *Myroides* isolated from the eggs of stable flies was considered essential, together with other bacteria, for the development of the larvae. This study evaluated a rearing procedure for the larvae to study insect-microbe interactions and evaluating microbial control agents.

The enzymes of *Myroides* spp. may also be useful. *Flavobacterium odoratum* (*M. odoratus*) was found to produce elastase, which is a commercially useful enzyme having clinical therapeutic applications and food processing applications as a meat-tenderizing agent or protein hydrolytic catalyst (Yan et al., 1994). An isoamylase from *F. odoratum* (*M. odoratus*) was found to greatly enhance the production of maltose, maltotriose, maltotetraose and trehalose under industrial conditions for starch processing in the presence of respective auxiliary enzymes (Takahashi et al., 1996). According to Labuschagne et al. (1997), the lipase of *F. odoratum* (*M. odoratus*) may be useful for industrial applications such as the hydrolysis of insoluble acylglycerols.

Literature Cited

Bachman, K. H., D. L. Sewell, and L. J. Strausbaugh. 1996. Recurrent cellulitis and bacteremia caused by Flavobacterium odoratum. Clin. Infect. Dis. 22:1112–1113.

Bergey, D. H., F. C. Harrison, R. S. Breed, B. W. Hammer, and F. M. Huntoon (Eds.). 1923. Bergey's Manual of Determinative Bacteriology, 1st ed.. Williams and Wilkins. Baltimore, MD.

Bernardet, J.-F., P. Segers, M. Vancanneyt, F. Berthe, K. Kersters, and P. Vandamme. 1996. Cutting a Gordian knot: Emended classification and description of the genus Flavobacterium, emended description of the family Flavobacteriaceae, and proposal of Flavobacterium hydatis nom. nov. (basonym, Cytophaga aquatilis Strohl and Tait 1978). Int. J. Syst. Bacteriol. 46:128–148.

Bernardet, J.-F., Y. Nakagawa, and B. Holmes. 2002. Proposed minimal standards for describing new taxa of the family Flavobacteriaceae and emended description of the family. Int. J. Syst. Evol. Microbiol. 52:1049–1070.

Bottone, E. J., R. M. Madayag, and M. N. Qureshi. 1992. Acanthamoeba keratitis: synergy between amebic and bacterial cocontamination in contact lens care systems as a prelude to infection. J. Clin. Microbiol. 30:2447–2450.

Bremer, P. J., I. Monk, and C. M. Osborne. 2001. Survival of Listeria monocytogenes attached to stainless steel surfaces in the presence or absence of Flavobacterium species. J. Food Protect. 64:1369–1376.

Bruun, B. 1982. Studies on a collection of strains of the genus Flavobacterium. Acta Path. Microbiol. Immunol. Scand. Sect. B 90:415–421.

Chester, F. D. 1897. Report of the mycologist: bacteriological work. Del. Agr. Exp. Sta. Bull. 9:38–145.

Chourasia, H. K. 1995. Kernel infection and aflatoxin production in peanut (Arachis hypogaea L.) by Aspergillus flavus in presence of geocarposphere bacteria. J. Food Sci. Technol. 32:459–464.

Christensen, P. J., and F. D. Cook. 1972. The isolation and enumeration of cytophagas. Can. J. Microbiol. 18:1933–1940.

Cornil, A.-V., and V. Babes. 1890. Les bactéries et leur role dans l'étiologie, l'anatomie et l'histologie pathogiques des maladies infectieuses, 3rd ed. Baillière. Paris, France.

Cowan, S. T. 1974. Cowan and Steel's Manual for the Identification of Medical Bacteria, 2nd ed. Cambridge University Press. Cambridge, UK.

Davis, J. M., M. M. Peel, and J. A. Gillians. 1979. Colonization of an amputation site by Flavobacterium odoratum after gentamycin therapy. Med. J. Australia 2:703–704.

Dees, S. B., C. W. Moss, D. G. Hollis, and R. E. Weaver. 1986. Chemical characterization of Flavobacterium odoratum, Flavobacterium breve, and Flavobacterium-like groups IIe, IIh, and IIf. J. Clin. Microbiol. 23:267–273.

Eisenberg, J. 1891. Bakteriologische Diagnostik. Leopold Voss. Hamburg, Germany.

Engelbrecht, K., P. J. Jooste, and B. A. Prior. 1996. Spoilage characteristics of Gram-negative genera and species isolated from Cape marine fish. S. Afr. J. Food Sci. Nutr. 8:66–71.

Frankland, P. F., and G. C. Frankland. 1894. Micro-organisms in Water. Longmans, Green & Co. London, UK.

Gherna, R., and C. R. Woese. 1992. A partial phylogenetic analysis of the "flavobacter-bacteroides" phylum: Basis for taxonomic restructuring. Syst. Appl. Microbiol. 15:513–521.

González, C. J., J. A. Santos, M.-L. García-López, and A. Otero. 2000. Psychrobacters and related bacteria in freshwater fish. J. Food Protect. 63:315–321.

Griffiths, M. W., J. D. Phillips, and D. D. Muir. 1981. Thermostability of proteases and lipases from a number of species of psychrotrophic bacteria of dairy origin. J. Appl. Bacteriol. 50:289–303.

Haburjak, J. J., and T. A. Schubert. 1997. Flavobacterium breve meningitis in a dog. J. Am. Anim. Hosp. Assoc. 33:509–512.

Hebbar, P., O. Berge, T. Heulin, and S. P. Singh. 1991. Bacterial antagonists of sunflower (Helianthus annuus L.) fungal pathogens. Plant Soil 133:131–140.

Herman, L. G. 1981. The slow growing Gram-negative pigmented water bacteria. *In:* H. Reichenbach, and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group, Proceedings of the International Symposium on Yellow-Pigmented Gram-negative Bacteria of the Flavobacterium-Cytophaga Group, Braunschweig, July 8 to 11, 1980. Verlag Chemie. Weinheim, Germany. 169–178.

Holmes, B., J. J. S. Snell, and S. P. Lapage. 1977. Revised description, from clinical isolates, of Flavobacterium odoratum Stutzer and Kwaschnina 1929, and designation of the neotype strain. Int. J. Syst. Bacteriol. 27:330–336.

Holmes, B., J. J. S. Snell, and S. P. Lapage. 1978. Revised description, from clinical strains, of Flavobacterium breve (Lustig) Bergey et al. 1923 and proposal of the neotype strain. Int. J. Syst. Bacteriol. 28:201–208.

Holmes, B., and R. J. Owen. 1979a. Proposal that Flavobacterium breve be substituted as the type species of the genus in place of Flavobacterium aquatile and emended description of the genus Flavobacterium: status of the named species of Flavobacterium. Int. J. Syst. Bacteriol. 29:416–426.

Holmes, B., J. J. S. Snell, and S. P. Lapage. 1979b. Flavobacterium odoratum: a species resistant to a wide range of antimicrobial agents. J. Clin. Pathol. 32:73–77.

Holmes, B., and R. J. Owen. 1981. Emendation of the genus Flavobacterium and the status of the genus: Developments after the 8th edition of Bergey's Manual. *In:* H. Reichenbach, and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group, Proceedings of the International Symposium on Yellow-Pigmented Gram-negative Bacteria of the Flavobacterium-Cytophaga Group, Braunschweig, July 8 to 11, 1980. Verlag Chemie. Weinheim Germany, 17–26.

Holmes, B., R. J. Owen, and T. A. McMeekin. 1984. Genus Flavobacterium Bergey, Harrison, Breed, Hammer and Huntoon, 1923, 97^{AL}. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:353–361.

Holmes, B. 1992. The genera Flavobacterium, Sphingobacterium and Weeksella. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. Berlin Germany, 4:3620–3630.

Hsueh, P.-R., J.-J. Wu, T.-Z. Hsiue, and W.-C. Hsieh. 1995. Bacteremic necrotizing fasciitis due to Flavobacterium odoratum. Clin. Infect. Dis. 21:1337–1338.

Hugo, C. J., and P. J. Jooste. 2003. Chapter 21: Culture media for genera in the family Flavobacteriaceae. *In:* J. E. L Corry, G. D. W. Curtis and R. M. Baird (Eds.) Handbook of Culture Media for Food Microbiology, 2nd ed. Elsevier Science Publishers. London, UK. 355–367.

Jooste, P. J., T. J. Britz, and J. De Haast. 1985. A numerical taxonomic study of Flavobacterium-Cytophaga strains from dairy sources. J. Appl. Bacteriol. 59:311–323.

Jooste, P. J., and C. J. Hugo. 1999. The taxonomy, ecology and cultivation of bacterial genera belonging to the family Flavobacteriaceae. Int. J. Food Microbiol. 53:81–94.

King, E. O. 1959. Studies on a group of previously unclassified bacteria associated with meningitis in infants. Am. J. Clin. Pathol. 31(3):241–247.

Kono, M., M. Sasatsu, and K. Makino. 1980. R-plasmid conferring resistance to ampicillin, carbenicillin and erythromycin in a Flavobacterium odoratum strain. Microbios Lett. 14:55–58.

Krasil'nikov, N. A. 1949. Guide to the Bacteria and Actinomycetes. Akad Nauk SSSR. Moscow, Russia.

Labuschagne, R. B., A. van Tonder, and D. Litthauer. 1997. Flavobacterium odoratum lipase: isolation and characterization. Enz. Microb. Technol. 21:52–58.

Lustig, A. 1890. Diagnostica dei batteri delle acque con una guida alle richerche batteriologiche e microscopiche. Rosenberg and Sellier. Torino, Italy.

Lysyk, T. J., L. Kalischuk-Tymensen, L. B. Selinger, R. C. Lancaster, L. Wever, and K.-J. Cheng. 1999. Rearing stable fly larvae (Diptera: Muscidae) on egg yolk medium. J. Med. Entomol. 36:382–388.

Macfarlane, D. E., P. Baum-Thureen, and I. Crandon. 1985. Flavobacterium odoratum ventriculitis treated with intraventricular cefotaxime. J. Infect. 11:233–238.

Mez, C. 1898. Mikroskopische Wasseranalyse. Julius Springer. Berlin, Germany.

Mori, R. 1888. Ueber pathogene Bacterien im Canalwasser. Zeitschr. Hyg. 4:47–54.

Nakagawa, Y., and K. Yamasato. 1993. Phylogenetic diversity of the genus Cytophaga revealed by 16S rRNA sequencing and menaquinone analysis. J. Gen. Microbiol. 139:1155–1161.

Owen, R. J., and B. Holmes. 1978. Heterogeneity in the characteristics of deoxyribonucleic acid from Flavobacterium odoratum. FEMS Microbiol. Lett. 48:41–46.

Owen, R. J., and B. Holmes. 1980. Differentiation between strains of Flavobacterium breve and allied bacteria by comparisons of deoxyribonucleic acids. Curr. Microbiol. 4:7–11.

Oyaizu, H., and K. Komagata. 1981. Chemotaxonomic and phenotypic characterization of the strains of species in the Flavobacterium-Cytophaga complex. J. Gen. Appl. Microbiol. 27:57–107.

Pickett, M. J., D. G. Hollis, and E. J. Bottone. 1991. Miscellaneous Gram-negative bacteria. *In:* A. Balows, W. J. Hausler Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (Eds.) Manual of Clinical Microbiology, 5th ed. American Society for Microbiology. Washington, DC. 410–428.

Prévot, A. R. 1961. Traité de systématique bactérienne. Dunod. Paris, France.

Rasoamananjara, D., F. Peladan, J. C. Turlot, H. Monteil, and C. Richard. 1986. Characterization of Flavobacterium species by analysis of volatile fatty acid production. J. Gen. Microbiol. 132:2723–2732.

Rasoamananjara, D., B. Korosec, and H. Monteil. 1987. Classification and identification of Flavobacterium species by carbon source utilization. J. Clin. Microbiol. 25:1285–1290.

Reichenbach, H. 1989. Order I: Cytophagales. *In:* J. T. Staley, M. P. Bryant, N. Pfennig and J. G. Holt (Eds.) Bergey's Manual of Determinative Bacteriology. Williams and Wilkins. Baltimore, MD. 2010–2082.

Rubin, S. J., P. A. Granato, and B. L. Wasilauskas. 1985. Glucose-nonfermenting Gram-negative bacteria. *In:*

E. H. Lennette, A. Balows, W. J. Hausler Jr., and H. J. Shadony (Eds.) Manual of Clinical Microbiology, 4th ed. American Society for Microbiology. Washington, DC. 330–349.

Shewan, J. M., and T. A. McMeekin. 1983. Taxonomy (and ecology) of Flavobacterium and related genera. Ann. Rev. Microbiol. 37:233–252.

Strandberg, D. A., J. H. Jorgensen, and D. J. Drutz. 1983. Activities of aztreonam and new cephalosporins against infrequently isolated Gram-negative bacilli. Antimicrob. Agents Chemother. 24:282–286.

Stutzer, M. J. 1923. Zur Frage Über die Fäulnisbakterien im Darm. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I. Orig. 91:87–90.

Stutzer, M., and A. Kwaschnina. 1929. In Aussaaten aus den Fäzes des Menschen gelbe Kolonien bildende Bakterien (Gattung Flavobacterium u.a.). Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I. Orig. 113:219–225.

Takahashi, K., J.-I. Abe, T. Kozuma, M. Yoshida, N. Nakamura, and S. Hizukuri. 1996. Production and application of an isoamylase from Flavobacterium odoratum. Enz. Microb. Technol. 19:456–461.

Tatum, H. W., W. H. Ewing, and R. E. Weaver. 1974. Miscellaneous Gram-negative bacteria. *In:* E. H. Lennette, E. H. Spaulding, and J. P. Truant (Eds.) Manual of Clinical Microbiology, 2nd ed. American Society for Microbiology. Washington, DC. 270–294.

Tide, C., S. R. Harkin, G. G. Geesey, P. J. Bremer, and W. Scholz. 1999. The influence of welding procedures on bacterial colonization of stainless steel weldments. J. Food Engin. 42:85–96.

Ursing, J., and B. Bruun. 1991. Genotypic heterogeneity of Flavobacterium Group IIb and Flavobacterium breve demonstrated by DNA-DNA hybridization. Acta Path. Microbiol. Immunol. Scand. Sect. B 99:780–786.

Vancanneyt, M., P. Segers, U. Torck, B. Hoste, J.-F. Bernardet, P. Vandamme, and K. Kersters. 1996. Reclassification of Flavobacterium odoratum (Stutzer 1929) strains to a new genus, Myroides, as Myroides odoratus comb. nov. and Myroides odoratimimus sp. nov. Int. J. Syst. Bacteriol. 46:926–932.

Vandamme, P., J.-F. Bernardet, P. Segers, K. Kersters, and B. Holmes. 1994. New perspectives in the classification of the flavobacteria: description of Chryseobacterium gen. nov., Bergeyella gen. nov., and Empedobacter nom. rev. Int. J. Syst. Bacteriol. 44:827–831.

Wayne, L. G. 1982. Actions of the Judicial Commission of the International Committee on Systematic Bacteriology on requests for opinions published between July 1979 and April 1981. Int. J. Syst. Bacteriol. 32:464–465.

Weeks, O. B. 1974. Genus Flavobacterium Bergey et al. 1923. *In:* R. E. Buchanan and N. E. Gibbons (Eds.) Bergey's Manual of Determinative Bacteriology, 8th ed. Williams and Wilkins. Baltimore, MD. 357–364.

Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1996. Identification of Unusual Pathogenic Gram-negative Aerobic and Facultatively Anaerobic Bacteria, 2nd ed. CDC. Atlanta, GA.

Yagci, A., N. Çerikçioglu, M. E. Kaufmann, H. Malnick, G. Söyletir, F. Babacan, and T. L. Pitt. 2000. Molecular typing of Myroides odoratimimus (Flavobacterium odoratum) urinary tract infections in a Turkish hospital. Eur. J. Clin. Microbiol. Infect. Dis. 19:731–732.

Yan, Z.-Y., G.-X. Guan, W.-Q. Zhang, and Z.-F. Lin. 1994. Elastolytic activity from Flavobacterium odoratum. Microbial screening and cultivation, enzyme production and purification. Process Biochem. 29:427–436.

Zahran, A. S., and A. A. Al-Saleh. 1997. Isolation and identification of protease-producing psychrotrophic bacteria from raw camel milk. Austral. J. Dairy Technol. 52:5–7.

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CHAPTER 6.12

The Genera *Chryseobacterium* and *Elizabethkingia*

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Introduction

Together with the genera *Bergeyella* and *Empedobacter*, the genus *Chryseobacterium* Vandamme, Bernardet, Segers, Kersters, and Holmes 1994 was built on the ruins of the genus *Flavobacterium*, shortly before the latter was thoroughly emended following extensive phylogenetic investigations (Vandamme et al., 1994; Bernardet et al., 1996; see also the following chapters in this edition: The Genus Flavobacterium in this Volume, The Genera Empedobacter and Myroides in this Volume, and The Genera Bergeyella and Weeksella in this Volume). For reasons that are explained in the section Phylogeny, the splitting of the genus *Chryseobacterium* was proposed very recently (Kim et al., 2005b); the organismpreviously known as *Chryseobacterium meningosepticum* and *C. miricola* were allocated to the new genus *Elizabethkingia* under the epithets *Elizabethkingia meningoseptica* and *E. miricola*. Among the currently recognized *Chryseobacterium* and *Elizabethkingia* species, those that were already described in the previous editions of *Bergey's Manual of Systematic Bacteriology* and *The Prokaryotes* were dealt with in the chapters Genus *Flavobacterium* Harrison, Breed, Hammer and Huntoon 1923 (Holmes et al., 1984) and The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* (*The Prokaryotes*, second edition and in this Volume), respectively.

The genera *Chryseobacterium* and *Elizabethkingia* belong to the family Flavobacteriaceae, the history and structure of which are presented in another chapter in this Volume: An Introduction to the Family Flavobacteriaceae. Currently, Flavobacteriaceae comprise 25 other valid genera; following extensive bacteriological surveys of various, mostly marine and polar environments, several additional genera are about to be published in the *International Journal of Systematic and Evolutionary Microbiology*. Taxonomic and nomenclatural issues concerning the family are dealt with by the Subcommittee on the taxonomy of *Flavobacterium* and *Cytophaga*-like bacteria of the International Committee on Systematics of Prokaryotes. This subcommittee has issued minimal standards for the description of new taxa in the family Flavobacteriaceae (Bernardet et al., 2002).

Many *Chryseobacterium* and *Elizabethkingia* strains occur in soil, freshwater, and marine environments, while others are found in dairy products; others are opportunistic pathogens of humans and animals. Most of the environmental species have not been extensively studied and some of them are only represented in culture collections by one or very few strains. Conversely, the clinical and dairy isolates have received more attention owing to their medical or economic significance, and collections comprising a reasonable number of strains are available.

Phylogeny

The 16S rRNA gene sequences of at least the type strains of all valid *Chryseobacterium* and *Elizabethkingia* species are now available, as well as the sequences of representative isolates of the taxa that are not validly published yet (see the section Taxonomy in this Chapter). The position of the genera within the family Flavobacteriaceae and the position of the family within the phylum "Bacteroidetes" (Garrity et al., 2003) or "Cytophaga-Flavobacterium-Bacteroides phylum" are presented in Figs. 1 and 2 in the chapter An Introduction to the Family Flavobacteriaceae in this Volume, together with comments on the denomination and delineation of higher taxa in the phylum. Figure 1 below is an updated phylogenetic tree of members of the genera *Chryseobacterium* and *Elizabethkingia*.

Since the 16S rRNA gene sequences of *Chryseobacterium balustinum*, *C. gleum* (the type species), *C. indologenes*, *C. indoltheticum* and *Elizabethkingia meningoseptica* deposited in databases and used to draw previously published phylogenetic trees were of poor quality, new sequences were recently determined and deposited by O. Matte-Tailliez, J.-F. Bernardet and B. Kerouault. These sequences were used to draw the current tree in combination with the high

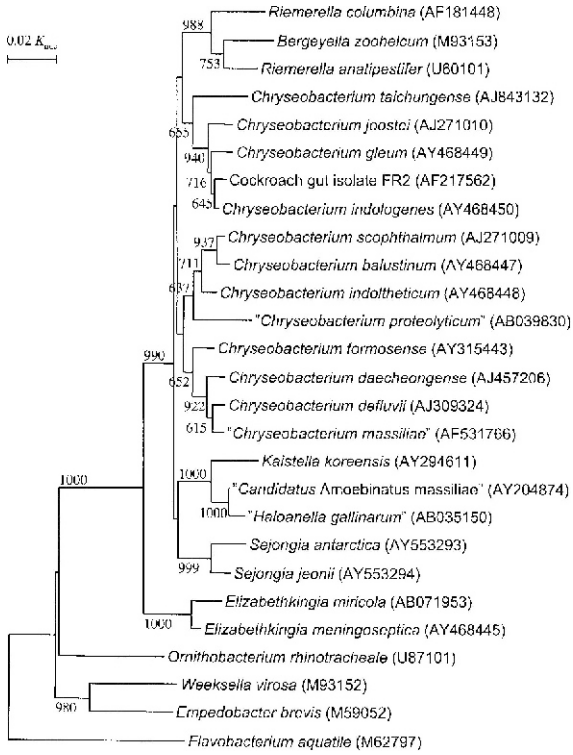

Fig. 1. Phylogenetic relationships among representatives of the genera *Chryseobacterium* and *Elizabethkingia* and of related organisms established on 16S rRNA gene sequence comparisons using the neighbor-joining method (Saitou and Nei, 1987). Sequences were taken from the GenBank nucleotide database; accession numbers of the sequences are given in parentheses. Scale bar, 0.02 *Knuc* (Kimura, 1980). The numbers on the branches represent the confidence limits (expressed as percentages rounded up to whole numbers) estimated by a bootstrap analysis (Felsenstein, 1985) of 1000 replicates; confidence limits less than 50% are not shown. Sequences were aligned using the CLUSTAL W version 1.8 software package (Thompson et al., 1994). The alignments were modified manually against the 16S rRNA secondary structure of *Escherichia coli* (Brosius et al., 1978). All species are included and represented by their type strain (except *Empedobacter brevis*). Representatives of invalid taxa for which the 16S rRNA sequences are available have also been included for information; their names are in quotation marks. *Flavobacterium aquatile* was used as outgroup.

quality sequences of all other organisms deposited by other authors. As can be seen by comparing the current tree with that in the chapter An Introduction to the Family Flavobacteriaceae in this Volume, the use of the new sequences actually had only a limited effect on the structure of the tree. In particular, regardless of the sequences used, *E. meningoseptica* occupies a separate position compared to all *Chryseobacterium* species, while *Bergeyella zoohelcum*, the two *Riemerella* species and members of the two recently described genera *Kaistella* (Kim et al., 2004) and *Sejongia* (Yi et al., 2005) are situated in between. Recently, *E. miricola* has linked to *E. meningoseptica* in its separate position (Li et al., 2003). The splitting of the genus *Chryseobacterium* into two branches was a consequence of the DNA-rRNA hybridization technique that was extensively used for phylogenetic investigations of the genus *Flavobacterium* and related genera during the 1980s and 1990s (Bauwens and De Ley, 1981; Segers et al., 1993; Mudarris et al., 1994; Vandamme et al., 1994; Vandamme et al., 1994; Vancanneyt et al., 1996). The resolution of this technique being lower than that achieved later on by 16S rRNA gene sequencing, the respective branching of the different *Chryseobacterium* species, *B. zoohelcum* and *Riemerella anatipestifer* was not accurately determined (Vandamme et al., 1994). Since distinct phenotypic and chemotaxonomic characteristics were found to differentiate all members of the new genus *Chryseobacterium* from *B. zoohelcum* and *R. anatipestifer* (see the sections Taxonomy and Identification in this Chapter), the organism known at that time as [*Flavobacterium*] *meningosepticum* was included in this genus although its separate position compared to other *Chryseobacterium* species was already recognized (Vandamme et al., 1994). When high-quality 16S rRNA gene sequencing finally replaced DNA-rRNA hybridization for phylogenetic studies, the separate position of *Chryseobacterium meningosepticum* became even more obvious. When a phylogenetic tree is drawn using the few sequences of the DNA gyrase large subunit (*gyrB*) gene available in this group of organisms (i.e., *E. meningoseptica*, *C. indologenes*, *B. zoohelcum*, *E. brevis* and *W. virosa*), the overall structure of the tree is similar to that of the 16S rRNA tree, except that *E. meningoseptica* is grouped with *C. indologenes* and separate from *B. zoohelcum* (Suzuki et al., 1999). The determination of the *gyrB* sequences of the other species will be necessary to assess whether the grouping (based on the sequences of a few species only) is actually sound.

Because *Chryseobacterium*, *Elizabethkingia*, *Kaistella*, *Sejongia*, *Bergeyella* and *Riemerella* species form a distinct separate branch, comparative studies of strains belonging to this group of organisms should preferably include reference strains of all of them. The invalid organism "*Haloanella gallinarum*", of which no isolates but only the 16S rRNA gene sequence is available, is also located between *Elizabethkingia* and *Chryseobacterium* species. *Ornithobacterium rhinotracheale*, *Empedobacter brevis* and *Weeksella virosa* are the closest phylogenetic neighbors of this large cluster, and the branch grouping all these organisms occupies the lowest position in the phylogenetic tree of the family Flavobacteriaceae (see the chapters An Introduction to the

Family Flavobacteriaceae, The Genera *Empedobacter* and *Myroides*, The Genera *Bergeyella* and *Weeksella*, and Capnophilic Bird Pathogens in the Family Flavobacteriaceae: *Riemerella*, *Ornithobacterium* and *Coenonia* all in this Volume).

Taxonomy

As shown in Table 1, the genus *Chryseobacterium* currently contains ten valid species; an additional species, "*C. proteolyticum*," has been published outside the *International Journal of Systematic and Evolutionary Microbiology* and has not been included yet in a validation list; the two "*C. proteolyticum*" strains are not available. The new genus *Elizabethkingia* only contains two species.

In addition to the *Chryseobacterium*, *Elizabethkingia*, *Kaistella*, *Sejongia*, *Bergeyella* and *Riemerella* species (see the section Phylogeny in this Chapter), phylogenetic studies showed that the *Chryseobacterium* branch also comprises two *Candidatus* (a provisional category created to classify organisms for which 16S rRNA gene sequences are available but that are not yet cultivated and described; Murray and Stackebrandt, 1995) species recovered from human nasal samples (Greub et al., 2004; Drancourt et al., 2004) and "*Haloanella gallinarum*," whose 16S rRNA gene sequence is deposited in databases (Fig. 1; see the section Pathogenicity and Epidemiology in this Chapter).

The reasons for creating a new genus to accommodate most of the organisms previously attributed to the genus *Flavobacterium* have been explained elsewhere (Vandamme et al., 1994; Bernardet et al., 1996) and will only be summarized here. After successive emendations, the genus *Flavobacterium* had been restricted to nonmotile and nongliding species (Holmes et al., 1984). The genus was restricted even further when it was recognized that the type species, *F. aquatile*, did not represent the genus and was consequently set aside in Holmes' next taxonomic review, i.e., in the second edition of *The Prokaryotes* (The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* in this Volume; see also Holmes, 1993). Following a decision of the Judicial Commission of the International Committee on Systematic Bacteriology, *F. aquatile* had to remain the type species of the genus *Flavobacterium* even though the genus had been thoroughly emended (Bernardet et al., 1996; see the chapter The Genus *Flavobacterium* in this Volume). Hence, the other bacterial species previously attributed to the genus *Flavobacterium* had to be relocated in other or new genera. Among the four natural groups of species recognized by Holmes in the second edition of *The Prokaryotes* on the basis of habitat, resistance to antimicrobial agents, production of yellow pigment and indole, oxidation of carbohydrates, and proteolytic activity, the groups B, C, and D rapidly became (or already were) the cores of the new genera *Myroides* (Vancanneyt et al., 1996), *Sphingobacterium* (Yabuuchi et al., 1983), and *Weeksella* and *Bergeyella* (Holmes et al., 1986a; Holmes et al., 1986b; Vandamme et al., 1994), respectively (see the chapters The Genera *Empedobacter* and *Myroides*, The Genera *Bergeyella* and *Weeksella*, and The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* all in this Volume).

Holmes' group A comprised [*Flavobacterium*] *balustinum*, [*F.*] *breve*, [*F.*] *indoltheticum*, [*F.*] *meningosepticum* (which grouped strains previously known as CDC group IIa), and the CDC group IIb defined by King (1959). The heterogeneity of the latter group had long been established, and [*F.*] *indologenes* (in spite of rather low levels of DNA relatedness between the strains; Yabuuchi et al., 1983) and [*F.*] *gleum* (Holmes et al., 1984) were among its members. Some authors also considered [*F.*] *balustinum* and [*F.*] *indoltheticum* to belong to group IIb (Hugo et al., 1999). However, many other strains in CDC group IIb could not be assigned to any of these four species; some of these strains formed small genomic groups that could be the cores of new species (Ursing and Bruun, 1991).

Phylogenetic studies by Vandamme et al. (1994) demonstrated: 1) [*F.*] *breve* occupied a separate position compared to other organisms in Holmes' group A; as a consequence, the generic epithet *Empedobacter* was revived for this species and the new combination *Empedobacter brevis* was established. 2) Other organisms in group A formed a tight cluster for which the new generic epithet *Chryseobacterium* was proposed and joined in the new combinations *C. balustinum*, *C. gleum*, *C. indologenes* and *C. indoltheticum*; [*F.*] *meningosepticum* in spite of its separate position was also proposed for inclusion in the genus *Chryseobacterium*. 3) The recently described fish pathogen [*F.*] *scophthalmum* (Mudarris et al., 1994) belonged to the same cluster and was consequently renamed *Chryseobacterium scophthalmum*. 4) *Riemerella anatipestifer* and *Weeksella zoohelcum* occupied separate positions at the base level between *E. meningoseptica* and the other *Chryseobacterium* species; however, their chemotaxonomic and other phenotypic characteristics were different enough from those of members of the genus *Chryseobacterium* to warrant their separate generic status. And 5) *W. zoohelcum* was only distantly related to the type species of the genus *Weeksella*, *W. virosa*; hence, the new generic

Table 1. Currently recognized species classified in the genera *Chryseobacterium* and *Elizabethkingia*.[a]

Species[b]	Type strain	G+C (mol%)	Source	Reference(s)	Number of strains
Chryseobacterium balustinum[AL,c]	NCTC 11212	33	Heart blood of freshwater fish (dace, *Leuciscus leuciscus*), Dordogne, France, 1959	Brisou et al., 1959 Holmes et al., 1984a	1, 3, ?
Chryseobacterium daecheongense	DSM 15235	37	Sediment, Lake Daecheong, Korea	Kim et al., 2005	1
Chryseobacterium defluvii[d]	DSM 14219	Not determined	Activated sewage sludge, Germany	Kämpfer et al., 2003	1
Chryseobacterium formosense	CIP 108367	Not determined	Rhizosphere of lettuce, Kuohsing, Taiwan	Young et al., 2005	1
Chryseobacterium gleum[T,AL,e]	ATCC 35910	37	Human vaginal swab, United Kingdom, 1979	Holmes et al., 1984b	12, 9, 15
Chryseobacterium indologenes[AL,f]	NCTC 10796	38	Human trachea at autopsy, 1958	Yabuuchi et al., 1983	13, 22, 65
Chryseobacterium indoltheticum[AL,g]	ATCC 27950	34	Marine mud	Campbell and Williams, 1951	2, 3, ?
Chryseobacterium joosteti[h]	LMG 18212	37	Raw cow milk, Kwazulu-Natal, South Africa, 1981	Hugo et al., 2003	11, 11, 11
"Chryseobacterium proteolyticum"	NIBHT P17664	37	Soil, rice field, Tsukuba, Japan	Yamaguchi and Yokoe, 2000	2, 0, 2
Chryseobacterium scophthalmum[i]	CCM 4109	34	Gills of diseased turbot (*Scophthalmus maximus*), Scotland, 1987	Mudarris et al., 1994	50, 7, 50
Chryseobacterium taichungense	CIP 108519	Not determined	Tar-contaminated soil, Taichung, Taiwan	Shen et al., in press	1
Elizabethkingia miricola[j]	JCM 11413	35	Condensation water, space station Mir, 1997	Li et al., 2003 Kim et al., in press	1, 2, 2
Elizabethkingia meningoseptica[AL,k]	ATCC 13253	37	Human cerebrospinal fluid, United States, 1949	King, 1959 Holmes et al., 1984a Kim et al., in press	?, 20, 150

Abbreviations: [T], type species; [AL], the species is cited on the Approved Lists of Bacterial Names (Skerman et al., 1980; Moore et al., 1985); ATCC, American Type Culture Collection, Manassas, Va., USA; CIP, Collection de l'Institut Pasteur, Paris, France; CCM, Czech Collection of Microorganisms, Brno, Czech Republic; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; JCM, Japanese Collection of Microorganisms, Tokyo, Japan; LMG, BCCM/LMG bacteria collection, Laboratorium voor Microbiologie, University of Gent, Gent, Belgium; NCTC, National Collection of Type Cultures, London, England; NIBHT, culture collection of the National Institute of Bioscience and Human Technology, Tsukuba, Japan.

[a]Names in quotation marks have not been validly published. Accession number is that in the recognized culture collection in which the type strain was first deposited. The origin and G+C content are those of the type strain. When more than one strain are known, three numbers are given, i.e., the number of strains on which the original description was based, the number of strains currently available in the largest recognized culture collections (i.e., ATCC, DSM, LMG, NCIMB [National Collection of Industrial, Marine and Food Bacteria, Aberdeen, Scotland] and NCTC), and the approximate number of strains which have been included in further studies and which are or may still be maintained in specialized culture collections, respectively.

[b]Previous names and corrected epithets of species (taken from Euzéby, 1997; List of Bacterial Names with Standing in Nomenclature) are as stated in footnotes c to k.

[c][*Flavobacterium*] *balustinum* Harrison 1929, "[*Empedobacter*] *balustinum*" Brisou et al. 1959.

[d]*Chryseobacterium defluvium*, name as deposited in the 16S rRNA sequence databases.

[e][*Flavobacterium*] *gleum* Holmes et al. 1984.

[f][*Flavobacterium*] *indologenes* Yabuuchi et al. 1983.

[g][*Flavobacterium*] *indoltheticum* Campbell and Williams 1951, "*Beneckea indolthetica*" (Campbell and Williams 1951) Campbell 1957.

[h]*Chryseobacterium joosteii*, name as deposited in the 16S rRNA sequence databases.

[i]"[*Cytophaga*] *scophthalmis*", name as listed in 1989 in the catalogue of strains of the Czech Collection of Microorganisms, [*Flavobacterium*] *scophthalmum* Mudarris et al. 1994.

[j][*Chryseobacterium*] *miricola*, name as deposited in the 16S rRNA sequence databases, [*Chrysebacterium*] miricola Li et al., 2003.

[k][*Flavobacterium*] *meningosepticum* King 1959, [*Chryseobacterium*] *meningosepticum* (King 1959) Vandamme et al. 1994.

epithet *Bergeyella* and the new combination *B. zoohelcum* were proposed for this species.

To minimize the inconvenience for clinical microbiologists, the etymology of the new generic epithet *Chryseobacterium* (a golden yellow rod) was chosen to be as similar as possible to that of *Flavobacterium* (a yellow rod). For the same reason, the name "[*Flavobacterium*] *aureum*" had previously been proposed, although not validly published, for CDC group IIb by Price and Pickett (1981). *Chryseobacterium balustinum* (Harrison, 1929) and *C. indoltheticum* (Campbell and Williams, 1951), the two oldest species, could not be selected as the type species of the genus *Chryseobacterium* owing to their meager characterization and to the fact that they were each represented by a single strain. *Chryseobacterium meningosepticum*, although clinically important and well characterized, was also not a good candidate since it was the most aberrant member of the genus. Both the phenotypic and genomic heterogeneity of *Chryseobacterium indologenes* (initially proposed to encompass all CDC group IIb strains) were known since its original description (Yabuuchi et al., 1983). *Chryseobacterium gleum* was chosen as the type species since its description was based on firm phenotypic and genomic grounds following an extensive comparative study of 12 strains (Holmes et al., 1984). As a matter of fact, since the identification of differentiating characteristics for *C. indologenes*, *C. gleum*, and the related strains was (and still is) problematic (see the section Identification in this Chapter) and since several genomic groups have been found within group IIb in addition to these two species (Ursing and Bruun, 1991), some authors proposed to continue referring to this taxon by the designation of "*Chryseobacterium* sp. CDC group IIb" (Ursing and Bruun, 1991; see the chapter The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* in this Volume).

Several new *Chryseobacterium* species have been described since the genus was established: "*C. proteolyticum*" (Yamaguchi and Yokoe, 2000), *C. defluvii* (Kämpfer et al., 2003), *C. joostei* (Hugo et al., 2003), [C.] *miricola* (Li et al., 2003), *C. daecheongense* (Kim et al., 2005a), *C. formosense* (Young et al., 2005), and *C. taichungense* (Shen et al., 2005) (see Table 1).

Recently, Kim et al. (2005b) performed a polyphasic study of several [C.] *meningoseptica* strains and of the only [C.] *miricola* strain available. All the techniques used confirmed the separate position of these two species compared to all *Chryseobacterium* species. Hence, the new genus *Elizabethkingia* was proposed to accomodate these two species (Kim et al., 2005b). Since the more simple epithets derived from the family name of Elizabeth O. King, who first described the bacterium (King, 1959), were already attributed to other bacteria or to various eukaryotes (J. Euzéby, personal communication), the epithet was based on her two names. Admittedly, this new nomenclatural change is a kind of tongue-twister and an additional inconvenience for clinical microbiologists after the change from *Flavobacterium* to *Chryseobacterium* proposed in 1994 for this clinically important organism (Vandamme et al., 1994). However, the study by Kim et al. (2005b) only confirmed and substantiated previous reports on the phenotypic and genomic discrepancies between *E. meningoseptica*/*E. miricola* and the other *Chryseobacterium* species, and some kind of decision had to be taken. Importantly, the study by Kim et al. (2005b) also resulted in the identifion as *E. miricola* of a strain previously considered *E. meningoseptica*, thus making a second strain of the former species available.

Habitat and Ecology

The variety of ecological niches represented by members of the genera *Chryseobacterium* and *Elizabethkingia* (Table 1) reflects that found in the whole family Flavobacteriaceae (see Table 1 in the chapter An Introduction to the Family Flavobacteriaceae in this Volume): they are widely distributed in nature and consequently found in clinical specimens and various food products (Jooste and Hugo [1999] and references therein). However, most recent surveys using a variety of techniques (either culture-independent or requiring previous bacterial isolation) to unravel the phylogenetic composition of complex bacterial communities were more generally aimed at revealing members of the phylum "Bacteroidetes", or at best, members of the family Flavobacteriaceae (see the chapters An Introduction to the Family Flavobacteriaceae and The Genus *Flavobacterium* both in this Volume). Some of these studies revealed the phylogenetic diversity of bacterial populations in Antarctic sea ice, Antarctic lakes, and mangrove environments by sequencing the 16S rRNA gene of many isolates; they identified several members of the genus *Flavobacterium* in these biotopes but failed to reveal any strain affiliated to the genus *Chryseobacterium* (Bowman et al., 1997; Nakagawa et al., 2001; Van Trappen et al., 2002). Similarly, members of the family Flavobacteriaceae were found in various soil and freshwater environments following the sequencing of cloned polymerase chain reaction (PCR)-amplified 16S rRNA genes, but no strain related to *Chryseobacterium* was found (Borneman et al., 1996; Crump et al., 1999).

Other studies used oligonucleotide probes for either in situ hybridization or probing of a 16S rDNA clone library to investigate bacterial communities. The 16S rRNA gene sequence of *Chryseobacterium* strains and related organisms was specifically taken into account in designing some of these probes, and their specific hybridization to reference strains of *Chryseobacterium* spp., *Bergeyella zoohelcum* and *Riemerella anatipestifer* was controlled (Weller et al., 2000). Bacterial strains with 16S rRNA sequences highly homologous to the same sequences in these species were subsequently detected in a river (Manz et al., 1996; O'Sullivan et al., 2002).

The main environments in which *Chryseobacterium* strains are known to occur are listed below. Although recent publications usually refer to *Chryseobacterium* strains specifically, many earlier reports should be interpreted with care since they deal with poorly identified "*Flavobacterium*" strains that might actually have belonged to other taxa in the family Flavobacteriaceae.

Environmental Isolates

Some members of the CDC group IIb, later described as *C. indologenes* and *C. gleum* (see the section Taxonomy in this Chapter), were found to occur in soil and water by Tatum et al. (1974). In his extensive phenotypic study of Gram-negative yellow-pigmented rods originating from various sources, Hayes (1977) delineated nine different phena. According to Owen and Holmes (1981), members of Hayes' phenon 1 could be attributed to the CDC group IIb and, although most members of this phenon were isolated from meat (see below), some originated from river water and soil samples (Hayes, 1977). *Chryseobacterium indologenes* strains recently recognized from their 16S rRNA gene sequence in soil samples in Indonesia and Spain have been shown to degrade various toxic compounds (Radianingtyas et al., 2003; Lopez et al., 2004; see the section Applications in this Chapter). The only bona fide *C. indoltheticum* strain available in culture collections was isolated from marine mud, but the exact location is unknown. Although most strains of the human pathogen *E. meningoseptica* were isolated from clinical specimens, strains isolated from soil or river water were also included in some studies (Owen and Holmes [1981] and references therein; Bruun, 1982; Bruun and Ursing, 1987). A *E. meningoseptica* strain (16S rRNA gene sequence no. AJ616734) was isolated from a eutrophic shallow lake in Eastern Asia (G. Wu et al., unpublished data). The description of "*Chryseobacterium proteolyticum*" was based on two strains retrieved from the soil of a rice field and from the bank of a brook in Japan (Yamaguchi and Yokoe, 2000) and *C. daecheongense* was isolated from the sediment of a shallow, freshwater lake in Korea (Kim et al., 2005). The recently described species *C. formosense* and *C. taichungense* were isolated from the rhizosphere of garden lettuce and from a sample of tar-contaminated soil, respectively, both in Taiwan (Young et al., 2005; Shen et al., 2005). In a recent survey of carbohydrate-degrading bacteria in various biotopes of subtropical islands, strains identified as *Chryseobacterium* spp. from their 16S rRNA gene sequences were found to predominate in terrestrial environments (Rosado and Govind, 2003). Among the very diverse bacterial community recovered from samples of penguin guano collected in Antarctica, several strains were related to *C. indologenes* according to the results of phenotypic tests and comparison of 16S rRNA gene sequences (Zdanowski et al., 2004). Interestingly, the same kind of habitat also harbored members of the recently described genus *Sejongia*, one of the close phylogenetic neighbors of the genera *Chryseobacterium* and *Elizabethkingia* (Yi et al., 2005). A *Chryseobacterium* sp. belonging to CDC group IIb was part of the normal microbiota associated with marigold flowers (Luis et al., 2004). Four *Chryseobacterium* sp. and "*Haloanella gallinarum*" strains were recovered among the biofilm microflora of a domestic sink drain (McBain et al., 2003), and *E. meningoseptica* strains were also isolated from the slimy brown deposit swabbed inside the spout of sink taps in a hospital during an outbreak (Hoque et al., 2001). Several *Chryseobacterium* strains were identified among the culturable bacterial population in the groundwater of a municipal water supply in Germany (Ultee et al., 2004). According to the results of several commercial identification galleries, *Chryseobacterium* spp. were also among the most common potentially pathogenic bacteria isolated from samples of treated and untreated drinking water in South Africa (Pavlov et al., 2004).

Since members of most *Chryseobacterium* and *Elizabethkingia* species exhibit varying degrees of tolerance to NaCl, as shown by their ability to grow on Difco Marine agar 2216 (1.95% NaCl; Bernardet et al., 2002; see the sections Isolation and Cultivation and Physiology in this Chapter), the environmental strains may occur both in freshwater and seawater, as well as in freshwater and marine fish (see below). In contrast with many *Flavobacterium* species recently recovered from cold environments (see the chapter The Genus *Flavobacterium* in this Volume), most *Chryseobacterium* species were not considered psychrophilic, except those associated with food and dairy products (see below). Also in contrast

to *Flavobacterium* strains, the role played in the degradation of biomacromolecules by the *Chryseobacterium* strains that occur in soil and water is not known.

Fish-associated members of the genera *Chryseobacterium* and *Elizabethkingia* have been repeatedly reported. They may represent pathogenic (see the section Pathogenicity and Epidemiology in this Chapter) or spoilage (see below in this section) organisms. They may also belong to the normal bacterial flora that colonize the mucus at the surface of the skin and gills and the intestine of healthy fish. Moreno et al. (1999) investigated the microflora in the mucus of farmed eels (*Anguilla anguilla*) using fluorescent in situ hybridization; however, among the range of oligonucleotide probes they used, the one targeting the "*Cytophaga-Flavobacterium* cluster" was not specific enough to distinguish the *Chryseobacterium* strains from related organisms. The identification techniques, namely fatty acid analysis and carbohydrate utilization profiles, used by Nedoluha and Westhoff (Nedoluha and Westhoff, 1997a; Nedoluha and Westhoff, 1997b) also proved inadequate to specifically detect *Chryseobacterium* strains in the skin, gills and intestine of farmed striped bass (*Morone saxatilis*) reared in three different aquaculture systems, although members of the phylum "Bacteroidetes" were indeed detected. The eel harboring the *Chryseobacterium* strain studied by Lijnen et al. (2000) was apparently healthy (I. Roelents, personal communication); hence, the strain was presumably a part of its normal microflora or a transient colonizer. Similarly, there was no mention of any disease in the salmon (*Oncorhynchus keta*) harboring the intestinal *C. balustinum* strain investigated by Morita et al. (1997). During a survey of bacteria associated with the skin mucus of farmed fish in Brittany (France), 10 *Chryseobacterium* sp. strains were identified from their phenotypic characteristics among 49 bacterial isolates (C. Michel and J.-F. Bernardet, unpublished data).

Recently, *Chryseobacterium* strains were also recognized from the gut of insects. After sequencing of 16S rDNA cloned directly from the gut content of an American cockroach (*Periplaneta americana*) revealed an organism affiliated to the family Flavobacteriaceae, a number of strains phenotypically resembling *C. indologenes* were isolated and characterized; the 16S rRNA gene sequence of one isolate proved highly related to those of the *C. indologenes* and *C. gleum* reference strains (Dugas et al., 2001). This organism, which was not found in the food supply, was considered an abundant and permanent symbiont of the cockroach. Investigating the influence of food-derived tannins on the gut microbiota of the larvae of the lepidopteran *Acentria ephemerella*, Walenciak et al. (2002) characterized a number of bacterial isolates from their 16S rRNA gene sequences; one of these gut isolates belonged to the genus *Chryseobacterium*. *Chryseobacterium* spp. were the most frequent organisms among the 36 bacterial genera identified from their 16S rRNA gene sequences in the midgut bacterial community of the biting mosquito *Culicoides variipennis* (Campbell et al., 2004). Members of the *Chryseobacterium*/*Elizabethkingia* group may also be associated with amoebae: approximately 50% of the free-living but potentially pathogenic amoebae in a Polish lake harbored endosymbiotic bacteria, including *E. meningoseptica* (Hadas et al., 2004).

Some *Chryseobacterium* strains were also recovered from particular industrial environments. For instance, *C. defluvii* was described from the bacterial flora in activated sludge (Kämpfer et al., 2003) and two *Chryseobacterium* sp. strains were detected among the complex filamentous microflora that occurs in paper mill slimes and frequently affects machine efficiency and paper quality (Oppong et al., 2003). A *E. meningoseptica* strain (16S rRNA gene sequence no. AY509953, E. Chicote et al., unpublished data) was isolated from spent nuclear fuel pools. The original strain of the recently described species *E. miricola* originated from the condensation water in a very special closed environment, the space station Mir (Li et al., 2003). Recently, the misidentified *E. meningoseptica* strain ATCC 33958 was attributed to *E. miricola* (Kim et al., 2005b); it was one of the bacterial species contaminating a commercial preparation of carboxypeptidase A (Elder and Alexander, 1982).

Food Isolates

Early studies indicated that isolates closely related to the CDC group IIb (see the section Taxonomy in this Chapter) had been isolated from a variety of food products such as milk and dairy products, canned products, poultry and poultry plants, meat and meat products, and vegetables during commercial processing (McMeekin et al., 1971; Hayes, 1977; McMeekin and Shewan, 1978; Holmes et al., 1984). Because the taxonomy of the flavobacteria has changed so drastically over the past few years, however, it is difficult to determine whether these isolates belong to the genera *Chryseobacterium* and *Elizabethkingia* or to another genus in the family Flavobacteriaceae (see the chapter An Introduction to the Family Flavobacteriaceae in this Volume). This is still true for some of the studies published nowadays in which the identification of the isolates is based on meager data. The

discussion that follows will focus on food products in which known and suspected *Chryseobacterium*/*Elizabethkingia* isolates played a role.

FISH *Chryseobacterium balustinum* has been isolated first from the scales of freshly caught halibut (*Hippoglossus hippoglossus*) in the Pacific Ocean (Harrison, 1929; Brisou et al., 1959); since this organism produced a yellowish slime on the skin, it was considered a fish spoilage agent rather than a pathogen (Austin and Austin, 1999). The only strain currently available in culture collections, however, may actually be pathogenic (see the section Pathogenicity and Epidemiology in this Chapter). Although recently isolated again from the skin and muscle of wild and farmed freshwater fish, *C. balustinum* was not regarded as an important contributor of the spoilage of the fish because of its low incidence (<1% of all isolates; González et al., 2000). The five *C. balustinum* strains, identified following a rather extensive phenotypic characterization, were not found in freshly caught fish but in fish stored more than three days in melting ice.

Gennari and Cozzolino (1989) isolated 39 strains of flavobacteria from the skin and gills of fresh and ice-stored Mediterranean sardines (*Sardina pilchardus*). Analysis of their phenotypic traits, however, could not place the isolates in any known species of the flavobacteria, but the authors found that four strains had characteristics resembling those of Holmes' group A; most of the species in this group nowadays are known as *Chryseobacterium* (see the section Taxonomy in this Chapter). The other 35 strains resembled members of the genus *Myroides* (see the chapter The Genera Empedobacter and Myroides in this Volume). In contrast to the finding of González et al., most of the flavobacteria isolates were from fresh sardines and their number decreased with time of storage in ice. Since proteolytic activity was found, the authors speculated that flavobacteria were involved in proteolytic spoilage of fresh fish early during chill storage, especially when chilling was slow or the temperature not sufficiently low. The contribution of flavobacteria to fish spoilage, however, was regarded as low compared to that of the other pigmented bacteria retrieved from the same fish (Gennari and Cozzolino, 1989).

Various proteolytic activities (i.e., degradation of skim milk, casein, and gelatin on agar media) and H_2S production were also found in *Chryseobacterium balustinum*, *C. gleum* and *C. indologenes* strains isolated from Cape marine fish in South Africa (*Empedobacter*, *Myroides* and *Weeksella* strains were also retrieved from the same fish; Engelbrecht et al., 1996). When off-odor production of the *Chryseobacterium* strains was evaluated on fish muscle extract, four of the eight *C. balustinum* strains were found to produce a pungent odor, two, a stale odor, and two, no odor. Four among the five *C. gleum* strains produced a stale odor, and one *C. indologenes* strain produced a fruity odor (Engelbrecht et al., 1996). These isolates were thought to be contaminants introduced during processing operations.

It is not known whether members of the genera *Chryseobacterium* and *Elizabethkingia* may occur in biofilms growing on the surface of seafood processing equipment as was shown for their close relative *Myroides odoratus* (Tide et al., 1999; Bremer et al., 2001).

MEAT PRODUCTS Flavobacteria have been frequently isolated from meat and poultry products (García-López et al., 1998), although they have seldom been accurately identified. When Hayes (1977) divided a large collection of flavobacteria and related Gram-negative yellow-pigmented rods into nine phena, the first five phena were found to belong to the genus *Flavobacterium*. In a study by Owen and Holmes (1981), the conclusion was drawn that Hayes' phenon 1 corresponded closely to CDC group IIb; some members of this taxon were later attributed to *C. indologenes* and *C. gleum* (see the section Taxonomy in this Chapter). The 53 strains in this phenon were isolated from the following sources: raw beef carcasses (6 isolates), raw lamb carcasses (7 isolates), raw pig carcasses (8 isolates), raw eviscerated chicken carcasses (13 isolates), raw milk (13 isolates), water from rivers or streams (2 isolates), and soil (3 isolates). Thus, *Chryseobacterium* strains clearly are found in a variety of meat products, but no mention was made about their role in these products.

POULTRY Recent studies, performed on poultry and on the air of poultry processing establishments, indicated the presence of *Flavobacterium* species (Geornaras et al., 1996; Ellerbroek, 1997). The identification procedures of these two studies were, however, insufficient to conclude whether these organisms belonged to the genus *Chryseobacterium* or to one of the other genera in the family Flavobacteriaceae.

DAIRY PRODUCTS The flavobacteria have long been known for their psychrotrophic growth and their potential to spoil milk and dairy products through proteolytic and lipolytic activities (Gilmour and Rowe, 1981; Cousin, 1982). They may also be associated with the presence of off-flavors in milk products, changes in the manufacturing characteristics of milk as a substrate for starter cultures, and even reduced

cheese yield (Cousin, 1982; Ellis and Marth, 1984; Jooste et al., 1986a).

In the review by Cousin (1982), *Flavobacterium* was listed as one of the psychrotrophic genera whose members were frequently isolated from raw and pasteurized milk as well as butter. Many studies were listed in this review (see Table 3 of Cousin, 1982) but, again, attribution of the flavobacteria reported in these studies to the genus *Chryseobacterium* or to other members of the family Flavobacteriaceae could not be determined.

Research in the Department of Food Science at the University of the Free State in South Africa has for a long time been focused on flavobacteria isolated from milk and dairy products. Jooste et al. (1985) were the first to isolate *Flavobacterium* CDC group IIb strains from milk and butter. In a subsequent study (Jooste et al., 1986a), it was suggested that these *Flavobacterium* species cause putrefaction in salted butter by growing in cream prior to churning. In another study in which *Flavobacterium* CDC group IIb and *C. balustinum* strains were isolated, Jooste et al. (1986b) found that the practical importance of dairy flavobacteria lies as much in their psychrotrophic growth and consequent proteinase production in refrigerated milk as in their contamination of milk via poorly sanitized pipelines and equipment. The numbers of flavobacteria isolated from raw milk were fairly constant during winter and summer (Fischer et al., 1987). A study by Welthagen and Jooste (1992) indicated that CDC group IIb isolates comprised the largest part of pigmented bacteria from raw milk. In subsequent investigations (Hugo and Jooste, 1997; Hugo et al., 1999), a large group of the CDC group IIb milk isolates evaluated in the abovementioned studies were identified as *C. indologenes* and one isolate as *C. gleum*. Among the remaining milk isolates, two new genomic groups (including the new species *C. joostei*; Hugo et al., 2003) were identified. A considerable number of the milk isolates could not be allocated to known or new species, however, and consequently remained classified as *Chryseobacterium* species CDC group IIb (Hugo et al., 1999). In a study by Venter et al. (1999), a metalloprotease from a strain of *C. indologenes* was purified and characterized; this protease was very heat-stable and its affinity for casein may play a role in the spoilage of milk and milk products.

OTHER PRODUCTS A *Chryseobacterium* sp. strain sharing a high degree of 16S rRNA gene sequence similarity with *C. indoltheticum* was identified in maple sap (Lagacé et al., 2004). The role of this organism in the contamination and quality of maple products has not been investigated.

Veterinary and Human Clinical Isolates

Chryseobacterium and *Elizabethkingia* strains have been isolated from a number of diseased animal species. Briefly, *E. meningoseptica* strains have been recovered from frogs (Chung, 1990; Green et al. [1999] and references therein; Mauel et al., 2002), turtles (Jacobson et al., 1989), birds (Vancanneyt et al., 1994), cats (Sims, 1974), and a dog (Bruun and Ursing, 1987). *Chryseobacterium indologenes* has also been reported from diseased frogs (Olson et al., 1992). *Chryseobacterium balustinum* and *C. scophthalmum* (Harrison, 1929; Brisou et al., 1959; Mudarris and Austin, 1989; Mudarris et al., 1994) and many strains affiliated to the CDC group IIb (J.-F. Bernardet et al., unpublished data) were isolated from diseased fish.

In humans, the most significant species is *E. meningoseptica*. Although historically associated with meningitis in premature neonates, it is now known to occur in several other types of infections. Members of the CDC group IIb have also been retrieved from a number of human cases; they were considered to be the most frequently isolated clinical flavobacteria (Holmes and Owen, 1981; Shewan and McMeekin, 1983). Even though there is usually no clear evidence of the pathogenic role of the *Chryseobacterium* and *Elizabethkingia* species other than *E. meningoseptica*, they do raise some concern since *Chryseobacterium*/*Elizabethkingia*-related infections frequently occur in immunocompromised patients and are resistant to a wide range of commonly used antimicrobial agents. Because their natural habitat is water, they may also be retrieved from hospital environments.

Extensive information on animal and human pathogenicity of members of the genera *Chryseobacterium* and *Elizabethkingia* is given in the section Pathogenicity and Epidemiology.

Isolation and Cultivation

Members of the genera *Chryseobacterium* and *Elizabethkingia* are generally not difficult to isolate and cultivate, since they are classic aerobic chemoorganotrophs. Hence, most of them grow readily on commercially available organic media, usually making unecessary the use of selective media and the addition of growth factors. The procedures used to isolate and cultivate the bacterial species now classified in the genera *Chryseobacterium* and *Elizabethkingia* were described in the previous editions of *Bergey's Manual* (Holmes et al., 1984) and *The Prokaryotes* (the chapter on The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* in this Volume). More recently, the general isolation and

maintenance procedures for members of the family Flavobacteriaceae were reviewed by Jooste and Hugo (1999). The same authors more specifically reviewed these procedures for the *Chryseobacterium* species that are usually associated with food (i.e., *C. balustinum*, *C. gleum*, *C. indologenes* and *C. joostei*; Hugo and Jooste, 2003; see the section Habitat and Ecology in this Chapter). Such classic media as brain heart infusion, nutrient, Mueller Hinton, and trypticase soy were all found suitable. Interestingly, although only two *Chryseobacterium* species originated from marine environments, most species exhibit a rather high degree of halotolerance and are consequently also able to grow on Difco marine 2216 agar (1.95% NaCl among several other salts; Bernardet et al., 2002); *C. balustinum* is the only known exception, but the growth of *C. defluvii*, "*C. proteolyticum*," *C. daecheongense*, *C. formosense* and *C. taichungense* on this medium has not been tested (Table 2). Conversely, the *Chryseobacterium* strains from marine origin grow as well on the above-mentioned non-marine media as on marine 2216 agar (J.-F. Bernardet, unpublished data). Strains of *E. meningoseptica* are also able to grow on marine 2216 agar but the growth of *E. miricola* on this medium has not been tested. *Chryseobacterium indoltheticum* was originally isolated on a chitin-containing media in the course of a study aimed at retrieving chitin-degrading bacteria from marine environments (Campbell and Williams, 1951), but it was later shown to grow well on other media. Similarly, "*C. proteolyticum*" was isolated on a medium that favored the growth of protein-deaminating enzyme producers from soil, but the strains were later maintained on nutrient agar (Yamaguchi and Yokoe, 2000). The isolation of *C. defluvii* was performed on R2A agar (Oxoid); the same medium, as well as nutrient and trypticase soy agars was used for further studies (Kämpfer et al., 2003). After an isolation step on several different media, *E. miricola* was cultivated on brain heart infusion agar (Li et al., 2003). *Chryseobacterium daecheongense*, *C. formosense* and *C. taichungense* were originally isolated on nutrient agar (Kim et al., 2005; Young et al., 2005; Shen et al., 2005). Most *Chryseobacterium* and *Elizabethkingia* species tested do not grow or grow poorly on cetrimide agar while the ability to grow on MacConkey agar varies among species (Table 2); most species, except *C. scophthalmum*, also grow on β-hydroxybutyrate (without producing β-hydroxybutyrate inclusion granules); *C. defluvii*, "*C. proteolyticum*," *C. daecheongense*, *C. formosense*, *C. taichungense* and *E. miricola* have not been tested for this characteristic.

The most often used temperature range for incubation is 20–30°C (Hugo and Jooste, 2003). Very poor growth or no growth at all occurs at 5°C, while members of four among the eleven *Chryseobacterium* species and of the two *Elizabethkingia* species are able to grow at 37°C (Table 2). At 42°C, most strains will not grow, except some *C. gleum* and some *E. meningoseptica* strains (J.-F. Bernardet, unpublished data). *Chryseobacterium gleum* strains were reported to grow at 41°C in contrast with *C. indologenes* strains (Yabuuchi et al., 1983). Approximately half of the *E. meningoseptica* strains studied by Bruun and Ursing (1987) were able to grow at 40°C; this trait was characteristic for strains derived from invasive neonatal infections. Good growth was reported for *C. daecheongense*, *C. formosense* and *C. taichungense* at 28–37°C, 25–32°C and 11–36°C, respectively (Kim et al., 2005; Young et al., 2005; Shen et al., 2005).

Fish-pathogenic members of the genera *Chryseobacterium* and *Elizabethkingia* (see the chapter Pathogenicity and Epidemiology) may be isolated from samples of external lesions or internal organs inoculated on one or several of the above-mentioned media and incubated at 20–25°C. A casein-containing medium (medium K) was first used to isolate and cultivate *C. scophthalmum* (Mudarris and Austin, 1989), but later studies demonstrated that this species grows equally well and even better on marine 2216, nutrient, and trypticase soy agars (Mudarris et al., 1994; J.-F. Bernardet, unpublished data).

The distinctive bright yellow to orange pigmentation and the shiny appearance of the colonies of members of the genus *Chryseobacterium* allow the selection of putative *Chryseobacterium* strains from agar plates with mixed bacterial growth, e.g., from external lesions of fish or from soil or water samples. Enhanced production of yellow pigment by strains of *C. daecheongense*, *C. formosense* and *C. taichungense* occurs on nutrient agar (Kim et al., 2005a; Young et al., 2005; Shen et al., 2005). The yellow pigmentation of the colonies of *C. defluvii* is weaker than that of the other *Chryseobacterium* species. This is also true for *Elizabethkingia miricola*, while the colonies of *E. meningoseptica* may be weakly pigmented or non-pigmented depending on the strains (see the section Identification in this Chapter).

Specific information on the isolation and cultivation of clinical *Chryseobacterium* and *Elizabethkingia* isolates is given in the section Pathogenicity and Epidemiology.

Identification

Characteristics which differentiate members of the genera *Chryseobacterium* and *Elizabethkingia* from members of other genera in the family Flavobacteriaceae are listed in Table 2 of the

Table 2. Differentiating characteristics of *Chryseobacterium* and *Elizabethkingia* species and closely related taxa.

Characteristic	*C. balustinum*	*C. daecheongense*	*C. defluvii*	*C. formosense*	*C. gleum*	*C. indologenes*	*C. indoltheticum*	*C. joostei*	*"C. proteolyticum"*	*C. scophthalmum*	*C. taichungense*	*E. meningoseptica*	*E. miricola*	*Bergeyella zoohelcum*	*Riemerella anatipestifer*	*Riemerella columbina*	*Kaistella koreensis*	*Sejongia Antarctica*	Sejongia jeonii
Habitat	Diseased freshwater fish	Sediment, freshwater lake	Activated sludge	Soil	Diseased humans, water, soil and hospital environments	Diseased humans, water, soil and hospital enviroments	Sea water, marine mud	Raw cow milk	Soil	Seawater, healthy and diseased marine fish	Soil	Diseased humans and animals, water, soil and hospital environments	Condensation water, contaminated commercial enzyme	Oral and nasal cavities of dogs and cats; diseased humans, dogs and cats	Respiratory tract of various healthy and diseased bird species (except pigeon)	Respiratory tract of diseased pigeons	Freshwater stream	Soil	Soil
Production of yellow flexirubin type pigment	+	+	(+)[a]	+	+	+	+	+	+	+	+	–[b]	–	–	–[c]	–[c]	+[a]	+[a]	+[a]
Capnophilic metabolism	–	ND	–	ND	–	–[d]	–	–	–	–	ND	–	–[e]	–	+	+	ND	–	–
Growth on																			
Marine agar 2216	–	ND	ND	ND	+	(+)	+	(+)	ND	+	ND	+	ND[f]	–	–	(+)	ND	+	+
Cetrimide agar	–	ND	ND	ND	–	(+)	–	(+)	ND	–	ND	(+)	(+)	–	–	–	ND	–	–
MacConkey agar	+	–	–	–	v	v	+	+	–	–	–	v	+	–	–	–	–	–	–
Growth at (°C)																			
5	(+)	–	–	–	–	–	+	+	ND	v	–	–	–	–	–	–	–	+	+
37	–	+	+	–	+	+	–	–	+	–	+	+	+	+	+	+	+	¬	¬
42	–	–	ND	–	v[g]	–[g]	–	–	–	–	–	v	–	–[h]	+[i]	+	v	–	–
Degradation of																			
Esculin	+	+	+	+	+[g]	+[g]	+	+	+	+	+	+[i]	+	–	–	+	+	+	+
DNA	+	ND	ND	ND	+	+	+	+	+	+	ND	+	+	–	–	–	ND	+	+
Starch	(+)	+	ND	ND	v	+	–	+	+	–	ND	–	v	–	+	–	–	+	+
Tween 80	+	+	ND	–	+	+	+	+	+	+	ND	v	+	–	ND	ND	ND	+	+

Tyrosine	(+)	ND	ND	ND	v	–	+	+	+	+	ND	(+)	–	$\underline{v}$	–	–	ND	(+)	–
Urea	–	–	–	–	$\underline{v}^{g}$	$\underline{v}^{g}$	–	v^{h}	–	+	–	$–^{h}$	+	+	v	v	v	–	–
Production of																			
Hydrogen sulphide	$\underline{v}$	–	ND	–	$\underline{v}$	+	+	–	(+)	–	–	–	$\underline{v}$	–	–	ND	–	–	–
Indole	+	–	+	+	+	+	+	+	+	–	(+)	$+^{i}$	v	+	v	–	+	+	+
β-Galactosidase	–	–	ND	–	v^{i}	–	–	–	+	+	(+)	+	+	–	–	–	–	–	–
L-Phenylalanine deaminase	–	ND	ND	ND	$\underline{v}^{g}$	$–^{g}$	–	–	–	v	ND	(+)	–	–	+	+	ND	+	+
Precipitate on 10% egg yolk agar	–	ND	ND	ND	+	+	+	+	ND	+	ND	–	+	$–^{h}$	–	–	ND	+	+
Reduction of																			
Nitrate	$\underline{v}$	+	–	–	v	v	–	–	–	–	–	–	–	–	–	–	v	–	–
Nitrite	–	–	ND	ND	v	v	–	–	–	–	ND	v	–	–	ND	ND	ND	–	–
Acid production from																			
L-Arabinose	–	–	–	–	$\underline{v}^{g,i}$	$–^{g}$	–	–	+	–	–	$–^{h}$	–	–	–	–	ND	–	–
Cellobiose	–	+	(+)	–	–	–	–	–	–	–	–	$\underline{v}$	–	–	–	–	ND	–	–
Ethanol	+	–	ND	ND	$\underline{v}^{g,h}$	$–^{g}$	–	–	–	+	ND	v	ND	–	–	–	ND	ND	ND
D-Fructose	+	+	ND	ND	+	(+)	–	+	ND	–	ND	$+^{i}$	+	–	(+)	(+)	ND	–	–
D-Glucose	+	–	+	(+)	+	+	+	+	+	–	(+)	+	+	–	+	+	–	+	+
Glycerol	–	+	ND	ND	v	(+)	–	v^{i}	(+)	–	ND	$+^{i}$	v	–	–	–	ND	ND	ND
Lactose	–	–	–	ND	–	–	–	–	–	–	–	v	+	–	–	–	ND	–	–
Maltose	–	–	+	–	+	+	+	+	+	–	(+)	+	+	–	+	+	ND	+	+
D-Mannitol	–	–	–	–	–	–	–	v^{i}	(+)	–	–	$+^{i}$	+	–	–	–	ND	–	–
Salicin	–	–	–	ND	$\underline{v}^{g}$	$–^{g}$	–	–	–	–	–	–	–	–	–	–	ND	ND	ND
Sucrose	v	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	ND	–	–
Trehalose	–	+	+	+	+	+	–	+	+	+	(+)	$+^{i}$	+	–	–	–	ND	–	–
D-Xylose	–	+	–	(+)	$\underline{v}^{g}$	$–^{g}$	–	–	ND	–	(+)	$–^{h}$	–	–	–	–	ND	–	–

Table 2. *Continued*

Characteristic	*C. balustinum*	*C. daecheongense*	*C. defluvii*	*C. formosense*	*C. gleum*	*C. indologenes*	*C. indoltheticum*	*C. joostei*	*"C. proteolyticum"*	*C. scophthalmum*	*C. taichungense*	*E. meningoseptica*	*E. miricola*	*Bergeyella zoohelcum*	*Riemerella anatipestifer*	*Riemerella columbina*	*Kaistella koreensis*	*Sejongia Antarctica*	Sejongia jeonii
Utilization of malonate	–	–	–	–	–	+	ND	–	–	ND	–	–	–	–	–	–	ND	ND	ND
Alkaline reaction on Christensen's citrate	–	ND	ND	–	v	+	v	v[i]	ND	+	–	v	+	–	–	–	ND	–	–
Range of G+C content (mol%)	35	37	ND	ND	36–39	37–39	34	36–37	37	33–35	ND	36–38	35	35–37	33–35	36–37	41–42	34	36

Symbols and abbreviations: +, positive reaction; –, netagive reaction; (+) weak or delayed reaction; v, varies within species; v, varies between references; and ND, not determined.

Since some of the characteristics listed were tested by several authors using different methods, the original publications should be consulted for direct comparison.

[a]The type of yellow pigment in *C. defluvii* and *K. koreensis* has not been determined (Kämpfer et al., 2003; Kim et al., 2004); the yellow pigment produced by *Sejongia* strains is not of the flexirubin type (Yi et al., 2005).

[b]*Elizabethkingia meningoseptica* strains either are not pigmented or produce a weak yellow nondiffusible pigment (e.g., the type strain; Bruun and Ursing, 1987). Members of all *Chryseobacterium* species produce a yellow to orange nondiffusible flexirubin type pigment.

[c]Strains of *Riemerella anatipestifer* and *R. columbina* grown on trypticase soy agar produce a light yellow-brown non-flexirubin pigment which diffuses in the agar and gives a beige color to the colonies (J.-F. Bernardet, unpublished data). On Columbia blood agar, colonies of *R. columbina* are grayish-white to beige, whereas those of *R. anatipestifer* are nonpigmented (Vancanneyt et al., 1999).

[d]*Chryseobacterium indologenes* strains can grow under anaerobic conditions in the presence of fumarate (Yabuuchi et al., 1983). This trait has not been tested in other *Chryseobacterium* species.

[e]The type strain of *Elizabethkingia miricola* can grow in the presence of 5% (v/v) carbon dioxide, although it has a strictly respiratory type of metabolism (Li et al., 2003). This trait has not been tested in *Elizabethkingia meningoseptica* and in *Chryseobacterium* species.

[f]Although its growth on marine agar has not been tested, the *Elizabethkingia miricola* type strain has been shown to tolerate seawater (Li et al., 2003).

[g]According to Yabuuchi et al. (1990), the strains of *Chryseobacterium gleum* and *C. indologenes* may be distinguished by their positive and negative reaction, respectively, for the following tests: growth at 41°C, production of phenylalanine deaminase, acid production from L-arabinose, ethanol, salicin, and D-xylose, hydrolysis of esculin after 4 h (strains of both species hydrolyze esculin after 24 h), and production of urease on Christensen's urea agar slant after 40 h. According to Ursing and Bruun (1991), members of *C. gleum* and of one allied genomic group could be differentiated from other CDC group IIb strains by their ability to grow at 40°C and to produce acid from arabinose, xylose and salicin.

[h]Most strains are negative for this characteristic.

[i]Most strains are positive for this characteristic.

[j]The two *Sejongia* species also differ by their ability to grow at 30°C (positive for *S. jeonii*, negative for *S. antarctica*).

Data taken from Bruun (1982); Yabuuchi et al. (1983); Yabuuchi et al. (1990); Holmes et al. (1984); Holmes et al. (1986b); Bruun and Ursing (1987); Ursing and Bruun (1991); Segers et al. (1993); Mudarris et al. (1994); Vandamme et al. (1994); Vancanneyt et al. (1999); Yamaguchi and Yokoe (2000); Hugo et al. (2003); Kämpfer et al. (2003); Li et al. (2003); Kim et al. (2004, 2005a,b); Yi et al. (2005); Young et al. (2005), J.-F. Bernardet (unpublished data); and Shen et al. (2005).

Table 3. Fatty acid composition (%) of *Chryseobacterium* and *Elizabethkingia* species and related bacteria.[a]

Fatty acid	*C. balustinum (1)*	*C. daechengense (1)*	*C. defluvii (1)*	*C. formosense (1)*	*C. gleum (5)*	*C. indologenes (45)*	*C. indoltheticum (1)*	*C. joostei (11)*	*C. scophthalmum (2)*	*C. taichungense (1)*	*E. meningoseptica (5)*	*E. miricola (2)*	*Bergeyella zoohelcum (1)*	*Riemerella anatipestifer (16)*	*Riemerella columbina (13)*	*Kaistella koreensis (3)*	*Sejongia antarctica (1)*	*Sejongia jeonii (1)*
12:0 *iso*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	tr	1
13:0 *iso*	tr	2	3	4	tr	tr	ND	tr	tr	tr	1	2	2	15	10	10	3	3
13:0 *anteiso*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2	ND	3	4
14:0 *iso*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1	2	2	5
Unknown 13.566[b]	2	2	tr	tr	1	2	2	1	3	7	2	2	2	2	ND	ND	tr	tr
15:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2	ND	3	2
15:0 2-OH	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1	ND	2	2
15:0 *iso*	32	51	59	52	35	34	29	35	35	35	44	46	48	53	45	50	14	12
15:0 *iso* 3-OH	3	2	3	2	3	3	2	3	3	4	3	3	4	8	4	3	1	1
15:0 *anteiso*	tr	1	3	2	tr	tr	6	tr	tr	tr	1	1	ND	6	22	13	15	24
15:1 *anteiso* A	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	7	ND
16:0	2	2	1	2	1	tr	1	tr	1	1	tr	1	tr	tr	ND	tr	ND	ND
16:0 3-OH	1	tr	tr	tr	1	1	tr	1	1	3	3	3	ND	ND	ND	ND	ND	ND
16:0 *iso*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	3	6
16:0 *iso* 3-OH	tr	tr	tr	1	ND	ND	1	ND	ND	1	tr	tr	ND	1	ND	2	5	9
16:1 *iso* H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	4	9
Unknow 16.580[b]	1	1	tr	1	2	2	1	2	2	2	2	1	1.4	tr	ND	ND	tr	tr
17:0 2-OH	tr	ND	tr	ND	ND	ND	3	ND	tr	ND	ND	ND	ND	ND	1	ND	3	2
17:0 *iso*	1	3	2	2	2	tr	tr	tr	tr	tr	tr	tr	ND	ND	ND	ND	ND	ND
17:0 *iso* 3-OH	17	16	14	11	22	19	14	20	16	22	15	15	13.5	13	7	9	6	4
17:1 *iso* ω 9c	27	8	5	4	20	24	26	23	25	9	8	7	17.5	ND	ND	6	21	9
17:1 *anteiso* ω 9c	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	3	2
18:1 *ω 5c*	tr	ND	ND	ND	tr	tr	tr	tr	tr	ND	tr	tr	1.5	ND	ND	ND	2	tr
Summed feature 4[c]	9	10	8	7	12	11	11	12	12	14	20	17	9	ND	3	3	3	3
Summed feature 5[c]	tr	ND	ND	ND	tr	tr	ND	tr	tr	ND	tr	ND	2	ND	ND	ND	ND	ND

Abbreviations: tr, trace (less than 1%); and ND, not detected.

[a]The fatty acid composition of "*Chryseobacterium proteolyticum*" has not been determined (Yamaguchi and Yokoe, 2000). Fatty acid percentages amounting to less than 1% of the total fatty acids in all species were not included; therefore, the percentages do not total 100%. When several strains were analyzed, rounded up means are given but standard deviations are not given; the number of strains analyzed is given in parentheses after the name of each species. Data taken from studies using different growth times, media and temperatures.

[b]Unknown fatty acid; numbers indicate equivalent chain length.

[c]Fatty acids that could not be separated by gas chromatography using the Microbial Identification System (Microbial ID) software were considered summed features. Summed feature 4 contains 15:0 *iso* 2-OH and/or 16:1ω7*c* and/or 16:1ω7*t*. Summed feature 5 contains 17:1 *iso* I and/or 17:1 *anteiso* B.

Modified from Hugo et al. (2003) and taken from Segers et al. (1993), Vancanneyt et al. (1999), Kämpfer et al. (2003), Li et al. (2003), Kim et al. (2004), Kim et al. (2005a,b), Yi et al. (2005), Young et al. (2005), Kim et al. (in press), and Shen et al. (2005).

chapter An Introduction to the Family Flavobacteriaceae in this Volume (see also Bernardet et al., 2002). Those characteristics that differ among species in the genus *Chryseobacterium* are listed in Table 2 (physiological and biochemical traits) and Table 3 (fatty acid composition) in the present chapter. Since *Bergeyella zoohelcum*, *Kaistella koreensis*, the two *Sejongia* species, the two *Riemerella* species and all members of the genera *Chryseobacterium* and *Elizabethkingia* form a distinct separate branch in phylogenetic trees (see the sections Phylogeny and Taxonomy in this Chapter), all these taxa have been included in Tables 2 and 3 for convenience.

The following description of the genus *Chryseobacterium*, based on the six species known at that time (i.e., including [*C.*] *meningosepticum* and [*C.*] *miricola*), is cited from Vandamme et al. (1994):

Description of *Chryseobacterium* gen. nov. *Chryseobacterium* (Chry.se.o.bac.te'ri.um. Gr. adj. *chryseos*, golden; Gr. neut. n. *bakterion*, a small rod; N. L. neut. n. *Chryseobacterium*, a yellow rod) cells are gram-negative, nonmotile, non-

spore-forming rods with parallel sides and rounded ends; typically the cells are 0.5 μm wide and 1 to 3 μm long. Intracellular granules of poly-β-hydroxybutyrate are absent. Aerobic. Chemoorganotrophic. All strains grow at 30°C; most strains grow at 37°C. Growth on solid media is typically pigmented (yellow to orange), but nonpigmented strains occur. Colonies are translucent (occasionally opaque), circular, convex to low convex, smooth, and shiny, with entire edges. Positive for catalase, oxidase, and phosphatase activities. Several carbohydrates, including glycerol and trehalose, are oxidized. Strong proteolytic activity occurs. Esculin is hydrolyzed. Agar is not digested. Resistant to a wide range of antimicrobial agents. Branched-chain fatty acids (i.e., 15:0 *iso*, *iso* 17:1ω9*c*, 17:0 *iso* 3-OH, and summed feature 4 [15:0 *iso* 2-OH or 16:1ω7*t* or both] are predominant (Segers et al., 1993). Sphingophospholipids are absent. Menaquinone 6 is the only respiratory quinone. Homospermidine and 2-hydroxyputrescine are the major polyamines in *Chryseobacterium indologenes*, whereas putrescine and agmatine are minor components (Hamana and Matsuzaki, 1991).

The type species is *Chryseobacterium gleum* comb. nov. The DNA base composition ranges from 33 to 38 mol% guanine plus cytosine. *Chryseobacterium* species are widely distributed in soil, water, and clinical sources.

The description of *Chryseobacterium balustinum* comb. nov. (basonym, *Flavobacterium balustinum* Harrison 1929), *Chryseobacterium gleum* comb. nov. (basonym, *Flavobacterium gleum* Holmes, Owen, Steigerwalt, and Brenner 1984), *Chryseobacterium indologenes* comb. nov. (basonym, *Flavobacterium indologenes* Yabuuchi, Kaneko, Yano, Moss, and Miyoshi 1983), *Chryseobacterium indoltheticum* comb. nov. (basonym, *Flavobacterium indoltheticum* Campbell and Williams 1951), *Chryseobacterium meningosepticum* comb. nov. (basonym, *Flavobacterium meningosepticum* King 1959), and *Chryseobacterium scophthalmum* comb. nov. (basonym, *Flavobacterium scophthalmum* Mudarris, Austin, Segers, Vancanneyt, Hoste, and Bernardet 1994) are the same as the original descriptions given in Harrison (1929), Holmes et al. (1984), Yabuuchi et al. (1983), Campbell and Williams (1951), King (1959), and Mudarris et al. (1994), respectively, as well as in Holmes et al. (1984).

Since 1994, seven additional species have been described, i.e., "*Chryseobacterium proteolyticum*" (Yamaguchi and Yokoe, 2000), *C. defluvii* (Kämpfer et al., 2003), *C. joostei* (Hugo et al., 2003), [C.] *miricola* (Li et al., 2003), *C. daecheongense* (Kim et al., 2005a), *C. formosense* (Young et al., 2005), and *C. taichungense* (Shen et al., 2005). The new genus *Elizabethkingia* was recently proposed to accomodate one of these new species (*E. miricola*) and one of the *Chryseobacterium* species included in the original description of the genus, [C.] *meningosepticum* (now the type species of the genus *Elizabethkingia*). Since most of the characteristics of the newly described *Chryseobacterium* species fitted the description of the genus, they did not make an emendation of its original description necessary. However, some additional information and slight discrepancies with the description are given below in the order of the above description. The formal description of the genus *Elizabethkingia* was given by Kim et al. (2005b). Since most phenotypic characteristics of members of the genera *Chryseobacterium* and *Elizabethkingia* are very similar, and since they were previously grouped in the genus *Chryseobacterium*, they will be dealt with together below. Those few characteristics which differentiate members of the two genera are listed in Table 2 in this chapter and in Kim et al. (2005b).

Concerning first the morphological characteristics, some *Chryseobacterium* strains may also produce filaments in addition to rods in liquid culture (e.g., *C. scophthalmum*, Mudarris et al., 1994). Electron microscopy studies have seldom been performed on *Chryseobacterium* strains; the presence of a thick cell wall (i.e., about 50 nm) was reported in *C. joostei* (Hugo et al., 2003) and *C. scophthalmum* (Mudarris et al., 1994). Cells of "*C. proteolyticum*" were studied using scanning electron microscopy, but no particular structure was evidenced (Yamaguchi and Yokoe, 2000). The production of extracellular slimy substances after prolonged incubation has been reported in *C. defluvii* (Kämpfer et al., 2003) and *C. formosense* (Young et al., 2005); it is likely responsible for the mucoid consistency of colonies after three days incubation reported in *C. daecheongense* (Kim et al., 2005a) and *C. formosense* (Young et al., 2005a) and in *Elizabethkingia* species (Kim et al., 2005). Colonies of *E. miricola* are very sticky on solid media (Kim et al., 2005). Some *E. meningoseptica* strains are encapsulated, especially following passage in mice (Holmes et al., 1984). A strong odor, reminiscent of that given off by some *Empedobacter*, *Myroides* and *Sphingobacterium* strains, is produced by most *Chryseobacterium* strains in liquid and solid culture; it has been described as "fruity" (Yabuuchi et al., 1983; Yabuuchi et al., 1990) or "cheesy" (Brisou et al., 1959). Other types of odor were reported when food products were spoiled by *Chryseobacterium* strains (Engelbrecht et al., 1996; see the section Habitat and Ecology in this Chapter).

As shown in Table 2, strains of *C. balustinum*, *C. indoltheticum*, *C. joostei*, *C. scopthalmum* and *C. formosense* do not grow at 37°C. The nondiffusible, nonfluorescent bright yellow to orange pigments produced by all *Chryseobacterium* strains belong to the flexirubin type (Reichenbach et al., 1981; Yabuuchi et al., 1983; Holmes et al., 1984; Mudarris et al., 1994; Yamaguchi and Yokoe, 2000; Hugo et al., 2003; Kim et al., 2005; Young et al., 2005; Shen et al., 2005; J.-F. Bernardet, unpublished data; see below). The sentence in the original description mentioning that nonpigmented *Chryseobacterium* strains occur was actually referring to *Elizabethkingia meningoseptica* strains and should be deleted. The type of yellow pigment in

C. defluvii has not been determined; contrary to the colonies of members of other *Chryseobacterium* species, those of *C. defluvii* were described as yellowish, not bright yellow (Kämpfer et al., 2003). The production of pigment may depend on the culture medium (Hugo and Jooste, 2003); it may also increase at low temperatures, in the presence of daylight, and by such compounds as casein, milk and starch (Holmes et al., 1984). While the original *E. miricola* strain was described as white-yellow (Li et al., 2003), the production of yellow pigment is strain-dependent in *E. meningoseptica*: 20 among the 52 strains studied by Bruun and Ursing (1987) were completely colorless while the other strains (including the type strain) produced a weak yellow pigment. Flexirubin type pigments were not detected in *E. meningoseptica* and *E. miricola* strains using the KOH technique (Kim et al., 2005b). However, these authors flooded the colonies with the KOH solution directly on agar, a procedure which makes weak color shifts difficult to detect. The procedure recommended in the minimal standards for describing new taxa in the family Flavobacteriaceae (Bernardet et al., 2002) was used on *E. meningoseptica* strains grown for several days at 37°C on trypticase soy agar. Three strains, including the type strain, produced pale cream-beige colonies that turned to a brownish color when flooded with 20% KOH, contrary to nine other strains whose colonies exhibited a weaker pigmentation; the pigmentation of the colonies and the color shift in the presence of KOH were stronger when the strains were grown in an illuminated incubator (J.-F. Bernardet, unpublished data). Hence, it seems some *Elizabethkingia* strains may indeed produce small amounts of flexirubin type pigment in the presence of light. Strains of *C. balustinum*, *C. gleum*, *C. indoltheticum*, *C. joostei* and *E. meningoseptica* also produce a pinkish-brown, diffusible pigment when grown on tyrosine agar (Hugo et al., 2003; J.-F. Bernardet, unpublished data); this characteristic is strain-dependent in *C. scophthalmum* (Mudarris et al., 1994). *Chryseobacterium daecheongense*, *C. formosense* and *C. taichungense* have not been tested for this characteristic. The production of catalase has not been tested in *C. defluvii*, *C. formosense* and *C. taichungense*, but *C. daecheongense* was shown to produce catalase (Kim et al., 2005). The phosphatase activity has not been searched for in *C. defluvii*, *E. miricola* (Li et al., 2003), and "*C. proteolyticum*" (Yamaguchi and Yokoe, 2000); this activity has been detected in *C. joostei* (Hugo et al., 2003). Although all *Chryseobacterium* species are able to oxidize some carbohydrates, several of them are actually unable to oxidize glycerol and/or trehalose, contrary to the statement in the description (Table 2). *Chryseobacterium indoltheticum* is the only *Chryseobacterium* species tested which is able to degrade chitin (Campbell and Williams, 1951; J.-F. Bernardet, unpublished data). The resistance to many antimicrobial agents of members of most *Chryseobacterium* and *Elizabethkingia* species has been repeatedly reported; however, data are unfortunately lacking for the recently described species "*C. proteolyticum*," *C. defluvii*, *C. daecheongense*, *C. formosense*, *C. taichungense* and *E. miricola* (Yamaguchi and Yokoe, 2000; Kämpfer et al., 2003; Li et al., 2003; Kim et al., 2005a; Young et al., 2005; Shen et al., 2005). This characteristic is discussed in the section Pathogenicity and Epidemiology. The list of predominant fatty acids given in the genus description is also valid for the new species (see below and Table 3). Homospermidine is the major polyamine in all *Chryseobacterium* species tested, but other polyamines may occur as minor components (see below). The range of DNA G+C content for the genus should be extended to 39 mol%, a value reached by some *C. gleum* and *C. indologenes* strains (Hugo et al., 1999). The base composition of *C. formosense* and *C. taichungense* has not been determined. Food products, particularly dairy products, should be added to the list of sources of members of the genus *Chryseobacterium* (Hugo et al., 1999; see the section Habitat and Ecology in this Chapter).

The meager original descriptions of *C. balustinum* (Harrison, 1929) and *C. indoltheticum* (Campbell and Williams, 1951) have actually not been taken into consideration in the formal genus description quoted above. There exists no *C. balustinum* strain dating back to the original isolation and description. The only bona fide strain currently available in culture collections, isolated much later from a freshwater fish in France, was attributed to this bacterial species because of an overall phenotypic similarity with Harrison's description; however, even the description of this isolate is imperfect (Brisou et al., 1959). The original description of *C. indoltheticum* was based on two isolates, one of which is the type and only bona fide strain currently available. Surprisingly, the original strains were described as "motile by means of peritrichous flagella" (Campbell and Williams, 1951); the type strain is nonmotile, however (Holmes et al., 1984). Hence, the characteristics currently taken into consideration for these two species actually result from later studies (Yabuuchi et al., 1983; Holmes et al., 1984; Holmes et al., 1984; J.-F. Bernardet, unpublished data; and the chapter The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* in this Volume).

In the minimal standards proposed for describing new genera and species in the family Flavobacteriaceae (Bernardet et al., 2002), par-

ticular methods were suggested to determine some of the phenotypic properties which can be used to differentiate members of the genera *Chryseobacterium* and *Elizabethkingia* from members of the other genera in the family and to differentiate among *Chryseobacterium* and *Elizabethkingia* species. These methods should preferably be used since the test conditions are critical. Some additional practical information is given below for the most problematic or delicate among those tests.

Detection of the Gliding Motility and Determination of the Pigment

The absence of gliding motility and the presence of flexirubin type pigments in members of the genus *Chryseobacterium* are two of the key characteristics which differentiate them from members of several other genera in the family Flavobacteriaceae. Information on the appropriate methods to detect gliding motility in liquid and solid cultures and to determine the type of yellow pigments was given in Bernardet et al. (2002) and in the chapters An Introduction to the Family Flavobacteriaceae and The Genus *Flavobacterium* in this Volume.

The pigments of *Chryseobacterium* strains have been identified as belonging to the flexirubin type (Reichenbach et al., 1981; see above) usually using the simple KOH test; the pigments in *C. indologenes* strains, however, have also been identified using a more sophisticated spectrophotometric method following extraction by different organic solvants (Yabuuchi et al., 1983).

The case of *C. scophthalmum* clearly illustrates the difficulty in detecting gliding motility, as well as the taxonomical and nomenclatural consequences a mistaken detection may have had before the advent of molecular methods. In the course of the original study, the fresh isolates were judged to exhibit gliding motility (Mudarris and Austin, 1989), and the new bacterial species was consequently deposited under the name "[*Cytophaga*] *scophthalmis*" in a culture collection. Gliding motility could not be confirmed, however, during a subsequent examination. The cultures were consequently considered nonmotile, and hence the new species was formally described as [*Flavobacterium*] *scophthalmum* (Mudarris et al., 1994). It was finally attributed to the genus *Chryseobacterium* after the extensive emendation of the genus *Flavobacterium* (Vandamme et al., 1994; Bernardet et al., 1996).

Production of β-D-Galactosidase

Precautions should be taken when detecting the presence of β-D-galactosidase, as it has been shown that results may differ depending on the type of test used. Nine *C. gleum* strains gave a positive result when tested using the conventional ONPG test (i.e., *o*-nitrophenyl-β-D-galactopyranoside on filter paper discs), whereas the production of enzyme was not detected on the substrate 2-naphthyl-β-D-galactopyranoside in API ZYM galleries (Holmes et al., 1984). Bruun and Ursing (1987) reported similar discrepancies between the two tests in a study of 52 *E. meningoseptica* isolates; similar to the ONPG test (Bülow, 1964), the Rosco enzyme assay system yielded positive results for all strains. The type strains of all *Chryseobacterium* and *Elizabethkingia* species and all milk isolates tested by Hugo et al. (2003) were also found negative for the production of β-D-galactosidase in API ZYM galleries and positive in the ONPG test. In an ongoing study of 70 *Chryseobacterium* fish isolates and reference strains, discrepancies in the detection of β-D-galactosidase were also noticed between the different tests used: the test included in API 20NE galleries (using *p*-nitrophenyl-β-D-galactopyranoside [PNPG] as the substrate) detected 14 positive strains. The ONPG disc test and the corresponding cupule in API 20E galleries (both using ONPG as the substrate) detected 10 positive strains; only 6 positive strains were detected using the test in API ZYM galleries (J.-F. Bernardet, unpublished data).

Acid Production from Carbohydrates

Those carbohydrates from which acid is produced are considered differentiating characteristics between *Chryseobacterium* and *Elizabethkingia* species (Table 2). The production of acid from carbohydrates should preferably be tested in ammonium salt medium (Bernardet et al., 2002). The discrepancies between the results obtained in different studies are probably at least partly due to the use of different media and incubation conditions. For instance, all three *C. gleum* strains studied by Yabuuchi et al. (1990) were able to produce acid from L-arabinose, ethanol, salicin and D-xylose. Ursing and Bruun (1991) also found all or most of their 9 strains positive or at least weakly positive for these tests, whereas all or most of the 12 *C. gleum* strains studied by Holmes et al. (1984) were considered negative for these characteristics.

Production of L-Phenylalanine Deaminase

The technique of Richard and Kiredjian (1995) has been recommended to detect the production of phenylpyruvic acid from L-phenylalanine (Bernardet et al., 2002). Briefly, 1 ml of very dense bacterial suspension is mixed with an equal volume of an aqueous, filter-sterilized solution of L-phenylalanine (0.5 g per 100 ml). After

stirring, the suspension is distributed in three tubes (0.5 ml per tube) and continuously stirred and maintained at room temperature. After 30 min, 4 h, and 18 h, 1 drop of $FeCl_3$ (2 volumes of distilled water and 1 volume of $FeCl_3$ solution, d = 1.26; the $FeCl_3$ solution included in the API galleries kit is convenient) is added to one of the tubes; a dark green color (which decreases after a few minutes) reveals the production of L-phenylalanine deaminase (Richard and Kiredjian, 1995). The intensity of the green color allows for a rough evaluation of the amount of L-phenylalanine deaminase produced, but weak reactions may be difficult to detect. Moreover, the reaction may be positive and subsequently negative; when this occurs, the strain should be considered positive (M. Kiredjian, personal communication). Using this technique, reference strains of all *Chryseobacterium* species were negative for this characteristic except the *C. scophthalmum* type strain, which was strongly positive; strains of *E. meningoseptica* were weakly positive (J.-F. Bernardet, unpublished data). Strains of "*C. proteolyticum*," *C. defluvii*, *C. daecheongense*, *C. formosense* and *C. taichungense* were not tested. Most *C. scophthalmum* strains had been found to produce L-phenylalanine deaminase on phenylalanine agar (Difco; Mudarris and Austin, 1989), while the type strain was the only positive among the six strains tested using the method of Richard and Kiredjian (J.-F. Bernardet, unpublished data). This method did not detect the production of L-phenylalanine deaminase in two *C. gleum* reference strains, whereas the three strains studied by Yabuuchi et al. (1990) were positive for this characteristic using an unspecified method. The diagnostic value of this test will only be established when several strains of each *Chryseobacterium* species become available and are tested.

Commercial Identification Galleries

Commercially available kits, strips and galleries have been extensively used over the last decade to identify bacterial strains in addition to or as a substitute for conventional biochemical tests, particularly in the clinical microbiology laboratory (see the section Pathogenicity and Epidemiology in this Chapter). Since these systems are primarily aimed at the identification of human pathogens, the temperature and time of incubation need to be adapted to the requirements of *Chryseobacterium* strains that do not grow at 37°C (Table 2). API galleries (bioMérieux) have frequently been used; API 20E, API 20NE, API ZYM, and API 50CH galleries have yielded interesting results when incubated at temperatures close to the optimum of the strains tested. Only *C. indologenes* and *E. meningoseptica* are included in the analytical profile index of API 20E and 20NE galleries. In API 20NE galleries, reference strains of *C. balustinum*, *C. gleum*, *C. indoltheticum*, *C. joostei* and *C. scophthalmum* all produced similar profiles, leading to their identification as *C. indologenes* with varying degrees of similarity; the identification of *Chryseobacterium* strains using API 20NE is usually not valid before 48 h of incubation at 30°C (J.-F. Bernardet, unpublished data). API ZYM galleries have been used following the manufacturer's instructions (i.e., 4 h of incubation at 37°C) for the *Elizabethkingia* isolates and the clinical *Chryseobacterium* isolates (e.g., *C. indologenes*; Yabuuchi et al., 1983) or at lower temperature (22–25°C) and for prolonged time of incubation (12–18 h) for environmental, fish and food isolates (Mudarris et al., 1994; Hugo et al., 2003; J.-F. Bernardet, unpublished data). Results yielded by API ZYM galleries may be interpreted either using the 0–5 color intensity scale suggested by the manufacturer or simply considering all grades from 1 to 5 as positive (Bruun and Ursing, 1987). Other commercial strips such as the Rosco system (Bruun and Ursing, 1987) and the Micronaut-E gallery (Merlin; Kämpfer et al., 2003; Shen et al., 2005) were also tested with *Chryseobacterium* strains. The *E. miricola* type strain was tested using the GN2 MicroPlate (Biolog), the Nonfergram S-1 kit (Wako Chemical), and the API 20NE and API 50CH galleries, but the authors did not discuss the respective practical value and advantage of these kits (Li et al., 2003). The descriptions of *C. daecheongense*, *C. formosense* and *C. taichungense* all included data from API galleries (Kim et al., 2005a; Young et al., 2005; Shen et al., 2005). Biotype 100 galleries (bioMérieux) have been tested on a collection of *Chryseobacterium* and *Elizabethkingia* reference strains and fish isolates; all strains were able to assimilate 4–22 substrates, and 38 substrates out of 99 were assimilated by at least one strain (J.-F. Bernardet and C. Bizet, unpublished data). Many strains of each species, however, should be tested on the Biotype 100 gallery to assess its value for the identification of new isolates.

Some *Chryseobacterium* species are differentiated with difficulty using the above-mentioned phenotypic tests. For instance, although members of the CDC group IIb (see the section Taxonomy in this Chapter) are relatively easily distinguished from other *Chryseobacterium* species and from members of the genera *Elizabethkingia*, *Bergeyella*, *Riemerella*, *Empedobacter*, *Myroides* and *Weeksella* (see above), the differentiation between members of the group, particularly between *C. indologenes* and *C. gleum*, is problematic. A list of phenotypic traits distinguishing *C. indologenes* and *C. gleum* from related taxa was proposed by Yabuuchi et al. (1983) and Holmes et al. (1984), respectively.

While the latter species was comprised of strains with very similar characteristics, there were many variable characteristics among the 13 original *C. indologenes* strains. According to Yabuuchi et al. (1990), *C. gleum* could be distinguished from *C. indologenes* on the basis of growth at 41°C, production of phenylalanine deaminase, acid production from L-arabinose, ethanol, salicin, and D-xylose, hydrolysis of esculin after 4 h (strains of both species hydrolyze esculin after 24 h), and production of urease on Christensen's urea agar slant after 40 h (Table 2). As reported above, the tests for the production of phenylalanine deaminase and for the production of acid from carbohydrates are not very easy to read and may yield different results depending on the procedure used. Moreover, the study by Yabuuchi et al. (1990) was based on only three *C. gleum* strains. Studying a larger number of strains (which had previously been identified by DNA-DNA hybridization experiments), Ursing and Bruun (1991) found that *C. gleum* formed a homogeneous group, but that it was very difficult or impossible to differentiate *C. gleum* and *C. indologenes* with certainty from other CDC group IIb strains using the characteristics proposed by Holmes et al. (1984), Yabuuchi et al. (1983), and Yabuuchi et al. (1990). The results of Ursing and Bruun (1991) agreed with those of Yabuuchi et al. with regard to the acid production from xylose and salicin, but agreed more with Holmes et al. with regard to urease production and esculin hydrolysis. As a consequence, they recommended that the name *Flavobacterium* (now *Chryseobacterium*) group IIb be used pending further investigation (Ursing and Bruun, 1991). Indeed, recent studies of collections of *Chryseobacterium* group IIb isolates from dairy products (Hugo, 1997; Hugo et al., 1999) and fish (J.-F. Bernardet et al., unpublished data) faced the same problem, even when polyphasic taxonomy was used: Only some of the strains could be grouped in delineated clusters, while the others remained isolated and classified as *Chryseobacterium* sp. CDC group IIb. Discrepancies between the results of different studies may also result from the use of different techniques or conditions; for instance, the critical growth temperature proposed for differentiating between *Chryseobacterium* species was 40°C (Ursing and Bruun, 1991), 41°C (Yabuuchi et al., 1990), or 42°C (Holmes et al., 1984; Holmes et al., 1984; Vandamme et al., 1994).

The use of other, usually more sophisticated, techniques to help identify members of the family Flavobacteriaceae was discussed by Bernardet et al. (2002) and in the chapter An Introduction to the Family Flavobacteriaceae in this Volume. Additional information on the application of these techniques to the genera *Chryseobacterium* and *Elizabethkingia* is provided below.

Fatty Acid Composition

The kind of taxonomic information resulting from the analysis of whole-cell fatty acid methyl esters (FAMEs) was discussed in the chapter An Introduction to the Family Flavobacteriaceae in this Volume. Table 3 lists the fatty acids that occur in *Chryseobacterium* and *Elizabethkingia* species and in related taxa. Hugo et al. (1999) considered that the *Chryseobacterium* species could not be differentiated from each other on the basis of fatty acid profiles, while the profiles of the related species *Elizabethkingia meningoseptica*, *Bergeyella zoohelcum* and *Empedobacter brevis* are distinct. The two *Riemerella* species differ from all other species by lacking two of their major fatty acids (i.e., 17:1 *iso* ω9*c* and summed feature 4) and by the highest proportion of 13:0 *iso*. The fatty acid composition of "*Chryseobacterium proteolyticum*" has not been determined (Yamaguchi and Yokoe, 2000), but the overall fatty acid composition of the recently described *Chryseobacterium* species (i.e., *C. defluvii*, *C. joostei*, *C. daecheongense*, *C. formosense*, and *C. taichungense*) is similar to that cited in the original genus description (see above). However, *C. defluvii*, *C. daecheongense* and *C. formosense* are characterized by a low proportion of 17:1 *iso* ω9*c* and a high proportion of 15:0 *iso* (Kämpfer et al., 2003; Kim et al., 2005a; Young et al., 2005). *Elizabethkingia meningoseptica* and *E. miricola* exhibit a low 17:1 *iso* ω9*c* content but contain the highest proportion of summed feature 4 (Kim et al., 2005b). Interestingly, the results of the new fatty acid analysis performed by Kim et al. (2005b) on the two *E. miricola* strains are quite different from those of the original analysis of the type strain (Li et al., 2003). The two *Sejongia* strains differ from all other species by distinct proportions of several fatty acids and by the presence of six additional fatty acids (Yi et al., 2005). Branched-chain fatty acids account for 80% of all fatty acids in *C. indologenes* and *E. meningoseptica* (Yabuuchi et al., 1983) and for 88% in *E. miricola* (Li et al., 2003). Therefore, although fatty acid profiles for most members of the genera *Chryseobacterium* and *Elizabethkingia* are not species specific, the determination of the fatty acid profile will help assign new isolates to the genera.

Whole-cell Protein Analysis

A good correlation between high DNA relatedness and high similarity of whole-cell protein patterns was shown for several members of the family Flavobacteriaceae (Vandamme et al.,

1996; Bernardet et al., 2002; see also the chapter An Introduction to the Family Flavobacteriaceae in this Volume). Therefore, the determination of whole-cell protein profiles may help the assignment of new isolates of *Chryseobacterium* and *Elizabethkingia* spp. or delineation of new species (Vandamme et al., 1994; Bernardet et al., 2002). However, whole-cell protein profiling requires the skill and equipment for highly standardized sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), numerical analysis of the resulting electrophoregrams, and database comparison. Hugo et al. (1999) reported that, unlike fatty acid analysis, SDS-PAGE analysis yielded a clear differentiation between the investigated *Chryseobacterium* and *Elizabethkingia* species; about half of the more than 100 dairy isolates studied could be clustered into nine major groups while the other half occupied separate positions. Similar results were obtained when the whole-cell protein profiles of a collection of *Chryseobacterium* strains isolated from diseased fish were analyzed (J.-F. Bernardet et al., unpublished data). *Bergeyella zoohelcum* and the two *Riemerella* species, which are phylogenetically related to the genera *Chryseobacterium* and *Elizabethkingia* (see the section Phylogeny in this Chapter), were also clearly differentiated using this technique (Segers et al., 1993; Vancanneyt et al., 1999). The whole-cell protein profiles of "*C. proteolyticum*" (Yamaguchi and Yokoe, 2000), *C. defluvii* (Kämpfer et al., 2003), *C. daecheongense* (Kim et al., 2005a), *C. formosense* (Young et al., 2005), *C. taichungense* (Shen et al., 2005), *Kaistella koreensis* (Kim et al., 2004), *Sejongia jeonii* and *S. antarctica* (Yi et al., 2005), and *E. miricola* (Li et al., 2003) have not been determined.

Other Chemotaxonomic Markers

Like other members of the family Flavobacteriaceae, those *Chryseobacterium* and *Elizabethkingia* species investigated for these characteristics contain menaquinone 6 as their only respiratory quinone and no sphingophospholipids (Vandamme et al., 1994; Bernardet et al., 2002; Kämpfer et al., 2003; Li et al., 2003; Kim et al., 2005a,b).

Homospermidine is the major polyamine in *C. balustinum*, *C. gleum*, *C. indologenes* and *E. meningoseptica*; trace amounts of putrescine and agmatine are also detected in the three former species (Hamana and Mastuzaki, 1990), whereas diaminopropane and 2-hydroxyputrescine are minor components in *C. indologenes* (Hamana and Matsuzaki, 1991). Recently described *Chryseobacterium* species have not been investigated for their polyamine composition, except *C. defluvii* and *C. daecheongense*, which contain *sym*-homospermidine as the major component and spermine and spermidine as minor components (Kämpfer et al., 2003; Kim et al., 2005a). Contrary to the statement by Kämpfer et al. (2003), previous publications have shown that no polyamine pattern can be considered characteristic of all members of the genus *Chryseobacterium*. Homospermidine is the only polyamine in *Bergeyella zoohelcum*, *Ornithobacterium rhinotracheale*, *Riemeralla anatipestifer* and *Weeksella virosa* (Hamana and Nakagawa, 2001).

The only *Chryseobacterium* species in which the polar lipid content was determined are *C. defluvii* and *C. daecheongense*; the major lipid was phosphatidylethanolamine, but several other, unknown polar lipids were also detected (Kämpfer et al., 2003; Kim et al., 2005a).

Multilocus Enzyme Electrophoresis

Multilocus enzyme electrophoresis (MEE) was used in an attempt to classify 103 *Chryseobacterium*, *Elizabethkingia* and *Empedobacter* dairy isolates and 27 reference strains (Hugo and Jooste, 1997). The isolates were placed in seven clusters; although the technique was successful in distinguishing the *E. meningoseptica* and *Empedobacter brevis* strains, it was not able to differentiate such closely related species as *C. indologenes*, *C. gleum* and *C. balustinum* and scattered the eleven isolates that would later be attributed to the new species *C. joostei* (Hugo et al., 2003) among several different MEE clusters (Hugo and Jooste, 1997).

DNA-DNA Hybridization

DNA-DNA relatedness, determined using quantitative DNA-DNA hybridization techniques, is still the acknowledged standard for the delineation of bacterial species. When the technique used allows the thermal stability of the DNA hybrids (D*Tm*) to be determined, this value should also be taken into consideration (Wayne et al., 1987; Stackebrandt et al., 2002). The different techniques available and a review of their application to members of the family Flavobacteriaceae were discussed extensively by Bernardet et al. (2002), and only additional information (especially focused on the genera *Chryseobacterium*- and *Elizabethkingia*) will be given here. Table 4 is an attempt to compile most of the published *Chryseobacterium*- and *Elizabethkingia*-related DNA-DNA hybridization results and to compare the relatedness values yielded by different techniques.

On the whole, Table 4 shows that the 70% DNA relatedness threshold value proposed by Wayne et al. (1987) does apply to *Chryseobacterium* and *Elizabethkingia* species. Strains

Table 4. Published DNA-DNA relatedness values within the genera *Chryseobacterium* and *Elizabethkingia* and between members of the genera *Chryseobacterium* and *Elizabethkingia* and members of related genera.

Species	Reference(s) and DNA-DNA hybridization technique(s) used	Intra-specific DNA relatedness (%)	Inter-specific DNA relatedness (%)	DNA relatedness with other genera (%)
Chryseobacterium balustinum	**A**: Non-labeled DNA on filters, ^{3}H-labeled DNA probe in solution **E**: Initial renaturation rate spectrophotometric method **G**: Quantitative microplate method	NA (one strain only)	**A**: *C. balustinum /C. indologenes*: 26 **E**: *C. balustinum /C. indoltheticum*: 35 **G**: *C. balustinum /C. indologenes*: 14	**A**: *C. balustinum /Sphingobacterium* spp.: 5–7; *C. balustinum /Empedobacter brevis*: 10 **G**: *C. balustinum /Empedobacter brevis* and *Myroides odoratus*: 4–8
Chryseobacterium daecheongense	**K**: Initial renaturation rate spectrophotometric method	NA (one strain only)	**K**: *C. daecheongense /C. defluvii*: 34	ND
Chryseobacterium gleum	**A**: Non-labeled DNA on filters, ^{3}H-labeled DNA probe in solution **B** and **D**: Hydroxyapatite method **E** and **F**: Initial renaturation rate spectrophotometric method **G**: Quantitative microplate method	**B**: 60–97 at optimal temperature (60°C), 63–92 at stringent temperature (75°C) **D**: 84–100 at 60°C, 72–100 at 75°C **F**: 73–96 at 65.8°C	**A**: *C. gleum /C. indologenes*: 49 **E**: *C. gleum /C. indoltheticum*: 14; *C. gleum / C. indologenes*: 25 **G**: *C. gleum /C. balustinum*: 14; *C. gleum / C. indologenes*: 31	**B**: *C. gleum /E. meningoseptica*: 14 **B**: *C. gleum /Empedobacter brevis*: 17 **G**: *C. gleum /Empedobacter brevis* and *Myroides ororatus*: 4–7
Chryseobacterium formosense	**J**: Micro-scale hydroxyapatite method with digoxigenin labelling	NA (one strain only)	**J**: *C. formosense /C. indoltheticum, C. scophthalmum, C. joostei* and *C. defluvii*: 7–27	ND
Chryseobacterium indologenes	**A**: Non-labeled DNA on filters, ^{3}H-labeled DNA probe in solution **D**: Hydroxyapatite method **F**: Initial renaturation rate spectrophotometric method **G**: Quantitative microplate method **I**: S1 nuclease method on DE81 filters	**A**: 35–87 at 55°C **D**: 88–96 at 60°C, 78–95 at 75°C **F**: 87–94 at 65.8°C	**A**: *C. indologenes /C. balustinum*: 26 **F:** *C. indologenes /C. gleum*: 43 **I**: *C. indologenes /C. gleum* and *C. joostei*: 22–25; *C. indologenes* /other *Chryseobacterium* species: 4–10	**A**: *C. indologenes /Sphingobacterium* spp. and *Flavobacterium johnsoniae*: 3–7; *C. indologenes /Myroides ororatus*: 15; *C. indologenes /Empedobacter brevis*: 7; *C. indologenes /E. meningoseptica*: 11–13; **G**: *C. indologenes /Empedobacter brevis* and *Myroides ororatus*: 4–8
Chryseobacterium joostei	**F**:Initial renaturation rate spectrophotometric method **I**: S1 nuclease method on DE81 filters	**F**: 80–83 at 65.8°C **I**: 70–88 at 62°C	**F:** *C. joostei IC. gleum* and *C. indologenes*: 19–23; *C. joostei* /other *Chryseobacterium species*: 15–19 **I**: *C. joostei* /other *Chryseobacterium* species: 5–20	**F**: *C. joostei /E. meningoseptica*: 10

"Chryseobacterium proteolyticum"	**G**: Quantitative microplate method	94 (only two strains)	**G**: *"C. proteolyticum" /C. balustinum, C. gleum,* and *C. indologenes*: 14–17	**G**: *"C. proteolyticum" /E. meningoseptica*: 7–8; *"C. proteolyticum" /Empedobacter brevis* and *Myroides odoratus*: 3–4
Chryseobacterium scophthalmum	**E-1**: initial renaturation rate spectrophotometric method **E-2**: S1 nuclease method on DE81 filters **I**: S1 nuclease method on DE81 filters	**E-1**: 91–100 at optimal temperature (65°C) **E-2**: 85–100 at 60°C	**E-1**: *C. scophthalmum /C. indoltheticum* and *C. balustinum*: 33–44; *C. scophthalmum / C. gleum* and *C. indologenes*: 10–13 **I**: *C. scophthalmum /C. indoltheticum* and *C. balustinum*: 39–45; *C. scophthalmum /* other *Chryseobacterium* species: 3–11	**E-2**: *C. scophthalmum /Cytophaga* spp., *Flexibacter* spp., *Flavobacterium* spp., *Sphingobacterium* spp; *Tenacibaculum* spp., *Microscilla* spp., and *Pedobacter* spp.: 0–8
Chryseobacterium taichungense	**L**: Micro-scale hydroxyapatite method with digoxigenin labelling	NA (one strain only)	**L**: *C. taichungense /C. indologenes* and *C. gleum*: 25–43 *C. taichungense / C. joostei:* 11–21	ND
Elizabethkingia miricola	**H** and **M**: Quantitative microplate method	**H**: NA (one strain only) **M:** 84 (two strains)	**H**: *E. miricola /E. meningoseptica*: 52 at optimal temperature (28.5°C), 45 at stringent temperature (38.5°C) **M**: *E. miricola /E. meningoseptica*: 23–54	**H**: *E. miricola /Chryseobacterium* spp.: 20–22 at optimal temperature, 11–16 at stringent temperature
Elizabethkingia meningoseptica	**C**: Hydroxyapatite method **G** and **M**: Quantitative microplate method	**C**: Two DNA relatedness groups related at 40–55; relatedness within each group: 71–94 **M**: Four strains related at 90–100, type strain related at 31–35 to other strains	**M**: see above	**C**: *E. meningoseptica /Chryseobacterium* spp.: 12–24; *E. meningoseptica /Empedobacter brevis*: 13–24; *E. meningoseptica /Myroides odoratus* and *Sphingobacterium* spp.: 4–12 **G**: *E. meningoseptica /Chryseobacterium* spp.: 8–10; *E. meningoseptica /Empedobacter brevis*: 7; *E. meningoseptica /Myroides odoratus*: 5

Abbreviations: NA, not applicable (only one strain available); ND, not determined.

Data from Yabuuchi et al., 1983 (A); Holmes et al., 1984 (B); Ursing and Bruun, 1987 (C); Ursing and Bruun, 1991 (D); Mudarris et al., 1994 (E-1 and E-2: two different hybridization techniques were used in this study); Hugo et al., 1999 (F); Yamaguchi and Yokoe, 2000 (G); Li et al., 2003 (H); J.-F. Bernardet, unpublished data (I); Young et al., 2005 (J); Kim et al., 2004, 2005a,b (K); and Shen et al., 2005 (M). DNA-DNA hybridization data are not available for *C. defluvii* (Kämpfer et al., 2003); the one available strain of *C. indoltheticum* has not been specifically tested for its DNA relatedness to other taxa.

belonging to the same species usually share DNA relatedness well above 70% (i.e., 75–100%), whereas DNA relatedness is distinctly below this value (5–50%) when the DNAs of strains belonging to different species are hybridized. In this latter case, the level of DNA relatedness usually reflects the phylogenetic distance between the species: strains of closely related species share about 50% DNA relatedness whereas they share 10–20% relatedness with strains of other, more distant species as reported for *C. gleum-C. indologenes* (Yabuuchi et al., 1983; Hugo et al., 1999) and for *C. scopthalmum-C. balustinum-C. indoltheticum* versus the other *Chryseobacterium* species (J.-F. Bernardet, unpublished data). However, significant discrepancies may occur when different hybridization techniques and temperatures (i.e., optimal or stringent) are used (Table 4). The only study comparing two different techniques on the same group of strains was performed on seven *C. scophthalmum* strains, and nearly identical DNA relatedness values (91–100% and 85–100%, respectively) were obtained using the initial renaturation rate spectrophotometric method (Mudarris et al., 1994) and the S1 nuclease method (J.-F. Bernardet, unpublished data). Unfortunately, such comparative data are lacking between the increasingly used quantitative microplate technique and the other techniques.

The most extensive DNA-DNA hybridization study published so far concerned a collection of 52 *E. meningoseptica* strains of varied pathological and geographic origin, including the type strain and five other reference strains (Ursing and Bruun, 1987). In a previous study of the six reference strains by Owen and Snell (1976), the type strain had already been shown to share only 29–65% DNA relatedness with the others. The study by Ursing and Bruun, using the hydroxyapatite method, confirmed that the type strain is indeed only distantly related to most other *E. meningoseptica* strains. When the optimal temperature for hybridization (60°C) was used, the 52 strains could be divided into two main hybridization groups I and II, with the former only comprising the type strain and three clinical isolates. The DNA relatedness values were 71–94% within each group, about 40–55% between members of the two groups, and <25% between members of the two groups and the type strains of *C. balustinum*, *C. gleum* and *C. indologenes*. The use of a more stringent hybridization temperature (75°C) and of additional labelled DNAs and the determination of ΔTm values resulted in the further delineation of four subgroups within the largest hybridization group II (Ursing and Bruun, 1987). When DNA-DNA experiments were performed within each of the subgroups, DNA relatedness values were very high at 60°C and still well above 75% at 75°C. On the other hand, DNA relatedness values between members of the subgroups were considerably lower at 75°C than at 60°C. These data are at variance with those of a study of *C. gleum* strains in which DNA relatedness values at 75°C could be higher than those at 60°C (Holmes et al., 1984); yet, the hydroxyapatite method was used in these two studies. Although intermediate DNA relatedness and high ΔTm values were found between members of the two major *E. meningoseptica* hybridization groups, these groups were still significantly more related to each other than to *Chryseobacterium* species. Moreover, differences between members of the two groups could not be found in phenotypic characteristics (Bruun and Ursing, 1987), antimicrobial susceptibility patterns (Bruun, 1987), and crossed immunoelectrophoresis results (Bruun and Høiby, 1987). Hence, the authors found no reason to propose a new bacterial species for members of the group that did not contain the type strain (Bruun and Ursing, 1987). As mentioned above, differentiating characteristics for some members of the genus *Chryseobacterium* (e.g., the CDC group IIb) are also difficult to find, making DNA relatedness studies even more necessary. Recently, new DNA-DNA hybridization experiments have been performed on seven *Elizabethkingia* strains using a quantitative microplate method (Kim et al., 2005b). One of the *E. meningoseptica* strains was actually shown to belong to *E. miricola* owing to its high DNA relatedness with the type strain of this species. Moreover, the *E. meningoseptica* type strain only shared 31–35% DNA relatedness with the five other strains. This result confirmed the distance between the type strain and the other strains studied, but again the authors found no phenotypic or ecological clue for splitting *E. meningoseptica* into two species (Kim et al., 2005b).

Sequence Analysis of Small Subunit rRNA and *gyrB* Genes

Sequences of the 16S rRNA of all valid *Chryseobacterium* and *Elizabethkingia* species are now available; their comparison, combined with earlier data from DNA-rRNA hybridization experiments (Bauwens and De Ley, 1981; Mudarris et al., 1994; Vandamme et al., 1994; Bernardet et al., 1996), provides a distinct view of the phylogenetic relationships within the genera *Chryseobacterium* and *Elizabethkingia* (Fig. 1) and between these genera and other members of the family Flavobacteriaceae and of the phylum "Bacteroidetes" (see Figs. 1 and 2 of the chapter An Introduction to the Family Flavobacteriaceae in this Volume). Stackebrandt and Goebel (1994) considered the 97% 16S rRNA sequence similarity value as a threshold below which two bacterial strains usually

share less than 60–70% DNA similarity and consequently belong to different species. Although these authors also clearly stated that DNA pairing studies need to be performed when strains share more that 97% 16S rRNA sequence similarity, this value has frequently been interpreted as a threshold above which no DNA reassociation study was necessary since the strains most probably belonged to the same species. However, several studies dealing with members of the family Flavobacteriaceae showed that the value of 97% 16S rRNA sequence similarity is frequently not correlated with DNA-DNA relatedness (see Bernardet et al. [2002], the references therein, and the chapter The Genus Flavobacterium in this Volume). As for members of the genera *Chryseobacterium* and *Elizabethkingia*, several examples of 16S rRNA gene sequence similarity values well above 97% between members of different species may be found in Table 3 in Li et al. (2003). For instance, the two *E. miricola* strains shared 97.8–98.4% sequence similarity with five *E. meningoseptica* strains (Kim et al., 2005b), and sequence similarities between *C. balustinum* and *C. scophthalmum* and between *C. gleum* and *C. indologenes* were as high as 98.9% and 99.0%, respectively (Li et al., 2003). The type strain of *C. joostei* also exhibited 97.7% and 97.6% 16S rRNA gene sequence similarity with those of *C. gleum* and *C. indologenes*, respectively, although DNA relatedness between the three strains was as low as 19–25% (Hugo et al., 2003). Similar values were reported in the descriptions of *C. daecheongense* and *C. formosense* (Kim et al., 2005a; Young et al., 2005). Hence, the fact that two *Chryseobacterium* strains share more than 97% 16S rRNA sequence similarity does not mean that they belong to the same species and should not be used as a reason not to perform DNA-DNA hybridization experiments. Conversely, no *Chryseobacterium* strains have been reported that simultaneously share DNA relatedness higher than 70% and 16S rRNA sequence similarity below 97%. In their description of *C. defluvii*, Kämpfer et al. (2003) decided that no DNA-DNA relatedness experiment was necessary since their isolate only shared <95.9% 16S rRNA sequence similarity with all other *Chryseobacterium* species and since the description of "*C. proteolyticum*" had previously demonstrated that low DNA relatedness was indeed correlated with 16S rRNA sequence similarity lower than 97% in the genus *Chryseobacterium* (although some species were not included in the study; Yamaguchi and Yokoe, 2000).

Sequences of *gyrB* genes, encoding the subunit B protein of DNA gyrase, are only available for a few *Chryseobacterium* species (Suzuki et al., 1999; see the section Phylogeny in this Chapter); hence, the correlation between *gyrB* sequence similarity and DNA relatedness or 16S rRNA sequencing cannot be evaluated yet.

Any combination of two or more of the above techniques constitutes a polyphasic taxonomic approach that could be used to characterize new *Chryseobacterium* and *Elizabethkingia* isolates (Vandamme et al., 1996). This approach has allowed the successful identification and description of all recently published *Chryseobacterium* species and the delineation of the new genus *Elizabethkingia*. To our knowledge, no PCR technique is available that would allow the detection of *Chryseobacterium* or *Elizabethkingia* strains in the environment and clinical samples, as well as the identification of cultured strains. The few methods currently available for typing *Chryseobacterium* and *Elizabethkingia* strain collections are discussed below.

Serotyping

Six serovars of *C. meningosepticum* A to F were initially described as being involved in human infections, mainly neonatal meningitis (King, 1959; Owen and Lapage, 1974; see the section Pathogenicity and Epidemiology in this Chapter), and serotyping has been used as an epidemiological marker in outbreaks of *E. meningoseptica* infections in neonatal intensive care units. Additional serovars G to N, not involving neonatal meningitis, have subsequently been described by Richard et al. (Richard et al. 1979, 1981), but representative strains of the serotypes I, J and L could actually belong to *Empedobacter brevis* (Owen and Holmes, 1981; Holmes et al., 1984). A serotype O was also reported (see the chapter The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* in this Volume). Serotype C, seen in about two-thirds of typed isolates from neonates, is the one most commonly found and associated with high mortality and serious neurological sequelae (Bloch et al., 1997). Typing sera are not commercially available. The serology of *Chryseobacterium* species has not been developed, but serological crossreactions between some members of CDC group IIb and *E. meningoseptica* have been reported (Price and Pickett, 1981).

Molecular Typing

Few molecular methods are currently available for typing *Elizabethkingia* strains. Although rare, infections caused by *E. meningoseptica* in newborn infants are severe (see the section Pathogenicity and Epidemiology in this Chapter), and ribotyping was used to type clinical isolates for epidemiological studies. The collection of 52 *E. meningoseptica* strains (previously studied using DNA-DNA hybridization; Ursing and Bruun, 1987; [see above] and phenotypic characteriza-

tion; Bruun and Ursing, 1987) was subjected to ribotyping using two different restriction enzymes to examine the taxonomic relevance of this technique; ribotyping patterns that could identify strains within a given DNA relatedness group (see above) were indeed found (Colding et al., 1994). Using four different enzymes, these authors also assessed the epidemiological value of ribotyping; three *E. meningoseptica* strains isolated from an outbreak in a neonatal intensive care unit (Bruun et al., 1989) had the same ribotype pattern, indicating a common source of infection, as expected. The banding patterns resulting from three different restriction enzyme digestions were combined and used in another study of 92 *E. meningoseptica* clinical isolates to search for possible correlation between ribotyping and serotyping (Quilici and Bizet, 1996). Ribotyping allowing differentiation between isolates belonging to the same serotype, as well as between isolates of different serotypes, provided a useful epidemiological tool. Six different patterns were found for 21 strains isolated during the same outbreak, suggesting unrelated sources of infection.

Recently, pulsed-field gel electrophoresis of bacterial DNAs digested by the restriction enzyme *Apa*I was used to demonstrate that patient strains and environmental strains of *E. meningoseptica* isolated during an outbreak in a neonatal intensive care unit were the same strain (Hoque et al., 2001; see the section Pathogenicity and Epidemiology in this Chapter). DNA macrorestriction analysis was also used to demonstrate relapse of a catheter-related *E. meningoseptica* bacteremia (Sader et al., 1995).

Preservation

Short term (i.e., several weeks or months) preservation of *Chryseobacterium* and *Elizabethkingia* clinical strains was successfully achieved using cystine-trypticase agar (BBL; see the chapter The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* in the second edition) or Dorset egg medium (Holmes et al., 1984) slants kept at 4°C. Strains of "*Chryseobacterium proteolyticum*" were maintained on nutrient agar at 4°C (Yamaguchi and Yokoe, 2000). In our experience, commercially available agar deeps (e.g., bacterial strain storage medium, Bio-Rad) are most convenient for short term storage of members of all *Chryseobacterium* and *Elizabethkingia* species and related taxa (J.-F. Bernardet, unpublished data).

Chryseobacterium and *Elizabethkingia* strains can be preserved for many years by simple cryopreservation at –70°C or less, using 10–30% (v/v) glycerol as a cryoprotectant within a routine growth medium. We have successfully cryopreserved all *Chryseobacterium* and *Elizabethkingia* species in CAS broth (i.e., 1% casitone [Difco], 0.1% $MgSO_4 \cdot 7H_2O$, pH 6.8 unadjusted) recommended by Reichenbach (1989) with 10% sterile glycerol (J.-F. Bernardet, unpublished data). Also advocated was deep freezing of *Chryseobacterium* and *Elizabethkingia* strains in defibrinated rabbit blood (Holmes et al., 1984) or in *Brucella* broth (BBL) containing 10% glycerol (see the chapter The Genera *Flavobacterium*, *Sphingobacterium*, and *Weeksella* in this Volume).

Lyophilization (freeze-drying) is also convenient for long-term preservation of *Chryseobacterium* and *Elizabethkingia* strains. We found this technique effective using a suspension medium consisting of two-thirds Bacto Brucella broth (Difco) and one-third filtered and heat-inactivated (1 h at 56°C) horse serum (C. Bizet, Collection de l'Institut Pasteur, personal communication). Lyophilization and deep freezing at –80°C were convenient for the long-term preservation of *C. joostei* strains; for shorter preservation, the strains were maintained in a freeze-dried state on filter-paper discs and stored in screw-capped tubes at –20°C (Hugo et al., 2003).

Physiology

Little is known about the biochemical physiology of *Chryseobacterium* and *Elizabethkingia*; most of the current knowledge appears in the formal description of the genus (Table 2) and in the other paragraphs of the section Identification. All *Chryseobacterium* and *Elizabethkingia* species are aerobic chemoorganotrophs; their metabolism is strictly respiratory, not fermentative, except for *C. scophthalmum*, which also exhibits a fermentative metabolism (Mudarris et al., 1994). However, some *C. gleum*, *C. indologenes* and other CDC group IIb strains can grow anaerobically in the presence of nitrate by using nitrate as a terminal electron acceptor and reducing it to N_2 (Holmes et al., 1984). *Chryseobacterium daecheongense* has been described as nitrate-reducing (Kim et al., 2005a) while the ability of *C. balustinum* to reduce nitrate has been variously assessed by different authors (Table 2). *Chryseobacterium indologenes* strains can also grow under anaerobic conditions in the presence of fumarate (Yabuuchi et al., 1983). The *Chryseobacterium* and *Elizabethkingia* strain retrieved from the gut of a cockroach by Dugas et al. (2001) is also able to use molecular oxygen and fumarate, but not nitrate. *Elizabethkingia miricola* is also aerobic with a strictly respiratory type of metabolism, although it can grow in the

presence of 5% (v/v) CO_2 (Li et al., 2003). Interesting physiological comparisons may be made with the capnophilic members of the family Flavobacteriaceae, i.e., the genera *Capnocytophaga* (see the chapter The Genus Capnocytophaga in this Volume), *Riemerella*, *Ornithobacterium* and *Coenonia* (see the chapter Capnophilic Bird Pathogens in the Family Flavobacteriaceae: Riemerella, Ornithobacterium and Coenonia in this Volume).

Most *Chryseobacterium* species exhibit a rather high tolerance to NaCl: Except *C. balustinum*, all *Chryseobacterium* species are able to grow on marine agar (e.g., Difco marine agar 2216 [1.95% NaCl in addition to several other salts]), although only members of two species were actually isolated from marine environments (Bernardet et al., 2002; Table 2). The most recently described species (i.e., "*C. proteolyticum*," *C. defluvii*, *C. daecheongense*, *C. formosense*, and *C. taichungense*) have not been tested for this characteristic. *Elizabethkingia meningoseptica* is able to grow on marine agar (J.-F. Bernardet, unpublished data) and *E. miricola* has been reported to tolerate seawater (Li et al., 2003).

Genetics

Excluding investigations targeting the whole genome (DNA-DNA hybridization and determination of the G+C content) or particular genes (sequencing of 16S rRNA and *gyrB* genes) for taxonomic and phylogenetic purposes or for the molecular typing of bacterial strains (see the section Identification in this Chapter), the genetics of *Chryseobacterium* and *Elizabethkingia* spp. has been poorly investigated. However, the gene transfer system, selectable marker system, suicide vector system, and transposon developed to genetically manipulate *Flavobacterium johnsoniae* were also used successfully on *E. meningoseptica* (McBride and Baker, 1996; McBride and Kempf, 1996). No plasmid has been reported in members of the genera *Chryseobacterium* and *Elizabethkingia* (Owen and Holmes, 1981); therefore, their widespread resistance to antimicrobial agents (see the section Pathogenicity and Epidemiology in this Chapter) is likely associated with chromosomal genes.

Pathogenicity and Epidemiology

Veterinary Medicine

Besides the *Chryseobacterium* strains that may be normal bacterial flora of various aquatic animals and those that may spoil fish products (see the section Habitat and Ecology in this Chapter), other strains have been isolated from a number of diseased animal species.

Since *Chryseobacterium balustinum* was originally isolated from the surface of dead marine fish, the current opinion is that this organism was a fish spoilage agent rather than a pathogen (Harrison, 1929; Austin and Austin, 1999); indeed, strains phenotypically similar to *C. balustinum* have recently been isolated again from the skin, muscle and intestine of apparently healthy freshwater fish (Morita et al., 1997; González et al., 2000; see the section Habitat and Ecology in this Chapter). However, the type and only bona fide strain currently available in culture collections, originally described under the name "[*Empedobacter*] *balustinum*," was isolated from the heart blood of a freshwater fish (dace, *Leuciscus leuciscus*) exhibiting signs of hemorrhagic septicemia; this strain (which cannot grow on marine agar) proved able to kill when injected into several other fish species (Brisou et al., 1959). Hence, *C. balustinum* probably occurs in freshwater as well as seawater and at the surface of fish; depending on circumstances, it may be saprophytic, become an opportunistic pathogen, or merely spoil the fish. It is also possible the neotype strain is actually different from the lost original strain.

The first strains of *C. scophthalmum* were recovered in Scotland from wild, healthy turbot (*Scophthalmus maximus*) and coastal water samples; similar organisms were subsequently isolated in 1987 from diseased turbot from the wild and during an outbreak in a local farm (Mudarris, 1989; Mudarris and Austin, 1989). The bacterium caused gill hyperplasia and hemorrhagic septicemia; diseased turbot exhibited hemorrhages in the eyes, skin and jaw, as well as several internal organs. Extensive phenotypic investigations were conducted on a large group of strains isolated in pure culture from the gills and internal organs (Mudarris and Austin, 1989). The organism was first considered a *Cytophaga* sp. after gliding motility was suspected on fresh isolates, but this character was not confirmed during later investigations. Growth occurred on marine media and also on media devoid of NaCl or seawater, demonstrating that the bacterium was halotolerant and not obligately marine. This trait, together with all other phenotypic and genomic characteristics, led to the description of the new species as [*Flavobacterium*] *scophthalmum* (Mudarris et al., 1994); it was subsequently transferred to the new genus *Chryseobacterium* (Vandamme et al., 1994). Histopathology of the disease was studied (Mudarris and Austin, 1992) and experimental infection was performed in juvenile turbot; immersion and intraperitoneal injection both resulted in high mortalities with

typical lesions (Mudarris and Austin, 1989). Interestingly, young rainbow trout kept in fresh water were also successfully infected by the same method. Control of the disease was possible by injection of or immersion in furazolidone solution, but sulfafurazole proved much less efficacious. As *C. scophthalmum* was also consistently recovered from the gills of healthy turbot, it was considered that it could actually belong to its normal microflora and that unknown factors could weaken the host and/or enhance the virulence of the bacterium (Mudarris and Austin, 1989). Immunization of turbot with the lipopolysaccharide (LPS) from *C. scophthalmum* was shown to induce high hemagglutination activities in the serum as well as in skin and gut mucus when fish were intraperitoneally injected, whereas administration of LPS by the oral route or by immersion was generally unsuccessful (Al-Harbi and Austin, 1992c). Skin and gut mucus of LPS-immunized turbot inhibited the growth of *C. scophthalmum* (Al-Harbi and Austin, 1992b). Immunohistochemical techniques were used to study the distribution of the bacterium and LPS following different routes of inoculation (Al-Harbi and Austin, 1992a). *Chryseobacterium scophthalmum* has never been isolated again since its original description. However, strains whose 16S rRNA gene sequences shared significant similarities with that of *C. scophthalmum* were found among the bacterial community in amoebae-infected gills of salmon (Bowman and Nowak, 2004).

Bacterial strains phenotypically similar to *C. indologenes* were isolated from leopard frogs (*Rana pipiens*) reared in laboratory facilities in the United States and suffering "red leg disease," a condition usually associated with *Aeromonas hydrophila* (Olson et al., 1992). In an ongoing study of CDC group IIb strains isolated from diseased fish reared under various conditions and in various geographic areas, many strains resembling *C. indologenes* were identified (J.-F. Bernardet et al., unpublished data). However, representative strains only exhibited <25% DNA relatedness with the type strain of *C. indologenes*. The degree of virulence of these isolates has not been evaluated yet by experimental infection of fish, but their susceptibility to a variety of antimicrobial agents has been tested using diffusion tests and the dilution method to assess minimal inhibitory concentrations (MICs). The results confirmed the frequency and unusual level of constitutive resistance to ampicillin, polymyxin B, chloramphenicol and oxytetracycline in fish-associated members of the genus *Chryseobacterium*; only oxolinic acid and associated sulfonamides seemed to allow the success of antibiotic therapy (Michel et al., 2005).

Strains of *E. meningoseptica* have apparently been retrieved from various animal species. This organism was the probable cause of a case of meningitis in a cat (Sims, 1974), and one of the strains studied by Bruun and Ursing (1987) was reportedly isolated from the blood of a dog. Cases were also reported from turtles (Jacobson et al., 1989) and snakes (Miller et al., 2004), and several strains were isolated in pure or nearly pure culture from internal organs of diseased birds belonging to different species (Vancanneyt et al., 1994). Several cases of *E. meningoseptica* infections were also reported in different frog species reared for food or laboratory purposes, such as leopard frog (*Rana pipiens*; Taylor et al., 1993), clawed frog (*Xenopus laevis*; Green et al., 1999), and bullfrog (*Rana catesbeiana*; Mauel et al., 2002). A bacterium responsible for hemorrhagic septicemia in farmed bullfrog in Taiwan was characterized and provisionally named "[*Flavobacterium*] *ranacida*" (Faung et al. [1996] and references therein); recently, this organism, as well as a similar isolate from clawed frogs (also with hemorrhagic septicemia) captured in South Africa and reared in a French laboratory, was also identified as *E. meningoseptica* (J.-F. Bernardet, unpublished data). The first *E. meningoseptica* infection in fish was diagnosed in June 2004 in France from farmed koi carps imported from China, and a second, similar case in carps imported from Africa was reported at the time of writing (February 2005); diseased carps displayed skin lesions and hemorrhagic septicemia (N. Keck, personal communication). "*Aegyptianella ranarum*" replicates in the red blood cells of various frog species and is transmitted by amphibian-feeding leeches; comparison of the sequence of its 16S rRNA gene showed a 91% similarity with the sequence of *E. meningoseptica* and the uncultured organism was renamed "*Candidatus* Hemobacterium ranarum" (Zhang and Rikihisa, 2004). In view of the presence of *E. meningoseptica* in different animal species reared for food or laboratory experiments or as pets, the possibility that infected animals might serve as reservoirs for human infection should be evaluated. However, since person-to-person infection has never been reported in *E. meningoseptica*, animal-to-person infection is very unlikely.

Human Medicine

Several members of the family Flavobacteriaceae (see the chapter An Introduction to the Family Flavobacteriaceae in this Volume) are known participants in human infections, although their pathogenic role is sometimes uncertain. Besides *Bergeyella zoohelcum* and to a lesser extent *Weeksella virosa*, *Empedobacter brevis*, and the three *Myroides* species (see the chapters The Genera *Bergeyella* and *Weeksella* and The Genera *Empedobacter* and *Myroides* in

this Volume), two *Chryseobacterium* species and *Elizabethkingia meningoseptica* may also be isolated from clinical specimens (Schreckenberger, 1998).

Isolation and Identification of *Chryseobacterium* and *Elizabethkingia* Strains in Clinical Microbiology Laboratories *Elizabethkingia meningoseptica* and bacteria originally designated *Flavobacterium* group IIb (*C. indologenes*, *C. gleum* and allied bacteria) by E. O. King (1959) are isolated from clinical specimens such as cerebrospinal fluids, blood, tracheal suctions, urine and wounds. They are rare isolates, occurring in less than 0.1–1.0% of positive cultures (Pickett and Pedersen, 1970; Matsen, 1975; Kirby et al., 2004). Organisms of group IIb are the most commonly isolated flavobacteria in the clinical microbiology laboratory, while *E. meningoseptica* is seen only occasionally (e.g., Holmes and Owen, 1981). *Elizabethkingia meningoseptica* is associated with often fatal neonatal meningitis, while the pathogenicity of group IIb is more doubtful.

Primary culture of *Chryseobacterium* and *Elizabethkingia* species can be done on blood, chocolate, McConkey and nutrient agar. Incubation is done at 35–37°C, but the organisms also grow well at room temperature. Assignment of a culture to the genus *Chryseobacterium* rests upon characteristics shared with *E. brevis*, *Myroides* spp., and *Sphingobacterium* spp.: strictly aerobic, nonmotile, oxidase positive, yellow pigmented, Gram-negative bacilli, highly resistant to a wide range of antimicrobial agents. Differentiation of the *Chryseobacterium* isolates from the above-mentioned taxa can be done on the basis of results for indole and urease production, gelatin hydrolysis, and acid production or assimilation of carbohydrates (see the section Identification and the chapters "The Genera *Empedobacter* and *Myroides*", "The Genera *Bergeyella* and *Weeksella*" and "The Genera *Flavobacterium*, *Sphingobacterium* and *Weeksella*" in this Volume). Differentiation of *E. meningoseptica*, members of group IIb, and *E. brevis* strains by conventional laboratory methods is difficult in the clinical microbiology laboratory. It rests mainly on the ability of *E. meningoseptica* strains to produce a weak yellow pigment or no pigment, acid from trehalose, and β-galactosidase (ONPG test) within 2 h (Bülow, 1964); members of group IIb produce a bright yellow pigment, also produce acid from trehalose, but are negative in the ONPG test after 2 h, and *E. brevis* strains produce a weak yellow pigment and are trehalose- and ONPG-negative (Bruun and Ursing, 1987; Ursing and Bruun, 1991). The use of automated identification systems, such as API and Vitek (bioMérieux), for identification has been steadily increasing for the last 15–20 years (see the section Identification in this Chapter). To the best of our knowledge, there have been no reports in the literature on the performance of automated systems to identify these organisms in large collections of clinical strains. As of May 2004, API 20E, 20NE, ID 32E and ID 32GN databases contain *E. meningoseptica* and *C. indologenes*, as does Vitek GNI+ and Vitek2 ID-GNB. Vitek GNI+ can also identify *C. gleum*, while *E. brevis* can be identified by API ID 32 GN and Vitek2 ID-GNB.

Elizabethkingia meningoseptica is the only species for which a serotyping scheme has been proposed, but it is of limited use for the identification of clinical isolates (see the section Identification in this Chapter).

Pathogenicity of *Elizabethkingia meningoseptica* in Neonates and Infants Neonatal disease, notably meningitis, caused by *E. meningoseptica*, is by far the best known example of pathogenicity in the *Chryseobacterium*/*Elizabethkingia* complex and has been reported from all over the world (Vandepitte et al., 1958; Solé-Vernin, 1960; Eeckels et al., 1965; Vaillant et al., 1974; Leitz et al., 1977; Samb et al., 1977; Torronteras-Santiago et al., 1978; Zappulla et al., 1979; Bø et al., 1986; Bloch et al., 1997 and references therein; Chiu et al., 2000; Hoque et al., 2001). Since the infection was first reported in 1944 by Shulman and Johnson (1944) and retrospectively identified by King (1959), Von Graevenitz (1985) estimated that about 120 cases had been reported in the literature. Bloch et al. (1997), in their comprehensive review of the English language literature up to 1994, found 308 reports of cultures positive for *E. meningoseptica*, of which 60% were judged to represent true infections. Neonatal infections, defined as occurring in infants 3 months of age, accounted for 115 cases, of which 97 (84%) were meningitis, sometimes with concomitant bacteremia or pneumonia (e.g., George et al., 1961; Madruga et al., 1970; Thong et al., 1981; Abrahamsen et al., 1989; Bruun et al., 1989; Heeg et al., 1994; Chiu et al., 2000). Prematurity occurred in more than half of the cases and accounted for two-thirds of the mortality of 57%. The majority of survivors suffered from post-meningitis sequelae, especially hydrocephalus. Serotype C was the predominant serotype, found in about two-thirds of the typed isolates. Since 1994, three more cases of neonatal meningitis have been reported in the literature (Chiu et al., 2000; Hoque et al., 2001).

In addition to the meningitis cases in infants <3 months of age reported by Bloch et al. (1997), there have been anecdotal reports of spontaneous meningitis cases in infants up to one and a half years of age (Samb et al., 1977; Zappulla et al., 1979; Johny et al., 1983; Sarvamangala et al.,

1983). These cases have been classified as community-acquired and presumably belong to the same disease entity as the community-acquired cases in infants <3 months of age (e.g., Sundin et al., 1991; Di Pentima et al., 1998). The outcome in these older infants appeared to be better, with survival without sequelae in most cases.

Neonatal disease also encompasses bacteremia and pneumonia, constituting 13% and 3% of cases in the review of Bloch et al. (1997). Meningitis had, however, not been excluded by lumbar puncture in all cases of bacteremia. Since 1994, at least nine cases of bacteremia have been reported in three outbreaks in neonatal wards (Hoque et al., 2001; Güngör et al., 2003; Tekerekoglu et al., 2003). Mortality in cases of bacteremia and pneumonia has been much lower than in cases of meningitis and in some instances difficult to relate to the *E. meningoseptica* infection. In a retrospective analysis of cases of nosocomial pneumonia in infants following cardiac surgery, *E. meningoseptica* strains were shown to represent 8.9% of all microbial isolates (Tan et al., 2004).

A characteristic for *E. meningoseptica* infections is that they often occur as small epidemics in neonatal wards, including intensive care units (e.g., Brody et al., 1958; Vandepitte et al., 1958; Cabrera et al., 1961; Madruga et al., 1970; Hazuka et al., 1977; Abrahamsen et al., 1989; Bruun et al., 1989; Heeg et al., 1994; Hoque et al., 2001; Güngör et al., 2003). Surveillance cultures of clinically uninfected infants hospitalized with infected infants often reveal extensive colonization with *E. meningoseptica*, sometimes accompanied by transient bacteremia.

The presence of *E. meningoseptica* in various hospital environments was reviewed by Bloch et al. (1997). Optimal growth conditions include moist, cool environments or standing water at approximately 21°C (see Green et al. [1999] and references therein). In some cases, the bacterium was isolated from such medical devices as the respirator, vaporizer and artificial ventilation tubing (e.g., Heeg et al., 1994). In other investigations, environmental cultures have demonstrated other reservoirs for the epidemic strain (e.g., Cabrera et al., 1961; Hazuka et al., 1977; Abrahamsen et al., 1989; Hoque et al., 2001; Güngör et al., 2003). Caution must, however, be exercised in the interpretation of these findings as closely related *Chryseobacterium* strains are often found in the hospital environment (Bruun et al., 1989). Hence, extensive characterization, including typing, is necessary to determine a certain source of infection. For instance, extensive screenings were performed in various hospital environments following the repeated isolation of multiresistant *E. meningoseptica* strains from neonates in an intensive care unit (Hoque et al., 2001). Using pulsed-field gel electrophoresis of DNA macrorestriction digests, several isolates recovered from sink taps and patient isolates were shown to be representatives of a single strain.

ADULTS Disease after the age of one year (here termed "adult form") is rare, with less than 100 cases reported in the literature (Bloch et al. [1997] and references therein; Chiu et al., 2000; Gunnarsson et al., 2002). It occurs either as outbreaks in hospitals after administration of contaminated medicine or use of devices contaminated via water or as more sporadic infections in immunocompromised patients or post-trauma and -surgery patients.

A number of outbreaks involving both colonized (i.e., asymptomatic carriers) and infected patients in intensive care units have been described (Olsen et al., 1965; Olsen, 1967a; du Moulin, 1979; Brown et al., 1989; Pokrywka et al., 1993). The involved patients were either colonized or infected in the lungs or suffered from transient bacteremia. The outbreaks were traced to parenteral anesthetics, aerosolized polymyxin B, respiratory tubes, or water and ice contaminated by *E. meningoseptica*. Mortality in these patients was low and could not be ascribed to *E. meningoseptica*.

More sporadic cases occurred in severely immunocompromised patients suffering from malignancy, end-stage hepatic and renal disease, extensive burns, AIDS, etc. (Sheridan et al., 1993; Sader et al., 1995; Bloch et al., 1997; Manfredi et al., 1999; Chiu et al., 2000). Again, by far the most common manifestations were pneumonia and bacteremia.

A few cases of infections in immunocompromised patients have been reported to be community-acquired (Chiu et al., 2000), but information about possible carriage of intravascular devices while at home was not given. Also, a close contact between these patients and hospitals must be presumed due to their debilitating underlying diseases. The exact role of *E. meningoseptica* infections in the mortality of immunocompromised patients is difficult to ascertain, as there are many cofactors for mortality involved. There are nine case reports of *E. meningoseptica* meningitis in adults in the literature (Madruga et al., 1970; Lapage and Owen, 1973; Mani et al., 1978; Rios et al., 1978; Harrington and Perlino, 1981; Kelsey et al., 1982; Chan et al., 1983; Sharma et al., 1984; Uchihara et al., 1988). All but one (Uchihara et al., 1988) involved known immunocompromised or neurosurgical patients. As bacterial identification pointed to CDC group IIb and not to *E. meningoseptica* in at least one of these reports, it can be concluded that meningitis in adults is extremely rare. Other rare manifes-

tations of adult *E. meningoseptica* infection include endocarditis (Werthamer et al., 1972; Sexton et al., 1985), cellulitis (Abter et al., 1993), abdominal infection (Pokrywka et al., 1993), septic arthritis (Gunnarsson et al., 2002), and eye infections (Bucci and Holland, 1991; Bloom et al., 2003).

Pathogenicity of *Chryseobacterium indologenes* and Allied Strains Included in CDC Group IIb

Strains belonging to CDC group IIb, i.e., *Chryseobacterium indologenes*, *C. gleum* and closely related strains, are the most commonly isolated flavobacteria (i.e., *Chryseobacterium*, *Elizabethkingia*, *Empedobacter*, *Myroides* and *Sphingobacterium*) in the clinical microbiology laboratory (e.g., Holmes and Owen, 1981; Yabuuchi et al., 1983; Holmes et al., 1984). Their pathogenicity for man is, however, less well documented.

There are about 35 cases of *C. indologenes*/group IIb bacteremias in the literature that, with the exception of sporadic case reports (Siegman-Igra et al., 1987; Nulens et al., 2001), have been published in three series, involving 14, 12 and 6 patients, respectively (Stamm et al., 1975; Hsueh et al., 1996b; Hsueh et al., 1996c). The first series of group IIb bacteremia cases occurred in an American intensive care unit over five months and was associated with indwelling arterial catheters (Stamm et al., 1975). Mortality between infected patients and control patients was not found to differ. The two other series of *C. indologenes* bacteremia cases (Hsueh et al., 1996b; Hsueh et al., 1996c) occurred in the National University Hospital in Taiwan and were associated with indwelling devices, such as central venous catheters, Port-a-Caths, endotracheal tubes, and drains. In six of these 18 cases, the bacteria were also isolated from tracheal aspirates or sputum, and the clinical diagnosis was pneumonia. Owing to the serious underlying conditions of these patients and the often polymicrobial nature of their infections, the pathogenic role of *C. indologenes* was difficult to determine. In two more recent cases of *C. indologenes* infection, bacteremia was associated with cellulitis in the absence of any form of immune compromise (Green and Nolan, 2001) and with chronic graft-versus-host disease in a leukemia patient after bone marrow transplantation (Lin et al., 2003).

A case of group IIb meningitis was reported in a patient after irrigation with tap water following exenteration of a paranasal sinus (Bagley et al., 1976). Susceptibility to and successful treatment with ampicillin, together with a negative indole test, do, however, throw some doubt on the identity of the causative organism.

Urinary tract infections with *C. indologenes* in connection with nephrostomy drainage and a urinary catheter, respectively, have also been reported from the National University Hospital in Taiwan (Hsueh et al., 1996b; Hsueh et al., 1996c). From the same hospital there have also been cases of ventilator-associated pneumonia, pyomyositis, and infected burn wounds (Hsueh et al., 1996a; Hsueh et al., 1997).

Antimicrobial Susceptibility of Clinical *Chryseobacterium* and *Elizabethkingia* Isolates and Treatment of *Chryseobacterium*/*Elizabethkingia* Infections

Members of *Chryseobacterium* and *Elizabethkingia* species are naturally resistant to polymyxins, aminoglycosides, chloramphenicol, and most β-lactams (Olsen, 1967b; Aber et al., 1978; Bruun, 1987; Chang et al., 1997; Fraser and Jorgensen, 1997; Kirby et al., 2004). *Elizabethkingia meningoseptica* produces at least two β-lactamases, a noninducible extended-spectrum β-lactamase and a carbapenem-hydrolyzing β-lactamase (Bellais et al., 2000). The latter enzyme, which is also produced by *C. indologenes* strains, has recently been identified in *Flavobacterium johnsoniae* (Naas et al., 2003). This means that *E. meningoseptica* is resistant to extended spectrum cephalosporins and carbapenems, but that some isolates are susceptible to ureidopenicillins. Data regarding susceptibility to piperacillin, with and without tazobactam, vary somewhat. In two recent investigations, both using the National Committee for Clinical Laboratory Standards (NCCLS) microdilution method, susceptibility to piperacillin-tazobactam was 29% and 71% of isolates, respectively (Fraser and Jorgensen, 1997; Kirby et al., 2004). Members of *Chryseobacterium* and *Elizabethkingia* species are mostly resistant to tetracyclines, erythromycin, and linezolid, while they display either an intermediate level of susceptibility or resistance to vancomycin and clindamycin (Bruun, 1987; Fraser and Jorgensen, 1997; Kirby et al., 2004). Susceptibility to trimethoprim-sulfamethoxazole also varies; in two studies (Fraser and Jorgensen, 1997; Kirby et al., 2004), 33% and 80% of susceptible isolates were found, respectively. The most active agents are minocycline, rifampicin and the newer quinolones (levofloxacin, gatifloxacin and sparfloxacin), while susceptibility to ciprofloxacin varies (Spangler et al., 1996; Fraser and Jorgensen, 1997; Kirby et al., 2004).

The high resistance of *Chryseobacterium* and *Elizabethkingia* species (and most other flavobacteria) to many antimicrobial agents and a susceptibility pattern unusual for Gram-negative bacteria have always attracted attention and been useful for their preliminary identification in

the clinical microbiology laboratory. Various studies have shown discrepancies between results of disk diffusion tests and MIC determinations, the former in general giving more sensitive results for erythromycin, clindamycin, vancomycin, tetracycline, chloramphenicol, and fusidic acid (Von Graevenitz and Grehn, 1977; Aber et al., 1978; Bruun, 1987; Fraser and Jorgensen, 1997). This has probably given rise to previous observations that *E. meningoseptica* is susceptible to antimicrobial agents traditionally used for treatment of infections caused by Gram-positive bacteria.

Elizabethkingia meningoseptica neonatal meningitis represents the greatest challenge in relation to treatment of infections caused by flavobacteria. Since cases of meningitis are rare, there are few reports of treatment experiences, and presumably the conduct of controlled trials to evaluate antimicrobial regimens will never be possible. Treatment will, therefore, depend on susceptibilities of individual strains and will probably include combinations of antimicrobials, and possibly intrathecal, as well as intravenous administration. Clinical microbiologists need to be aware of the fact that false sensitivities may be shown by disk diffusion (vide supra) and that resistance may rapidly develop during antimicrobial treatment (Hazuka et al., 1977; Ferlauto and Wells, 1981). Survival of neonatal meningitis without development of hydrocephalus is associated with time to sterilization of cerebrospinal fluid and presumably to age at presentation of meningitis and length of gestation.

A summary of antimicrobial treatment and outcomes of neonatal *E. meningoseptica* meningitis for the years 1973–1995 has been published by Di Pentima et al. (1998). At present (May 2004), there seems to be two schools of thought regarding the best choice of antimicrobial regimens for treatment, exemplified by the *Sandford Guide to Antimicrobial Therapy* (Gilbert et al. 2003) and *Antibiotic Essentials* (Cunha, 2003). *The Sandford Guide*s recommendation of vancomycin rests on the report of Di Petima et al. (1998) of two cases and on one case cited by them (Tizer et al., 1995). These three cases were successfully treated with vancomycin and rifampicin without development of hydrocephalus. The combination of vancomycin and rifampicin was shown to act synergistically in vitro by Di Petima et al. (1998). However, note that the three cases involved full-term infants 3–4 weeks of age at the time of presentation of meningitis. Other anecdotal reports have shown the successful use of rifampicin in conjunction with other antimicrobials in the treatment of meningitis (Bruun et al., 1989; Hoque et al., 2001). In *Antibiotic Essentials* (Cunha, 2003), trimethoprim-sulfamethoxazole is recommended for meningitis, while vancomycin and rifampicin is advocated for non-central nervous system infections. Again, the evidence for successful treatment is anecdotal (Ferlauto and Wells, 1981; Johny et al., 1983; Di Petima, 1995). The newer quinolones are, as mentioned above, promising agents for the treatment of meningitis. There is, however, at present no clinical experience with their use in neonatal meningitis, as they have not been approved for use in children, let alone neonates.

A recent case of *C. indologenes* bacteremia was successfully treated with piperacillin and tazobactam and the infection did not recur (Lin et al., 2003).

The resistance of *E. meningoseptica* strains to chlorine and other disinfectants has also been reported (see Green et al. [1999] and references therein). For instance, during the above-mentioned outbreak in a neonate intensive care unit, screening cultures remained positive following chlorination of the water tanks and sink taps; only after the replacement of the taps did the cultures become negative (Hoque et al., 2001).

Besides the above-mentioned, well-characterized bacterial species, other organisms belonging to the *Chryseobacterium* phylogenetic branch (Fig. 1; see the sections Phylogeny and Taxonomy in this Chapter) may prove pathogenic to humans. For instance, three potentially pathogenic organisms, "*Candidatus* Chryseobacterium timonae," "*Chryseobacterium massiliae*" and "*Candidatus* Amoebinatus massiliae," were recently recovered from human nasal samples using amoebal coculture (Drancourt et al., 2004; Greub et al., 2004). Similarly, two of the "*Haloanella gallinarum*" strains listed in databases were labelled as "undescribed bacterial pathogens isolated from human tissue" and "new pathogenic bacterium" (sequence nos. [AY244776] [Drancourt et al., 2004] and [AB035150] [H.-S. Pham et al., unpublished data], respectively).

Applications

Some *Chryseobacterium* strains have potential applications in the cleanup of various environmental contaminants. For instance, the cleanup of pentachlorophenol (PCP), a pesticide that has been associated with wood preservation and a disinfectant in the food industry, is essential since PCP poses significant health hazards. Yu and Ward (1996) used *C. gleum*, *Agrobacterium radiobacter* and *Pseudomonas* sp. as a mixed culture to degrade PCP. Although rates of PCP degradation by individual isolates were lower than

that observed for the mixed culture, *C. gleum* manifested the highest PCP degradative ability. Another bacterial consortium including *C. indologenes* and three other bacterial species was found to degrade aniline and 4-chloroaniline in a contaminated agricultural soil in Indonesia (Radianingtyas et al, 2003). A *C. indologenes* strain was also identified among the soil bacteria that were able to degrade the toxic furan and phenolic compounds resulting from the acid treatment of lignocellulose (Lopez et al., 2004). Another *C. indologenes* strain (16S rRNA sequence no. [AJ288294]; D. G. Karpouzas, unpublished data) was able to degrade the insecticide and nematocide carbofuran, a member of the carbamate group.

Other potential applications of *Chryseobacterium* strains may result from the presence of particular enzymes. For instance, Yamaguchi et al. (2001) recently described a novel enzyme from "*C. proteolyticum*" that deamidates glutaminyl residues in proteins. Protein deamidation is regarded as a promising method to improve protein functionality (e.g., emulsifiers or foaming agents) in food systems (Hamada, 1994). A *Chryseobacterium* strain isolated from an apparently healthy eel (*Anguilla anguilla*) and exhibiting a high 16S rRNA sequence similarity with *C. gleum* and *C. indologenes* was shown to secrete a novel endopeptidase that specifically cleaves the human plasminogen and could consequently reduce bacterial invasion (Lijnen et al., 2000). Cold-active proteases were characterized from a *Chryseobacterium* isolate from salmon (*Oncorhynchus keta*) intestine; analysis of its 16S rRNA sequence revealed a high similarity with that of *C. balustinum* (Morita et al., 1997). Activity of the proteases was highest at 10°C although the optimal growth temperature of the strain was 20°C. In the course of a screening of various subtropical environments for bacteria exhibiting novel carbohydrate-degrading activities, *Chryseobacterium* strains were shown to predominate in the terrestrial environments (Rosado and Govind, 2003).

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Literature Cited

Aber, R. C., C. Wennersten, and R. C. Moellering Jr. 1978. Antimicrobial susceptibility of Flavobacteria. Antimicrob. Agents Chemother. 14:483–487.

Abrahamsen, T. G., P. H. Finne, E. Lingaas. 1989. Flavobacterium meningosepticum infections in a neonatal intensive care unit. Acta Paediatr. Scand. 78:51–55.

Abter, E. I. M., L. I. Lutwick, M. J. Torrey, and R. Mann. 1993. Cellulitis associated with bacteraemia due to Flavobacterium meningosepticum. Clin. Infect. Dis. 17:929–930.

Al-Harbi, A. H., and B. Austin. 1992a. Distribution of formalized cells and lipopolysaccharide (LPS) of a fish pathogenic Cytophaga-like bacterium in tissues of turbot (Scophthalmus maximus L.) following uptake by injection, immersion and the oral route. Bull. Eur. Assoc. Fish Pathol. 12:100–103.

Al-Harbi, A. H., and B. Austin. 1992b. Influence of skin and gut mucus from turbot (Scophthalmus maximus L.) on the survival of a fish pathogenic Cytophaga-like bacterium. Bull. Eur. Assoc. Fish Pathol. 12:80–82.

Al-Harbi, A. H., and B. Austin. 1992c. The immune response of turbot, Scophthalmus maximus (L.) to lipopolysaccharide from a fish pathogenic Cytophaga-like bacterium. J. Fish Dis. 15:449–452.

Altinok, I. 2004. Toxicity and therapeutic effects of chloramine-T for treating Flavobacterium columnare infection in goldfish. Aquaculture. 239:47–56.

Austin, B., and D. A. Austin. 1999. Bacterial Fish Pathogens: Disease of Farmed and Wild Fish, 3rd (revised) ed. Praxis Publishing. Chichester, UK.

Bagley, D. H., J. C. Alexander, V. J. Gill, R. Dolin, and A. S. Ketcham. 1976. Late Flavobacterium species meningitis after craniofacial exenteration. Arch. Intern. Med. 136:229–231.

Bauwens, M., and J. De Ley. 1981. Improvements in the taxonomy of Flavobacterium by DNA:rRNA hybridization. *In:* H. Reichenbach and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group. Verlag Chemie. Weinheim, Germany. Gesellschaft für Biotechnologische Forschung Monograph Series, No. 5:27–31.

Bellais, S., L. Poirel, T. Naas, D. Girlich, and P. Nordmann. 2000. Genetic-biochemical analysis and distribution of the Ambler Class A beta-lactamase CME-2, responsible for extended-spectrum cephalosporin resistance in Chryseobacterium (Flavobacterium) meningosepticum. Antimicrob. Agents Chemother. 44:1–9.

Bernardet, J.-F., P. Segers, M. Vancanneyt, F. Berthe, K. Kersters, and P. Vandamme. 1996. Cutting a Gordian knot: Emended classification and description of the genus Flavobacterium, emended description of the family Flavobacteriaceae, and proposal of Flavobacterium hydatis nom. nov. (basonym, Cytophaga aquatilis Strohl and Tait 1978). Int. J. Syst. Bacteriol. 46:128–148.

Bernardet, J.-F., Y. Nakagawa, and B. Holmes. 2002. Proposed minimal standards for describing new taxa of the family Flavobacteriaceae and emended description of the family. Int. J. Syst. Evol. Microbiol. 52:1049–1070.

Bloch, K. C., R. Nadarajahn, and R. Jacobs. 1997. Chryseobacterium meningosepticum: an emerging pathogen in immunocompromised adults: Report of 6 cases and literature review. Medicine (Baltimore) 76:30–41.

Bloom, A. H., H. D. Perry, E. D. Donnefeld, and R. G. Davis. 2003. Chryseobacterium meningosepticum keratitis. Am. J. Ophthalmol. 136:356–357.

Bø, B. I., T. J. Gutteberg, and G. Størvold. 1986. Neonatal meningitt forårsaket av Flavobakterium meningoseptikum. Tidsskr. Nor. Lægeforen 106:751–753.

Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W.

Triplett. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. 62:1935–1943.

Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin. 1997. Diversity and association of psychrophilic bacteria in Antarctic sea ice. Appl. Environ. Microbiol. 63:3068–3078.

Bowman, J. P., and B. Nowak. 2004. Salmonid gill bacteria and their relationship to amoebic gill disease. J. Fish Dis. 27:483–492.

Bremer, P. J., I. Monk, and C. M. Osborne. 2001. Survival of Listeria monocytogenes attached to stainless steel surfaces in the presence or absence of Flavobacterium spp. J. Food Protect. 64:1369–1376.

Brisou, J., C. Tysset, and B. Vacher. 1959. Etude de trois souches microbiennes, famille des Pseudomonadaceae, dont la synergie provoque une maladie de caractère septicémique chez les poissons blancs de la Dordogne, du Lot et de leurs affluents. Ann. Inst. Pasteur 96:689–696.

Brody, J. A., H. Moore, and E. O. King. 1958. Meningitis caused by an unclassified Gram-negative bacterium in newborn infants. Am. J. Dis. Child. 96:1–5.

Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli. Proc. Natl. Acad. Sci. USA 75:4801–4805.

Brown, R. B., D. Phillips, M. J. Barker, R. Pieczarka, M. Sands, and D. Teres. 1989. Outbreak of nosocomial Flavobacterium meningosepticum respiratory infections associated with use of aerosolized polymyxin B. Am. J. Infect. Control 17:121–125.

Bruun, B. 1982. Studies on a collection of strains of the genus Flavobacterium I: Biochemical studies. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 90:415–421.

Bruun, B. 1987. Antimicrobial susceptibility of Flavobacterium meningosepticum strains identified by DNA-DNA hybridization. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 95:95–101.

Bruun, B., and Høiby. 1987. Crossed immunoelectrophoretic analysis of Flavobacterium meningosepticum and allied Flavobacterium taxa. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 95:245–252.

Bruun, B., and J. Ursing. 1987. Phenotypic characterization of Flavobacterium meningosepticum strains identified by DNA-DNA hybridization. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 95:41–47.

Bruun, B., E. Tvenstrup Jensen, K. Lundstrøm, and G. E. Andersen. 1989. Flavobacterium meningosepticum infection in a neonatal ward. Eur. J. Clin. Microbiol. Infect. Dis. 8:509–514.

Bucci Jr., F. A., and E. J. Holland. 1991. Flavobacterium menigosepticum keratitis succesfully treated with topical Trimetroprim-Sulfamethoxazole. Am. J. Opthalmol. 111:116–118.

Bülow, P. 1964. The ONPG test in diagnostic bacteriology. Acta Pathol. Microbiol. Scand. 60:376–402.

Cabrera, H. A., and G. H. Davis. 1961. Epidemic meningitis of the newborn caused by Flavobacteria. Am. J. Dis. Child. 101:289–295.

Campbell, L. L., and O. B. Williams. 1951. A study of chitin-decomposing micro-organisms of marine origin. J. Gen. Microbiol. 5:894–905.

Campbell, L. L. 1957. Genus Beneckea Campbell. *In:* R. S. Breed, E. G. D. Murray, and N. R. Smith (Eds.) Bergey's Manual of Determinative Bacteriology, 7th ed. Williams & Wilkins. Baltimore, MD. 328–332.

Campbell, C. L., D. L. Mummey, E. T. Schmidtmann, and W. C. Wilson. 2004. Culture-independent analysis of midgut microbiota in the arbovirus vector Culicoides sonorensis (Diptera: Ceratopogonidae). J. Med. Entomol. 41:340–348.

Cepeda, C., S. García-Márquez, and Y. Santos. 2004. Improved growth of Flavobacterium psychrophilum using a new culture medium. Aquaculture. 238:75–82.

Chan, K. H., P. Y. Chou, R. Y. Wang, and C. Y. Huang. 1983. Meningitis caused by Flavobacterium meningosepticum after transsphenoidal hypophysectomy with recovery. Surg. Neurol. 20:294–296.

Chang, J.-C., P.-R. Hsueh, J.-J. Wu, S.-W. Ho, W.-C. Hsieh, and K.-T. Luh. 1997. Antimicrobial susceptibility of flavobacteria as determined by agar dilution and disk diffusion methods. Antimicrob. Agents Chemother. 41:1301–1306.

Chiu, C.-H., M. Waddingdon, W.-S. Hsieh, D. Greenberg, P. C. Schreckenberger, and A. M. Carnahan. 2000. Atypical Chryseobacterium meningosepticum and meningitis and sepsis in newborns and the immunocompromised. Emerg. Infect. Dis. 6:481–486.

Chung, H.-Y. 1990. On the bacterial disease of captive bullfrog. *In:* G.-H. Kou, H. Wakabayashi, I-C. Liao, S.-N. Chen, and C.-F. Lo (Eds.) Proceedings of the Republic of China-Japan Symposium on Fish Diseases. National Science Council, Taiwan NSC Symposium Series No. 16:81–89.

Colding, H., J. Bangsborg, N.-E. Fiehn, T. Bennekov, and B. Bruun. 1994. Ribotyping for differentiating Flavobacterium meningosepticum isolates from clinical and environmental sources. J. Clin. Microbiol. 32:501–505.

Cousin, M. A. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. J. Food Protect. 45:172–207.

Crump, B. C., E. V. Armbrust, and J. A. Baross. 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. Appl. Environ. Microbiol. 65:3192–3204.

Cunha, B. 2003. Antibiotic Essentials. Physician's Press. Royal Oak, MI.

Di Pentima, M. C., E. O. Mason Jr., and S. L. Kaplan. 1998. In vitro antibiotic synergy against Flavobacterium meningosepticum: Implications for therapeutic options. Clin. Infect. Dis. 26:1169–76.

Drancourt, M., P. Berger, and D. Raoult. 2004. Systematic 16S rRNA gene sequencing of atypical clinical isolates identified 27 new bacterial species associated with humans. J. Clin. Microbiol. 42:2197–2202.

Dugas, J. E., L. Zurek, B. J. Paster, B. A. Keddie, and E. R. Leadbetter. 2001. Isolation and characterization of a Chryseobacterium strain from the gut of the American cockroach, Periplaneta americana. Arch. Microbiol. 175:259–262.

Du Moulin, G. C. 1979. Airway colonization by Flavobacterium in an intensive care unit. J. Clin. Microbiol. 10:155–160.

Eeckels, R., J. Vandepitte, and V. Seynhaeve. 1965. Neonatal infections with Flavobacterium meningosepticum. Belg. Tijdschr. Geneeskde. 5:244–256.

Elder, J. H., and S. Alexander. 1982. Endo-β-N-acetylglucosaminidase F: endoglycosidase from Flavobacterium meningosepticum that cleaves both high-mannose and complex glycoproteins. Proc. Natl. Acad. Sci. USA 79:4540–4544.

Ellerbroek, L. 1997. Airborne microflora in poultry slaughtering establishments. Food Microbiol. 14:527–531.

Ellis, B. R., and E. H. Marth. 1984. Growth of Pseudomonas or Flavobacterium in milk reduced yield of cheddar cheese. J. Food Protect. 47:713–716.

Engelbrecht, K., P. J. Jooste, and B. A. Prior. 1996. Spoilage characteristics of Gram-negative genera and species isolated from Cape marine fish. S. Afr. J. Food Sci. Nutr. 8:66–71.

Euzéby, J. P. 1997. List of bacterial names with standing in nomenclature: A folder available on the Internet. Int. J. Syst. Bacteriol. 47:590–592.

Faung, S.-T., L. Chiu, and C.-T. Wang. 1996. Platelet lysis and functional perturbation by 13-methyl myristate, the major fatty acid in Flavobacterium ranacida. Thrombosis Res. 81:91–100.

Felsenstein, J. 1985. Confidence limits of phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Ferlauto, J. J., and D. H. Wells. 1981. Flavobacterium meningosepticum in the neonatal period. South. Med. J. 74:757–759.

Fischer, P. L., P. J. Jooste, and J. C. Novello. 1987. The seasonal distribution of psychrotrophic bacteria in Bloemfontein raw milk supplies. S. Afr. J. Dairy Sci. 19:73–76.

Fraser, S. L., and J. H. Jorgensen. 1997. Reappraisal of the antimicrobial susceptibilities of Chryseobacterium and Flavobacterium species and methods for reliable susceptibility testing. Antimicrob. Agents Chemother. 41:2738–2741.

García-López, M. L., M. Prieto, and A. Otero. 1998. The physiological attributes of Gram-negative bacteria associated with spoilage of meat and meat products. *In:* A. Davies and R. Board (Eds.) The Microbiology of Meat and Poultry. Blackie Academic & Professional. London, UK. 1–34.

Garrity, G. M., K. L. Johnson, J. A. Bell, and D. B. Searles. 2003. Taxonomic outlines of the Prokaryotes. *In:* G. M. Garrity (Ed.) Bergey's Manual of Systematic Bacteriology, 2nd ed., Release 4.0. Springer-Verlag. New York, NY.

Gennari, M., and C. Cozzolino. 1989. Observations on Flavobacterium, Cytophagaceae and other pigmented bacteria isolated from fresh and ice stored sardines. Arch. Vet. Ital. 40:372–384.

George, R. M., C. P. Cochran, and W. E. Wheeler. 1961. Epidemic meningitis of the newborn caused by Flavobacteria. Am. J. Dis. Child. 101:296–304.

Georgnaras, I., A. E. de Jesus, E. van Zyl, and A. von Holy. 1996. Bacterial populations associated with poultry processing in a South African abattoir. Food Microbiol. 13:457–465.

Gilbert, D. N., R. C. Moellering Jr., and M. A. Sande (Eds.). 2003. The Sandford Guide to Antimicrobial Therapy. Antimicrobial Therapy. Hyde Park, VT.

Gilmour, A., and M. T. Rowe. 1981. Micro-organisms associated with milk. *In:* R. K. Robinson (Ed.) Dairy Microbiology. Applied Science Publishers. London, UK. 1:35–75.

González, C. J., J. A. Santos, M.-L. García-López, and A. Otero. 2000. Psychrobacters and related bacteria in freswater fish. J. Food Protect. 63:315–321.

Grabowski, L. D., S. E. LaPatra, and K. D. Cain. 2004. Systemic and mucosal antibody response in tilapia, Oreochromis niloticus (L.), following immunization with Flavobacterium columnare. J. Fish Dis. 27:573–581.

Green, S. L., D. M. Bouley, R. J. Tolwani, K. S. Waggie, B. D. Lifland, G. M. Otto, and J. E. Ferrell. 1999. Identification and management of an outbreak of Flavobacterium meningosepticum infection in a colony of South African clawed frogs (Xenopus laevis). J. Am. Vet. Med. Assoc. 214:1833–1838.

Green, B. T., and P. E. Nolan. 2001. Cellulitis and bacteraemia due to Chryseobacterium indologenes. J. Infect. 42:219–220.

Greub, G., B. La Scola, and D. Raoult. 2004. Amoebae-resisting bacteria isolated from human nasal swabs by amoebal coculture. Emerg. Infect. Dis. 10:470–477.

Güngör, S., M. Özen, A. Akinci, and R. Durmaz. 2003. A Chryseobacterium meningosepticum outbreak in a neonatal ward. Infect. Control Hosp. Epidemiol. 24:613–617.

Gunnarsson, G., H. Baldursson, and I. Hilmarsdottir. 2002. Septic arthritis caused by Chryseobacterium meningosepticum in an immunocompetent male. Scand. J. Infect. Dis. 34:299–315.

Hadas, E., M. Derda, J. Winiecka-Krusnell, and A. Sutek. 2004. Acanthamoeba spp. as vehicles of pathogenic bacteria. Acta Parasitol. 49:276–280.

Hamada, J. S. 1994. Deamidation of food proteins to improve functionality. Crit. Rev. Food Sci. Nutr. 34:283–292.

Hamana, K., and S. Matsuzaki. 1990. Occurrence of homospermidine as a major polyamine in the authentic genus Flavobacterium. Can. J. Microbiol. 36:228–231.

Hamana, K., and S. Matsuzaki. 1991. Polyamine distribution in the Flavobacterium-Cytophaga-Sphingobacterium complex. Can. J. Microbiol. 37:885–888.

Hamana, K., and Y. Nakagawa. 2001. Polyamine distribution profiles in newly validated genera and species within the Flavobacterium-Flexibacter-Cytophaga-Sphingobacterium complex. Microbios 106(S2):105–116.

Harrington, S. P., and C. A. Perlino. 1981. Flavobacterium meningosepticum sepsis: disease due to bacteria with unusual antibiotic susceptibility. South. Med. J. 74:764–766.

Harrison, F. C. 1929. The discolouration of halibut. Can. J. Res. 1:214–239.

Hayes, P. R. 1977. A taxonomic study of flavobacteria and related Gram-negative yellow pigmented rods. J. Appl. Bacteriol. 43:345–367.

Hazuka, B. T., A. S. Dajani, K. Talbot, and B. M. Keen. 1977. Two outbreaks of Flavobacterium meningosepticum type E in a neonatal intensive care unit. J. Clin. Microbiol. 6:450–455.

Heeg, P., W. Heizmann, and H. Mentzel. 1994. Infections caused by Flavobacterium meningosepticum in neonates of an intensive care unit. Zbl. Hyg. Umweltmed. 195:282–287.

Holmes, B., and R. J. Owen. 1981. Emendation of the genus Flavobacterium and the status of the genus. Developments after the 8th edition of Bergey's Manual. *In:* H. Reichenbach and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group. Verlag Chemie. Weinheim, Germany. Gesellschaft für Biotechnologische Forschung Monograph Series, No. 5:17–26.

Holmes, B., R. J. Owen, and T. A. McMeekin. 1984. Genus Flavobacterium Bergey, Harrison, Breed, Hammer and Huntoon 1923, 97^{AL}. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 1:353–361.

Holmes, B., R. J. Owen, A. G. Steigerwalt, and D. J. Brenner. 1984. Flavobacterium gleum, a new species found in human clinical specimens. Int. J. Syst. Bacteriol. 34:21–25.

Holmes, B., A. G. Steigerwalt, R. E. Weaver, and D. J. Brenner. 1986a. Weeksella virosa gen. nov., sp. nov. (formerly group IIf), found in human clinical specimens. System. Appl. Microbiol. 8:185–190.

Holmes, B., A. G. Steigerwalt, R. E. Weaver, and D. J. Brenner. 1986b. Weeksella zoohelcum sp.nov. (formerly group IIj), from human clinical specimens. System. Appl. Microbiol. 8:191–196.

Holmes, B. 1993. Recent developments in Flavobacterium taxonomy. *In:* P. J. Jooste (Ed.) Advances in the Taxonomy and Significance of Flavobacterium, Cytophaga and Related Bacteria. University of the Orange Free State Press. Bloemfontein, South Africa. 6–15.

Hoque, S. N., J. Graham, M. E. Kaufmann, and S. Tabaqchali. 2001. Chryseobacterium (Flavobacterium) meningosepticum outbreak associated with colonization of water taps in a neonatal intensive care unit. J. Hosp. Infect. 47:188–192.

Hsueh, P.-R., T.-R. Hsiue, and W.-C. Hsieh. 1996a. Pyomyositis in intravenous drug abusers: Report of a unique case and review of the literature. Clin. Infect. Dis. 22:858–860.

Hsueh, P.-R., T.-R. Hsiue, J.-J. Wu, L.-J. Teng, S.-W. Ho, W.-C. Hsieh, and K.-T. Luh. 1996b. Flavobacterium indologenes bacteraemia: Clinical and microbiological characteristics. Clin. Infect. Dis. 23:550–555.

Hsueh, P.-R., L.-J. Teng, S.-W. Ho, W.-C. Hsieh, and K.-T. Luh. 1996c. Clinical and microbiological characteristics of Flavobacterium indologenes infections associated with indwelling devices. Clin. Microbiol. 34:1908–1913.

Hsueh, P.-R., L.-J. Teng, P.-C. Yang, S.-W. Ho, W.-C. Hsieh, and K.-T. Suh. 1997. Increasing incidence of nosocomial Chryseobacterium indologenes infections in Taiwan. Eur. J. Clin. Microbiol. Infect. Dis. 16:568–574.

Hugo, C. J. 1997. A Taxonomic Study of the Genus Chryseobacterium from Food and Environmental Sources [PhD thesis]. University of the Orange Free State. Bloemfontein, South Africa.

Hugo, C. J., and P. J. Jooste. 1997. Preliminary differentiation of food strains of Chryseobacterium and Empedobacter using multilocus enzyme electrophoresis. Food Microbiol. 14:133–142.

Hugo, C. J., P. J. Jooste, P. Segers, M. Vancanneyt, and K. Kersters. 1999. A polyphasic taxonomic study of Chryseobacterium strains isolated from dairy sources. Syst. Appl. Microbiol. 22:586–595.

Hugo, C. J., and P. J. Jooste. 2003. Culture media for genera in the family Flavobacteriaceae. *In:* J. E. L. Corry, G. D. W. Curtis, and R. M. Baird (Eds.) Handbook of Culture Media for Food Microbiology, 2nd ed. Elsevier Science Publishers. London, UK. 355–367.

Hugo, C. J., P. Segers, B. Hoste, M. Vancanneyt, and K. Kersters. 2003. Chryseobacterium joostei sp. nov., isolated from the dairy environment. Int. J. Syst. Evol. Microbiol. 53:771–777.

Izumi, S., and F. Aranishi. 2004a. Plasmid profiling of Japanese Flavobacterium psychrophilum isolates. J. Aquat. An. Health. 16:99–103.

Izumi, S., and F. Aranishi. 2004b. Relationship between gyrA mutations and quinolone resistance in Flavobacterium psychrophilum isolates. Appl. Environ. Microbiol. 70:3968–3972.

Jacobson, E. R., C. H. Gardiner, and S. L. Barten. 1989. Flavobacterium meningosepticum infection of a Barbour's map turtle. J. Zool. Wildl. Med. 20:474–477.

Johny, M., F. A. F. Khuffash, and K. M. Elhag. 1983. Antimicrobial treatment of Flavobacterium meningosepticum infection. Ann. Trop. Paediatr. 3:125–128.

Jooste, P. J., T. J. Britz, and J. De Haast. 1985. A numerical taxonomic study of Flavobacterium-Cytophaga strains from dairy sources. J. Appl. Bacteriol. 59:311–323.

Jooste, P. J., T. J. Britz, and P. M. Lategan. 1986a. The prevalence and significance of Flavobacterium strains in commercial salted butter. Milchwissenschaft 41:69–73.

Jooste, P. J., T. J. Britz, and P. M. Lategan. 1986b. The significance of flavobacteria as proteolytic psychrotrophs in milk. Milchwissenschaft 41:618–621.

Jooste, P. J., and C. J. Hugo. 1999. The taxonomy, ecology and cultivation of bacterial genera belonging to the family Flavobacteriaceae. Int. J. Food Microbiol. 53:81–94.

Kämpfer, P., U. Dreyer, A. Neef, W. Dott, and H.-J. Busse. 2003. Chryseobacterium defluvii sp. nov., isolated from wastewater. Int. J. Syst. Evol. Microbiol. 53:93–97.

Kelsey, M. C., A. M. Emmerson, and Y. Drabu. 1982. Flavobacterium meningosepticum ventriculitis: in vivo and in vitro results with the combinations Rifampicin-Erythromycin and Mezlocillin-Cefoxitin. Eur. J. Clin. Microbiol. 1:138–143.

Kim, M. K., W.-T. Im, Y. K. Shin, J. H. Lim, S.-H. Kim, B. C. Lee, M.-Y. Park, K. Y. Lee, and S.-T. Lee. 2004. Kaistella koreensis gen. nov., sp. nov., a novel member of the Chryseobacterium-Bergeyella-Riemerella branch. Int. J. Syst. Evol. Microbiol. 54:2319–2324.

Kim, K. K., H.-S. Bae, P. Schumann, and S.-T. Lee. 2005a. Chryseobacterium daecheongense sp. nov., isolated from freshwater lake sediment. Int. J. Syst. Evol. Microbiol. 55:133–138.

Kim, K. K., M.-K. Kim, J. H. Lim, H. Y. Park, and S.-T. Lee. 2005b. Transfer of *Chryseobacterium meningosepticum* and *Chryseobacterium miricola* to *Elizabethkingia* gen. nov. as *Elizabethkingia meningoseptica* comb. nov. and *Elizabethkingia miricola* comb. nov. Int. J. Syst. Evol. Microbiol. 55:1287–1293. DOI:10.1099/ijs.0.63541-0.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Molec. Evol. 16:111–120.

King, E. O. 1959. Studies on a group of previously unclassified bacteria associated with meningitis in infants. Am. J. Clin. Pathol. 31:241–247.

Kirby, J. T., H. S. Sader, T. R. Walsh, and R. N. Jones. 2004. Antimicrobial susceptibility and epidemiology of a worldwide collection of Chryseobacterium spp.: Report from the SENTRY antimicrobial surveillance program (1997–2001). J. Clin. Microbiol. 42:445–448.

Kumagai, A., C. Nakayasu, and N. Oseko. 2004. Effect of tobramycin supplementation to medium on isolation of Flavobacterium psychrophilum from ayu Plecoglossus altivelis. Fish Pathol. 39:75–78.

LaFrentz, B. R., S. E. LaPatra, G. R. Jones, and K. D. Cain. 2004. Protective immunity in rainbow trout Oncorhynchus mykiss following immunization with distinct molecular mass fractions isolated from Flavobacterium psychrophilum. Dis. Aquat. Org. 59;17–26:

Lagacé, L., M. Pitre, M. Jacques, and D. Roy. 2004. Identification of the bacterial community of maple sap by using amplified ribosomal DNA (rDNA) restriction analysis and rDNA sequencing. Appl. Environ. Microbiol. 70:2052–2060.

Lapage, S. P., and R. J. Owen. 1973. Flavobacterium meningosepticum from cases of meningitis in Botswana and England. J. Clin. Pathol. 26:747–749.

Leitz, R., W. Handrick, V. Steinert, and H. Uhlig. 1977. Flavobacterium meningosepticum als meningitiserreger

bei einem frühgeborenen. Pediatrie und Grenzgebiete 16:363–370.

Li, Y., Y. Kawamura, N. Fujiwara, T. Naka, H. Liu, X. Huang, K. Kobayashi, and T. Ezaki. 2003. Chryseobacterium miricola sp. nov., a novel species isolated from condensation water of space station Mir. Syst. Appl. Microbiol. 26:523–528.

Lijnen, H. R., B. van Hoef, F. Ugwu, D. Collen, and I. Roelants. 2000. Specific proteolysis of human plasminogen by a 24 kDa endopeptidase from a novel Chryseobacterium sp. Biochemistry 39:479–488.

Lin, J. T., W. S. Wang, C. C. Yen, J. H. Liu, T. J. Chiou, M. H. Yang, T. C. Chao, and P. M. Chen. 2003. Chryseobacterium indologenes bacteremia in a bone marrow transplant recipient with chronic graft-versus-host disease. Scand. J. Infect. Dis. 35:882–883.

Lopez, M. J., N. N. Nichols, B. S. Dien, J. Moreno, and R. J. Bothast. 2004. Isolation of microorganisms for biological detoxification of lignocellulosic hydrolysates. Appl. Microbiol. Biotechnol. 64:125–131.

Luis, N. B. J., J. I. Hugo, B. A. Enrique, R. M. Ramiro, and P. L. Octavio. 2004. An optimization study of solid-state fermentation: xanthophylls extraction from marigold flowers. Appl. Microbiol. Biotechnol. 65:383–390.

Madruga, M., U. Zanon, G. M. N. Pereira, and A. C. Galvão. 1970. Meningitis caused by Flavobacterium meningosepticum: The first epidemic outbreak of meningitis in the newborn in South America. J. Infect. Dis. 121:328–330.

Manfredi, R., A. Nanetti, M. Ferri, A. Mastroianni, O. V. Coronado, and F. Chiodo. 1999. Flavobacterium spp. organisms as opportunistic bacterial pathogens during advanced HIV disease. J. Infect. 39:146–152.

Mani, R. M., K. C. Kuruvila, P. M. Batliwala, P. N. Damle, G. V. Shirgaonkar, R. P. Soni, and P. R. Vyas. 1978. Flavobacterium meningosepticum as an opportunist. J. Clin. Pathol. 31:220–222.

Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K.-H. Schleifer. 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga-Flavobacter-Bacteroides in the natural environment. Microbiology 142:1097–1106.

Martínez, J. L., A. Casado, and R. Enríquez. 2004. Experimental infection of Flavobacterium psychrophilum in fins of Atlantic salmon Salmo salar revealed by scanning electron microscopy. Dis. Aquat. Org. 59:79–84.

Massias, B., F. Dumetz, M.-C. Urdaci, and M. Le Hénaff. 2004. Identification of P18, a surface protein produced by the fish pathogen Flavobacterium psychrophilum. J. Appl. Microbiol. 97; 574–580:

Matsen, J. M. 1975. Hospital infections with pigmented water bacteria. Health Lab. Sci. 12:305–310.

Mauel, M. J., D. L. Miller, K. S. Frazier, and M. H. Hines II. 2002. Bacterial pathogens isolated from cultured bullfrogs (Rana catesbeiana). J. Vet. Diagn. Invest. 14:431–433.

McBain, A. J., R. G. Bartolo, C. E. Catrenich, D. Charbonneau, R. G. Ledder, A. H. Rickard, S. A. Symmons, and P. Gilbert. 2003. Microbial characterization of biofilms in domestic drains and the establishment of stable biofilm microcosms. Appl. Environ. Microbiol. 69:177–185.

McBride, M. J., and S. A. Baker. 1996. Development of techniques to genetically manipulate members of the genera Cytophaga, Flavobacterium, Flexibacter, and Sporocytophaga. Appl. Environ. Microbiol. 62:3017–3022.

McBride, M. J., and M. J. Kempf. 1996. Development of techniques for the genetic manipulation of the gliding bacterium Cytophaga johnsonae. J. Bacteriol. 178:583–590.

McMeekin, T. A., J. T. Patterson, and J. G. Murray. 1971. An initial approach to the taxonomy of some gram negative yellow pigmented rods. J. Appl. Bacteriol. 34:699–716.

McMeekin, T. A., and J. M. Shewan. 1978. Taxonomic strategies for Flavobacterium and related genera. J. Appl. Bacteriol. 45:321–332.

Michel, C., O. Matte-Tailliez, B. Kerouault, and J.-F. Bernardet. Resistance pattern and assessment of phenicol agents' minimum inhibitory concentration in multiple drug resistant Chryseobacterium isolates from fish and aquatic habitats. J. Appl. Microiol. 99:323–332.

Miller, D. L., Z. A. Radi, S. L. Stiver, and T. D. Thornhill. 2004. Cutaneous and pulmonary mycosis in green anacondas (Eunectes murinus). J. Zoo Wildl. Med. 35:557–561.

Moore, W. E. C., E. P. Cato, and L. V. H. Moore. 1985. Index of the bacterial and yeast nomenclatural changes published in the International Journal of Systematic Bacteriology since the 1980 approved lists of bacterial names (1 January 1980 to 1 January 1985). Int. J. Syst. Bacteriol. 35:382–407.

Moreno, Y., C. R. Arias, H. Meier, E. Garay, and R. Aznar. 1999. In situ analysis of the bacterial communities associated to farmed eel by whole-cell hybridization. Lett. Appl. Microbiol. 29:160–165.

Morita, Y., T. Nakamura, Q. Hasan, Y. Murakami, K. Yokoyama, and E. Tamiya. 1997. Cold-active enzymes from cold-adapted bacteria. J. American Oil Chemists' Society 74:441–444.

Mudarris, M. 1989. Studies on the Microflora of Turbot (Scophthalmus maximus L.) with Emphasis on Bacterial Gill Disease [PhD thesis]. Heriott-Watt University. Edinburgh, UK.

Mudarris, M., and B. Austin. 1989. Systemic disease in turbot Scophthalmus maximus caused by a previously unrecognised Cytophaga-like bacterium. Dis. Aquat. Org. 6:161–166.

Mudarris, M., and B. Austin, P. 1992. Histopathology of a gill and systemic disease of turbot (Scophthalmus maximus L.) caused by a Cytophaga-like bacterium. Bull. Eur. Assoc. Fish Pathol. 12:120–123.

Mudarris, M., B. Austin, P. Segers, M. Vancanneyt, B. Hoste, and J.-F. Bernardet. 1994. Flavobacterium scophthalmum sp.nov., a pathogen of turbot (Scophthalmus maximus L.). Int. J. Syst. Bacteriol. 44:447–453.

Murray, R. G. E., and E. Stackebrandt. 1995. Taxonomic note: implementation of the provisional status Candidatus for incompletely described procaryotes. Int. J. Syst. Bacteriol. 45:186–187.

Naas, T., S. Bellais, and P. Nordmann. 2003. Molecular and biochemical characterization of a carbapenem-hydrolyzing beta-lactamase from Flavobacterium johnsoniae. J. Antomicrob. Chemother. 51:267–273.

Nagai, T., Y. Lida, and T. Yoneji. 2003. Field trials of a vaccine with water-soluble adjuvant for bacterial coldwater disease in ayu Plecoglossus altivelis. Fish Pathol. 38:63–65.

Nakagawa, Y., M. Suzuki, and K. Hatano. 2001. Phylogenetic diversity of Cytophaga-like strains isolated from the sub-tropical zone of Japan. IFO Res. Commun. 20:61–71.

Nedoluha, P. C., and D. Westhoff. 1997a. Microbiological analysis of striped bass (Morone saxatilis) grown in a recirculating system. J. Food Protect. 60:948–953.

Nedoluha, P. C., and D. Westhoff. 1997b. Microbiology of striped bass grown in three aquaculture systems. Food Microbiol. 14:255–264.

Nulens, E., B. Bussels, A. Bols, B. Gordts, and H. W. Van Landuyt. 2001. Recurrent bacteraemia by Chryseobacterium indologenes in an oncology patient with a totally implanted intravascular device. Clin. Microbial. Infect. 7:369–373.

Olsen, H., W. C. Frederiksen, and K. E. Siboni. 1965. Flavobacterium meningosepticum in 8 non-fatal cases of postoperative bacteræmia. Lancet 285:1294–1296.

Olsen, H. 1967a. A clinical analysis of 10 cases of postoperative infection with Flavobacterium meningosepticum. Danish Med. Bull. 14:1–5.

Olsen, H. 1967b. An in vitro study of the antibiotic sensitivity of Flavobacterium meningosepticum. Acta Path. Microbiol. Scand. 70:601–612.

Olson, M. E., S. Gard, M. Brown, R. Hampton, and D. W. Morck. 1992. Flavobacterium indologenes infection in leopard frogs. J. Am. Vet. Med. Assoc. 201:1766–1770.

Oppong, D., V. M. King, and J. A. Bowen. 2003. Isolation and characterization of filamentous bacteria from paper mill slimes. Int. Biodeter. Biodegrad. 52:53–62.

O'Sullivan, L. A., A. J. Weightman, and J. C. Fry. 2002. New degenerate Cytophaga-Flexibacter-Bacteroides-specific 16S ribosomal DNA-targeted oligonucleotide probes reveal high bacterial diversity in River Taff epilithon. Appl. Environ. Microbiol. 68:201–210.

Owen, R. J., and S. P. Lapage. 1974. A comparison of strains of King's group IIb of Flavobacterium with Flavobacterium meningosepticum. Ant. v. Leeuwenhoek 40:255–264.

Owen, R. J., and J. J. S. Snell. 1976. Deoxyribonucleic acid reassociation in the classification of flavobacteria. J. Gen. Microbiol. 93:89–102.

Owen, R. J., and B. Holmes. 1981. Identification and classification of Flavobacterium species from clinical sources. *In:* H. Reichenbach and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group. Verlag Chemie. Weinheim, Germany. Gesellschaft für Biotechnologische Forschung Monograph Series, No. 5:39–50.

Pavlov, D., C. M. E. de Wet, W. O. K. Grabow, and M. M. Ehlers. 2004. Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. Int. J. Food Microbiol. 92:275–287.

Pickett, M. J., and M. M. Pedersen. 1970. Non-fermentative bacilli associated with man: II. Detection and identification. Am. J. Clin. Pathol. 54:164–177.

Pokrywka, M., K. Viazanko, J. Medvick, S. Knabe, S. McCool, A. W. Pasculle, and J. N. Dowling. 1993. A Flavobacterium meningosepticum outbreak among intensive care patients. Am. J. Infect. Control 21:139–145.

Price, K. W., and M. J. Pickett. 1981. Studies of clinical isolates of flavobacteria. *In:* H. Reichenbach and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group. Verlag Chemie. Weinheim, Germany. Gesellschaft für Biotechnologische Forschung Monograph Series, No. 5:63–77.

Quilici, M.-L., and C. Bizet. 1996. Ribotyping of Chryseobacterium meningosepticum: its use as an epidemiological tool and its correlation with serovars. Res. Microbiol. 147:415–425.

Radianingtyas, H., G. K. Robinson, and A. T. Bull. 2003. Characterization of a soil-derived bacterial consortium degrading 4-chloroaniline. Microbiology-SGM 149:3279–3287.

Rahman, M. H., M. Ototake, and T. Nakanishi. 2003. Water-soluble adjuvants enhance the protective effect of Flavobacterium psychrophilum vaccines in ayu Plecoglossus altivelis. Fish Pathol. 38:171–176.

Reichenbach, H., W. Kohl, and H. Achenbach. 1981. The flexirubin-type pigments, chemosystematically useful compounds. *In:* H. Reichenbach and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group. Verlag Chemie. Weinheim, Germany. Gesellschaft für Biotechnologische Forschung Monograph Series, No. 5:101–108.

Reichenbach, H. 1989. Order I. Cytophagales . *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. The Williams and Wilkins Co. Baltimore, MD. 3:2011–2013.

Richard, C., H. Monteil, A. Le Faou, and B. Laurent. 1979. Etude de souches de Flavobacterium isolées à Strasbourg dans un service de réanimation médicale. Individualisation d'un nouveau sérotype (G) de Flavobacterium meningosepticum. Méd. Mal. Infect. 9:124–128.

Richard, C., H. Monteil, and B. Laurent. 1979. Individualisation de six nouveaux types antigéniques de Flavobacterium meningosepticum. Ann. Microbiol. Inst. Pasteur 130B:141–144.

Richard, C., and B. Laurent. 1981. Occurrence of Flavobacterium meningosepticum serotypes in France. *In:* H. Reichenbach and O. B. Weeks (Eds.) The Flavobacterium-Cytophaga Group. Verlag Chemie. Weinheim, Germany. Gesellschaft für Biotechnologische Forschung Monograph Series, No. 5:165–168.

Richard, C., and M. Kiredjian. 1995. Laboratory Methods for the Identification of Strictly Aerobic Gram-negative Bacilli. Institut Pasteur. Paris, France.

Rios, I., J. J. Klimek, E. Maderazo, and R. Quintiliani. 1978. Flavobacterium meningosepticum meningitis: report of selected aspects. Antimicrob. Agents Chemother. 14:444–447.

Rosado, W., and N. S. Govind. 2003. Identification of carbohydrate-degrading bacteria in sub-tropical regions. Rev. Biol. Trop. 51:205–210.

Sader, H. S., R. N. Jones, and M. A. Pfaller. 1995. Relapse of catheter-related Flavobacterium meningosepticum bacteraemia demonstrated by DNA macrorestriction analysis. Clin. Infect. Dis. 21:997–1000.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molec. Biol. Evol. 4:406–425.

Samb, A., J.-P. Chiron, F. Denis, A. Sow, and I. D. Mar. 1977. Méningite à Flavobacterium meningosepticum. Bull. Soc. Méd. Afr. Noire Langue Française 22:84–91.

Sanni A. I., A. A. Onilude, I. F. Fadahunsi, and R. O. Afolabi. 1999. Microbial deterioration of traditional alcoholic beverages in Nigeria. Food Res. Int. 32:163–167.

Sarvamangala, J. N., A.Venkatesh, and P. Shivananda. 1983. Flavobacterial meningitis in an infant. Indian J. Pediatr. 50:93–95.

Schreckenberger, P. C. 1998. Emended classification and description of the family Flavobacteriaceae and the genus Sphingobacterium. Clin. Microbiol. Newslett. 20:115–124.

Segers, P., W. Mannheim, M. Vancanneyt, K. De Brandt, K.-H. Hinz, K. Kersters, and P. Vandamme. 1993. Riemerella anatipestifer gen. nov., the causative agent of septicemia anserum exsudativa, and its phylogenetic

affiliation within the Flavobacterium-Cytophaga rRNA homology group. Int. J. Syst. Bacteriol. 43:768–776.

Sexton, D. J., P. C. Houk, and R. N. Grantham. 1985. Successful treatment of prosthetic valve endocarditis due to Flavobacterium meningosepticum. South. Med. J. 78:1267–1268.

Shan, L., T. Martin, L. M. Sollid, G. M. Gray, and C. Khosla. 2004. Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coelic sprue. Biochem. J. 383:311–318.

Sharma, S., S. Srinivasan, R. S. Rao, and R. K. Puri. 1984. Flavobacterium meningitis. Indian Pediatr. 21:582–583.

Shen, F. T., P. Kämpfer, C. C. Young, W. A. Lai, and A. B. Arun. 2005. *Chryseobacterium taichungense* sp. nov., isolated from contaminated soil. Int. J. Syst. Evol. Microbiol. 55:1301–1304.

Sheridan, R. L., C. M. Ryan, M. S. Pasternack, J. M. Weber, and R. G. Tompkins. 1993. Flavobacterial sepsis in massively burned pediatric patients. Clin. Infect. Dis. 17:185–187.

Shewan, J. M., and T. A. McMeekin. 1983. Taxonomy and ecology of Flavobacterium and related genera. Ann. Rev. Microbiol. 37:233–252.

Shulman, B. H., and M. S. Johnson. 1944. A case of meningitis in a premature infant due to a proteolytic Gram-negative bacillus. J. Lab. Clin. Med. 29:500–507.

Siegman-Igra, Y., D. Schwartz, G. Soferman, and N. Konforti. 1987. Flavobacterium group IIb bacteraemia: Report of a case and review of Flavobacterium infections. Med. Microbiol. Immunol. 176:103–111.

Sims, M. A. 1974. Flavobacterium meningosepticum, a probable cause of meningitis in a cat. Vet. Rec. 95:567–569.

Skerman, V. B. D., V. McGowan, and P. H. A. Sneath (Eds.). 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225–420.

Solé-Vernin, C., C. M. Ulson, and M. Zuccolotto. 1960. Verificacão de Flavobacterium meningosepticum King, 1959, em Saõ Paulo (Brasil). Rev. Inst. Med. Trop. São Paulo 2:54–58.

Spangler, S. K., M. A. Visalli, M. R. Jacobs, and P. C. Appelbaum. 1996. Susceptibilities of non-Pseudomonas aeruginosa Gram-negative nonfermentative rods to ciprofloxacin, ofloxacin, levofloxacin, d-ofloxacin, sparfloxacin, ceftazidime, piperacillin, piperacillin-tazobactam, trimethoprim-sulfamethoxazole, and imipenem. Antimicrob. Agents Chemother. 40:772–775.

Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44:846–849.

Stackebrandt, E., W. Frederiksen, G. M. Garrity, P. A. D. Grimont, P. Kämpfer, M. C. J. Maiden, X. Nesme, R. Rosselló-Mora, J. Swings, H. G. Trüper, L. Vauterin, A. C. Ward, and W. B. Whitman. 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. 52:1043–1047.

Stamm, W. E., J. J. Colella, R. L. Anderson, and R. E. Dixon. 1975. Indwelling arterial catheters as a source of nosocomial bacteraemia: An outbreak caused by Flavobacterium species. N. Engl. Med. J. 292:1099–1102.

Sundin, D., B. D. Gold, F. E. Berkowitz, D. A. Schwartz, and D. Goo. 1991. Community-acquired Flavobacterium meningosepticum meningitis, pneumonia, and septicemia in a normal infant. Pediatr. Infect. Dis. J. 10:73–76.

Suzuki, M., Y. Nakagawa, S. Harayama, and S. Yamamoto. 1999. Phylogenetic analysis of genus Marinilabilia and related bacteria based on the amino acid sequences of GyrB and emended description of Marinilabilia salmonicolor with Marinilabilia agarovorans as its subjective synonym. Int. J. Syst. Evol. Microbiol. 49:1551–1557.

Tan, L. H., X. N. Sun, X. K. Zhu, Z. W. Zhang, P. H. Li, and Q. Shit. 2004. Epidemiology of nosocomial pneumonia in infants after cardiac surgery. Chest 125:410–417.

Tatum, H. W., W. H. Ewing, and R. E. Weaver. 1974. Miscellaneous Gram-negative bacteria. *In:* E. H. Lenette, E. H. Spalding, and J. P. Truant (Eds.) Manual of Clinical Microbiology, 2nd ed. American Society for Microbiology. Washington, DC. 270–294.

Taylor, F. R., R. C. Simmonds, and D. G. Loeffler. 1993. Isolation of Flavobacterium meningosepticum in a colony of leopard frogs (Rana pipiens). Lab. Anim. Sci. 43:105.

Taylor, P. W. 2004. Detection of Flavobacterium psychrophilum in eggs and sexual fluids of Pacific salmonids by a polymerase chain reaction assay: implications for vertical transmission of bacterial coldwater disease. J. Aquat. An. Health. 16:104–108.

Tekerekoglu, M. S., R. Durmaz, M. Ayan, Z. Cizmeci, and A. Akinci. 2003. Analysis of an outbreak due to Chryseobacterium meningosepticum in a neonatal intensive care unit. Microbiologica 26:57–63.

Thomas-Jinu, S., and A. E. Goodwin. 2004. Acute columnaris infection in channel catfish, Ictalurus punctatus (Rafinesque): efficacy of practical treatments for warmwater aquaculture ponds. J. Fish Dis. 27:23–28.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680.

Thong, M. L., S. D. Puthucheary, and E. L. Lee. 1981. Flavobacterium meningosepticum infection: an epidemiological study in a newborn nursery. J. Clin. Pathol. 34:429–433.

Tide, C., S. R. Harkin, G. G. Geesey, P. J. Bremer, and W. Scholz. 1999. The influence of welding procedures on bacterial colonization of stainless steel weldments. J. Food Engin. 42:85–96.

Tizer, K. B., J. S. Cervia, A.-M. Dunn, J. J. Stavola, and G. J. Noel. 1995. Successful combination of vancomycin and rifampicin therapy in a newborn with community acquired Flavobacterium meningosepticum neonatal meningitis. Pediatr. Infect. Dis. J. 14:916–917.

Torronteras-Santiago, R., C. Cintado-Bueno, R. Garcia-Gomez, P. Macias-Mardones, and M. Menendez-Ruiz. 1978. Pyoventriculite par Flavobacterium meningosepticum. Pédiatrie 33:393–398.

Uchihara, T., T. Yakota, S. Watabiki, M. Ueki, S. Miyake, and H. Tsukagoshi. 1988. Flavobacterium meningosepticum meningitis in an adult. Am. J. Med. 85:738–739.

Ultee, A., N. Souvatzi, K. Maniadi, and H. König. 2004. Identification of the culturable and nonculturable bacterial population in ground water of a municipal water supply in Germany. J. Appl. Microbiol. 96:560–568.

Ursing, J., and B. Bruun. 1987. Genetic heterogeneity of Flavobacterium meningosepticum demonstrated by

DNA-DNA hybridization. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 95:33–39.

Ursing, J., and B. Bruun. 1991. Genetic heterogeneity of Flavobacterium group IIb and Flavobacterium breve demonstrated by DNA-DNA hybridization. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 99:780–786.

Vaillant, V. S.-B., R. B. Salvador, and M. S. M. Rams. 1974. Estudio de dos casos de infeccion por Flavobacterium meningosepticum. Rev. Clin. Española 133:455–456.

Vancanneyt, M., P. Segers, L. Hauben, J. Hommez, L. A. Devriese, B. Hoste, P. Vandamme, and K. Kersters. 1994. Flavobacterium meningosepticum, a pathogen in birds. Int. J. Syst. Evol. Microbiol. 32:2398–2403.

Vancanneyt, M., P. Segers, U. Torck, B. Hoste, J.-F. Bernardet, P. Vandamme, and K. Kersters. 1996. Reclassification of Flavobacterium odoratum (Stutzer 1929) strains to a new genus, Myroides, as Myroides odoratus comb. nov. and Myroides odoratimimus sp. nov. Int. J. Syst. Bacteriol. 46:926–932.

Vancanneyt, M., P. Vandamme, P. Segers, U. Torck, R. Coopman, K. Kersters, and K.-H. Hinz. 1999. Riemerella columbina sp. nov., a bacterium associated with respiratory disease in pigeons. Int. J. Syst. Bacteriol. 49:289–295.

Vandamme, P., J.-F. Bernardet, P. Segers, K. Kersters, and B. Holmes. 1994. New perspectives in the classification of the flavobacteria: description of Chryseobacterium gen. nov., Bergeyella gen. nov., and Empedobacter nom. rev. Int. J. Syst. Bacteriol. 44:827–831.

Vandamme, P., P. Segers, M. Vancanneyt, K. Van Hove, R. Mutters, J. Hommez, F. Dewhirst, B. Paster, K. Kersters, E. Falsen, L. A. Devriese, M. Bisgaard, K.-H. Hinz, and W. Mannheim. 1994. Ornithobacterium rhinotracheale gen. nov., sp.nov., isolated from the avian respiratory tract. Int. J. Syst. Bacteriol. 44:24–37.

Vandamme, P., M. Vancanneyt, A. van Belkum, P. Segers, W. G. V. Quint, K. Kersters, B. Paster, and F. Dewhirst. 1996. Polyphasic analysis of strains of the genus Capnocytophaga and Center for Disease Control group DF-3. Int. J. Syst. Bacteriol. 46:782–791.

Vandepitte, J., C. Beeckmans, and R. Buttiaux. 1958. Quatre cas de méningite à Flavobacterium chez des nouveaunés. Ann. Soc. Belge Méd. Trop. 38:563–570.

Van Trappen, S., J. Mergaert, S. Van Eygen, P. Dawynt, M. C. Cnockaert, and J. Swings. 2002. Diversity of 746 heterotrophic bacteria isolated from microbial mats from ten Antarctic lakes. System. Appl. Microbiol. 25:603–610.

Venter, H., G. Osthoff, and D. Litthauer. 1999. Purification and characterization of a metalloprotease from Chryseobacterium indologenes Ix9a and determination of the amino acid specificity with electrospray mass spectrometry. Protein Expr. Purif. 15:282–295.

Von Graevenitz, A., and M. Grehn. 1977. Susceptibility studies on Flavobacterium meningosepticum II-b. FEMS Microbiol. Lett. 2:289–292.

Von Graevenitz, A. 1985. Ecology, clinical significance and antimicrobial susceptibility of infrequently encountered glucose-nonfermenting Gram-negative rods. *In:* G. S. Gilardi (Ed.) Non-fermentative Gram-negative Rods. Laboratory Identification and Clinical Aspects. Marcel Dekker. New York, NY. 181–232.

Walenciak, O., W. Zwisler, and E. M. Gross. 2002. Influence of Myriophyllum spicatum-derived tannins on gut microbiota of its herbivore Acentria ephemerella. J. Chem. Ecol. 28:2045–2056.

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. Int. J. Syst. Bacteriol. 37:463–464.

Weller, R., F. O. Glöckner, and R. Amann. 2000. 16S rRNA-targeted oligonucleotide probes for the in situ detection of members of the phylum Cytophaga-Flavobacterium-Bacteroides. System. Appl. Microbiol. 23:107–114.

Welthagen, J. J., and P. J. Jooste. 1992. Isolasie en karakterisering van gepigmenteerde psigrotrofe bakterieëuit verkoelde roumelk. S. Afr. J. Dairy Sci. 24:47–52.

Werthamer, S., and M. Weiner. 1972. Subacute bacterial endocarditis due to Flavobacterium meningosepticum. Am. J. Clin. Pathol. 57:410–412.

Yabuuchi, E., T. Kaneko, I. Yano, C. W. Moss, and N. Miyoshi. 1983. Sphingobacterium gen. nov., Sphingobacterium spiritivorum comb. nov., Sphingobacterium multivorum comb. nov., Sphingobacterium mizutae sp. nov., and Flavobacterium indologenes sp. nov.: Glucose-nonfermenting Gram-negative rods in CDC groups IIk-2 and IIb. Int. J. Syst. Bacteriol. 33:580–598.

Yabuuchi, E., Y. Hashimoto, T. Ezaki, Y. Ido, and N. Takeuchi. 1990. Genotypic and phenotypic differentiation of Flavobacterium indologenes Yabuuchi et al. 1983 from Flavobacterium gleum Holmes et al. 1984. Microbiol. Immunol. 34:73–76.

Yamaguchi, S., and M. Yokoe. 2000. A novel protein-deamidating enzyme from Chryseobacterium proteolyticum sp. nov., a newly isolated bacterium from soil. Appl. Environ. Microbiol. 66:3337–3343.

Yamaguchi, S., D. J. Jeenes, and D. B. Archer. 2001. Protein-glutaminase from Chryseobacterium proteolyticum, an enzyme that deamidates glutaminyl residues in proteins. Eur. J. Biochem. 268:1410–1421.

Yi, H., H. I. Yoon, and J. Chun. 2005. Sejongia antarctica gen. nov., sp. nov., Sejongia jeonii sp. nov., isolated from the Antarctic. Int. J. Syst. Evol. Microbiol. 55:409–416.

Young, C. C., P. Kämpfer, F.-T. Shen, W.-A. Lai, and A. B. Arun. 2005. Chryseobacterium formosense sp. nov., isolated from the rhizosphere of Lactuca sativa L. (Garden lettuce). Int. J. Syst. Evol. Microbiol. 55:423–426.

Yu, J., and O. Ward. 1996. Investigation of the biodegradation of pentachlorophenol by the predominant bacterial strains in a mixed culture. Int. Biodeter. Biodegrad. 5:181–187.

Zappulla, F., M. Gatti, G. Rocchi, R. Tanas, O. Varoli, and A. Beccari. 1979. Infezione da Flavobacterium meningosepticum in una bambina de 19 mesi. Minerva Pediatrica 31:679–682.

Zdanowski, M. K., P. Weglenski, P. Golik, J. M. Sasin, P. Borsuk, M. J. Zmuda, and A. Stankovic. 2004. Bacterial diversity in Adélie penguin, Pygoscelis adeliae, guano: molecular and morpho-physiological approaches. FEMS. Microbiol. Ecol. 50:163–173.

Zhang, C., and Y. Rikihisa. 2004. Proposal to transfer 'Aegyptianella ranarum', an intracellular bacterium of frog red blood cells, to the family Flavobacteriaceae as 'Candidatus Hemobacterium ranarum' comb. nov. Environ. Microbiol. 6:568–573.

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CHAPTER 6.13

The Marine Clade of the Family Flavobacteriaceae: The Genera Aequorivita, Arenibacter, Cellulophaga, Croceibacter, Formosa, Gelidibacter, Gillisia, Maribacter, Mesonia, Muricauda, Polaribacter, Psychroflexus, Psychroserpens, Robiginitalea, Salegentibacter, Tenacibaculum, Ulvibacter, Vitellibacter and Zobellia

JOHN P. BOWMAN

Introduction

The family Flavobacteriaceae represents a major branch of the Gram-negative phylum *Bacteroidetes* that is also popularly referred to as the "*Cytophaga-Flavobacterium-Bacteroides* group or phylum" (see The List of Bacterial Names with Standing in Nomenclature website for official taxonomic names). In the last decade the Flavobacteriaceae has undergone rapid expansion incorporating many new species and genera, mostly from marine and polar ecosystems. A standardized minimal set of criteria has been recently established for the description of new members of this taxonomic group (Bernardet et al., 2002). Several genera within the family, most obviously *Flavobacterium* (Bernardet et al., 1996), have undergone substantial taxonomic revision. This heightened activity has at least partially come in response to the increasing evidence that this homogenously chemoheterotrophic group has environmental significance in aquatic ecosystems. Recent research indicates the marine genera of the family are a major component of the ocean pelagic zone microbial biomass, strongly coupled to phytoplanktonic primary production (photosynthesis), and, on the basis of at least anecdotal research, responsible for a major fraction of organic matter remineralization in the oceans (reviewed by Kirchman, 2002). The marine clade of genera in the Flavobacteriaceae is phylogenetically distinct, notable for containing a large number of cold adapted (psychrophilic and psychrotolerant) species, and in this respect, it parallels marine genus clusters in phylum *Proteobacteria*, which are the only other consistently major component of surface ocean bacterioplankton. As of early 2004, 19 validly described genera, listed in the title of the chapter, belong to the marine clade of the family Flavobacteriaceae. A general coverage of the biology of these genera is the focus of this chapter.

Evolution of the Marine Clade

Currently, analysis of 16S rRNA gene sequence is used as the major criterion in establishing new taxa amongst the existing hierarchy of bacteria. This has been the case for the marine clade of the Flavobacteriaceae in which all the official names have been either created or revised only since 1997, with phylogeny used initially to designate new taxa. A large number of validly described genera and species reside within the marine clade (Table 1), which collectively forms a distinct phylogenetic branch as shown in Fig. 1. The next closest related bacterial taxon to this clade is the genus *Flavobacterium* which inhabits a wide range of aquatic ecosystems and, though found in marine ecosystems, is not halophilic. *Flavobacterium* seems to represent an ecological transition between the marine clade and a distinct cluster of soil, freshwater and warm-blooded parasitic species represented by genus *Chryseobacterium* (Fig. 1) that also belong to the family Flavobacteriaceae. This phylogenetic arrangement of genera provides a hypothesis for reconstructing the evolutionary history of the Flavobacteriaceae marine clade. Ancestral species appear to have undergone an evolutionary expansion, moving into many non-marine environments, including the human intestinal tract, which is dominated by the Bacteroidaceae (a family of anaerobic bacteria just adjacent to family Flavobacteriaceae). It can be hypothesized that the Flavobacteriaceae marine clade itself is comprised of lineages descended from ancestral species that remained within marine ecosystems. This possible long occupation of marine habitats

Table 1. Known habitats, isolation media, and incubation temperatures for Flavobacteriaceae marine clade species.

Genus	Known habitat(s)	Isolation medium	Incubation temperature (°C)	Reference(s)
Aequorivita				
A. antarctica, A. lipolytica	Seawater, sea-ice, and quartz stone subliths	MA or SW	20	Bowman and Nichols, 2002
A. crocea	Seawater, and sea-ice	MA or SW	20	Bowman and Nichols, 2002
A. sublithincola	Quartz stone subliths	R2A + sea salts, MA	20	Bowman and Nichols, 2002
Arenibacter				
A. latericius	Marine sediment, seaweed, and holothurians	Medium B or MA	25	Ivanova et al., 2001
A. troitsensis	Marine sediment	MA	25	Nedashkovskaya et al., 2003b
Cellulophaga				
C. algicola	Sea-ice algal assemblage, and seaweed	MA	10–15	Bowman, 2000
C. baltica, C. fucicola	Seaweed, and adjacent seawater (Baltic Sea)	CYT	20	Johansen et al., 1999
C. lytica	Marine sediment, seawater, and seaweed	MA	20–25	Lewin, 1969
Croceibacter atlanticus	Seawater	LMHN (HTC tech.)	20–25	Cho and Giovannoni, 2003
[C.] latercula	Seawater (aquarium)	MA	20	Lewin, 1969
[C.] marinoflava	Seawater	MA	20–25	Reichenbach, 1989
Formosa algae	Seaweed	MA	20–25	Ivanova et al., 2004
Gelidibacter				
G. algens	Sea-ice, and quartz stone subliths	MA	15–20	Bowman et al., 1997b
G. mesophilus	Seawater (Mediterranean Sea)	MA	25	Maciá et al., 2002
Gillisia limnaea	Microbial mats, and low salinity polar lakes	R2A or MA	20–25	Van Trappen et al., 2002; Van Trappen et al., 2003
Maribacter spp.				
M. orientalis, M. aquivivus	Seawater	MA	25–30	Nedashkovskaya et al., 2004a

M. sedimenticola	Marine sediment	MA	25–30	Nedashkovskaya et al., 2004a
M. ulvicola	Seaweed	MA	25–30	Nedashkovskaya et al., 2004a
Mesonia algae	Seaweed	MA	25–30	Nedashkovskaya et al., 2003a
Muricauda ruestringensis	Hexadecane enriched marine sediment	Modified MA + hexadecane	25	Bruns et al., 2001
Psychroflexus				
P. gondwanensis	Hypersaline lakes, and Antarctic	MA	20–25	Dobson et al., 1991; Dobson et al., 1993
P. torquis	Sea-ice algal assemblage	MA	4–10	Bowman et al., 1997a; Bowman et al., 1997c; Bowman et al., 1998
P. tropicus	Hypersaline lakes, and Hawaii	MA	30–37	Donachie et al., 2004
Psychroserpens burtonensis	Seawater (pycnocline of fjord)	MA (+Tween 80)	4–10	Bowman et al., 1997b
Polaribacter				
P. filamentus, P. irgensii, P. franzmannii	Seawater, and sea-ice	MA	4–10	Gosink et al., 1998
P. glomeratus	Seawater	MA	10–15	McGuire et al., 1987
Robiginitalea biformata	Seawater	LNHM (HTC tech.)	20–25	Cho and Giovannoni, 2004
Salegentibacter salegens	Hypersaline lakes, and Antarctic	MA	20–25	Dobson et al., 1991; Dobson et al., 1993
Tenacibaculum				
T. amylolyticum, T. mesophilum	Sponge tissue, seaweed	1/5 LMB	25	Suzuki et al., 2001
T. ovolyticum	Fish (Halibut) eggs	MA	20–25	Hansen et al., 1992
T. skaggerakense	Seawater	1/10 MA	20	Frette et al., 2004
T. maritimum	Fish (lesions, tissue)			Hikida et al., 1979
Ulvibacter litoralis	Seaweed	MA	25–30	Nedashkovskaya et al., 2004b
Vitellibacter vladivostokensis	Holothurian	MA	25–30	Nedashkovskaya et al., 2003c
Zobellia				
Z. galactanivorans	Seaweed	Mineral salts + carrageenan	20	Barbeyron et al., 2001
Z. uliginosa	Marine sediment	MA	25	ZoBell and Upham, 1944

Growth Media Abbreviations: MA, marine agar; SW, seawater medium; CYT, casein yeast medium; 1/5 LMB, high salt Luria marine broth; and LNHM, low nutrient heterotrophic m.

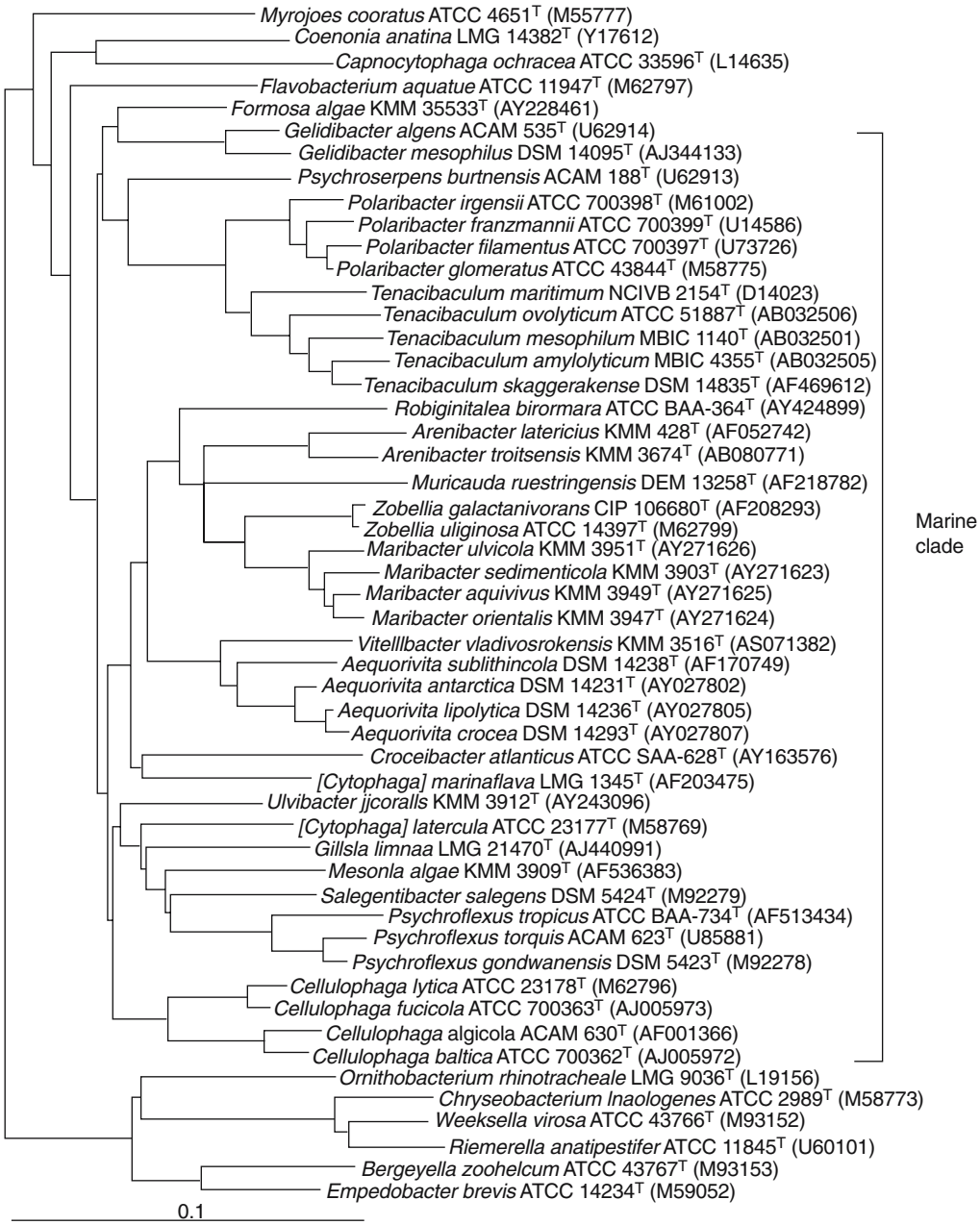

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the position of marine clade genera and species of the family Flavobacteriaceae.

may explain why they make up such a large proportion of the marine microbial biomass (see the Ecology and Habitats section in this Chapter) and have adapted to practically all oxygenated marine econiches.

Taxonomy

Apart from 16S rRNA gene sequences, polyphasic criteria detailed in Bernardet et al. (2002) define the taxonomy of the marine clade. Characteristics that differentiate the various genera in the marine clade are shown in Tables 2–6. Almost all taxa in the marine clade are quite new and only six nomenclaturally valid species have existed for any significant length of time, and of these, four have been reclassified. Two species, *Cytophaga marinoflava* (Reichenbach, 1989) and *Cytophaga latercula* (Lewin, 1969), are misclassified at the genus-level by 16S rRNA analysis and quite distinct from the other genera within

the marine clade (Fig. 1). The genus *Cytophaga*, represented by type species *C. hutchinsonii* (a nonmarine soil species), is unrelated as it belongs to a completely different family of the phylum *Bacteroidetes*. The four other "old" species are *Tenacibaculum maritimum*, *Tenacibaculum ovolyticum*, *Cellulophaga lytica* and *Zobellia uliginosa*.

Tenacibaculum maritimum strains were originally isolated from diseased fish by Hikida et al. (1979), but the official description was not made until later by Wakabayashi et al. (1986), who called the fish strains "*Flexibacter maritimus*." The species description was then later emended by Bernardet and Grimont (1989). In *Bergey's Manual* (Volume 3, 1st edition), Reichenbach (1989) renamed *F. marinus Cytophaga marina*. Along with this species, Hansen et al. (1992) described a phenotypically similar species from diseased Atlantic halibut eggs, called "*Flexibacter ovolyticus*." Subsequent 16S rRNA gene data clearly indicated these species belonged to neither *Flexibacter* or *Cytophaga* and thus the confusion of names was finally resolved when Suzuki et al. (2001) created the genus *Tenacibaculum*. *Tenacibaculum* now contains five species (Table 1).

Cytophaga lytica, originally isolated by Lewin (1969) from a marine aquarium outflow and from the surface of marine algal species, was designated the type species of the genus *Cellulophaga* (Johansen et al., 1999). The name is a misnomer as none of the species in *Cellulophaga* actually degrade or utilize cellulose (in the form of filter paper or crystalline powdered cellulose), though they can degrade the derivative carboxymethylcellulose.

Cytophaga uliginosa (originally *Flavobacterium uliginosum*) was renamed by Reichenbach (1989) and only one strain is known. ZoBell and Upham isolated this strain, described it in a seminal paper on marine bacteria (ZoBell and Upham, 1944), and called it "*Agarbacterium uliginosa*" as it rapidly decomposes agar. Subsequently Barbeyron et al. (2001) created a new genus called appropriately "*Zobellia*," which included *Zobellia uliginosa* as the type species renamed from *Cytophaga uliginosa*.

All the remaining named species belonging to the marine clade are relatively new, have not undergone any taxonomic revision, and should remain stable nomenclatural entities. A strong and reliable taxonomic framework has thus been fortunately developed for this environmentally abundant group, unlike the case for other marine and nonmarine groups within the *Bacteroidetes* (e.g., *Flexibacter* and *Microscilla*), which still require heavy taxonomic revision.

Morphological Characteristics

Phenotypically, the marine clade has several common phenotypic properties (many listed in Tables 2–6) which can aid in initial identification.

Marine clade species are generally characterized (with some exceptions) as strictly aerobic chemoheterotrophic, halophilic, Gram-negative, rod-shaped cells, which are either nonmotile or able to glide and produce colonies pigmented yellow to a deep orange. Some species may form filaments though very long filament formation (>100 μm) is rare. The filaments of some species may be coiled or helical when cells are from log phase cultures. In older cultures, the rods and filaments of some species (such as *Gelidibacter algens* and *Croceibacter atlanticus*) may degenerate into still viable coccoidal cells, possibly spheroplasts, which are usually 1–2 μm in diameter. Some *Polaribacter* species form gas vesicles, though this trait has not been observed in any other species of the clade and thus could be idiosyncratic for the genus. *Muricauda ruestringensis* is unusual in forming long thin appendages which have at the tip a bulbous vesicle-like structure. The appendages are a continuum of the outer membrane (Bruns et al., 2001). The species also undergoes an asymmetrical life cycle in which cells contain different chromosomal DNA contents (Muller et al., 2001).

Gliding motility can sometimes be discerned directly from colonial morphology; however, in the marine clade, gliding is generally not very noticeable and often requires very close microscopic examination in wet preparations. Gliding motility is fairly obvious in some species belonging to the genera *Gelidibacter*, *Cellulophaga* and *Zobellia* but is much less obvious in the other species, especially *Cytophaga marinoflava* and *Psychroflexus torquis*, which only glide under certain conditions. See Reichenbach (1989) and individual description papers for detailed assessments of gliding motility in particular species.

Cell biomass is pigmented either yellow or orange (sometimes almost red) owing to carotenoid and/or flexirubin pigments. Flexirubin pigments produced by three species of the marine clade (Table 2) can be detected easily by addition of a strong base (e.g., 20% [w/v] KOH) to cell masses which as a result turn instantly red-brown if flexirubins are present (Reichenbach, 1989). Carotenoids can be assessed in a general way by examining the absorbance spectra of hexane or chloroform cell extracts by spectrophotometry and thin layer chromatography against known standards such as zeaxanthin and saproxanthin. Accurate molecular identification of carotenoids requires more advanced organic chemical analysis against standards,

Table 2. Morphological characteristics of Flavobacteriaceae marine clade species.

Genus	Cell mass color	Flexirubins	Cell shape(s)	Cell size range (width × length μm)	Gliding motility
Aequorivita	Y or O	–	Rods, filaments	0.2–0.5 × 0.5–20.0	–
Arenibacter	O	–	Rods	0.4–0.7 × 0.3–5.0	–
Cellulophaga	Y-O	–	Rods	0.4–0.8 × 1.5–4.7	+
Croceibacter	Y	–	Rods	0.3–0.6 × 1.5–5.0	–
[C.] latercula	O-R	–	Rods, filaments	0.3–0.4 × 1.0–40	+
[C.] marinoflava	O-Y	–	Rods	0.5–0.6 × 1.0–3.0	+
Formosa	Y	–	Rods	0.4–0.9 × 0.8–1.8	+
Gelidibacter	Y	–	Rods, filaments[a]	0.4–0.5 × 0.5–4.0	V
Gillisia	Y	–	Rods	0.7 × 3.0	–
Maribacter	Y	–	Rods	0.3–0.7 × 1.2–10	–
Mesonia	Y	–	Rods	0.4–0.5 × 1.6–2.3	–
Muricauda	Y	–	Rods (appendages)[b]	0.3–0.6 × 1.1–2.7	+
Polaribacter[c]	O	–	Rods, coils, filaments	0.3–0.8 × 0.8–48.0	–
Psychroflexus	O	–	Rods, coils, filaments[a]	0.2–2 × 2–>100.0	V
Psychroserpens	Y	–	Rods, vibrioids, coils, helices	0.5–0.6 × 2.0–20.0	–
Robiginitalea	O	–	Rods[a]	0.3–0.7 × 1.6–5.6	–
Salegentibacter	Y	–	Rods, filaments	0.5–0.8 × 1.2–12	–
Tenacibaculum	Y	–	Rods, filaments	0.4–0.5 × 1.5–30	+
Ulvibacter	O	+	Rods	0.4–0.5 × 2.5–7.3	–
Vitellibacter	Y-O	+	Rods	0.3–0.5 × 3–10	–
Zobellia	Y-O	+	Rods	0.3–0.4 × 3.0–8.0	+

Symbols and abbreviations: Y, yellow; O, orange: R, red; +, present; –, absent; and V, varies between species.
[a]Some genera convert to almost entirely coccoid bodies in stationary growth phase.
[b]*Muricouda ruestringensis* strains form appendages which only emerge from the cell outer membrane and thus are quite unlike prostheca, which are contiguous with the cell membrane and cell wall.
[c]Some but not all species can form gas vesicles (Gosink et al., 1998).

and this has been rarely done for species in the marine clade.

Physiology

Overall, marine clade species are mostly adapted to cold or cool saline, typically oceanic ecosystems. None have been found to grow in excess of 45°C, and tolerance to elevated temperatures (>50°C) is very limited. Some species, including *Polaribacter* spp., *Psychroserpens burtonensis* and *Psychroflexus torquis*, are highly cold adapted, can only grow below room temperature, and will rapidly lose viability at temperatures over 20–25°C. Marine clade species are otherwise psychrotolerant or mesophilic (Table 3), growing well at 20–25°C. The temperature requirements clearly relate to the environment from which the species were isolated. Thus tropical environments will usually yield mesophilic species while species from polar environments do not in general grow at 30°C.

Sodium is generally required by the marine clade. Certain species (Table 3) that inhabit transitional marine ecosystems with variable or low salt levels, e.g., estuarine waters or the Baltic Sea, can grow with without sodium. Some species are quite demanding and also require divalent cations for growth (Table 3). These species only grow in a narrow range of salinity concentrations and thus are classic stenohaline species. A few species, such as *Salegentibacter salegens*, *Psychroflexus gondwanensis* and *Psychroflexus tropicus*, are very broadly salt tolerant and are found in hypersaline lakes. These are unusual phylum *Bacteroidetes* species in requiring high Na ion concentrations (50–100 practical salinity units [psu]) for optimal growth. The only other known example in the *Bacteroidetes* is the species *Salinibacter ruber* (Anton et al., 2002); however, this species is only distantly related to the Flavobacteriaceae marine clade. *Psychroflexus* is also unusual in that the species have widely variant ecophysiology even though they form a distinct and coherent genus (Table 3).

All marine clade species grow aerobically and are homogenously chemoheterotrophic in nature. Facultative phototrophic, lithotrophic or autotrophic growth of any sort does not occur. In general, little specific information is available on catabolic pathways in the group, though it is suspected that most use the Embden-Parnas-Meyerhof pathway for D-glucose catabolism. A few species can grow anaerobically (Table 4) though the mechanisms and extent of this are mostly unknown. *Muricauda ruestringensis* can grow under anoxic conditions in complex media, but neither ferments carbohydrates nor uses nitrate as an alternative electron acceptor. On the other hand, *Formosa algae* and *Polaribacter* species can grow fermentatively on various

Table 3. Basic ecophysiological traits of the marine clade of the family Flavobacteriaceae.

Genus	Growth temperature (°C)						Requires divalent cations	Salinity requirement and tolerance (psu)				
	4	20	25	30	37	42		0	25	80	120	200
Aequorivita	+[a]	+	+	–	–	–	–	V	+	V	–	–
Arenibacter	–	+	+	+	+	V	–	–	+	+/–	–	–
Cellulophaga	+	+	+	V	V	–	V	–	+	V	–	–
Croceibacter	–	+	+	–	–	–	–	–	+	+	+	–
[C.] latercula	nd	+	+	+	–	–	nd	–	+	–	–	–
[C.] marinoflava	+	+	+	+	–	–	–	+/–	+	+/–	–	–
Formosa	–	+	+	+	–	–	–	+	+	–	–	–
Gelidibacter	+	+	+	V	V	–	–	–	+	–	–	–
Gillisia	+	+	+	+/–	–	–	–	+	+	–	–	–
Maribacter	+	+	+	+	–	–	–	–	+	–	–	–
Mesonia	+	+	+	+	–	–	–	–	+	+	+	–
Muricauda	–	+	+	+	+	–	–	–	+	+	–	–
Psychroflexus												
P. torquis	+	–	–	–	–	–	+	–	+	–	–	–
P. gondwanensis	+	+	+	+	–	–	–	+	+	+	+	+/–
P. tropicus	–	+	+	+	+	+	–	–	+	+	+	+
Psychroserpens	+	–	–	–	–	–	+	–	+	–	–	–
Polaribacter	+	V	–	–	–	–	+	–	+	–	–	–
Robiginitalea	–	+	+	+	+	+	–	–	+	+	–	–
Salegentibacter	+	+	+	+	V	–	–	+	+	+	+	+
Tenacibaculum	V	+	+	V	V	–	V	–	+	+	V	–
Ulvibacter	+	+	+	+	–	–	–	–	+	–	–	–
Vitellibacter	+	+	+	+	+	+	–	V	+	–	–	–
Zobellia	–	+	+	+	+	V	–	–	+	–	–	–

Symbols and abbreviations: psu, practical salinity units; +, good growth; +/–, weak or delayed growth; V, growth varies between species; –, no growth; and nd, no data available.

sugars such as D-glucose and D-mannose. Denitrification is rare in the clade, and where it occurs, none of the denitrifying species appear capable of coupling nitrate to anaerobic respiration. Further analysis is required to confirm denitrification in these strains, including detection of nitrite reductase genes and gas chromatography. A small proportion of species do not form catalase (e.g., *M. ruestringensis*) or form cytochrome *c* oxidase; otherwise most are positive (Table 5), reflecting their strictly aerobic nature. All species which have been tested use menaquinone-6 as the primary isoprenoid quinone in respiratory electron transport chains.

Nutritional Characteristics

Several species require organic sources of nitrogen and in many cases other unknown growth factors found in yeast extract (Table 4). *Psychroserpens burtonensis* also requires vitamins for growth in defined media and is stimulated by the addition of Tweens. Thus generalized defined mineral salts media (see example below) are supplemented with 0.05% (w/v) yeast extract. Optionally vitamin solution such as the one formulated by Balch and Wolfe (1976) can be added as well as Tween 80 (~0.025% [v/v]). The medium may not be effective for absolutely every strain, owing to lack of knowledge in specific growth requirements. Single amino acids (such as L-glutamate or L-asparagine) may serve as sole nitrogen sources, though data on these requirements are lacking. Overall, marine clade species do not have unusual or idiosyncratic substrate utilization patterns to obtain energy and carbon. Most species can use an array of carbohydrates, including D-glucose, a few organic acids (typically tricarboxylic acid intermediates), and some amino acids. Sugar alcohols, hydrocarbons, aromatic compounds, and nitrogenous compounds (such as polyamines) are rarely used for growth. *Muricauda ruestringensis* is an interesting case in point since it was isolated from a continuous hexadecane-fed chemostat but is unable to utilize hexadecane or other hydrocarbons. Several species are asaccharolytic, preferring amino acids as sole carbon and energy sources (Table 4). Many species can use complex macromolecular substrates as carbon and/or nitrogen sources for growth. The extent of this ability varies widely between the genera (Table 4). Cellulose may even be degraded by some strains, such as *Tenacibaculum skaggerakense*, even though this polymer is not normally found in abundance in marine ecosystems. By comparison several species (especially *Cellulophaga* and *Zobellia* spp.)

Table 4. Nutritional and metabolic aspects of members of the Flavobacteriaceae marine clade.

Genus	Growth factor requirements	Anaerobic growth	Utilization of carbohydrates (D-glucose)	Polysaccharide hydrolysis (substrate)	Protein hydrolysis (substrate)
Aequorivita	YE[a]	–	V/w (V/w)	V (starch)[b]	+ (gelatin, casein, elastin)[b]
Arenibacter	–	–	+ (V)	–	V (gelatin)
Cellulophaga	–	–	+ (+)	+ (agar, CMC, starch)	+ (gelatin, casein, elastin)
Croceibacter	–	–	+ (–)	+ (starch)	+ (gelatin, casein)
[C.] latercula	–	–	+ (+)	+ (starch, agar, alginate, chitin)	+ (gelatin)
[C.] marinoflava	nd	–	+ (+)	+ (starch)	nd
Formosa	nd	+	+ (+)	V/w (starch)	V/w (gelatin)
Gelidibacter	YE or AA	–	+ (+)	+ (starch)	+ (gelatin)
Gillisia	nd	–	–	–	+ (gelatin)
Maribacter	nd	–	+ (V)	+ (agar, alginate)	+ (gelatin)
Mesonia	nd	–	–	–	+ (gelatin, casein)
Muricauda	–	+	+ (–)	–	–
Polaribacter[c]	YE	+	+ (+)	+ (starch)	V (gelatin)
Psychroflexus	YE or AA	–	+ (V)	+ (starch)	–
Psychroserpens	YE, vitamins	–	–	–	+ (gelatin, casein)
Robiginitalea	–	–	+ (+)	+ (starch)	–
Salegentibacter	YE or AA	–	+ (V)	+ (starch)	+ (gelatin)
Tenacibaculum[c]	YE or AA	–	V (V)	V (starch, chitin, cellulose)	+ (casein, gelatin)
Ulvibacter	nd	–	–	–	+ (gelatin)
Vitellibacter	–	–	–	–	+ (gelatin, casein, elastin)
Zobellia	nd	–	+ (+)	+ (agar, starch, carrageenan, CMC, chitin)	+ (gelatin, casein)

Symbols and abbreviations: YE, yeast extract; IN, inorganic nitrogen (nitrate or ammonium salts) needed; AA, amino acids; +, test positive; V, test varies between species; w, weakly positive; V/w, test results are variable and weak; –, test negative; nd, no data available; and CMC, carboxymethylcellulose.

[a]Yeast extract (YE) requirement indicates at least one amino acid and other unknown factors are required for growth in a defined mineral salts medium. If only amino acids (AA) are listed, the genus needs an organic nitrogen source (such as a single amino acid like L-glutamate) but does not have any other special nutritional requirements. Vitamins may also be required when grown on a semi-defined mineral salts medium containing 0.05% (w/v) yeast extract.

[b]Substrate indicated is hydrolyzed by most strains of the respective genus.

[c]*Tenacibaculum skaggerakense* also degrades collagen, hydroxyethylcellulose, barley β-glucans and pullulan.

Table 5. Biochemical characteristics and DNA base composition of genera belonging to the Flavobacteriaceae marine clade.

Genus	Catalase	Cytochrome *c* oxidase	NO_3 reduction to NO_2 (N_2, N_2O)	H_2S reduction	Urease	DNAse	Esculin hydrolysis	Mol% G + C
Aequorivita	+	V/w	–	–	+	V	V	38–39
Arenibacter	+	+	V (denitrification)	–	ND	–	ND	37–40
Cellulophaga	+	+	V	–	V	V	+	32–38
Croceibacter	+	–	–	ND	+	+	–	35
[C.] latercula	–	+	–	+	ND	+	ND	32
[C.] marinoflava	+	+	±	–	ND	ND	+	37
Formosa	+	–	+ (denitrification)	–	+	ND	ND	34
Gelidibacter	+	+	–	–	–	V	+	36–38
Gillisia	+	+	–	–	–	–	+	38–39
Maribacter	+	+	–	–	ND	V	ND	35–39
Mesonia	+	+	–	+	–	–	ND	32–34
Muricauda	–	ND	–	–	–	ND	–	41
Polaribacter[e]	+	V/W	–	–	–	ND	V	31–34
Psychroflexus	+	V	–	–	–	V	V	32–36
Psychroserpens	+	–	–	–	–	–	–	27–29
Robiginitalea	+	+	–	–	–	–	+	55–56
Salegentibacter	+	+	+	–	–	+	+	37–38
Tenacibaculum	+	+	V	–	ND	+	ND	29–32
Ulvibacter	+	+	+	–	–	–	ND	37–38
Vitellibacter	+	+	–	–	–	+	ND	41
Zobellia	+	+	+	–	–	+	+	42–43

Symbols and abbreviations: +, test positive; V, test varies between species; w, test is weakly positive; ±, result varies between literature sources; –, test negative; and ND, no data.

can degrade agar and carrageenan, polymers produced by many marine macrophytes. Some species produce an array of strong proteases (which can be demonstrated by the degradation of insoluble proteins like elastin), a feature of various *Aequorivita* and *Vitellibacter* strains (Table 4). *Aequorivita lipolytica* also has the ability to digest a wide array of lipids and lipid-esters including tributyrin, Tweens and olive oil (Bowman and Nichols, 2002). The above metabolic tendencies indicate that marine clade members collectively can decompose and utilize a large fraction of organic substances produced in ocean surface waters and coastal environments (Cottrell and Kirchman, 2000; Kirchman, 2002).

Defined Medium for Carbon Source Testing (Nedashkovskaya et al., 2003a)

$NaNO_3$	0.02 g
NH_4Cl	0.02 g
Yeast extract	0.005 g
Carbon source	0.4% (w/v)
Artificial sea salts	100 ml

The carbon source may include carbohydrates, amino acids, and sodium salts of organic acids. Autoclave 121°C, 15 min. Filter sterilize some substrates (especially carbohydrates) because of their heat lability.

Marine Agar (MA)

Bacteriological peptone	5 g
Yeast extract	2 g
Ferric pyrophosphate	10 mg
Sea salts	35 g
Agar (optional)	15 g
Distilled water	1000 ml

Alternatively, replace sea salts and distilled water with natural seawater. Adjust pH to 7.3–7.5. Autoclave 121°C, 15 min.

Marine 2216 agar or broth can also be purchased dehydrated from Difco Laboratories.

Chemotaxonomic and Genotypic Properties

All species of the marine clade contain a high proportion of branched chain fatty acids (Table 6). The distribution of other types of fatty acids is much more genus or species specific, especially monounsaturated fatty acids and hydroxy fatty acids (Table 6), while psychrophilic species produce a greater proportion of *anteiso*-branched chain fatty acids components. *Anteiso*-branched fatty acids produce more fluid membranes at low temperature (Kaneda, 1991) and thus help maintain a constant equilibrium in membrane viscosity. Only *Psychroflexus torquis* forms polyunsaturated fatty acids (PUFA), including eicosapentaenoic acid (20:5ω3) and arachidonic acid (20:4ω6), an exceptionally rare ability amongst the Bacteria (Russell and Nichols, 1999). PUFA-synthesis ability has only been documented for various marine, cold-adapted members of order *Alteromonadales* (Gammaproteobacteria). Eicosapentaenoic acid is also produced by the species *Flexibacter polymorphus* (Johns and Perry, 1977), which is only distantly related to the

Table 6. Whole fatty acid profiles of the genera of the Flavobacteriaceae marine clade.

	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Fatty acid	% of total fatty acids:																% of total fatty acids:				
15:0	1–3	**13–29**	**9–14**	**17–27**	**5**	**9**	**4**	**6–11**	ND	**6–15**	**5**	1	**3–8**	2–4	**8–13**	**5**	**5–6**	**5**	**4**	**4**	**7–10**
16:0	1–3	3	1–3	1	1	2	1	1–2	ND	t	t	1	1–2	t	t	4	1–2	3	ND	2	**2–6**
i14:0	t	t	ND	t	t	t	ND	t	ND	ND	1	ND	t–4	1–5	t	ND	ND	t	2	ND	ND
i15:0	**8–17**	**17–18**	**7–21**	**13–22**	**18**	**15**	**13**	**4–12**	+	**11–21**	**21**	**16**	**26–51**	**1–30**	**10–11**	**24**	**24–28**	**23**	**21**	**69**	**23–31**
i16:0	1–5	1	1–3	1–2	2	1	1	**6–10**	+	t	**6**	ND	t	**4–11**	t	**8**	ND	ND	3	3	t
i17:0	ND	1	t	ND	**5**	ND	t	ND	ND	ND	ND	ND	t	t	ND	2	1	2	ND	3	t
i15:1	**9–13**	**8–19**	**7–10**	**15–21**	**7**	**17**	**9**	**8–15**	+	**10–19**	**8**	**16**	**9–15**	**t–10**	**12–17**	**4**	**14–21**	**15**	**12**	**2**	**8–10**
i16:1	**3–7**	t	1–2	ND	ND	t	2	**5–10**	**8**	ND	4	ND	**4–7**	t	**7–11**	1	ND	**7**	**4**	t	t
i17:1	**5–10**	**6**	**5–7**	1–3	**13**	**17**	**9**	**3–7**	**7**	2–4	**5**	1	**0–1**	ND	**1–2**	**13**	ND	2	**4**	**4**	**6–8**
a15:0	**16–21**	**7**	1–3	**7–9**	t	**5**	2	**10–16**	+	1–2	**4**	2	**1**	**10–35**	**10–11**	**12**	3–4	**3**	2	**8**	**3–4**
a17:0	ND	t	ND	ND	ND	ND	ND	1–2	ND	ND	ND	ND	t	ND	ND	2	ND	ND	ND	t	ND
a15:1	**8-23**	2	t	ND	ND	2	t	**6–12**	ND	ND	t	1	t	**2–17**	**7–10**	ND	ND	1	ND	ND	ND
a17:1	2–5	1	1–2	1	t	2	1	**5–6**	7	ND	2	ND	ND	ND	t	**7**	ND	2	ND	ND	t
15:1	0–1	3	1–3	**10–14**	t	2	ND	**4–5**	ND	2–5	2	3	**5–13**	ND	**16–19**	1	ND	**9**	1	t	1–2
16:1	**1–8**	**10–11**	**9–19**	**3–7**	t	**7**	**5**	**7–8**	ND	**6–13**	**5**	ND	1–3	ND	**6–10**	**9**	**3–4**	**8**	6	t	**11–12**
17:1	ND	2	t	ND	1	ND	2	**3–5**	ND	t-2	3	ND	ND	ND	2–3	1	ND	t	1	ND	t
18:1	ND	ND	ND	ND	ND	ND	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
20:4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0–2	ND	ND	ND	ND	ND	ND	ND
20:5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0–5	ND	ND	ND	ND	ND	ND	ND
2-OH 17:0	ND	ND	ND	ND	ND	ND	ND	ND	**13**	ND	2	3	ND	ND	ND	ND	**1**	ND	2	ND	ND
3-OH 14:0	ND	ND	ND	1–3	t	ND	ND	ND	ND	ND	ND	ND	ND	t	ND	ND	ND	ND	ND	ND	ND
3-OH i15:0	2–5	ND	**3–9**	ND	**7**	2	**4**	t	ND	**3–5**	3	**5**	**7–18**	t–5	ND	ND	**4**	**8**	**3**	2	1–6
3-OH a15:0	t–6	ND	t–2	ND	ND	1	ND	ND	ND	ND	ND	ND	ND	t	ND	1	ND	ND	ND	ND	ND
3-OH 15:0	ND	ND	t	ND	ND	ND	ND	ND	ND	1–2	ND	1	ND	1–3	ND	ND	ND	1	1	ND	ND
3-OH i16:0	**2–9**	ND	**5–7**	ND	t	2	**4**	t	ND	2–3	**6**	3	t	**15–19**	ND	t	ND	4	9	ND	0–6
3-OH 16:0	ND	ND	t–2	ND	ND	ND	ND	t	ND	2–4	ND	3	**1–8**	1	ND	t	ND	ND	ND	ND	2
3-OH i17:0	2–5	ND	**4–21**	ND	**35**	**9**	**28**	t	ND	**12–20**	**15**	**29**	t	t	ND	3	**25–27**	1	**17**	1	**12–15**
3-OH a17:0	3–4	ND	t	ND	1	**5**	3	t	ND	ND	ND	ND	t–4	7–11	ND	2	ND	2	ND	ND	t
cy 17:0	ND	ND	ND	3–7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Symbols and abbreviations: +, present; ND, not detected; fatty acid nomenclature: N: n—carbon chain length: number of double bonds; suffixes: i, *iso*-branched; a, *anteiso*-branched; 2-OH, 2(α)-hydroxy; 3-OH, 3(β)-hydroxy; cy, cyclopropane group. Fatty acids in bold type are the major components present; t, trace component present at less than 0.5%.

[a]1. *Aequorivita*, 2. *Arenibacter*, 3. *Cellulophaga*, 4. *Croceibacter*, 5. *[C.] latercula*, 6. *[C.] marinoflava*, 7. *Formosa*, 8. *Gelidibacter*, 9. *Gillisia* (only major or differentiating fatty acids are shown), 10. *Maribacter*, 11. *Mesonia*, 12. *Muricauda*, 13. *Polaribacter*, 14. *Psychroflexus*, 15. *Psychroserpens*, 16. *Robiginitalea*, 17. *Salegentibacter*, 18. *Tenacibaculum* (data only available for T. maritimum), 19. *Ulvibacter*, 20. *Vitellibacter* and 21. *Zobellia*.

Flavobacteriaceae marine clade. Overall, fatty acid profiles are very useful for identifying genera within the marine clade and are important criteria for the description of new taxa. The wide range of profiles clearly reflects the evolutionary and ecological diversification of the marine clade members.

Polyamines are another useful chemotaxonomic marker which can be applied to characterize bacterial strains at approximately the family or genus level. In the marine clade, the only major polyamine present was homospermidine (Hamana and Nakagawa, 2001a). *Psychroflexus torquis* was also found to produce a large amount of the aromatic amine 2-phenylethylamine (Hamana and Niitsu, 2001b).

Almost all marine clade members have genomic DNA G+C contents falling into a relatively narrow range of 27–43 mol% (Table 5). A significant exception is the species *Robiginitalea biformata*, which has 55–56 mol% (Table 5), among the highest for the *Bacteroidetes phylum*. Further genomic analysis will be required to ascertain the genetic and evolutionary significance of this difference, which is probably linked to codon usage.

Pathogenicity

The only species within the Flavobacteriaceae marine clade known to have demonstrable pathogenicity impacting on commercial fishing are *Tenacibaculum maritimum* and *Tenacibaculum ovolyticum* (reviewed by Bernardet [1998] and Kusuda and Kawai [1998]). *Tenacibaculum maritimum* causes flexibacteriosis of several commercial fish species, resulting in serious erosion of the mouth, tail and fins from expanding lesions. Juvenile fish are especially susceptible and the disease results in high levels of mortality; 30% population losses are not unusual. *Tenacibaculum maritimum* was first isolated from Japanese sea bream (Hikida et al., 1979); however, most commercial fish species are affected, including Atlantic salmon. *Tenacibaculum ovolyticum* appears much less of a problem though it has been implicated in the disease and loss of viability of fish eggs, including those of Atlantic halibut (Hansen et al., 1992) and sardines (Miguez and Combarro, 2003). Polymerase chain reaction (PCR) techniques are available that detect as little as a single cell of *Tenacibaculum maritimum* and other bacterial fish pathogens in fish tissue samples (Wilson et al., 2002), thus enabling detection of "covert infections" in aquaculture farms.

Preservation

Almost all marine clade members form stable cultures and so can be maintained on agar plates or slants at normal growth temperature for 1–3 weeks, or when kept at 2°C, for 3–12 months. A temperature of 2°C is more ideal than 4–5°C as fungal contamination is reduced and survival for cold-adapted species is enhanced. Mesophilic species usually display better survival at slightly higher temperatures (typically 5–12°C) and thus may be contaminated by fungi. Antifungal compounds, such as nystatin, should be added to media to prevent this. Marine 2216 agar is an excellent media for agar storage. Low nutrient media or media containing a large amount of carbohydrates should not be used. The most notable exception to agar storage is the storage of *Cytophaga latercula*, which undergoes rapid autolysis and death on agar and so needs to be transferred every 1–3 days. Higher incubation temperatures require more frequent transfers. This species can be conveniently maintained as a barely visible suspension in seawater or artificial sea salts (30–35 $g \cdot liter^{-1}$) at 10–15°C for several months or even years. This method can be applied theoretically for other marine clade species though alternative storage preservation procedures should be employed as a general precaution. All marine clade species can be preserved by cryopreservation in marine 2216 broth mixed with 10–30% (v/v) glycerol. This involves creating heavy cell suspensions of strains mixed in the cryopreserving medium, which has been pre-chilled on ice, and then direct transfer to a –70°C or colder freezer. The suspensions can also be frozen directly in liquid nitrogen at –196°C. The cultures should be replaced on an annual basis if possible. Multiple vials of cryopreserved cells should be stored in one freezer in addition to a back-up set stored in a separate freezer. Lyophilization can also be employed using a variety of desiccants. Desholme and Bernardet (1998) found fetal bovine serum was an excellent desiccant for storing *Tenacibaculum maritimum*. Horse serum and skim milk can also be used, though results may be variable for some species, and lyophilized strains should have a cryopreserved back-up. Marine 2216 broth and agar (equivalent formula given below) as well as general routine growth media can be used as resuscitation media for either cryopreserved or lyophilized cultures.

Artificial Seawater Formula (ZoBell, 1946)

NH_4NO_3	2 mg
H_3BO_3	27 mg
$CaCl_2$	1.14 g
$FePO_4$	1 mg

$MgCl_2$	5.14 g
KBr	0.1 g
KCl	0.7 g
$NaHCO_3$	0.2 g
NaCl	24.3 g
NaF	3 mg
Na_2SiO_3	2 mg
Na_2SO_4	4.1 g
SrCl	26 mg
Distilled water	1000 ml

Sea salts can also be purchased from chemical companies such as Sigma-Aldrich or from aquarium suppliers (the latter source is far cheaper and has a similar level of quality).

Isolation and Cultivation

The media useful for isolation of Flavobacteriaceae marine species are shown in Table 1. In most cases, strains are obtained by directly plating samples onto agar. Seaweed and marine faunal samples (e.g., holothurians) are usually first homogenized in a solution of sea salts or natural seawater before plating. Some species, such as *Mesonia algae*, can be obtained easily by first allowing the source material to decay in natural seawater, e.g., seaweed thallus samples, thus elevating the populations of heterotrophic decomposers dramatically. Psychrophilic strains have enhanced isolation after first pre-enriching samples, such as thawed sea-ice, in a suitable medium (e.g., marine 2216 broth) at a low temperature (0–4 °C) for 24–48 h before plating. The low temperature encourages the multiplication of psychrophilic species responding to the media nutrients. Some species (including *Croceibacter atlanticus* and *Robiginitalea biformata*) have been isolated with the High Throughput Cultivation (HTC) technique (Connon and Giovannoni, 2002; Table 1). The HTC method has been used successfully for isolating strains adapted to highly oligotrophic (low nutrient) ecosystems such as open ocean seawater (e.g., Rappé et al., 2002). In this procedure the sample, usually seawater, is diluted in 96- or 384-well trays to <10 cells per well. Cultures grown from each well are then screened using fluorescent in situ hybridization (FISH) and 16S rRNA gene sequencing. In the case of *C. atlanticus* and *R. biformata*, the strains were isolated initially using low nutrient heterotrophic medium (LNHM) with the HTC technique but subsequently grown on marine 2216 agar. Not all strains obtained by HTC will grow on agar; so far it is unknown if this feature extends to the Flavobacteriaceae marine clade.

Zobellia galactanivorans was isolated using carrageenan as the sole carbon source (Table 1) and thus complex substrates may be ideal for the isolation of many species. Suitable substrates are listed in Table 4. As most species have been obtained on standard complex growth media such as marine 2216 agar or dilutions of this media, it is possible novel species may be isolated using more selective substrates as has been done successfully for isolating novel soil bacteria (Joseph et al., 2003). The incubation temperature for primary isolation plates and media usually matches the habitat temperature which for most marine ecosystems can range from sub-zero temperatures up to 30°C.

All strains grow well on marine 2216 agar at the incubation temperatures listed in Table 1.

High Salt Luria Marine Broth (1/5 LMB) (Suzuki et al., 2001)

Tryptone	2 g
Yeast extract	1 g
Artificial seawater	1000 ml

Adjust pH to 7.2. Autoclave 121°C, 15 min.

Medium B (Ivanova et al., 1996)

Bacteriological peptone	2 g
Casein hydrolysate	2 g
Yeast extract	2 g
D-Glucose	1 g
KH_2PO_4 (anhyd.)	20 mg
$MgSO_4 \cdot 7H_2O$	50 mg
Natural seawater	500 ml
Distilled water	500 ml

Adjust pH to 7.5–7.8. Autoclave 121°C, 15 min.

Low Nutrient Heterotrophic Medium (LNHM) (Rappé et al., 2002)

NH_4Cl	1.0 mg
KH_2PO_4	0.1 mg
D-Glucose	10 mg
D-Ribose	10 mg
Sodium succinate	10 mg
Sodium pyruvate	10 mg
Glycerol	10 mg
N-Acetylglucosamine	10 mg
Ethanol	0.002% (v/v)
Natural seawater	1000 ml

Filter sterilize (0.2 mm) and autoclave the natural seawater. Do not add agar to this medium.

CYT Medium (Johansen et al., 1999)

Casein	1 g
Yeast extract	0.5 g
$CaCl_2 \cdot H_2O$	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Agar (optional)	15 g
Sea salts	20 g
Distilled water	1000 ml

Adjust pH to 7.3. Autoclave 121°C, 15 min.

Modified (Hexadecane) Marine Agar (adapted from Bruns et al., 2001)

Bacteriological peptone	2.5 g
Yeast extract	1.5 g
Hexadecane (optional)	0.2% (v/v)
Agar	15 g
Sea salts	22.5 g
Distilled water	1000 ml

Adjust pH to 7.2. Autoclave 121°C, 15 min.Used for isolation of *Muricauda ruestringensis*, which does not actually utilize or benefit from hexadecane.

Ecology and Habitats

Studies using 16S rRNA-based clone libraries, denaturing gradient gel electrophoresis, and FISH indicate most *Bacteroidetes* in sea-ice and seawater belong to the Flavobacteriaceae marine clade (Brown and Bowman, 2001; Bano and Hollibaugh, 2002; Junge et al., 2002; Brinkmeyer et al., 2003; Kirchman et al., 2003). Other lineages from different families of the *Bacteroidetes* also occur though often at smaller and more variable levels.

From various culture-independent studies, marine clade members occur in all marine ecosystems which have at least some access to oxygen. They also are common in some marine-derived environments including saline lakes (formed by uplifting of the seabed above sea level, e.g., Vestfold Hills, Antarctica) and lithic habitats, which act as protective environments from harsh climatic conditions including desiccation and freezing (Smith et al., 2000). This is especially the case for genus *Gelidibacter*, which appears broadly distributed, detected in sea-ice at both poles (Bowman et al., 1997c; Staley and Gosink, 1999; Brown and Bowman, 2001), in cyanobacterial communities living under semi-translucent quartz stones in Antarctic deserts (Smith et al., 2000), widely in seawater (Kelly and Chistoserdov, 2001; Macián et al., 2002), and in the surface of tropical marine sediments (Madrid et al., 2001). By comparison, *Polaribacter* has only been isolated and detected in sea-ice and seawater of polar oceans, where it is one of the dominant taxa present (Staley and Gosink, 1999; Brown and Bowman, 2001; Bano and Hollibaugh, 2002; Junge et al., 2002; Brinkmeyer et al., 2003). In regards to the benthos, the marine clade only concentrates in sediment surface mixed layers in which faunal bioturbation and oceanic currents provide sufficient oxygenation. The surface mixed layer of continental shelf sediments and marine clade members appears to be relatively common, making up about 10% of the bacterial population (Bowman and McCuaig, 2003a). Further down in marine sediment layers, which are fully anoxic, different mostly uncultured Bacteroidetes lineages lying outside of the Flavobacteriaceae predominate instead. Marine clade members (especially *Cellulophaga* species) may be encountered in some transitional marine ecosystems such as estuaries or brackish water (e.g., that of the Baltic Sea). Marine clade species (described or undescribed) have never been isolated from freshwater ecosystems. Related genera such as *Flavobacterium*, *Myroides* and *Chryseobacterium* are abundant in there instead, suggesting these genera may play an analogous role in freshwater aquatic ecosystems.

Since they are nonmotile or move by gliding, marine clade members benefit most when given access to surfaces for colonization. Surfaces in turn maximize access to nutrients through adsorption of nutrients and formation of biofilms. Nutrients are the most important factor driving life in the oceans, and marine clade organisms have characteristics which are well adapted to growth with marine biota, especially algae, living on exudates and detritus (including both simple compounds and complex macromolecules). The primarily epiphytic nature of the Flavobacteriaceae marine clade and efficient colonization of marine living surfaces have been shown by various FISH analyses. For example, marine clade members may make up as much as 70% of bacterioplankton associated with algal blooms and algal surface biofilms (e.g., Glöckner et al., 1999). Various other studies also indicate that members of the *Bacteroidetes* can proliferate on or in most marine macrobiota (Table 1) and in marine aggregates (Grossart and Ploug, 2001; Ploug et al., 2002). Psychrophilic species have been detected in or isolated from sea-ice algal assemblages, with the incidence for isolation much lower in sea-ice lacking algae (Bowman et al., 1997c; Brown and Bowman, 2001). The most likely reason for this is that algae simply provide surfaces and nutrients for bacterial proliferation. Recent studies by Junge et al. (2004) indicate that particle-associated Flavobacteriaceae marine clade species make up the bulk of bacterial populations within the brine channels of winter sea-ice. The brine channels are an unusual extreme environment combining intense cold (–1°C to <–20°C) and high salinity (up to 150 psu). Thus marine clade species in brine channels represent a special variety of extremophiles not found anywhere else. Data from such ecosystems may have significance for astrobiological research in which studies are directed toward possible extraterrestrial life in our solar system, which may well be mostly extremophilic in nature. Many of these extremophilic species remain to be cultured, and the biochemical adaptations required to survive in such environments are also only just now being investigated (Junge et al., 2004). It has been suggested that the algal phycosphere, the region around algal cells enriched with photosynthetically derived exudates, perhaps held in partially by algal exopolysaccharide gel envelopes (Krembs et al., 2003),

is a substrate concentration zone supporting enhanced growth at sub-zero temperatures. A major hurdle for cold-adapted bacteria is lack of cytoplasmic membrane permeability resulting in less efficient and slower transport of nutrients; enzyme catalysis is also impeded owing to cold denaturation. Though the permeability of cytoplasmic membranes is improved by modulation of fatty acid components (Kaneda, 1991; Russell and Nichols, 1999) and cell membrane proteins (e.g., cold-shock-like proteins), high substrate and ionic concentration in the external medium provide a favorable gradient for nutrient transport (Tokai et al., 2000) and particularly for Na^+-substrate coupled co-transport (Wilson and Ding, 2001). Sea-ice bacteria are also adept at making cold-active enzymes (Nichols et al., 1999; Deming, 2003).

The marine clade appears to have ecological impacts beyond simple mineralization-based commensalism (secondary production); some evidence suggests that algal associated species may enhance algal growth (Grossart, 1999). This could be considered a mutualistic relationship. By colonizing the surfaces of some algae or even metazoans, some bacterial species can also produce compounds which inhibit the settlement and colonization of other organisms (e.g., Egan et al., 2000). In this respect, toxic secondary metabolites allow bacteria to play an aggressive, predatory role in food webs. For example several strains (mostly related to *Cellulophaga*, *Psychroserpens* and *Formosa*) can actively attack and kill various algal species (Doucette et al., 1999; Kondo et al., 1999; Nagasaki et al., 2000; Skerratt et al., 2000; Adachi et al., 2002), in particular dinoflagellates, by producing extracellular enzymes and toxic compounds. The lysis of algal cells in blooms causes a rapid local increase in dissolved and particulate organic matter which supports bacterial growth and thus marine clade species are probably important in the instigation of algal bloom crashes and subsequent decomposition. This extends to marine aggregates (when large enough to see they are referred to as "marine snow"), which are exopolysaccharide conglomerated clumps of organic detritus generated from algal blooms and metazoan activity (e.g., zooplankton sloppy feeding and fecal pellets). As the aggregates sink to the nepheloid layer (a layer of suspended particles above the ocean bottom) and benthos (biota that live at or near the bottom), marine clade members and other bacteria actively degrade organic matter, thus making the aggregates hot spots of biological activity in an otherwise apparently quiescent pelagic zone. The break up of aggregates owing to ocean currents and upwelling would help distribute cells throughout the ocean to repeat the cycle.

Applications

Marine clade members have a substantial biotechnological potential which so far is virtually untapped. Cold-adapted species produce cold-active enzymes which have substantial industrial utility. The applications in industry are manifold and have been extensively reviewed (see, e.g., Bull et al. [2000] and Rothschild and Mancinelli [2001]); important focus areas for cold active enzymes include janitorial processes, modification of heat labile substances (such as various foods) and in energy conservation. For example, *Cellulophaga algicola* isolated from Antarctic algae produces a wide range of active extracellular enzymes capable of breaking down proteins and polysaccharides with temperature optima of only 20–30°C and remaining active at subzero temperatures (Nichols et al., 1999). Interesting uses for these enzymes are beginning to emerge. For example, *Arenibacter latericius* produces an enzyme (α-*N*-acetylgalactosaminidase IV), which can specifically modify human erythrocytes (Bakunina et al., 2002). Various marine clade species produce specialist fucoidan and agarase enzymes (Sakai et al., 2002; Allouch et al., 2003) that cleave specific polysaccharide chemical bonds and so may have utility in creating tailored oligosaccharides for the food and pharmaceutical industries.

The psychrophile, *Psychroflexus torquis*, as previously mentioned produces PUFA, which has direct applicability to the nutriceutical industry. Omega-3 PUFA (including eicosapentaenoic acid) is essential for the development of healthy infants, provides significant health benefits for cardiovascular systems and nervous systems (Li and Sinclair, 2002), and may reduce cancer and arthritis (Shahidi and Kim, 2002; Tavani et al., 2003). Arachidonic acid, an omega-6 PUFA, also produced by *P. torquis*, also has value as a fine chemical and applications as a pharmaceutical.

Several species of the marine clade, mostly undescribed, have been shown to produce bioactive compounds of pharmaceutical potential (e.g., tumor formation inhibitors, neuroactive agents; Umezawa et al., 1983; Perovic et al., 1998; Yan et al., 2002). Algicidal strains (indicated previously) have also been touted as a biocontrol for toxic algal blooms, such as the infamous "red tide," which plagues various coastlines and can lead to fish kills, damage to the aquaculture industry, human health problems, and overall degradation of the coastal marine environment.

Literature Cited

Adachi, M., K. Fukami, R. Kondo, and T. Nishijima. 2002. Identification of marine algicidal Flavobacterium sp. 5

N-3 using multiple probes and whole-cell hybridization. Fisheries Sci. 68(4):713–720.

Allouch, J., M. Jam, W. Helbert, T. Barbeyron, B. Kloareg, B. Henrissat, and M. Czjzek. 2003. The three-dimensional structures of two beta-agarases. J. Biol. Chem. 278(47):47171–47180.

Anton, J., A. Oren, S. Benlloch, F. Rodriguez-Valera, R. Amann, and R. Rossello-Mora. 2002. Salinibacter ruber gen. nov., sp. nov., a novel, extremely halophilic member of the Bacteria from saltern crystallizer ponds. Int. J. Syst. Evol. Microbiol. 52:485–491.

Bakunina, I. Y., R. A. Kuhlmann, L. M. Likhosherstov, M. D. Martynova, O. I. Nedashkovskaya, V. V. Mikhailov, and L. A. Elyakova. 2002. Alpha-N-acetylgalactosaminidase from marine bacterium Arenibacter latericius KMM 426(T) removing blood type specificity of A-erythrocytes. Biochemistry 67(6):689–695.

Balch, W. E., and R. S. Wolfe. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of Methanobacterium ruminantium in a pressurized atmosphere. Appl. Environ. Microbiol. 32:781–791.

Bano, N., and J. T. Hollibaugh. 2002. Phylogenetic composition of bacterioplankton assemblages from the Arctic Ocean. Appl. Environ. Microbiol. 68(2):505–518.

Barbeyron, T., S. L'Haridon, E. Corre, B. Kloareg, and P. Potin. 2001. Zobellia galactanovorans gen. nov., sp. nov., a marine species of Flavobacteriaceae isolated from a red alga, and classification of [Cytophaga] uliginosa (ZoBell and Upham 1944) Reichenbach 1989 as Zobellia uliginosa gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol. 51(3):985–997.

Bernardet, J. F., and P. A. D. Grimont. 1989. Deoxyribonucleic acid relatedness and phenotypic characterization of Flexibacter columnaris sp. nov., nom. rev., Flexibacter psychrophilus sp. nov., nom. rev., and Flexibacter maritimus Wakabayashi, Hikida, and Masumura 1986. Int. J. Syst. Bacteriol. 39:346–354.

Bernardet, J. F., P. Segers, M. Vancanneyt, F. Berthe, K. Kersters, and P. Vandamme. 1996. Cutting a Gordian knot: emended classification and description of the genus Flavobacterium, emended description of the family Flavobacteriaceae, and proposal of Flavobacterium hydatis nom. nov. (basonym, Cytophaga aquatilis Strohl and Tait 1978). Int. J. Syst. Bacteriol. 46:128–148.

Bernardet, J. F. 1998. Cytophaga, Flavobacterium, Flexibacter and Chryseobacterium infections in cultured marine fish. Fish Pathol. 33(4):229–238.

Bernardet, J. F, Y. Nakagawa, B. Holmes, and the Subcommittee on the Taxonomy of Flavobacteria. 2002. Proposed minimal standards for describing new taxa of the family Flavobacteriaceae and emended description of the family. Int. J. Syst. Evol. Microbiol. 52(3):1049–1070.

Bowman, J. P., M. V. Brown, and D. S. Nichols. 1997a. Biodiversity an ecophysiology of bacteria associated with Antarctic sea-ice. Antarct. Sci. 9:134–142.

Bowman, J. P., S. A. McCammon, J. L. Brown, P. D. Nichols, and T. A. McMeekin. 1997b. Psychroserpens burtonensis gen. nov., sp. nov. and Gelidibacter algens gen. nov., sp. nov., psychrophilic bacteria isolated from Antarctic lacustrine and sea ice habitats. Int. J. Syst. Bacteriol. 47(3):670–677.

Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin. 1997c. Diversity and association of psychrophilic bacteria in Antarctic sea-ice. Appl. Environ. Microbiol. 63:3068–3078.

Bowman, J. P., S. A. McCammon, T. Lewis, J. H. Skerratt, J. L. Brown, D. S. Nichols, and T. A. McMeekin. 1998. Psychroflexus torquis gen. nov., sp. nov., a psychrophilic species from Antarctic sea ice, and reclassification of Flavobacterium gondwanense (Dobson et al., 1993) as Psychroflexus gondwansensis gen. nov., comb. nov. Microbiology 144:1601–1609.

Bowman, J. P. 2000. Description of Cellulophaga algicola sp. nov., isolated from the surfaces of Antarctic algae, and reclassification of Cytophaga uliginosa (ZoBell and Upham 1944) Reichenbach 1989 as Cellulophaga uliginosa comb. nov. Int. J. Syst. Evol. Microbiol. 50(5):1861–1868.

Bowman, J. P., and D. S. Nichols. 2002. Aequorivita gen. nov., a member of the family Flavobacteriaceae isolated from terrestrial and marine Antarctic habitats. Int. J. Syst. Evol. Microbiol. 52:1533–1541.

Bowman, J. P., and R. M. McCuaig. 2003a. Diversity and biogeography of prokaryotes dwelling in Antarctic continental shelf sediment. Appl. Environ. Microbiol. 69(5):2463–2484.

Bowman, J. P., C. Mancuso Nichols, and J. A. E. Gibson. 2003b. Algoriphagus ratkowskyi gen. nov., sp. nov., Brumimicrobium glaciale gen. nov., sp. nov., Cryomorpha ignava gen. nov., sp. nov. and Crocinitomix catalasitica gen. nov., sp. nov., novel flavobacteria isolated from various polar habitats. Int. J. Syst. Evol. Microbiol. 53:1343–1355.

Brinkmeyer, R., K. Knittel, J. Jürgens, H. Weyland, R. Amann, and E. Helmke. 2003. Diversity and structure of bacterial communities in Arctic versus Antarctic pack ice. Appl. Environ. Microbiol. 69(11):6610–6619.

Brown, M. V., and J. P. Bowman. 2001. A molecular phylogenetic survey of sea-ice microbial communities (SIMCO). FEMS Microbiol. Ecol. 35:267–275.

Bruns, A., M. Rohde, and L. Berthe-Corti. 2001. Muricauda ruestringensis gen. nov., sp. nov., a facultatively anaerobic, appendaged bacterium from German North Sea intertidal sediment. Int. J. Syst. Evol. Microbiol. 51(6):1997–2006.

Bull, A. T., A. C. Ward, and M. Goodfellow. 2000. Search and discovery strategies for biotechnology: The paradigm shift. Microbiol. Molec. Biol. Rev. 64:573–606.

Cho, J.-C., and S. J. Giovannoni. 2003. Croceibacter atlanticus gen. nov. sp. nov., a novel marine bacterium in the family Flavobacteriaceae. Syst. Appl. Microbiol. 26(1):76–83.

Cho, J.-C., and S. J. Giovannoni. 2004. Robiginitalea biformata gen. nov., sp. nov., a new marine bacterium in the family Flavobacteriaceae that contains higher G+C composition. Int. J. Syst. Evol. Microbiol.

Connon, S. A., and S. J. Giovannoni. 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl. Env. Microbiol. 68:3878–3885.

Cottrell, M. T., and D. L. Kirchman. 2000. Natural assemblages of marine proteobacteria and members of the Cytophaga-Flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. Appl. Environ. Microbiol. 66(4):1692–1697.

Deming, J. W. 2003. Psychrophiles and polar regions. Biofutur 229:43–50.

Desholme, B., and J. F. Bernardet. 1998. Freeze-drying of Flavobacterium columnare, Flavobacterium psychrophilum and Flexibacter maritimus. Dis. Aquat. Org. 27:77–80.

Dobson, S. J., S. R. James, P. D. Franzmann, and T. A. McMeekin. 1991. A numerical taxonomic study of some pigmented bacteria isolated from Organic Lake, an Antarctic hypersaline lake. Arch. Microbiol. 156(1):56–61.

Dobson, S. J., R. R. Colwell, T. A. McMeekin, and P. D. Franzmann. 1993. Direct sequencing of the polymerase chain reaction amplified 16S rRNA gene of Flavobacterium gondwanense sp. nov. and Flavobacterium salegens sp. nov., two new species from a hypersaline Antarctic lake. Int. J. Syst. Bacteriol. 43(1):77–83.

Donachie, S. P., J. P. Bowman, and M. Alam. 2004. Psychroflexus tropicus sp. nov., a new obligately halophilic Cytophaga-Flavobacterium-Bacteroides group bacterium isolated from an Hawaiian hypersaline lake. Int. J. Syst. Evol. Microbiol.

Doucette, G. J., E. R. McGovern, and J. A. Babinchak. 1999. Algicidal bacteria active against Gymnodinium breve (Dinophyceae). I: Bacterial isolation and characterization of killing activity. J. Phycol. 35(6):1447–1454.

Egan, S., T. Thomas, C. Holmström, and S. Kjelleberg. 2000. Phylogenetic relationship and antifouling activity of bacterial epiphytes from the marine alga Ulva lactuca. Environ. Microbiol. 2(3):343–347.

Frette, L., N. O. G. Jørgensen, H. Irming, and N. Kroer. 2004. Tenacibaculum skaggerrakense sp. nov., a marine bacterium isolated from the pelagic zone in Skaggerrak, Denmark. Int. J. Syst. Evol. Microbiol.

Glöckner, F. O., B. M. Fuchs, and R. Amann. 1999. Bacterioplankton compositions of lakes and oceans: A first comparison based on fluorescence in situ hybridization. Appl. Env. Microbiol. 65:3721–3726.

Gosink, J. J., C. R. Woese, and J. T. Staley. 1998. Polaribacter gen. nov., with three new species, P. irgensii sp. nov., P. franzamannii sp. nov. and P. filamentus sp. nov., gas vacuolate polar marine bacteria of the Cytophaga-Flavobacterium-Bacteroides group and reclassification of "Flectobacillus glomeratus" as Polaribacter glomeratus comb. nov. Int. J. Syst. Bacteriol. 48(1):223–235.

Grossart, H. P. 1999. Interactions between marine bacteria and axenic diatoms (Cylindrotheca fusiformis, Nitzschia laevis, and Thalassiosira weissflogii) incubated under various conditions in the lab. Aquat. Microb. Ecol. 19(1):1–11.

Grossart, H.P. and, Ploug, H. 2001. Microbial degradation of organic carbon and nitrogen on diatom aggregates. Limnol. Oceanogr. 46 (2):267–277.

Hamana, K., and Y. Nakagawa. 2001a. Polyamine distribution profiles in newly validated genera and species within the Flavobacterium-Flexibacter-Cytophaga-Sphingobacterium complex. Microbios 106:105–116.

Hamana, K., and M. Niitsu. 2001b. Large production of an aromatic amine, 2-phenylethylamine, in a psychrophilic marine bacterium, Psychroflexus torquis. J. Gen. Appl. Microbiol. Tokyo 47(2):103–105.

Hansen, G. H., Ø. Bergh, J. Michaelsen, and N. Knappskog. 1992. Flexibacter ovolyticus sp. nov., a pathogen of eggs and larvae of Atlantic Halibut, Hippoglossus hippoglossus L. Int. J. Syst. Bacteriol. 42(3):451–458.

Hikida, M., H. Wakabayashi, S. Egusa, and K. Masumura. 1979. Flexibacter sp., a gliding bacterium pathogenic to some marine fishes in Japan. Bull. Jpn. Soc. Sci. Fish. 45:421–428.

Ivanova, E. P., E. A. Kiprianova, V. V. Mikhailov, F. G. Levanova, A. G. Garagulya, N. M. Gorshkova, N. Yumoto, and S. Yoshikawa. 1996. Characterization and identification of marine Alteromonas nigrifaciens strains and emendation of the description. Int. J. Syst. Bacteriol. 48(1):247–256.

Ivanova, E. P., O. I. Nedashkovskaya, J. Chun, A. M. Lysenko, G. M. Frolova, V. I. Svetashev, M. V. Vysotskii, V. V. Mikhailov, A. Huq, and R. R. Colwell. 2001. Arenibacter gen. nov., new genus of the family Flavobacteriaceae and description of a new species, Arenibacter latericius sp. nov. Int. J. Syst. Evol. Microbiol. 51(6):1987–1995.

Ivanova, E. P., Y. A. Alexeeva, S. Flavier, J. P. Wright, N. V. Zhukova, V. V. Gorshkova, D. V. Nicolau, and R. Christen. 2004. Formosa algae gen. nov., a novel member of the family Flavobacteriaceae. Online: (http://ijs.sgmjournals.org/misc/pip.shtml). Int. J. Syst. Bacteriol.

Johansen, J. E., P. Nielsen, and C. Søjholm. 1999. Description of Cellulophaga baltica gen. nov., sp. nov. and Cellulophaga fucicola gen. nov., sp. nov. and reclassification of [Cytophaga] lytica to Cellulophaga lytica gen. nov., comb. nov. Int. J. Syst. Bacteriol. 49:1231–1240.

Johns, R. B., and G. J. Perry. 1977. Lipids of the marine bacterium Flexibacter polymorphus. Arch. Microbiol. 114:267–271.

Joseph, S. J., P. Hugenholtz, P. Sangwan, C. A. Osborne, and P. H. Janssen. 2003. Laboratory cultivation of widespread and previously uncultured soil bacteria. Appl. Env. Microbiol. 69:7210–7215.

Junge, K., F. Imhoff, T. Staley, and J. W. Deming. 2002. Phylogenetic diversity of numerically important arctic sea-ice bacteria cultured at subzero temperature. Microb. Ecol. 43(3):315–328.

Junge, K., H. Eicken, and J. W. Deming. 2004. Bacterial activity at –2 to –20°C in Arctic wintertime sea ice. Appl. Env. Microbiol. 70:550–557.

Kaneda, T. 1991. Iso- and anteiso-fatty acids in bacteria: Biosynthesis, function and taxonomic significance. Microbiol. Rev. 55:288–302.

Kelly, K. M., and A. Y. Chistoserdov. 2001. Phylogenetic analysis of the succession of bacterial communities in the Great South Bay (Long Island). FEMS Microbiol. Ecol. 35(1):85–95.

Kirchman, D. L. 2002. The ecology of Cytophaga-Flavobacteria in aquatic environments. FEMS Microbiol. Ecol. 39(2):91–100.

Kirchman, D. L., L. Y. Yu, and M. T. Cottrell. 2003. Diversity and abundance of uncultured Cytophaga-like bacteria in the Delaware Estuary. Appl. Environ. Microbiol. 69(11):6587–6596.

Kondo, R., I. Imai, K. Fukami, K. Minami, and A. Hiroishi. 1999. Phylogenetic analysis of algicidal bacteria (Family Flavobacteriaceae) and selective detection by PCR using a specific primer set. Fisheries Sci. 65(3):432–435.

Krembs, C., J. W. Deming, and H. Eicken. 2003. Can Microorganisms, Especially Melosira arctica, Significantly Alter the Physical Properties of Sea-Ice and to What Biological Advantage? Online: (http://psc.apl.washington.edu/CKrembs/AAAS.pdf). Polar Science Center, University of Washington. Seattle, WA.

Kusuda, R., and K. Kawai. 1998. Bacterial diseases of cultured marine fish in Japan. Fish Pathol. 33(4):221–227.

Lewin, R. A. 1969. A classification of flexibacteria. J. Gen. Microbiol. 58:189–206.

Li, D., and A. J. Sinclair. 2002. Macronutrient innovations: The role of fats and sterols in human health. Asia Pac. J. Clin. Nutr. 11 (Suppl. 6):155–162.

Macián, M. C., M. J. Pujalte, M. C. Márquez, W. Ludwig, A. Ventosa, E. Garay, and K.-H. Schleifer. 2002. Gelidibacter mesophilus sp. nov., a novel marine bacterium in the family Flavobacteriaceae. Int. J. Syst. Evol. Microbiol. 52:1325–1329.

Madrid, V. M., J. Y. Aller, R. C. Aller, and A. T. Chistoserdov. 2001. High prokaryote diversity and analysis of community structure in mobile mud deposits off French Guiana: identification of two new bacterial candidate divisions. FEMS Microbiol. Ecol. 37(3):197–209.

McGuire, A. J., P. D. Franzmann, and T. A. McMeekin. 1987. Flectobacillus glomeratus sp. nov., a curved, nonmotile, pigmented bacterium isolated from Antarctic marine environments. Syst. Appl. Microbiol. 9:265–272.

Miguez, B., and M. P. Combarro. 2003. Bacteria associated with sardine (Sardina pilchardus) eggs in a natural environment (Ria de Vigo, Galicia, northwestern Spain). FEMS Microbiol. Ecol. 44(3):329–334.

Morita, R. Y. 1975. Psychrophilic bacteria. Bacteriol. Rev. 39:144–167.

Muller, S., B. Kiesel, and L. Berthe-Corti. 2001. Muricauda ruestringensis has an asymmetric cell cycle. Acta Biotechnol. 21(4):343–357.

Nagasaki, K., M. Yamaguchi, and I. I. Imai. 2000. Algicidal activity of a killer bacterium against the harmful red tide dinoflagellate Heterocapsa circularisquama isolated from Ago Bay, Japan. Nippon Guisan Gakk. 66(4):666–673.

Nedashkovskaya, O. I., S. B. Kim, S. K. Han, A. M. Lysenko, M. Rohde, N. V. Zhukova, E. Falsen, G. M. Frolova, V. V. Mikhailov, and K. S. Bae. 2003a. Mesonia algae gen. nov., sp. nov., a novel marine bacterium of the family Flavobacteriaceae isolated from the gree alga Acrisiphonia sonderi (Kürtz) Kornm. Int. J. Syst. Evol. Microbiol. 53(6):1967–1971.

Nedashkovskaya, O. I., M. Suzuki, M. V. Vysotskii, and V. V. Mikhailov. 2003b. Arenibacter troitsensis sp. nov., isolated from marine bottom sediment. Int. J. Syst. Evol. Microbiol. 53(5):1287–1290.

Nedashkovskaya, O. I., M. Suzuki, M. V. Vysotskii, and V. V. Mikhailov. 2003c. Vitellibacter vladivostokensis gen. nov., sp. nov., a new member of the phylum Cytophaga-Flavobacterium-Bacteroides. Int. J. Syst. Evol. Microbiol. 53(5):1281–1286.

Nedashkovskaya, O. I., S. B. Kim, S. K. Han, A. M. Lysenko, M. Rohde, M. S. Rhee, G. M. Frolova, E. Falsen, V. V. Mikhailov, and K. S. Bae. 2004a. Maribacter gen. nov., a new member of the family Flavobacteriaceae, isolated from marine habitats, containing the species Maribacter sedimenticola sp. nov., Maribacter aquivivus sp. nov., Maribacter orientalis sp. nov. and Maribacter ulvicola sp. nov. Int. J. Syst. Bacteriol.

Nedashkovskaya, O. I., S. B. Kim, M. S. Rhee, A. M. Lysenko, E. Falsen, G. M. Frolova, V. V. Mikhailov, and K. S. Bae. 2004b. Ulvibacter litoralis gen. nov., sp. nov., a novel member of the family Flavobacteriaceae isolated from the green alga Ulva fenestrata. Int. J. Syst. Evol. Microbiol.

Nichols, D. S, J. P. Bowman, K. Sanderson, C. Mancuso Nichols, T. Lewis, T. A. McMeekin, and P. D. Nichols. 1999. Developments with Antarctic microorganisms: culture collections, bioactivity screening, taxonomy, PUFA production and cold-adapted enzymes. Curr. Opin. Biotechnol. 10:240–246.

O'Sullivan, L.A., K. E. Fuller, E. M. Thomas, C. M. Turley, J. C. Fry, and A. J. Weightman. 2004. Wide distribution and culturability of the uncultivated "AGG58 cluster" of the Bacteroidetes phylum in aquatic environments. FEMS Microbiol. Ecol. 47:359–370.

Perovic, S, A. Wichels, C. Schutt, G. Gerdts, S. Pahler, R. Steffen, and W. E. G. Muller. 1998. Neuroactive compounds produced by bacteria from the marine sponge Halichondria panicea: activation of the neuronal NMDA receptor. Env. Toxicol. Pharmacol. 6(2):125–133.

Ploug, H., Hietanen, S., and, Kuparinen, J. 2002. Diffusion and advection within and around sinking, porous diatom aggregates. Limnol. Oceanogr. 47 (4):1129–1136.

Rappé, M. S., S. A. Connon, K. L. Vergin, and S. J. Giovannoni. 2002. Cultivation of the ubiquitous SAR 11 marine bacterioplankton clade. Nature 418(6898):630–633.

Reichenbach, H. 1989. Genus I: Cytophaga Winogradsky 1929, 577^{AL} emend. *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology, 1st ed. Williams & Wilkins. Baltimore, MD. 3:2015–2050.

Rothschild, L. J., and R. L. Mancinelli. 2001. Life in extreme environments. Nature 409:1092–1101.

Russell, N. J., and D. S. Nichols. 1999. Polyunsaturated fatty acids in marine bacteria—a dogma rewritten. Microbiology (UK) 145(4):767–779.

Sakai, T., H. Kimura, and I. Kato. 2002. A marine strain of Flavobacteriaceae utilizes brown seaweed fucoidan. Mar. Biotechnol. 4(4):399–405.

Shahidi, F., and S. K. Kim. 2002. Marine lipids as affected by processing and their quality preservation by natural antioxidants. ACS Symp. Ser. 816:1–13.

Skerratt, J. H., J. P. Bowman, G. Hallegraeff, S. James, and P. D. Nichols. 2002. Algicidal bacteria associated with blooms of a toxic dinoflagellate in a temperate Australian estuary. Mar. Ecol. Prog. Ser. 244:1–15.

Smith, M. C., J. P. Bowman, F. J. Scott, and M. A. Line. 2000. Sublithic bacteria associated with Antarctic quartz stones. Antarct. Sci. 12:177–184.

Staley, J. T., and J. J.Gosink. 1999. Poles apart: Biodiversity and biogeography of sea ice bacteria. Ann. Rev. Microbiol. 53:189–215.

Suzuki, M., Y. Nakagawa, S. Harayama, and S. Yamamoto. 2001. Phylogenetic analysis and taxonomic study of marine Cytophaga-like bacteria: proposal for Tenacibaculum gen. nov. with Tenacibaculum maritimum comb. nov. and Tenacibaculum ovolyticum comb. nov., and description of Tenacibaculum mesophilum sp. nov. and Tenacibaculum amylolyticum sp. nov. Int. J. Syst. Evol. Microbiol. 51(5):1639–1652.

Tavani, A., C. Pelucchi, M. Parpinel, E. Negri, S. Franceschi, F. Levi, and C. La Vecchia. 2003. n-3 polyunsaturated fatty acid intake and cancer risk in Italy and Switzerland. Int. J. Cancer 105(1):113–116.

Tokai, M., H. Kawasaki Y. Kikuchi, and K. Ouchi. 2000. Cloning and characterization of the CSF1 gene of Saccharomyces cerevisiae, which is required for nutrient uptake at low temperature. J. Bacteriol. 182(10):2865–2868.

Umezawa, H., Y. Okami, S. Kurasawa, T. Ohnuki, M. Ishizuka, T. Takeuchi, T. Shiio, and Y. Yugari. 1983. Marinactan, antitumor polysaccharide produced by marine bacteria. J. Antibiot. (Tokyo) 36(5):471–477.

Van Trappen, S., J. Mergaert, S. Van Eygen, P. Dawyndt, M. C. Cnokaert, and J. Swings. 2002. Diversity of 746 heterotrophic bacteria isolated from microbial mats from ten Antarctic lakes. Syst. Appl. Microbiol. 25:603–610.

Van Trappen, S., I. Vandecandelaere, J. Mergaert, and J. Swings. 2003. Gillisia limnaea gen. nov., sp. nov., a new member of the family Flavobacteriaceae isolated from a microbial mat in Lake Fryxell, Antarctica. Int. J. Syst. Evol. Microbiol.

Wakabayashi, H., M. Hikida, and K. Masumura. 1986. Flexibacter maritimus sp. nov., a pathogen of marine fishes. Int. J. Syst. Bacteriol. 36:396–398.

Wilson, T. H., and P. Z. Ding. 2001. Sodium-substrate cotransport in bacteria. BBA Bioenergetics 1505(1):121–130.

Wilson, T., J. Carson, and J. Bowman. 2002. Optimisation of one-tube PCR-ELISA to detect femtogram amounts of genomic DNA. J. Microbiol. Meth. 51(2):163–170.

Yan, L. M., K. G. Boyd, and J. G. Burgess. 2002. Surface attachment induced production of antimicrobial compounds by marine epiphytic bacteria using modified roller bottle cultivation. Mar. Biotechnol. 4(4):356–366.

ZoBell, C. E., and H. C. Upham. 1944. A list of marine bacteria including descriptions of sixty new species. Bull. Scripps Inst. Oceanogr. Univ. Calif. (Techn. Ser.) 5:239–292.

ZoBell, C. E. 1946. Marine Microbiology. Chronica Botanica. Waltham, MA.

Prokaryotes (2006) 7:695–708
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CHAPTER 6.14

Capnophilic Bird Pathogens in the Family Flavobacteriaceae: Riemerella, Ornithobacterium and Coenonia

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Introduction

Riemerella columbina, *Ornithobacterium rhinotracheale* and *Coenonia anatina* were initially recognized in the course of long-term studies on the etiology of respiratory tract infections in birds as phenotypically unusual isolates. The reclassification of the organism known as *Pasteurella anatipestifer* and *Moraxella anatipestifer* (Segers et al., 1993) triggered a series of taxonomic studies leading to the stepwise characterization and description of *O. rhinotracheale* (Vandamme et al., 1994), *C. anatina* (Vandamme et al., 1999) and *R. columbina* (Vancanneyt et al., 1999). There have been no additional studies since the original description of the latter two organisms. However, the formal description of *O. rhinotracheale* led to a wealth of studies on this bacterium because of its recognition as an economically important pathogen in turkey and chicken husbandry. Rather unexpectedly, although not closely related, *Riemerella*, *Ornithobacterium* and *Coenonia* turned out to belong to the same major phylogenetic lineage, now known as the family Flavobacteriaceae (Bernardet et al., 1996).

Taxonomy

Overview

This group of capnophilic bird pathogens comprises four species. Within the genus *Riemerella*, *R. anatipestifer* is the type species with *R. columbina* as a second species. *Ornithobacterium rhinotracheale* and *C. anatina* are the type and only species of their respective genera.

Riemerella anatipestifer has been isolated worldwide from domestic and wild birds. The organism causes a disease named "exudative septicemia" (Riemer, 1904), which particularly affects ducks, less frequently geese and turkeys, and sporadically chickens and wild birds (Brogden, 1989; Hinz et al., 1998b). This pathogen has been classified in the genera *Moraxella* and *Pasteurella* but was considered species incertae sedis in *Bergey's Manual of Systematic Bacteriology* (Bøvre, 1984; Mannheim, 1984). Its affiliation with the *Flavobacterium-Cytophaga* lineage was suggested by the presence of menaquinones as sole respiratory quinones and of branched-chain fatty acids in high percentages, the absence of carbohydrate fermentation, a similar pattern of hydrolytic enzymes, the low DNA base ratio, and the lack of flagellation (Piechulla et al., 1986; Rossau et al., 1991). In 1993, DNA-rDNA hybridization experiments unequivocally demonstrated that this bacterium indeed belonged to the *Flavobacterium-Cytophaga* lineage (Segers et al., 1993). A novel genus, *Riemerella*, was proposed to accommodate this bacterium because of its genomic divergence from allied taxa and because of its unique chemotaxonomic and phenotypic properties, which included a capnophilic metabolism (Segers et al., 1993). Subsequent 16S rRNA gene sequence analysis confirmed these initial rRNA hybridization-based findings (Subramaniam et al., 1997; Ryll et al., 2001).

A group of thirteen *R. anatipestifer*-like isolates from diseased pigeons was included in a polyphasic taxonomic study which demonstrated that they represented a distinct *Riemerella* species for which the name *R. columbina* sp. nov. was proposed (Vancanneyt et al., 1999). Shortly thereafter, a second group of *R. anatipestifer*-like bacteria, known as "taxon 1502" (Hinz et al., 1998b), was described using a similar polyphasic approach (Vandamme et al., 1999). This study entailed the proposal of *C. anatina* to accommodate taxon 1502 isolates, which originated from pekin ducks, muscovy ducks, geese and pigs.

In the late 1980s the isolation of a pleomorphic Gram-negative rod-shaped bacterium from turkey and chicken samples in Belgium by Jef

Hommez (Provinciaal Verbond voor Dierenziektenbestrijding, Torhout, Belgium) triggered another polyphasic taxonomic study. Similar bacteria had been isolated in Germany, the United Kingdom, France, the Republic of South Africa and the United States (Charlton et al., 1993; Vandamme et al., 1994) and were first reported in 1993 (Charlton et al., 1993; Hafez et al., 1993). The study collection included 21 isolates from the respiratory tract of turkeys, chickens, rooks, and a partridge, and most isolates were associated with various respiratory tract infections including tracheitis, pericarditis, sinusitis, airsacculitis, and pneumonia. The taxonomic study revealed that they represented a novel bacterium for which the name *O. rhinotracheale* was proposed. Subsequent studies identified *O. rhinotracheale* as a respiratory pathogen in birds worldwide.

Phylogeny

Together with about 20 validly named genera, several generically misclassified organisms, and a number of invalid taxa, the genera *Riemerella*, *Ornithobacterium* and *Coenonia* form the family Flavobacteriaceae (Bernardet et al., 1996; Fig. 1). *Riemerella anatipestifer* and *R. columbina* isolates share a 16S rDNA sequence similarity level of about 95% and are closely related to *Bergeyella zoohelcum*, a bacterium isolated from cats, dogs and various human clinical specimens (Holmes et al., 1986). The average 16S rDNA sequence similarity level between *Riemerella* and *Bergeyella* strains is about 93.5%. *Ornithobacterium rhinotracheale* represents a rather distinct line of descent within this family, with only about 87.5% 16S rDNA sequence similarity towards its closest neighbors (the genera *Empedobacter* and *Weeksella*). *Coenonia anatina* is closely related to members of the genus *Capnocytophaga* (about 89.5% 16S rDNA sequence similarity), which are capnophilic bacteria occurring in the oral cavity of man and animals.

Identification

One-dimensional Whole-cell Protein Electrophoresis

In bacterial taxonomy, one-dimensional whole-cell protein electrophoresis has been used as a most useful primary screening tool for the rapid delineation of closely related bacteria (Vandamme et al., 1996). The technique is primarily a research tool but has been used to recognize members of the genera *Riemerella*, *Ornithobacterium* and *Coenonia* and was used as the key instrument to delineate the four species (Segers et al., 1993; Vandamme et al., 1994; Vandamme et al., 1999; Vancanneyt et al., 1999). Figure 2 shows the dendrogram derived from the numerical analysis of the unweighted pair group average linkage of correlation coefficients between whole-organism protein patterns of strains representing the four taxa and illustrates that they are readily distinguishable.

Whole-cell Fatty Acid Methyl Ester Analysis

The average cellular fatty acid methyl ester composition of *Riemerella*, *Ornithobacterium* and *Coenonia* species after cultivation for 48 h on tryptic soy agar and preparation and analysis of the profiles using the Microbial Identification System (Microbial ID, Inc., Newark, DE, USA) is given in Table 1.

The branched chain fatty acids 13:0 *iso*, 15:0 *iso*, 15:0 *anteiso*, 15:0 *iso* 3OH, and 17:0 *iso* 3OH are predominant in all species. Overall, the four average fatty acid profiles are clearly different and allow a straightforward differentiation of the four taxa, including both *Riemerella* species. The fatty acid profiles and in particular the high percentage of 15:0 *iso* 3OH and 17:0 *iso* 3OH and an unidentified fatty acid with equivalent chain length value of 13.566 also differentiate *O. rhinotracheale* from *Capnocytophaga* spp. (Vandamme et al., 1994).

DNA Base Compositions

The DNA base ratio of *R. anatipestifer*, *R. columbina*, *O. rhinotracheale* and *C. anatina* is similar: 33–35, 36–37, 37–39, and 35–36 mol%, respectively (Segers et al., 1993; Vandamme et al., 1994; Vandamme et al., 1999; Vancanneyt et al., 1999).

Isolation and Growth Conditions

Riemerella anatipestifer and *R. columbina*

During acute septicemic stage of the disease, *R. anatipestifer* can be grown from heart blood, brain, airsacs, bone marrow, liver, pneumonic lung and fibrinous exudate. *Riemerella columbina* has been isolated mostly from samples of the respiratory tract. In more chronic cases it is often difficult to isolate the agent from the lesions. Swabs from the nasal cavity, pharynx and upper part of the trachea should be taken from birds with clinically inapparent infections.

Riemerella anatipestifer and *R. columbina* are chemoorganotrophic, mesothermophilic and microaerophilic to aerophilic. Trypticase soy agar and Columbia agar base with 5–10% defi-

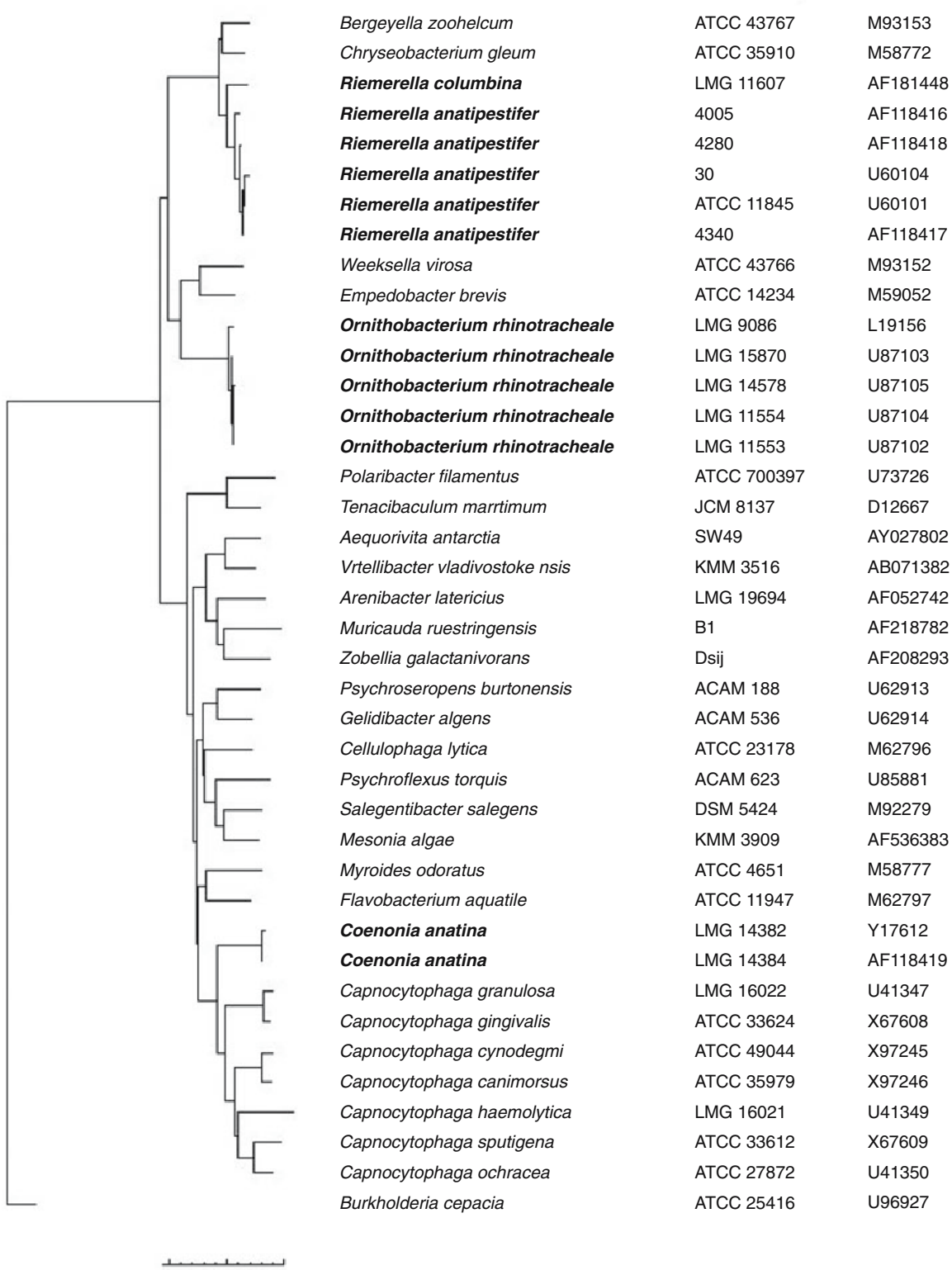

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of *Riemerella*, *Ornithobacterium* and *Coenonia* strains and the type species of the other genera belonging to the family Flavobacteriaceae. The scale bar represents 10% difference in nucleotide sequence as determined by measuring the lengths of the horizontal lines connecting any two species. *Burkholderia cepacia*, a beta-proteobacterium, was used as an outgroup species.

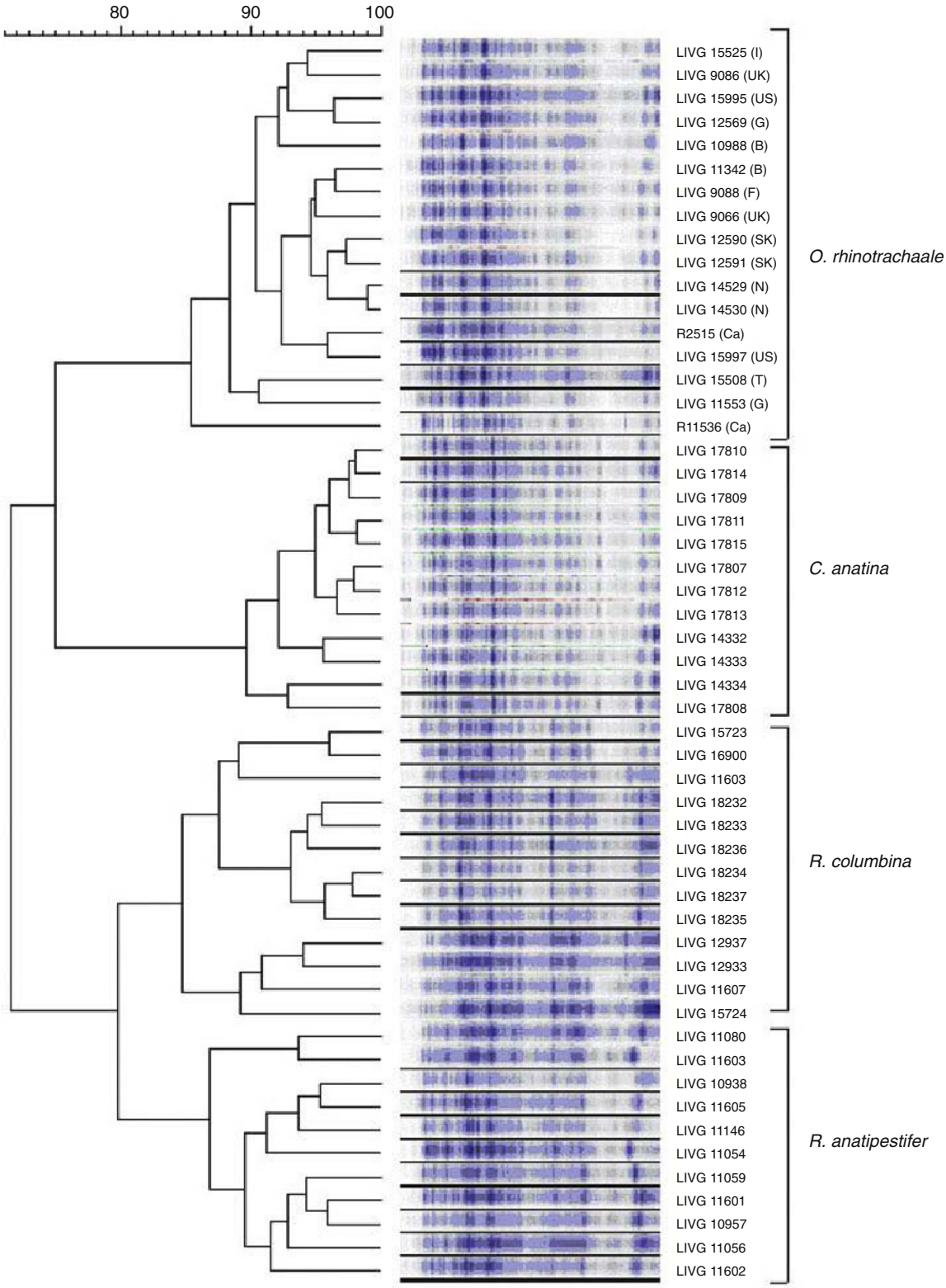

Fig. 2. Computer-reproduced images and dendrogram derived from the unweighted pair group average linkage of correlation coefficients between whole-organism protein patterns (fingerprints) of representative *Riemerella*, *Ornithobacterium* and *Coenonia* strains. For *O. rhinotracheale*, the geographic origin of the isolates is given: B (Belgium), Ca (Canada), F (France), G (Germany), I (Israel), N (the Netherlands), SA (Republic of South Africa), UK (United Kingdom), and US (United States). The numerical analysis was performed using the GelCompar 4.0 software package (Applied Maths, Kortrijk, Belgium). The profiles were recorded and stored on a PC computer. The similarity between all pairs of traces was expressed by the Pearson product moment correlation coefficient converted for convenience to a percent value.

Table 1. Whole-cell fatty acid composition of *Riemerella*, *Ornithobacterium* and *Coenonia* species.[a]

Species	13:0 *iso*	ECL[b] 13.566	15:0 *iso*	15:0 *anteiso*	15:0 *iso*3OH	16:0 3OH	17:0 *iso*3OH
R. anatipestifer[c]	15.1 ± 3.8	1.4 ± 0.5	52.4 ± 4.6	5.4 ± 1.1	8.2 ± 2.4	?	13.6 ± 3.4
R. columbina[c]	10.1 ± 1.8	Tr	45.3 ± 3.3	22.1 ± 3.3	3.9 ± 0.9	?	7.0 ± 1.5
C. anatina[d]	33.9 ± 4.6	Tr	40.8 ± 4.7	3.2 ± 0.7	6.4 ± 1.3	4.1 ± 0.9	6.3 ± 2.3
O. rhinotracheale[e]	Tr	4.2 ± 1.5	57.4 ± 6.1	Tr	8.1 ± 1.9	2.8 ± 1.8	20.2 ± 5.0

Abbreviations: ECL, equivalent chain length; and Tr, trace amount (less than 1%).

[a]Those fatty acids for which the average amount for all taxa was less than 1% are not given. Therefore, the percentages for each group do not total 100%. Mean percentages and standard deviations are given.

[b]The identity of the fatty acid is not known.

[c]Data from Vancanneyt et al. (1999). *Riemerella columbina* strains also contain 2.0 ± 0.7% of 13:0 *anteiso*, 1.0 ± 0.2% of 14:0 *iso*, 1.0 ± 0.4% of 15:0 2OH, 1.0 ± 0.1% of 17:0 2OH, and 2.9 ± 1.3% of summed feature 4, which consists of one or more of the following fatty acids that cannot be separated by the Microbial Identification System: 15:0 *iso* 2OH, 16:1 ω7c, and 16:1 ω7t.

[d]Data from Vandamme et al. (1999). *Coenonia anatina* strains also contain 2.8 ± 2.4% of 14:0.

[e]Data from Vandamme et al. (1994). *Ornithbacterium rhinotracheale* strains also contain 1.5 ± 1.0% of 17:0 *iso*, 2.9 ± 1.3% of 16:0, and 1.1 ± 0.5% of ECL 16.580.

brinated sheep blood are the only media by which riemerellae have been successfully isolated. At present, a selective medium (containing 10% defibrinated sheep blood, trypticase soy agar, or Columbia agar as the agar base and gentamycin [80-200 µg/ml] and neomycin [25-40 µg/ml]) is the medium of choice for the primary isolation because other bacterial agents, contaminants or secondary invaders may inhibit or overgrow the fragile organisms of riemerellae (Köhler et al., 1995). A comparative study revealed significantly higher isolation rates with the selective medium (Köhler et al., 1995).

Furthermore, a microaerobic atmosphere comprising 5–10% CO_2 and high humidity greatly increases the recovery of the riemerellae during primary isolation (e.g., candle jar conditions). After subculturing, some isolates can be adapted to grow on conventional media with or without additional CO_2 and with or without serum. Under strict anaerobic conditions, no growth has been observed.

Although some *R. anatipestifer* strains grew at an incubation temperature of 45°C, no growth was obtained at 4 or 55°C; maximum growth usually occurs after 48–72 h when incubated at 37°C on blood agar in a candle jar. The colonies are 2–3 mm in diameter, circular, convex, entire, transparent to grayish, glistening and butyrous.

Colonies on transparent medium (e.g., blood agar base + 5% serum) show iridescence when observed with obliquely transmitted light.

Coenonia anatina

This species can be grown from heart blood, fibrinous exudate from the surface of the heart, brain, and parenchymatous organs of birds with symptoms of a septicemic running disease. If the respiratory tract is the only affected organ system, swabs from the nasal cavity, infraorbital sinus, or trachea and/or samples of pneumonic lungs and fibrinous exudate of the air sacs should be cultured.

Coenonia anatina is a chemoorganotrophic, mesothermophilic and capnophilic microorganism. Similar to *R. anatipestifer*, the routine medium for the isolation is trypticase soy agar or Columbia agar base containing 7–10% defibrinated sheep blood. Selective medium supplemented with gentamycin (80 µg/ml) and neomycin (40 µg/ml) should be used for the primary isolation because fast growing bacteria like *Escherichia coli* or *Pseudomonas aeruginosa* may overgrow or inhibit *Canatina* (Köhler et al., 1995).

Growth is optimal at 37°C in a microaerobic and CO_2-enriched atmosphere with high humidity. *Coenonia anatina* does not grow under aerobic conditions and yields weak growth under strict anaerobic conditions. Colonies are convex, circular, opaque and glistening with entire edges and smooth surface on the 24-h blood agar culture. After 48–72 h incubation, colonies on selective medium may appear more whitish and mottled.

Ornithobacterium rhinotracheale

Samples for bacterial culture should be collected at an early stage of the disease. Trachea, lungs, and air sacs are the best tissues from which to isolate *O. rhinotracheale*. Infraorbital sinus and nasal cavity are also suitable for culture, but *O. rhinotracheale* can easily be masked by overgrowth of other bacteria.

Blood agar with 5–10% sheep blood is commonly used for primary isolation. *Ornithobacterium rhinotracheale* also grows readily on tryptose soy agar as well as in peptone water and

Pasteurella broth, both aerobically and anaerobically. There is no growth on MacConkey agar. In samples with fast growing bacteria such as *E. coli*, *Proteus* or *Pseudomonas*, *O. rhinotracheale* may be overgrown and therefore cannot be detected in routine investigation. Most *O. rhinotracheale* isolates are resistant to gentamycin (Hafez et al., 1993). Back et al. (1998) recommended the use of 10 µg of gentamycin per ml of blood agar medium to isolate *O. rhinotracheale* from contaminated samples. Blood agar containing 5 µg of gentamycin and 5 µg of polymyxin per ml of medium can also be used as selective medium.

Incubation of primary plates for 48 hours under anaerobic or, preferentially, microaerobic (5–10% CO_2) conditions is recommended. The optimal growth temperature is 37°C. On blood agar, the colonies are small, gray-white, opaque, nonhemolytic and differ in diameter (1–3 mm).

Detection and Identification

Riemerella anatipestifer and *R. columbina*

The history of the disease and its clinical signs and gross lesions may allow a presumptive diagnosis which has to be confirmed by the detection of the causal agent. After isolation, riemerellae can be detected and identified using direct and indirect methods.

Among the direct detection methods, immunofluorescent procedures can be used to identify riemerellae in tissues and exudate from infected birds (Marshall et al., 1961). More traditionally, isolated colonies should be selected and subcultured for further identification procedures. Riemerellae are usually identified by their cultural and physiological characteristics.

Riemerellae cells are Gram-negative capsulated, nonmotile, nonsporeforming rods occurring singly, in pairs, or as filamentous forms. In 16–24-h old cultures on blood agar, the rods vary from 0.2–0.4 µm in width and 1–5 µm in length.

The absence of species-specific characteristics in conventional macrotests makes reliable identification difficult. *Riemerella anatipestifer* has been described as an organism that does not produce indole and does not ferment carbohydrates (Bangun et al., 1981). However, there is no doubt that indole-producing strains exist (Pathanasophon et al., 1994; Hinz et al., 1998b). Using the buffered single substrate test (BSS-test), *R. anatipestifer* and *R. columbina* oxidatively catabolize several carbohydrates to acid (Table 2). Weak acid production by *R. anatipestifer* from carbohydrates may be masked by alkaline breakdown products from peptone when peptone-containing media are used (Hinz et al., 1998a).

Commercially available micro-test strips, especially those by which the enzyme activity of resting cells can be examined, are useful identification procedures for *R. anatipestifer* and *R. columbina* (e.g., API galleries such as 20NE, ID 32 E, and ZYM). However, it should be considered that the detection sensitivity of these commercial systems are different (Hinz et al., 1998b).

Table 3 lists differential diagnostic characteristics that can be used to distinguish *R. anatipestifer* and *R. columbina* from other bacterial species that may be encountered in specimens from birds: *C anatina*, *O. rhinotracheale*, *Chryseobacterium meningosepticum* and *Pasteurella* species. Only two characteristics clearly differentiate *R. columbina* from *R. anatipestifer*: pigment formation and esculin hydrolysis (β-glucosidase). Esculin hydrolysis, gelatinase, hyaluronidase (chondroitin sulfatase), and β-galactosidase activities are of high differential diagnostic value.

Indirect detection of riemerellae in ducks can be achieved through several enzyme-linked immunosorbent assays (ELISAs). Hatfield et al. (1987) reported on an ELISA that was more sensitive than agglutination tests to detect infection with *R. anatipestifer* by specific serum antibodies. Huang et al. (2002) developed an ELISA using a recombinant 41-kDa partial protein (P45N′) for the detection of *R. anatipestifer* infections in ducks. More recently, Lobbedey and Schlatterer (2003) reported on a direct and an indirect ELISA, which were applied for the detection of duck antibodies against *R. anatipestifer* antigens in egg yolk of vaccinees and in serum of their offspring.

In epidemiological studies, *R. anatipestifer* isolates have been serotyped using agglutination and agar-gel precipitation reactions. Both of these procedures involve surface antigens

Table 2. Acid production from carbohydrates in buffered single substrate tests by *R. anatipestifer* and *R. columbina*.

	R. anatipestifer		
Acid from[a]	Indole –	Indole +	*R. columbina*
D-(+)-Glucose	+/–	+	+
D-(–)-Fructose	–/+	+	(+)/–
D-(+)-Mannose	+/–	+	+
Maltose	+	+	+
Dextrin	+	+	+
Trehalose	–/+	–	–
Saccharose	–	–/+	–
L-(–)-Sorbose	–/(+)	–	–/(+)

Symbols used: +, >90% positive; –, >90% negative; +/–, >80% positive; –/+, >20% negative; and (+), weak or delayed positive.

[a]No acid was produced from: adonitol, dulcitol, D-(+)-galactose, *N*-acetyl-D-glucosamine, lactose, lactulose, *myo*-inositol, D-(–)-mannitol, salicin, D-(–)-sorbitol, and D-(+)-xylose.

Table 3. Main differential characteristics of capnophilic bird pathogens.

Characteristic	RA	RC	CA	OR	CM	Pas
Aerobic growth on blood agar	+	+	–	–	+	+
Growth on MacConkey agar	–	–	–	–	+	–
Colony pigmentation	–	+	–	–	+	–
Motility	–	–	–	–	–	–
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	–	+	+
Urease	V	V	–	+	V	–
Indole production	V	–	–	–	V	V
Nitrate reduction	–	–	–	–	–	+
Esculin hydrolysis[a] (β-D-glucosidase)	–	+	+	–	+	–
Gelatinase	+	+	–	–	+	–
Hyaluronidase[b]	–	–	+	+	ND	V
β-D-Galactosidase[a]	–	–	+	+	–	V
α-D-Glucosidase[c]	+	+	+	+	+	V
N-Acetyl-β-glucosaminidase[c]	–	–	+	+	–	ND
α-L-Fucosidase [c]	–	–	+	–	–	ND
Utilization of carbon sources for growth[a]	–	–	–	–	+	–
Host spectrum	Birds and pigs	Pigeon	Anatine birds	Birds	Humans and birds	Mammals and birds

Symbols used: +, >90% positive; –, >90% negative; and (+), weak and/or delayed positive.
Abbreviations: RA, *R. anatipestifer*; RC, *R. columbina*; CA, *C. anatina*; OR, *O. rhinotracheale*; CM, *C. meningosepticum*; and Pas, *Pasteurella* species; V, variable, and ND, not done.
[a]API-20NE test results.
[b]Identical with the chondroitin sulfatase test results.
[c]API-ZYM test results.

that are presumed to be polysaccharides. To date, 21 serovars have been reported (Pathanasophon et al., 2002). DNA based fingerprinting has been performed using restriction endonucleases analysis (Rimler and Nordholm, 1998), repetitive sequence PCR (Huang et al., 1999), and restriction fragment length polymorphism analysis (Subramaniam et al., 1997). Weng et al. (1999) reported that a preliminary analysis of hybridization patterns of genomic DNA with an insertion element-based probe indicated that it might also be a useful tool for epidemiological studies.

Coenonia anatina

Isolated colonies should be selected and subcultured for further identification procedures. *Coenonia anatina* is identified by morphological, cultural and physiological characteristics. *Coenonia anatina* cells are Gram-negative, nonsporeforming, nonmotile rod-shaped bacteria. In 16-h-old blood agar cultures the mean cell size is 0.2–0.4 μm in width and 1.25–2.5 μm in length. Some cells are spherically swollen, spindle-lemon-shaped and arranged in short chains.

The main differential diagnostic characteristics distinguishing *C. anatina* from *O. rhinotracheale*, *C. meningosepticum*, *R. anatipestifer* and *R. columbina* as well as *Pasteurella* species are given in Table 3. Differential features towards *Capnocytophaga* species, the close phylogenetic neighbors of *C. anatina*, have been reported (Vandamme et al., 1999) but are not presented here because *Capnocytophaga* has not been detected in birds up to now.

The enzyme-based activity profile of *C. anatina* has a higher degree of similarity to *O. rhinotracheale* than to the riemerellae. The main features that allow the differentiation of *C. anatina* from *O. rhinotracheale* are the presence of catalase and α-L-fucosidase activity, esculin hydrolysis, and the absence of urease activity. However, it should be considered that 1–4% of the *O. rhinotracheale* field strains give a positive catalase reaction.

In contrast to *R. anatipestifer* and *R. columbina*, *C. anatina* strains display nearly identical carbohydrate acidification patterns when the test results in conventional biochemical and buffered single substrate test systems are compared. *Coenonia anatina* can be distinguished from riemerellae in being gelatinase negative and hyaluronidase, chondroitin sulfatase, *N*-acetyl-β-glucosaminidase and α-L-fucosidase positive.

Ornithobacterium rhinotracheale

Clinical signs and lesions are of little value for diagnosis since many other infectious agents can produce similar clinical signs and post-mortem lesions. Accurate diagnosis must be substantiated by direct detection or cultural isolation of the causative bacteria and/or indirectly through

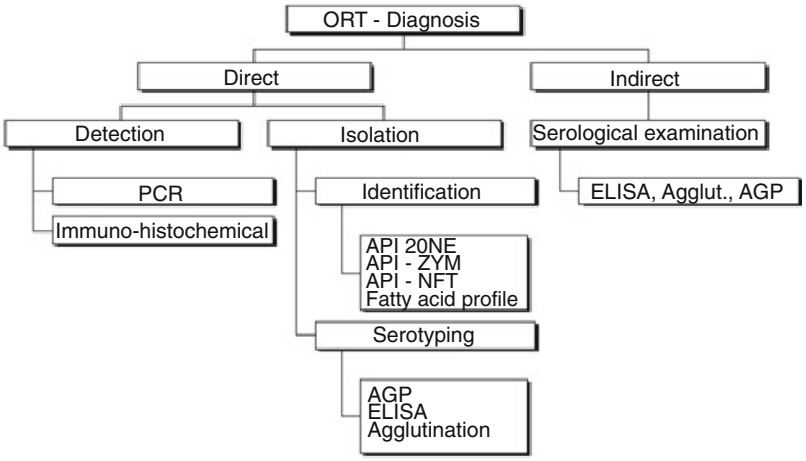

Fig. 3. Algorithm for specific laboratory diagnosis of *O. rhinotracheale* (Hafez, 2002). PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; ORT, *Ornithobacter rhinotrachaele*; and AGP, agar gel precipitation.

detection of antibodies using serological examination (Fig. 3).

Ornithobacterium rhinotracheale cells are Gram-negative pleomorphic rods. They produce oxidase but not indole. Nearly all isolates are β-galactosidase (ortho-nitrophenyl-β-D-galactoside; ONPG) positive and catalase negative, and most of them reacted positively in the urease test. However, Ryll et al. (2002) were able to isolate and identify a cytochrome-oxidase negative strain of *O. rhinotracheale* from turkeys.

Biochemical examination using commercial biochemical API 20 NE test-kit (Bio-Mérieux, France, or API 20 NFT, United States) revealed for 99.5% of *O. rhinotracheale* strains a reaction code of 022 000 4 (61%) or 002 000 4 (38.5%). Further identification could be carried out using API ZYM test system or fatty acid profile. Another commercial identification system, the RapID NF Plus system (Innovative Diagnostics, United States), gave high identification scores (Biocodes 4-7-2-2-6-4, 4-7-6-2-6-4, 6-7-6-2-6-4 or 7-2-2-6-4) when 110 strains were tested.

A specific polymerase chain reaction (PCR) test can be performed for the direct detection of *O. rhinotracheale* using the primer combination OR16S-F1 (5′-GAG AAT TAA TTT ACG GAT TAA G) and OR16S-R1 (5′-TTC GCT TGG TCT CCG AAG AT). This combination amplifies a 784-bp fragment of the 16S rRNA gene of *O. rhinotracheale,* but not of other closely related bacteria with which *O. rhinotracheale* could be confused (Van Empel, 1998a; Hung and Alvarado, 2001). This PCR assay needs optimization for the direct demonstration of *O. rhinotracheale* in diagnostic material. A sensitive immunohistochemical staining procedure was described by Van Empel et al. (1999b). Using this procedure, it was found that *O. rhinotracheale* was the cause of 70% of the cases with respiratory symptoms in broiler chickens, while by cultural and/or serological examination only 30% of the cases could be associated to *O. rhinotracheale* (Van Empel et al., 1999b).

Serological examination for indirect detection of *O. rhinotracheale* through the presence of antibodies in blood serum of birds can be carried out using a slide agglutination test prepared from different serovars, ELISA-tests, or dot-immunobinding assay (Erganis et al., 2002). The serotype specificity of the ELISA depends on the method of antigen extraction used for coating the ELISA plates. Boiled extract antigens are serotype-specific (Van Empel et al., 1997), while antigen extraction with sodium dodecyl sulfate (SDS-antigen) results in more cross-reactions (Hafez and Sting, 1999a). Self made ELISA (SDS-extraction) as well as two commercially available ELISA-kits (Biocheck and IDEXX) allowed detection of antibodies against all tested *O. rhinotracheale* serovars (Hafez et al., 2000). Generally, using ELISA, antibodies against *O. rhinotracheale* can be detected in serum and egg yolk shortly after infection, and titers will peak between 1 and 4 weeks postinfection (Van Empel et al., 1996). Because titers decline rapidly after peaking, serum samples for flock screening should be taken frequently.

Ornithobacterium rhinotracheale isolates have been subtyped through serology and DNA fingerprinting (Hafez and Beyer, 1997). Serotyping approaches included specific antisera in agar gel precipitation, ELISA, and rapid slide agglutination (Back et al., 1998; Bock et al., 1997; Van Empel et al., 1997; Van Empel, 1998a; Hafez and Sting, 1999a). Currently 18 serovars designated "A" to "R" have been reported. Most chicken isolates belong to serovar A, whereas turkey isolates are more heterogeneous and belong to serovars A, B and D (Van Empel and Hafez, 1999a). *Ornithobacterium rhinotracheale* isolates have also been typed by using several DNA fingerprinting methods including multilocus enzyme electrophoresis, ribotyping, random-

amplified-polymorphic-DNA (RAPD) and rep-PCR (Amonsin et al., 1997; Hafez and Beyer, 1997; Leroy-Sétrin et al., 1998; Hung and Alvarado, 2001).

Habitat, Epidemiology and Disease

Riemerella anatipestifer and *R. columbina*

NATURAL HABITAT. *Riemerella anatipestifer* is distributed worldwide in a wide spectrum of hosts. It has been isolated from domesticated ducks, geese, turkeys, chickens and budgerigars as well as from wild Anatidae but also from pheasants, guinea fowl, quail, partridges, guillemots, gulls and the lungs of domesticated pigs with severe pneumonia. *Riemerella anatipestifer* may persist on the mucosa of the upper respiratory tract and pharynx of clinically healthy birds (Ryll et al., 2001).

There are no indications that this organism can multiply outside the host in the environment.

EPIDEMIOLOGY. Riemerellosis is primarily a disease of young domestic ducks and geese, but affects also turkeys and sporadically chickens and pheasants. It is unknown how *R. anatipestifer* is introduced into the flock. The microorganism is probably egg-transmitted, with lateral spread most commonly via the respiratory route. Adverse environmental conditions and concomitant infections often predispose birds to outbreaks of the disease. At present, there is no information on the epidemiology of *R. columbina*.

DISEASE. The clinical signs reflect the involvement of *R. anatipestifer* with both the respiratory tract and the nervous system. In duckling flocks, outbreaks of the disease appear mostly between the second and eighth, seldom up to tenth, week of age. Signs most observed in an acute case are ocular and nasal discharge and greenish diarrhea. As the disease progresses, leg weakness, ataxia, tremor of the head and neck, loss of equilibrium, and torticollis become the prominent signs.

The mortality ranges between 1 and 10%. Surviving birds may be stunted.

The most obvious gross lesion is fibrinous exudate, which covers serosal surfaces in general, but is most evident in pericardial cavity and over the surface of liver and airsac. The fibrin contains a few inflammatory cells, primarily mononuclear cells and heterophils. The spleen may be enlarged and mottled. Fibrinous meningitis in both acute and subacute phase may be found. Localized inflammations usually occur in the skin and joints.

The present knowledge on the significance of *R. columbina* as a pathogen is limited. The clinical signs and gross lesions indicate the involvement of *R. columbina* as a pathogenic agent in the genesis of severe respiratory disease of pigeons.

Coenonia anatina

NATURAL HABITAT. *Coenonia anatina* has been found in anatine birds from Germany, England and Thailand. It has been grown from samples of commercial ducks, muscovy ducks, geese, and in one case, also from a single pigeon (B. Köhler, personal communication). *Coenonia anatina* may persist on the mucosa of the upper respiratory tract. There are no indications for its multiplication outside the host in the environment.

The isolation of *C. anatina* as pure culture, or together with *R. anatipestifer*, *Salmonella enteritidis* or *E. coli*, from samples of the respiratory tract (pneumonic lungs, air sac fibrin and pericardium) and brain of ducks and geese was always associated with signs similar to those of the *R. anatipestifer*-associated exudative septicemia.

Coenonia anatina was recovered from over 30% of the cases of septicemia-related losses in commercial duck flocks of the northern part of Germany in the period 1994–1995. Vaccination with inactivated whole-cells induced specific protection in ducklings. All these data and observations support its etiological involvement in the pathological process of diseases, which have been seen under field conditions (Vandamme et al., 1999).

With regard to the epidemiology, pathogenicity, disease and treatment, no further scientific findings are available at present.

Ornithobacterium rhinotracheale

NATURAL HABITAT. *Ornithobacterium rhinotracheale* has been isolated from chickens, chukar partridges, ducks, geese, guinea fowl, gulls, ostriches, partridges, pheasants, pigeons, quail, rooks and turkeys. Results of molecular epidemiological investigation indicate that the majority of *O. rhinotracheale* isolates recovered from domesticated poultry worldwide are represented by a small group of closely related clones and suggest that it was recently introduced to domesticated poultry from wild bird population (Amonsin et al., 1997).

EPIDEMIOLOGY. The disease is spread horizontally by direct and indirect contact through aerosols or drinking water. Vertical transmission is suspected, since some workers have isolated *O. rhinotracheale* in isolated cases from reproductive organs and hatching eggs, infertile eggs, and dead embryos after experimental infection.

DISEASE. The severity of clinical signs, duration of the disease and mortality are extremely variable and are influenced by many environ-

mental factors such as poor management, inadequate ventilation, high stocking density, poor litter conditions, poor hygiene, high ammonia level, concurrent diseases, and the type of secondary infection. Outbreaks in turkey flocks have been observed mostly in male birds over 14 weeks of age. However, in many cases, the disease also affected young poults (2–8 weeks old). The mortality ranges between 1–15% during the acute phase (8 days). Initial symptoms are coughing, sneezing and nasal discharge followed in some cases by severe respiratory distress, dyspnea, prostration and sinusitis. The symptoms are accompanied with a reduction in feed consumption and water intake. In turkey breeder flocks, clinical signs are accompanied mostly with drops in egg production and increases in the number of unsettable hatching eggs. Clinical signs in broilers generally appear between the third and fourth week of age with a mortality rate of 2–10%. The clinical signs are depression, decrease in food intake, reduced weight gains, transit nasal discharge, and sneezing, followed by facial edema. In broiler breeders and layers, the disease primarily affects the birds at the peak of production, mostly between age 24 and 52 weeks. The first signs are mild respiratory symptoms. The mortality is variable and relatively low in uncomplicated cases. Gross lesions are similar to those seen in fowl cholera in turkeys but to lesser extent. They are characterized by uni- or bilateral fibrinopurulent pneumonia and airsacculitis. Mild tracheitis, pericarditis and enteritis may also be detected.

Pathogenicity

Riemerella anatipestifer is a bacterial pathogen that causes a fatal septicemic disease primarily in ducklings and less frequently in goslings and turkey poults and only sporadically in chicks. Possibly it is also pathogenic for domesticated pigs.

The disease can experimentally be reproduced in healthy ducks by exposure to *R. anatipestifer*. The age of the bird, its genetically determined susceptibility and immune status, as well as the potential virulence of *R. anatipestifer* play an important role in the severity of the disease, which can be established most consistently by parenteral (e.g., intravenous, subcutaneous, or airsac) injection of the microorganism. On the basis of the difference in incidence, severity and extent of the disease, strains can be classified into four categories: highly virulent, virulent, slightly virulent, and avirulent.

To date, little is known on the possible virulence factors of *R. anatipestifer* involved in the pathogenesis of the disease. Obviously, factors exist that correlate with virulence as not all strains have the capacity to induce a disease. The production of a fibrinolytic enzyme (Bangun et al., 1981) and hemolysin by some strains may increase their invasiveness. Chang et al. (1998) described five different plasmid DNA profiles among *R. anatipestifer* strains. A 3.9-kb plasmid, designated "pCFC 1," seemed to be a virulence factor. This plasmid contained at least four open reading frames (ORFs) designated "*vap*D," two of which (*vap*D1 and *vap*D2) encoded proteins homologous to virulence-associated proteins of chromosomal origin that have been found in other pathogens such as *Dichelobacter nodosus*, *Haemophilus influenzae*, *Actinobacillus actinomycetemcomitans* and *Neisseria gonorrhoeae*. Weng et al. (1999) subsequently identified a virulence-associated protein homolog gene and a novel insertion sequence element, designated "ISRa1," in a second plasmid of 5.6 kb, pCFC2. The complete sequence of pCFC2 had a 28% G+C content and three large ORFs. ISRa1 was flanked by 15-bp imperfect inverted repeats and contained an ORF encoding a putative transposase of 292 amino acids. Southern blot analysis indicated that in *R. anatipestifer* strains examined, ISRa1 was present with at least 2–20 copies. Crasta et al. (2002) identified and characterized the genetic determinant (*cam*) of the cyclic AMP (CAMP) cohemolysin as a potential virulence factor.

Ornithobacterium rhinotracheale is a severe respiratory pathogen. Strains isolated from turkeys, chickens or partridges were used for aerosol challenges of both turkeys and broilers of various ages (Van Empel et al., 1996). Under experimental conditions, the most severe symptoms and pneumonic lesions were produced in turkeys when *O. rhinotracheale* was inoculated following exposure to *Bordetella avium*, *E. coli* O78:H9, turkey rhinotracheitis virus, Newcastle disease virus, infectious bronchitis virus as well as *Chlamydophila psittaci*. Neither the origin nor the serovar of the *O. rhinotracheale* strains had an effect on the pathogenicity (Van Empel and Hafez, 1999a). In other studies, airsacculitis, pneumonia, and increased mortality were observed after aerosol, intratracheal, intravenous and/or intrathoracal inoculation without a primer (Ryll et al., 1997; Sprenger et al., 1998).

Treatment

Riemerella anatipestifer and *R. columbina*

ANTIMICROBIAL SUSCEPTIBILITY. Most strains of *R. anatipestifer* are susceptible to enrofloxacin, amoxicillin, chloramphenicol, novobiocin, spiramycin, lincomycin and tetracyclines. Strains were often resistant to penicillin G, streptomycin and sulfonamides. Recently, an increase

in multiresistant strains has been observed (Köhler et al., 1995). More than 90% of the strains are resistant to kanamycin, colistin, gentamycin, neomycin and polymyxin B.

There are indications that the susceptibility of *R. columbina* is similar to that of *R. anatipestifer*.

ANTIMICROBIAL TREATMENT. The bacterial agent responds to drugs added to drinking water or parenterally injected. However, the degree of success varies among antibacterial drugs. Satisfactory treatment of acute riemerellosis is achieved if medication is started immediately after onset of clinical signs. Treatment is of considerable value in preventing disastrous losses when other control measures fail.

VACCINATION. Formalin-inactivated bacterin has been found effective for immunization of ducklings. Since immunity induced by bacterin is serovar-specific, bacterin should contain cells of predominant serovars (i.e., serovar 1) to provide an effective protection. Ducklings are vaccinated at 2–3 weeks of age.

The best results in ducklings are obtained by vaccination of the female breeder duck flocks. This induces maternally derived antibody levels in their progeny, resulting in significant protection for 2–3 weeks.

A live vaccine has been reported to provide significant protection against riemerellosis when administered to 1-day-old ducklings by aerosol or drinking water. A single vaccination provided protection for at least 42 days (Sandhu, 1991). The generation of protective immunity against *R. anatipestifer* infection in ducks has been investigated by immunizations with recombinant glutathione sulfatransferase (GST) fusion proteins consisting of OmpA (a 42-kDa major outer membrane protein) and P45N′ (a 41-kDa N-terminal fragment of a newly identified 45-kDa potential surface protein from *R. anatipestifer*; Huang et al., 2002). The DNAs encoding OmpA and P45N′ were isolated from *R. anatipestifer* serotype 15 (field strain 110/89) and serotype 19 (reference strain 30/90), respectively. Immunoblotting and ELISA results showed that the purified recombinant proteins induced the production of antibodies in immunized ducks. However, neither was protective against subsequent challenge with the virulent serotype 15 strain 34/90. All the five ducks immunized with formalinized *R. anatipestifer* strain 34/90 survived the challenge with the homologous strain, whereas six out of seven ducks in the nonimmunized control group died within a week following the challenge.

Ornithobacterium rhinotracheale

ANTIMICROBIAL SUSCEPTIBILITY. Several studies on antimicrobial resistance profiles of *O. rhinotracheale* have been published (Hafez et al., 1993; Devriese et al., 1995; Malik et al., 2003; Soriano et al., 2003).

In 1995, Devriese et al. reported on exceptionally high levels of acquired resistance to various groups of antibiotics including penicillin-cephalosporin, lincosamide and macrolide, and fluoroquinolone antibiotics in Belgium. Malik et al. (2003) examined in vitro antibiotic resistance profiles of 125 isolates of *O. rhinotracheale* isolated from turkeys in Minnesota during 1996–2002. A majority of isolates was sensitive to clindamycin, erythromycin, spectinomycin and ampicillin. Resistance against sulfachloropyridazine decreased from 1996 to 2002, but an increase in resistance was seen against gentamycin, ampicillin, trimethoprim sulfa, and tetracycline. The resistance against penicillin remained constant from year to year. Soriano et al. (2003) determined the minimal inhibitory concentrations of 10 antimicrobial drugs for Mexican isolates and found a marked resistance trend. The susceptibility of *O. rhinotracheale* to amoxicillin, enrofloxacin, and oxytetracycline was variable. However, consistent higher minimal inhibitory concentration values were obtained for gentamycin, fosfomycin, trimethoprim, sulfamethazine, sulfamerazine, sulfaquinoxaline, and sulfachloropyridazine.

In Germany (own results), all tested *O. rhinotracheale* isolates (100%) showed high sensitivity to amoxicillin, chloramphenicol and chlortetracycline. About 90 and 36% of the isolates were found to be sensitive to erythromycin and furazolidone, respectively. In addition, only 6% of tested isolates were found to be sensitive to enrofloxacin. None of isolates were susceptible to apramycin, neomycin, gentamycin and sulfonamide/trimethoprine (Hafez, et al., 1993). The sensitivity to enrofloxacin seems to be origin-related, since most of turkey isolates from Germany and the Netherlands are resistant, while 98% of strains from France and 71% of isolates from Belgium are sensitive to enrofloxacin.

ANTIMICROBIAL TREATMENT. The treatment of infections with antibiotics is very difficult because of the inconsistent sensitivity of the strains and regional variation in the sensitivity of *O. rhinotracheale* to antibiotics. It is advisable in all cases to estimate the sensitivity of strain involved. Under field conditions, however, water medication using amoxicillin at a dose level of 250 ppm for 3–7 days gives satisfactory results.

CONTROL. *Ornithobacterium rhinotracheale* is highly sensitive to different chemical disinfectants. Preparations of VENNO-VET 1 super (based on different organic acids [formic and glyoxyl acids]) and VENNO-FF super (containing different aldehydes) allowed in vitro inactivation

of *O. rhinotracheale* at concentrations of 0.5% within 15 minutes (Hafez and Schulze, 2003). However, currently, *O. rhinotracheale* infection appears to have become endemic and can affect every new restocking even in previously cleaned and disinfected houses, especially in areas with intensive poultry production as well as on multiple age farms. Failure to clean and disinfect properly after an infected flock has left can cause infection of neighboring flocks and the continuous cycling of the causative agent from house to house.

VACCINATION. Several attempts to combat the disease using vaccines were carried out with different results. Vaccination trials using autogenous bacterins successfully reduced the number of outbreaks of *O. rhinotracheale* infections in turkey flocks in the field (Bock et al., 1997). Vaccination trials using inactivated vaccine in mineral oil adjuvant in broiler, in broiler breeders as well as in turkey flocks were carried out. The early results are promising, and the vaccine gives good protection (Van Empel and Van den Bosch, 1998b; Hafez et al., 1999b). Live vaccination is also feasible, but up to now, no nonvirulent strains of *O. rhinotracheale* have been found. A temperature sensitive mutant of *O. rhinotracheale* has some protective properties, but more tests are needed to evaluate the efficacy and safety of this strain (Lopes et al., 2002). Currently, inactivated vaccine in mineral oil adjuvant for broiler breeders (Intervet) is commercially available.

Literature Cited

Amonsin, A., J. F. X. Wellehan, L.-L. Li, P. Vandamme, C. Lindeman, M. Edman, R. A. Robinson, and V. Kapur. 1997. Molecular epidemiology of Ornithobacterium rhinotracheale. J. Clin. Microbiol. 35:2894–2898.

Back, A., D. Halvorson, G. Rajashekara, and K. Nagaraja. 1998. Development of a serum plate agglutination test to detect antibodies to Ornithobacterium rhinotracheale. J. Vet. Diagn. Invest. 10:84–86.

Bangun, A., D. N. Tripathy, and L. E. Hanson. 1981. Studies of Pasteurella anatipestifer: An approach to its classification. Avian Dis. 25:326–337.

Bernardet, J.-F., P. Segers, M. Vancanneyt, F. Berthe, K. Kersters, and P. Vandamme. 1996. Cutting a Gordian knot: Emended classification and description of the genus Flavobacterium, emended description of the family Flavobacteriaceae, and proposal of Flavobacterium hydatis nom. nov. (basonym Cytophaga aquatilis Strohl and Tait 1978). Int. J. Syst. Bacteriol. 46:128–148.

Bock, R., P. Freidlin, M. A. ManoimInbar, A. Frommer, P. Vandamme, and P. Wilding. 1997. Ornithobacterium rhinotracheale (ORT) associated with a new turkey respiratory tract infectious agent in Israel. *In:* Proceedings of the 11th International Congress of the World Veterinary Poultry Association (WVPA), 18-22 August. Hungarian Branch of the WVPA and Veterinary Medical Research Institute of the Hungarian Academy of Science. Budapest, Hungary. 120.

Bøvre, K. 1984. Genus II. Moraxella Lwoff 1939, 173 emend. Henriksen and Bøvre 1968, 391^{AL}. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1:296–303.

Brogden, K. A. 1989. Pasteurella anatipestifer infection. *In:* C. Adlam and J. M. Rutter (Eds.) Pasteurella and pasteurellosis. Academic Press. London, UK. 115–129.

Chang, C. F., P. E. Hung, and Y. F. Chang. 1998. Molecular characterization of a plasmid isolated from Riemerella anatipestifer. Avian Pathol. 27:339–345.

Charlton, B. R., S. E. Channing-Santiago, A. A. Bickford, C. J. Cardona, R. P. Chin, G. L. Cooper, R. Droual, J. S. Jeffrey, C. U. Meteyer, H. L. Shivaprasad, and R. W. Walker. 1993. Preliminary characterization of a pleomorphic Gram-negative rod associated with avian respiratory disease. J. Vet. Diagn. Invest. 5:47–51.

Crasta, K. C., K.-L. Chua, S. Subramaniam, J. Frey, H. Loh, and H. M. Tan. 2002. Identification and characterization of CAMP cohemolysin as a potential virulence factor of Riemerella anatipestifer. J. Bacteriol. 184:1932–1939.

Devriese, L. A., J. Hommez, P. Vandamme, K. Kersters, and F. Haesebrouck. 1995. In vitro antibiotic sensitivity of Ornithobacterum rhinotracheale strains from poultry and wild birds. Vet. Rec. 137:435–436.

Erganis, O., H. H. Hadimili, K. Kav, M. Corlu, and D. Öztürk. 2002. A comparative study on detection of Ornithobacterium rhinotracheale antibodies in meat-type turkeys by dot immunobinding assay, rapid agglutination test and serum agglutination. Avian Pathol. 31:201–204.

Hafez, H. M., W. Kruse, J. Emele, and R. Sting. 1993. Eine Atemwegsinfektion bei Mastputen durch Pasteurella-ähnliche Erreger: Klinik, Diagnostik und Therapie. *In:* Proceedings of the International Conference on Poultry Diseases, Potsdam. German Veterinary Medical Society Publisher. Giessen, Germany. 105–112.

Hafez, H. M., and W. Beyer. 1997. Preliminary investigation on Ornithobacterium rhinotracheale (ORT) isolates using PCR-fingerprints. *In:* XIth International Congress of the World Veterinary Poultry Association (WVPA), 18-22 August. Hungarian Branch of the WVPA and Veterinary Medical Research Institute of the Hungarian Academy of Science. Budapest, Hungary. 51.

Hafez, H. M., and R. Sting. 1999a. Investigations on different Ornithobacterium rhinotracheale "ORT" isolates. Avian Dis. 34:1–7.

Hafez, H. M., S. Jodas, A. Stadler, and P. Van Empel. 1999b. Efficacy of Ornithobacterium rhinotracheale inactivated vaccine in commercial turkey under field condition. *In:* H. M. Hafez and A. Mazaheri (Eds.) Proceedings of the 2nd International Symposium on Turkey Diseases, Berlin. German Veterinary Medical Society Publisher. Giessen, Germany. 107–117.

Hafez, H. M., A. Mazaheri, and R. Sting. 2000. Efficacy of ELISA for detection of antibodies against several Ornithobacterium rhinotracheale serotypes. Deutsche Tierärztliche Wochenschrift 107:142–143.

Hafez, H. M. 2002. Diagnosis of Ornithobacterium rhinotracheale. Int. J. Poultry Sci. 1:114–118.

Hafez, H. M. and D. Schulze. 2003. Examinations on the efficacy of chemical disinfectants on Ornithobacterium rhinotracheale in vitro. Archiv für Geflügelkunde 67:153–156.

Hatfield, R. M., B. A. Morris, and R. R. Henry. 1987. Development of enzyme-linked immunosorbent assay for the detection of humoral antibody to Pasteurella anatipestifer. Avian Pathol. 16:123–140.

Hinz, K. H., M. Ryll, and B. Köhler. 1998a. Detection of acid production from carbohydrates by Riemerella anatipestifer and related organisms using the buffered single substrate test. Vet Microbiol. 60:277–284.

Hinz, K.-H., M. Ryll, B. Köhler, and G. Glünder. 1998b. Phenotypic characteristics of Riemerella anatipestifer and similar micro-organisms from various hosts. Avian Pathol. 27:33–42.

Holmes, B., A. G. Steigerwalt, R. E. Weaver, and D. J. Brenner. 1986. Weeksella zoohelcum sp. nov. (formerly Group IIj), from human clinical specimens. Syst. Appl. Microbiol. 8:191–196.

Huang, B., S. Subramaniam, K.-L. Chua, J. Kwang, H. Loh, J. Frey, and H. M. Tan. 1999. Molecular fingerprinting of Riemerella anatipestifer by repetitive sequence PCR. Vet. Microbiol. 67:213–219.

Huang, B., J. Kwang, H. Loh, J. Frey, H. M. Tan, and K.-L. Chua. 2002. Development of an ELISA using a recombinant 41 kDa partial protein (P45N′) for the detection of Riemerella anatipestifer infections in ducks. Vet. Microbiol. 88:339–349.

Hung, A. L., and A. Alvarado. 2001. Phenotypic and molecular characterization of isolates of Ornithobacterium rhinotracheale from Peru. Avian Dis. 45:999–1005.

Köhler, B., R. Heiss, and K. Albrecht. 1995. Riemerella anatipestifer as pathogen for geese in the northern and central parts of Germany. *In:* Proceeding of the 49th Symposium on Poultry Diseases of the German Veterinary Medicine Society, Hannover. German Veterinary Medical Society Publisher. Giessen, Germany. 50–71.

Leroy-Sétrin, S., G. Flaujac, K. Thenaisy, and E. Chaslus-Dancla. 1998. Genetic diversity of Ornithobacterium rhinotracheale strains isolated from poultry in France. Lett. Appl. Microbiol. 26:189–193.

Lobbedey, L., and B. Schlatterer. 2003. Development and application of an ELISA for the detection of duck antibodies against Riemerella anatipestifer antigens in egg yolk of vaccinees and in serum of their offspring. J. Vet. Med. B Infect. Dis. Vet. Public Health 50:81–85.

Lopes, V., A. Back, H. J. Shin, D. Halvorson, and K. V. Nagaraja. 2002. Development, characterization and preliminary evaluation of a temperature-sensitive mutant of Ornithobacterium rhinotracheale for potential use as a live vaccine in turkeys. Avian Dis. 46:162–168.

Malik, Y. S., K. Olsen, K. Kumar, and S. M. Goyal. 2003. In vitro antibiotic resistance profiles of Ornithobacterium rhinotracheale strains from Minnesota turkeys during 1996–2002. Avian Dis. 47:588–593.

Mannheim, W. 1984. Family III: Pasteurellaceae Pohl 1981a, 382^{VP}. *In:* N. R. Krieg and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore MD, 1:550–557.

Marshall, J. D., P. A. Hansen, and W. C. Eveland. 1961. Histobacteriology of the genus Pasteurella: 1. Pasteurella anatipestifer. Cornell Vet. 51:24–34.

Pathanasophon, P., T. Tanticharoenyos, and T. Sawada. 1994. Physiological characteristics, antimicrobial susceptibility and serotypes of Pasteurella anatipestifer isolated from ducks in Thailand. Vet. Microbiol. 39:179–185.

Pathanasophon, P., P. Phuektes, T. Tanticharoenyos, W. Narongsak, and T. Sawada. 2002. A potential new serotype of Riemerella anatipestifer isolated from ducks in Thailand. Avian Pathol. 31:267–270.

Piechulla, K., S. Pohl, and W. Mannheim. 1986. Phenotypic and genetic relationships of so-called Moraxella (Pasteurella) anatipestifer to the Flavobacterium/Cytophaga group. Vet. Microbiol. 11:261–270.

Riemer. 1904. Kurze Mitteilung über eine bei Gänsen beobachtete exsudative Septikämie und deren Erreger. Zbl. Bakt. I. Abt. 37:641–648.

Rimler, R. B., and G. E. Nordholm. 1998. DNA fingerprinting of Riemerella anatipestifer. Avian Dis. 42:101–105.

Rossau, R., A. Van Landschoot, M. Gillis, and J. De Ley. 1991. Taxonomy of Moraxellaceae fam. nov., a new bacterial family to accomodate the genera Moraxella, Acinetobacter, and Psychrobacter and related organisms. Int. J. Syst. Bacteriol. 41:310–319.

Ryll, M., K. H. Hinz, K. P. Behr, W. Kruse, U. Löhren, and M. Südbeck. 1997. Zu Pathogenität und Verbreitung der Ornithobacterium rhinotracheale-Infektion bei der Pute. *In:* Proceedings of the 52nd Meeting of the Fachgruppe "Geflügelkrankheiten" der Deutsche Veterinärmedizinische Gesellschaft, Hannover. German Veterinary Medical Society Publisher. Giessen, Germany. 39–60.

Ryll, M., H. Christensen, M. Bisgaard, J. P. Christensen, K. H. Hinz, and B. Köhler. 2001. Studies on the prevalence of Riemerella anatipestifer in the upper respiratory tract of clinically healthy ducklings and characterization of untypable strains. J. Vet. Med. B Infect. Dis. Vet. Public Health 48:537–546.

Ryll, M., R. Günther, H. M. Hafez, and K. H. Hinz. 2002. Isolierung und Differenzierung eines Cytochromoxidase-negativen Ornithobacterium-rhinotracheale-Stamms aus Puten. Berliner und Münchener Tierärztlichen Wochenschrift 115:274–277.

Sandhu, T. S. 1991. Immunogenicity and safty of live Pasteurella anatipestifer vaccine in Pekin ducklings: Laboratory and field trials. Avian Pathol. 20:423–432.

Segers, P., W. Mannheim, M. Vancanneyt, K. De Brandt, K.-H. Hinz, K. Kersters, and P. Vandamme. 1993. Description of Riemerella anatipestifer gen. nov., comb. nov., the causative agent of septicaemia anserum exsudativa and its phylogenetic affiliation within the Flavobacterium-Cytophaga rRNA homology group. Int. J. Syst. Bacteriol. 43:768–776.

Soriano, V. E., N. A. Vera, C. R. Salado, R. P. Fernandez, and P. J. Blackall. 2003. In vitro susceptibility of Ornithobacterium rhinotracheale to several antimicrobial drugs. Avian Dis. 47:476–480.

Sprenger, J., A. Back, P. Shaw, K. Nagaraja, D. Roepke, and D. Halvorson. 1998. Ornithobacterium rhinotracheale infection in turkeys: Experimental reproduction of the disease. Avian Dis. 42:154–161.

Subramaniam, S., K.-L. Chua, H.-M. Tan, H. Loh, P. Kuhnert, and J. Frey. 1997. Phylogenetic position of Riemerella anatipestifer based on 16S rRNA gene sequences. Int. J. Syst. Bacteriol. 47:562–565.

Vancanneyt, M., P. Vandamme, P. Segers, U. Torck, R. Coopman, K. Kersters, and K.-H. Hinz. 1999. Riemerella columbina sp. nov., a bacterium associated with respiratory disease in pigeons. Int. J. Syst. Bacteriol. 49:289–295.

Vandamme, P., P. Segers, M. Vancanneyt, K. Van Hove, R. Mutters, J. Hommez, F. Dewhirst, B. Paster, K. Kersters, E. Falsen, L. Devrieze, K.-H. Hinz, and W. Mannheim.

1994. Description of Ornithobacterium rhinotracheale gen. nov., sp. nov. isolated from the avian respiratory tract. Int. J. Syst. Bacteriol. 44:24–37.

Vandamme, P., B. Pot, M. Gillis, P. De Vos, K. Kersters, and J. Swings. 1996. Polyphasic taxonomy, a consensus approach to bacterial classification. Microbiol. Rev. 60:407–438.

Vandamme, P., M. Vancanneyt, P. Segers, M. Ryll, B. Köhler, W. Ludwig, and K. H. Hinz. 1999. Coenonia anatina gen. nov., sp. nov., a novel bacterium associated with respiratory disease in ducks and geese. Int. J. Syst. Bacteriol. 49:867–874.

Van Empel, P., H. van den Bosch, D. Goovaerts, and P. Storm. 1996. Experimental infection in turkeys and chickens with Ornithobacterium rhinotracheale. Avian Dis. 40:858–864.

Van Empel, P., H. van den Bosch, P. Loeffen, and P. Storm. 1997. Identification and serotyping of Ornithobacterium rhinotracheale. J. Clin. Microbiol 35:418–421.

Van Empel, P. 1998a. Ornithobacterium rhinotracheale [thesis]. University of Utrecht. Utrecht, The Netherlands.

Van Empel, P., and H. van den Bosch. 1998b. Vaccination of chickens against Ornithobacterium rhinotracheale infection. Avian Dis. 42:572–578.

Van Empel, P., and H. M. Hafez. 1999a. Ornithobacterium rhinotracheale: A review. Avian Pathol. 28:217–227.

Van Empel, P., M. Vrijenhoek, D. Goovaerts, and H. van den Bosch. 1999b. Immuno-histochemical and serological investigation of experimental Ornithobacterium rhinotracheale infection in chickens. Avian Pathol. 28:187–193.

Weng, S., W. Lin, Y. Chang, and C. Chang. 1999. Identification of a virulence-associated protein homolog gene and ISRa1 in a plasmid of Riemerella anatipestifer. FEMS Microbiol. Lett. 179:11–19.

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CHAPTER 6.15

The Genus *Capnocytophaga*

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Introduction/Habitat

Bacteria included in the genus *Capnocytophaga* typically are aerotolerant anaerobes with a requirement for significant quantities of carbon dioxide (CO_2) for initiation of growth—aerobic as well as anaerobic—and with the ability to form thin, spreading colonies (i.e., exhibit surface translocation) on solid media. They are common inhabitants of dental plaque in the oral cavities of mammals, from which they can be isolated in instances of periodontal diseases as well as from healthy individuals (Paster et al., 2001); they are less frequently isolated from lesions resulting from dog and cat bites and from inadvertant oral microbial contamination of open wounds (e.g., Chadha and Brady, 1999; Alexandrakis et al., 2000; Phibbs et al., 2002).

Seven species of the genus are presently recognized (*C. ochracea*, *gingivalis*, *sputigena*, *canimorsus*, *cynodegmi*, *hemolytica* and *granulosa*) on the basis of several polyphasic analyses including phenotypic trait analysis, 16S rRNA analysis, DNA-DNA hybridization, fatty acyl composition of cell lipids and cellular protein profiles (Williams and Hammond, 1979a; Vandamme et al., 1996).

Shortly after the initial characterization of isolates and grouping into three species (*C. ochracea*—the type strain, *C. gingivalis* and *C. sputigena*; Leadbetter et al., 1979), it was noted that strains otherwise uncategorized (known as "dysgonic fermenters," e.g., group DF-1 of the United States Centers for Disease Control and Prevention [CDC]) should properly be considered *Capnocytophaga* (Williams et al., 1979b).

Several DF-2 and DF-2-like isolates came to be considered identical to strains isolated from the mouths of dogs and were termed "*C. canimorsus*" and "*C. cynodegmi*" (Brenner et al. 1989), even though these latter species (unlike the type strain and other species) possessed catalase and oxidase activities. The properties of the Gram-negative fusiform cells in CDC group DF-3 resemble but are not identical to those of DF-1 and DF-2; these strains are not presently considered capnocytophagas (Vandamme et al., 1996).

Human oral cavity isolates labeled originally *Sphaerocytophaga* (Graf, 1961; Graf, 1962) and *Bacteroides oralis* var. *elongatus* (Loesche et al., 1964) were so regarded probably because their traits were incompletely described, unrecognized, or of mistaken significance; such strains are now regarded as *Capnocytophaga*. Their DNA G+C content is 36–44 mol%.

As revealed by phylogenetic analyses of 16S rRNA, the seven species are quite closely related (see Bernadet et al., 2002) and constitute a cohesive subgroup of the proposed family Flavobacteriaceae.

As originally noted (Holt et al., 1979b), *Capnocytophaga* cells are judged Gram-negative both from actual staining results and from transmission electron microscopic analysis of the cell envelope. Surface appendages such as flagella or pili have not been observed, nor have significant intracellular inclusions regularly been reported.

The slender rods (ca. 0.6 μm × 4–7 μm) appear to have smooth, blunt, rounded poles when unstained preparations are viewed by phase-contrast optics, but cells stained for light microscopic examination appear with pointed ends and irregular surface "blebs" apparently resulting from cell distortion during the drying and staining procedures (Holt et al., 1979b).

Physiological, Biochemical and Chemotaxonomic Properties

As noted, these bacteria are able to initiate colonial growth in air, or in its absence, provided elevated concentrations (5–10%, v/v) of CO_2 are available. Samples streaked from dental plaques form thin "lens-paper" gray colonial growth (best observed with oblique illumination) that spreads beyond the streak-lines as a result of gliding motility. Such growth is usually seen best on trypticase soy, brain heart infusion, or Todd-Hewitt agar supplemented with sheep or other blood (Leadbetter et al., 1979; Vandamme et al.,

1996). Upon transfer of such growth, more luxuriant growth occurs, and growth on the streaklines appears as gray, pink or iridescent, but cell masses removed by scraping are yellow. The different colonial hues are considered to result from the arrangement of cells of different sizes and this arrangement is characteristic for each of the species (Holt et al., 1979a). Cell masses collected from liquid cultures are likewise yellow; the pigment is considered to be of flexirubin-type although detailed chemical analysis of the capnocytophaga pigment has not been reported, and not all species have been shown to become red or pink when treated with 10% (w/v) KOH; this latter color change is considered indicative of flexirubins.

The growth requirement for CO_2, added as bicarbonate to liquid media, is not readily apparent when inocula are large masses of cells from colonies or from another liquid culture. This seemingly occurs because the inoculated population is sufficiently large and metabolically active to generate enough CO_2 to enable growth.

Acetate and succinate are the other major metabolic end products. The relationship between the need for larger-than-normal amounts of CO_2 and a role for CO_2 in a truncated propionic-type fermentation (accumulation of succinate) was at first inferred (Leadbetter et al., 1979) and later experimentally established (Calmes et al., 1980). In addition, the decarboxylation of pyruvate was experimentally established to account for acetate and CO_2 production (Kapke et al., 1980).

Although it is generally regarded that capnocytophagal growth in the presence of air is a result of fermentation rather than respiration, a single persuasive study (Spratt et al., 1996) demonstrates that the cell yield of *C. gingivalis* growth with glucose is comparable to that of other aerobic heterotrophs growing via respiration. Given this result, and the probable reduction of fumarate in accounting for succinate formation, it may be more appropriate to consider *Capnocytophaga* physiology not as fermentative but rather respiratory (either aerobic or anaerobic). The presence of benzidine-reactive components (cytochromes?) along with the assumed activities of respiratory quinone (primarily menaquinone-6) seems consistent with such an argument for respiration.

The capnocytophagas were first noted to contain unusually (at that time) large amounts of odd-number, *iso* and *anteiso*-branched, hydroxylated fatty acyl moieties. These have been shown to be characteristic components of the novel sulfonolipids ("capnoids: capnine and *N*-acyl capnine") that constitute as much as 20% of the total cell lipid (Godchaux and Leadbetter, 1980). Lipid of this type and quantity is a common feature of many cytophaga and flavobacters and may be a useful chemotaxonomic marker for certain flavobacterial subgroups although little attention has been given this possibility. In *Flavobacterium johnsoniae* the dominant *N*-acyl capnine (Godchaux and Leadbetter, 1984) is located in the cells' outer membrane (Godchaux and Leadbetter, 1988), where it may function in interacting with the solid substrata over which cells glide. The presence of *N*-acyl capnine has been shown to be a molecular correlate of gliding motility in this bacterium (Abbanat et al., 1986). The capnoids are largely localized in the cell envelope of *C. gingivalis*, where a similar role in gliding motility was postulated (Godchaux and Leadbetter, 1980).

The capnocytophagas, like so many other surface-translocating ("gliding") bacteria (Dworkin, 1969), are susceptible to growth inhibition by the antibiotic actinomycin D; this susceptibility is not common in nongliding Gram-negative bacteria.

The growth-inhibiting ability of an array of other antibiotics has been examined repeatedly (see Holt and Kinder [1989] and Roscoe et al. [1992]), reflecting the significance of capnocytophagas in various infections in the oral cavity, certain septicemal infections, and a few other uncommon disease states (Paster et al., 2002).

β-Lactam antibiotics have been considered efficacious therapeutic agents, but the emergence of novel β-lactamases frequently has rendered these drugs ineffective. Employing β-lactamase inhibitors in conjunction with β-lactam antibiotics and others (such as clindamycin, imipenem or ciprofloxin) is now considered desirable therapy.

Isolation and Maintenance

Isolation from mammalian oral cavities and infection sites (Rolph et al., 2001) such as the eye, abscesses, and bloodstream is best achieved by streak-plating samples on a medium such as trypticase soy-sheep blood agar, incubating in an anaerobic environment with an elevated CO_2 content (a classic candle-jar, for example) at 35–37°C for 3–5 days and then visually examining the agar surface for very thin (not typical, elevated) colonies that spread beyond the streakline where cells had been deposited. Such growth often is not apparent unless oblique illumination is employed. Not all blood agar media support colony spreading and instead only quite small (1 mm) colonies develop (see Holt and Kinder [1989] and Leadbetter et al. [1979]); the factors affecting colony spread have not been studied extensively, although addition of blood to media is not essential.

Literature Cited

Abbanat, D. R., E. R. Leadbetter, W. Godchaux 3rd, and A. Escher. 1986. Sulphonolipids are molecular determinants of gliding motility. Nature 324:367–369.

Alexandrakis, G., L. A. Palma, D. Miller, and E. C. Alfonso. 2000. Capnocytophaga keratitis. Am. J. Opthamol. 107:1503–1506.

Bernadet, J.-F., Y. Nakagawa, and B. Holmes. 2002. Proposed minimal standards for describing new taxa of the family Flavobacteriaceae and emended description of the family. Int. J. Syst. Evol. Microbiol. 52:1049–1070.

Brenner, D. J., D. G. Hollis, G. R. Fanning, and R. E. Weaver. 1989. Capnocytophaga canimorsus sp.nov. (formerly CDC group DF-2), a cause of septicemia following dog bite, and C. cynodegmi sp. nov., a cause of localized wound infection following dog bite. J. Clin. Microbiol. 27:231–235.

Calmes, R., G. W. Rambicure, W. Gorman, and T. T. Lillich. 1980. Energy metabolism in Capnocytophaga ochracea. Infect. Immun. 29:551–560.

Chadha, V., and B. A. Warady. 1999. Capnocytophaga canimorsus peritonitis an a pediatric peritoneal dialysis patient. Pediatr. Nephrol. 13:646–648.

Godchaux 3rd, W., and E. R. Leadbetter. 1980. Capnocytophaga spp. contain sulfonolipids that are novel in prokaryotes. J. Bacteriol. 144:592–602.

Godchaux 3rd, W., and E. R. Leadbetter. 1984. Sulfonolipids of gliding bacteria: Structures of the N-acylaminosulfonates. J. Biol. Chem. 259:2982–2990.

Godchaux 3rd, W., and E. R. Leadbetter. 1988. Sulfonolipids are localized in the outer membrane of the gliding bacterium Cytophaga johnsonae. Arch. Microbiol. 150:42–47.

Graf, W. 1961. Anaerobe Myxobakterien, neue Mikroben in der menschlichen Mundhole. Arch. Hyg. Bakteriol. 145:405–459.

Graf, W. 1962. Die Pathogenitat anaerober Myxobakterien (Sphaerocytophaga) im Tieversuch. Arch. Hyg. Bakteriol. 146:492–500.

Holt, S. C., G. Forcier, and B. J. Takacs. 1979a. Fatty acid composition of gliding bacteria: Oral isolates of Capnocytophaga compared with Sporocytophaga. Infect. Immun. 26:298–304.

Holt, S. C., E. R. Leadbetter, and S. S. Socransky. 1979b. Capnocytophaga: New genus of Gram-negative gliding bacteria. II: Morphology and ultrastructure. Arch. Microbiol. 122:17–27.

Holt, S. C. and S. A. Kinder. 1989. Genus Capnocytophaga Leadbetter, Holt, and Socransky 1982. *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 3:2050–2057.

Kapke, P. A., A. T. Brown, and T. T. Lillich. 1980. Carbon dioxide metabolism by Capnocytophaga ochracea: Identification, characterization, and regulation of phosphoenolpyruvate carboxykinase. Infect. Immun. 27:756–766.

Leadbetter, E. R., S. C. Holt, and S. S. Socransky. 1979. Capnocytophaga: New genus of Gram-negative gliding bacteria. I: General characteristics, taxonomic considerations and significance. Arch. Microbiol. 122:9–16.

Loesche, W. J., S. S. Socransky, and R. J. Gibbons. 1964. Bacteroides oralis, proposed new species isolated from the oral cavity of man. J. Bacteriol. 88:1323–1327.

Newman, M. G., V. L. Sutter, M. J. Pickett, U. Blachman, J. R. Greenwood, V. Grinenko, and D. Citron. 1979. Detection, identification, and comparison of Capnocytophaga, Bacteroides ochraceus, and DF-1. J. Clin. Microbiol. 10:557–562.

Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabude, and F. E. Dewhirst. 2001. Bacterial diversity in human subgingival plaque. J. Bacteriol. 183:3770–3788.

Paster, B. J., W. A. Falkler Jr., C. O. Enwonwu, E. O. Indighe, K. O. Savage, V. A. Levanos, M. A. Tamer, R. L. Ericson, C. N. Lau, and F. E. Dewhirst. 2002. Prevalent bacterial species and novel phylotypes in advanced noma lesions. J. Clin. Microbiol. 40:2213–2216.

Phibbs, S. E., D. M. Tamblyn, and P. R. Badenoch. 2002. Capnocytophaga canimorsus endophthalmitis following cataract surgery. Clin. Exp. Opthamol. 30:375–377.

Rolph, H. J., A. Lennon, M. P. Riggio, W. P. Saunders, D. MacKenzie, L. Coldero, and J. Bagg. 2001. Molecular identification of microorganisms from endodontic infections. J. Clin. Microbiol. 39:3282–3289.

Roscoe, D. L., S. J. Demcov, D. Thornber, R. Wise, and A. M. Clark. 1992. Antimicrobial susceptibilities and beta lactamase characterization of Capnocytophaga species. Antimicrob. Agents Chemother. 36:2197–2200.

Socransky, S. S., S. C. Holt, E. R. Leadbetter, A. C. R. Tanner, E. D. Savitt, and B. F. Hammond. 1979. Capnocytophaga: New genus of Gram-negative gliding bacteria. III: Physiological characterization. Arch. Microbiol. 122:29–33.

Speck, H., R. M. Kroppenstedt, and W. Mannheim. 1987. Genomic Relationships and Species Differentiation in the Genus Capnocytophaga. Zbl. Bakteriol. Hyg. A266:390–402.

Spratt, D. A., J. Greenman, and A.G. Schaffer. 1996. Capnocytophaga gingivalis: Effects of glucose concentration on growth and hydrolytic enzyme production. Microbiology 142:2161–2164.

Vandamme, P., M. Vancanneyt, A. van Belkum, P. Segers, W. G. V. Quint, K. Kersters, B. J. Paster, and F. E. Dewhirst. 1996. Polyphasic analysis of strains of the genus Capnocytophaga and Center for Disease Control Group DF-3. Int. J. Syst. Bacteriol. 46:782–791.

Williams, B. L., and B. F. Hammond. 1979a. Capnocytophaga: New genus of Gram-negative gliding bacteria. IV: DNA base composition and sequence homology. Arch. Microbiol. 122:35–39.

Williams, B. L., D. Hollis, and L. V. Holdeman. 1979b. Synonymy of strains of Center for Disease Control Group DF-1 with species of Capnocytophaga. J. Clin. Microbiol. 10:550–556.

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CHAPTER 6.16

The Genera *Rhodothermus*, *Thermonema*, *Hymenobacter* and *Salinibacter*

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Introduction

The genera *Rhodothermus*, *Thermonema*, *Hymenobacter*, and *Salinibacter* all consist of Gram-negative, red- or yellow-pigmented obligatory aerobic chemoheterotrophic bacteria. The first two genera are thermophilic, with temperature optima around 60–65°C; *Hymenobacter* and related strains described in the past as "Taxeobacter" species are oligotrophic soil bacteria, some of them isolated from cold environments in the Antarctic. Finally, *Salinibacter* is a genus of extremely halophilic red bacteria that are among the most salt-tolerant and salt-requiring strains within the bacterial domain. Phylogenetically they all form deep branches affiliated with the *Cytophaga-Flavobacterium-Bacteroides* branch of the Bacteria (Fig. 1 shows *Thermonema* and *Rhodothermus*, Figs. 2 and 3 show *Hymenobacter* and *Salinibacter*).

While relatively little is known about the properties of *Thermonema* and *Hymenobacter*, extensive research has been devoted to *Rhodothermus* in view of its production of thermotolerant cellulases, xylanases, and other biotechnologically potentially valuable thermophilic enzymes. The extremely halophilic *Salinibacter* was only recently discovered, but it has become evident that *Salinibacter* makes a significant contribution to the biota of NaCl-saturated saltern crystallizer ponds. The species is of considerable interest in view of its mode of osmotic adaptation, which differs from that of the other aerobic halophiles within the domain Bacteria. Unexpectedly, it shares many properties with the extremely halophilic representatives of the archaeal order Halobacteriales.

The Genus *Rhodothermus*

Rhodothermus marinus, the type species of the genus *Rhodothermus* and presently the only recognized species of the genus, was first isolated from shallow-water submarine hot springs in Iceland (Alfredsson et al., 1988; Kristjánsson and Alfredsson, 1992). It is a thermophile that grows optimally at 65°C and tolerates up to 77°C for growth. Similar isolates have since been obtained from a variety of thermal environments on the Azores (Nunes et al., 1992a) and in Japan (Sako et al., 1996). Since it was discovered that *Rhodothermus* strains possess thermophilic cellulase and xylanase activities, as well as other thermophilic enzymatic activities of potential biotechnological interest, much study has been devoted to the genus.

Taxonomy and Phylogeny

The type strain of *Rhodothermus marinus* (Alfredsson et al., 1988) is DSM 4252, ATCC 43812 (16S rDNA accession number X80994). The G+C content of its DNA is 64.4–64.7 mol%.

A second species, *R. obamensis*, was proposed by Sako et al. (1996) to accommodate a new isolate from a shallow marine hydrothermal environment in Tachibana Bay, Nagasaki Prefecture, Japan. The proposed type strain (OKD7 = JCM 9785) was claimed to differ sufficiently from *R. marinus* to warrant its description as a new species. The G+C content of its DNA was higher (66.6 mol%), and its 16S rDNA sequence was claimed to be only 95% similar to that of *R. marinus*. In addition, many additional differences were described, including differences in optimum temperature, cell size, and substrate utilization pattern (Table 1).

Silva et al. (2000) performed a critical comparative study of the type strains of *R. marinus* and *R. obamensis*. The higher salt tolerance of *R. marinus* as compared to *R. obamensis* was confirmed. However, no significant differences in optimum growth temperature were detected. Moreover, a great degree of similarity in the DNAs was found (DNA-DNA hybridization 78%), and renewed sequencing of the 16S rDNA

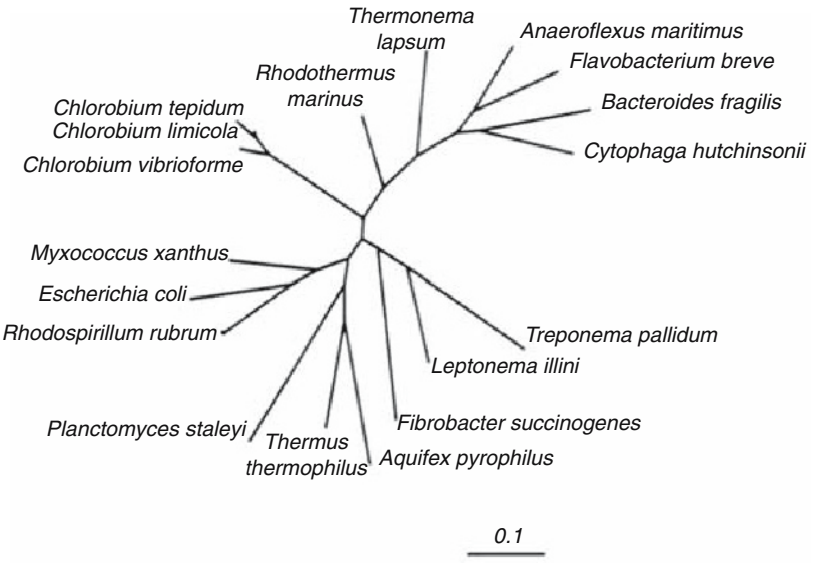

Fig. 1. Maximum-likelihood analysis of selected 16S rRNA sequences, showing the phylogenetic position of the genera *Rhodothermus* and *Thermonema* as deep branches related to the *Cytophaga-Flavobacterium-Bacteroides* branch of the domain Bacteria. The bar indicates the expected number of nucleotide substitutions per site. From Andrésson and Friðjónsson (1994); reproduced with permission.

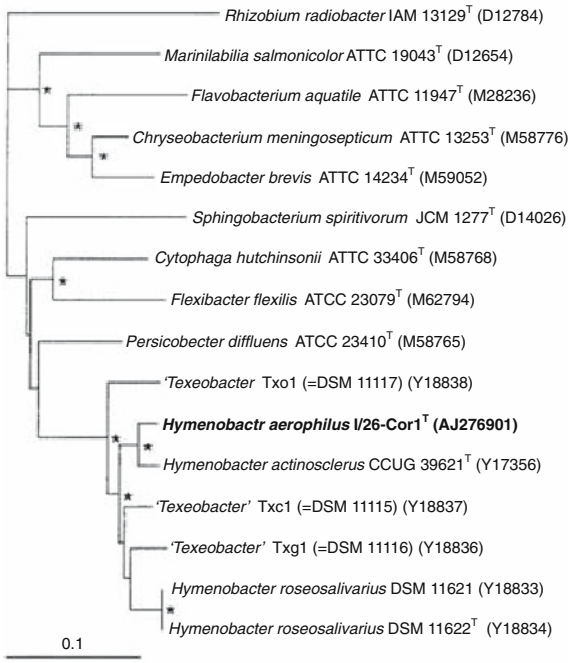

Fig. 2. Dendrogram indicating the estimated phylogenetic relationship among *Hymenobacter*, "Taxeobacter," and other members of the *Cytophaga-Flavobacterium-Bacteroides* group. The reconstruction is based on comparison of 16S rDNA sequences. Branching points that displayed significant bootstrap values (>75%) are indicated with asterisks. *Rhizobium radiobacter* was included as an outgroup. From Buczolits et al. (2002); reproduced with permission.

showed a much higher similarity (99.5%) than the value of 95% previously reported by Sako et al. (1996). The fatty acid profiles were also highly similar. In view of these findings, *R. obamensis* Sako et al. 1996 should be considered a junior heterotypic synonym of *Rhodothermus marinus* Alfredsson et al. 1995.

Table 1. Comparison of *Rhodothermus marinus* and "*R. obamensis*."

	R. marinus	*R. obamensis*
Cell length (μm)	2–2.5	4–10
Optimum temperature (°C)	65	80
Generation time (min)	80	90
Growth in 6% NaCl	+	–
Growth on		
Xylose	+	–
Sorbitol	–	+
Casein	–	+
Casamino acids	–	+
Glutamine	+	–
Serine	–	+
Proline	–	+
DNA G+C content DNA (mol%)	65	66.6

Symbols: +, growth; and –, no growth.
Data from Sako et al. (1996).

Ten strains very similar to the type strain of *R. marinus* were isolated from São Miguel, Azores (Nunes et al., 1992a). These isolates, together with 20 strains from shallow marine hot springs in Iceland and additional isolates obtained from Stufe di Nerone, Naples, Italy, were subjected to a comparative genomic study. Whole genome DNA-DNA hybridization confirmed that all strains investigated may be classified in a single species (77–89% similarity). Pulsed field gel electrophoresis using restriction enzymes *Ase*I and *Pme*I generated distinct electrophoretic patterns for most strains. Genome sizes of 3.3–3.6 MBp were calculated from the electrophoresis patterns. Ribotyping (restriction fragment length polymorphism of the rRNA genes) with restric-

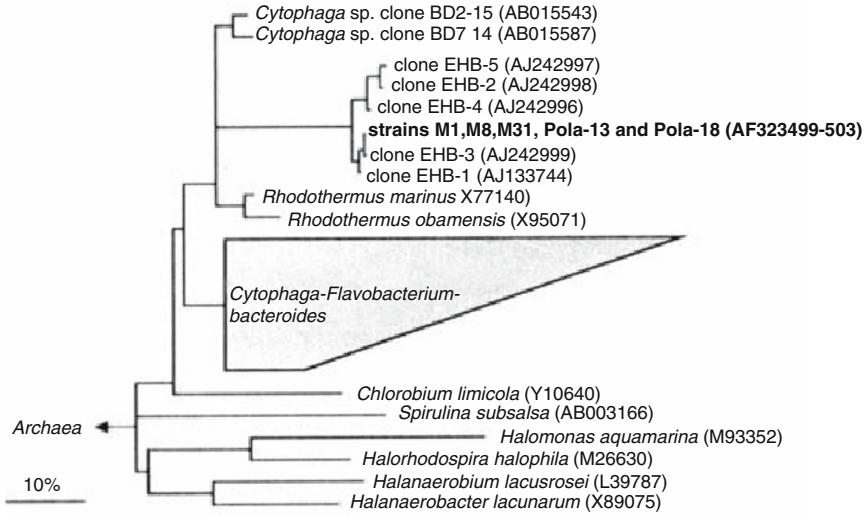

Fig. 3. Phylogenetic reconstruction based on 16S rDNA sequences from the strains and clones affiliated to *Salinibacter*. The tree is based on the results of a parsimony analysis including only complete or almost complete 16S rDNA sequences of all sequences of the *Cytophaga-Flavobacterium-Bacteroides* phylum available (over 400) and representatives of the domain Bacteria. The topology of the tree was corrected according to the results of distance matrix, maximum parsimony, and maximum likelihood analyses of various datasets. Multifurcations indicate where a topology could not be resolved unambiguously. The bar indicates 10% of estimated sequence divergence. From Antón et al. (2002); reproduced with permission.

tion enzymes *Eco*RI, *Pst*I, *Bam*HI, and *Hind*III produced several distinct ribotypes (Moreira et al., 1996).

The sequence of the single 16S rRNA gene of *R. marinus* reveals a distant relationship to the *Flexibacter-Cytophaga-Bacteroides* group (Andrésson and Friðjónsson, 1994; Fig. 1). The genome contains a single rRNA operon with an intergenic region coding for $tRNA^{Ile}$ and $tRNA^{Ala}$ between the 16S and 23S (Andrésson and Friðjónsson, 1994).

Morphological and Cultural Characteristics

Rhodothermus marinus is a Gram-negative rod-shaped bacterium (Fig. 4). Cells are generally 2–3 × 0.5 μm in size. When grown on carbohydrate-rich media, slime capsules may be formed around the cells (Alfredsson et al., 1988). Motility is rarely observed in liquid cultures, but the presence of a single polar flagellum was reported in strains isolated from São Miguel, Azores (Nunes et al., 1992a).

Rhodothermus marinus is a thermophile. The type strain grows up to 77°C, with an optimum at 65°C. No growth was observed below 54°C (Alfredsson et al., 1988). A claim for a higher temperature optimum for the strain originally designated "*R. obamensis*" (optimum 80°C, maximum 85°C; Sako et al., 1996) was not confirmed in a controlled comparative study (Silva et al., 2000). Under optimal growth conditions, growth rates of about 0.5 h^{-1} are reached (Alfredsson et al., 1988).

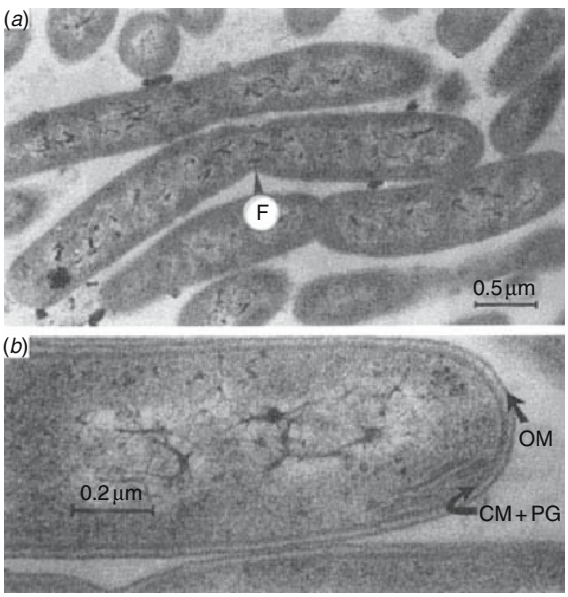

Fig. 4. Electron micrographs of *Rhodothermus marinus* strain R-18. (a) Thin longitudinal and cross-sections showing the width and length of typical cells as well as dividing cells with furrowing (F). (b) The cell envelope, consisting of a cytoplasmic membrane plus peptidoglycan layer (CM + PG) and a well-separated outer membrane (OM). From Alfredsson et al. (1988); reproduced with permission.

Rhodothermus strains require salt for growth. Optimal growth is achieved at 1–3% NaCl. Some strains, including the type strain, can grow above 6% NaCl (Alfredsson et al., 1988), while the isolate formerly designated "*R. obamensis*" toler-

ates up to 5% only (Sako et al., 1996; Silva et al., 2000). Growth is optimal in the neutral pH range.

Rhodothermus is an obligatory aerobic heterotroph that does not reduce nitrate. Substrates used for growth include common sugars, like glucose, mannose, galactose, sucrose, lactose, maltose, raffinose, cellobiose, sorbitol, mannitol and starch, and some carboxylic acids (acetate, lactate and pyruvate). Cellulose, xylans, and other complex polysaccharides can be broken down as well (see the section on biotechnological Applications in this Chapter). Proteins (casein and gelatin) and protein hydrolysates (casein, peptone, and casamino acids) can also serve as carbon and nitrogen sources, as can certain amino acids. Glutamate and aspartate are used by most strains examined, and some strains use other amino acids as well (Alfredsson et al., 1988; Nunes et al., 1992a; Sako et al., 1996).

Physiological, Biochemical and Chemotaxonomic Properties

With an optimum growth temperature of around 65°C and the ability to grow up to about 80°C, *Rhodothermus* is a true thermophile. When "*R. obamensis*" was exposed to supraoptimal temperatures (90–95°C), stress proteins were induced that improved survival. Putative heat shock proteins and high temperature growth-specific proteins have been detected. The putative heat shock proteins were similar to the ATP binding subunits of ATP-binding cassette (ABC) transporters, and the high temperature growth-specific proteins corresponded to domains II and III of elongation factor Tu (Takai et al., 1998a).

The thermal properties of many enzymes of *Rhodothermus* have been characterized, also in view of possible biotechnological applications. Tables 2 and 3 summarize some of these data.

The ability of *Rhodothermus* to produce thermophilic xylanases, cellulases, and other enzymes that hydrolyze complex polysaccharides has become the subject of in-depth studies because of their biotechnological potential. Table 3 summarizes some of the properties of the enzymes involved in polysaccharide metabolism, and further details are given below.

Rhodothermus marinus grows on xylan as carbon and energy source with a high rate (growth rate up to 0.43 h^{-1}). Under these conditions at least two thermostable xylanolytic enzymes are secreted into the medium, an endo-1,4-β-xylanase and a xylan 1,4-β-xylosidase (Dahlberg et al., 1993; Manelius et al., 1994). A multidomain endo-β-1,4-xylanase encoded by the gene *xyn1* was cloned and expressed in *Escherichia coli*. This protein contains five domains. Two carbohydrate-binding domains (CBDs) are repeated in tandem at the N-terminus. The *xyn1* gene is the first example encoding a CBD family IV in combination with a xylan hydrolyzing catalytic domain of the glycosyl hydrolase family 10. The enzyme binds specifically to xylan, to β-glucan, and to amorphous, but not to crystalline, cellulose (Nordberg Karlsson et al., 1996; Nordberg Karlsson et al., 1997; Nordberg Karlsson et al., 1998a). A truncated enzyme (411 amino acids + a 22-amino acid leader peptide) contains the catalytic domain only. The products of xylose degradation are mainly xylobiose and xylotetrose. The enzyme is optimally active at 80°C and pH 7.5. Under these conditions, its half-life is 100 min, but it can be further stabilized by 1 mM Ca^{2+}. The enzyme has no exo- or endo-cellulase activity (Nordberg Karlsson et al., 1998b). An efficient production protocol of the truncated *Rhodothermus* xylanase in *E. coli* has been designed, using fed-batch cultures (Nordberg Karlsson et al., 1999; Ramchuran et al., 2002). Optimization of the growth conditions enabled a yield of 35% of the intracellular protein being recombinant xylanase (Nordberg Karlsson et al., 1999). The two N-terminal carbohydrate-binding domains (CBM4-1 and CBM4-2) of the native xylanase have also been investigated in further depth, and they have been cloned and produced in *E. coli*. They bind to insoluble xylan and to phosphoric-acid-swollen cellulose, but not to Avicel or to crystalline cellulose (Abou Hachem et al., 2000). Binding of two ions of calcium to CBM4-2 increases its unfolding temperature by approximately 23°C (Abou Hachem et al., 2002). CBM4-2 has a β-sandwich structure formed by 11 strands and contains a prominent cleft which is the binding site for xylan (Simpson et al., 2002). High quality nuclear magnetic resonance (NMR) spectra of the domain now exist, enabling the assignment of virtually all ^{1}H, ^{13}C and ^{15}N resonances (Jamieson et al., 2002).

Rhodothermus marinus produces α-L-arabinofuranosidase when grown on yeast extract + birch wood xylan or some other polysaccharides and low-molecular-weight carbohydrates. This is the most thermostable arabinofuranosidase reported so far (Gomes et al., 2000).

Rhodothermus can also make a thermostable extracellular endocellulase. This endo-β-1,4-glucanase is active on carboxymethylcellulose but does not degrade Avicel, Sigmacell 10 cellulose, granular cellulose, *p*-nitrophenyl-β-D-cellobioside, or 4-methylumbelliferyl-β-D-cellobioside. Carboxymethylcellulose is split into glucose, cellobiose, cellotriose, and larger oligosaccharides (Hreggvidsson et al., 1996). A thermostable cellulase of glycosyl hydrolase family 12 from *R. marinus*, encoded by the gene *celA*, was cloned and overexpressed in *E. coli*. The recombinant enzyme showed activity on

Table 2. Thermal properties of selected enzymes of *Rhodothermus marinus* (including the strain formerly designated *R. obamensis*).

Enzyme	Thermal properties	Further details	References
Phosphoenol-pyruvate carboxylase	Optimum activity at 75°C; is rapidly inactivated at 100°C and gradually inactivated at 93°C; 50% of activity is left after 30 h at 80°C; the enzyme is stabilized at 93°C by the positive effector acetyl CoA and by the substrate phosphoenolpyruvate	The gene *ppc* coding for the enzyme was cloned and overexpressed in *Escherichia coli*; the native enzyme is a tetramer of 107.8-kDa subunits (each 937 amino acids); activity is optimal at pH 8.0	Takai et al., 1997a; Takai et al., 1997b; Takai et al., 1998b
Citrate synthase	Optimally active at 85°C; 20–30% of the optimal activity remains at 40°C; half life at 80°C, 2.9 and 28 min for the hexameric and the dimeric form, at 90°C, 18 and 12 min, respectively	There is a hexameric (280 kDa) and a dimeric (100 kDa) enzyme; the dimeric enzyme also has 2-methylcitrate synthase activity	Nordberg Karlsson et al., 2002
Ferredoxin	Highly stable; the midpoint for thermally induced unfolding is 102°C	A $[3Fe\text{-}4S]^{+1/0}$ ferredoxin with an apparent molecular mass of 9 kDa and an unusually low reduction potential of –650 mV; the molecule is very sensitive to oxygen; the function of this ferredoxin in *Rhodobacter* is yet unclear	Pereira et al., 2002
DNA polymerase I	Optimally active at 55°C; has a half life of 2 min at 90°C; four out of the 45 *Rhodothermus* strains tested had DNA Polymerase activity with >10% activity remaining after 30 min at 95°C	The 924-amino acid, 104.8-kDa protein has been cloned; it has DNA polymerization activity, 5′ → 3′ exonuclease activity, and 3′ → 5′ proofreading activity	Blöndal et al., 2000; Hjörleifsdóttir et al., 1997b
DNA ligase	None out of 9 strains tested had DNA ligase activity that was active after 30 min at 90°C. The purified enzyme was most active from 55 to 75°C. Its half life at 91°C was 7 min	The 712 amino acid, 79.5-kDa enzyme was cloned and overexpressed in *E. coli*; it has a higher DNA ligation efficiency than the enzyme from *Thermus scotoductus* and *Thermus thermophilus* at temperatures between 37 and 46°C	Hjörleifsdóttir et al., 1997b; Housby and Southem, 2002; Housby et al., 2000; Thorbjamardóttir et al., 1995
Type II thymidine kinase	Optimally active at 65°C; has a half life of 15 min at 90°C	The gene of this first thymidine kinase described from a thermophilic bacterium encodes a 213-amino acid, 23.6-kDa protein; the enzyme functions in the pyrimidine salvage pathway	Blöndal et al., 1999
Mannosylglycerate synthase	Optimally active at 85–90°C; upon incubation at 90°C, 50% of activity is lost in 30 min. At 65°C, the specific activity was fivefold lower than at the optimum temperature, and the half life for inactivation increased to 170 min.	The 46.1-kDa enzyme, encoded by the gene *mgs*, has been overexpressed in *E. coli*	Martins et al., 199

Table 3. Thermal properties of *Rhodothermus* enzymes involved in degradation of complex polysaccharides.

Enzyme	Thermal properties	Further details	References
Endo-1,4-β-xylanase	Half life 14h at 90°C and pH 7	None	Dahlberg et al., 1993
Xylan-1,4-β-xylosidase	Highest initial activity at 90°C; half life 45min at 90°C and pH 7, and 14h at 85°C	Apparent size 169kDa by gel filtration	Dahlberg et al., 1993; Manelius et al., 1994
Cellulase (extracellular endo-β-1,4-glucanase)	50% of activity remains after 3.5h at 100°C and 80% after 16h at 90°C	Apparent monomer of 49kDa; pH optimum 7	Hreggvidsson et al., 1996
Cellulase of glycosyl hydrolase family 12	Highest initial activity at 100°C; 75% of activity left after 8h at 90°C	A 260-amino acid, 28.8-kDa protein, encoded by the *celA* gene; pH optimum 6–7	Halldórsdóttir et al., 1988
Amylase	Optimally active at 85°C, pH 6.5–7; half life at 85°C 3h	None	Gomes et al., 2003
Pullulanase	Optimally active at 80°C, pH 6.5–7; half life at 85°C 30min	None	Gomes et al., 2003
β-Glucanase	Optimally active at 85°C, pH 7.0; fully active after 16h at 80°C, with a half life of 3h at 85°C	The mature enzyme (29.7kDa) is made with a 30-amino acid signal peptide	Spilliaert et al., 1994
Laminariase	Optimally active at 88°C and pH 5.5; stable for more than 2 days at 80°C	Encoded by the *lamR* gene	Krah et al., 1998 Petersen et al., 2000
Mannanase	Optimally active at 85°C and pH 5–6.5; half life of 45.3h at 85°C and 4.2h at 90°C	A 997-amino acid, 113-kDa protein	Gomes and Steiner, 1998; Politz et al., 2000
Branching enzyme (transglucosidase)	Optimally active at 65°C and pH 6–6.5; stable after 30 min at 80°C; 50% of activity is retained after 16 h at 80°C	The predicted protein of 621 amino acids, 72kDa, is probably an intracellular enzyme	Shinohara et al., 2001
α-Galactosidase	Optimally active at 85°C, with a broad pH range of 4–8; half life 2h at 75°C	The protein is a tetramer of 50-kDa subunits	Blücher et al., 2000
α-L-Arabinofuranosidase	Optimally active at 85°C and pH 5.5–7; half life 8.3 h at 85°C and 17 min at 90°C	None	Gomes et al., 2000

carboxymethylcellulose and lichenan, but not on birch xylan or laminarin (Halldórsdóttir et al., 1988). The catalytic module is preceded by a linker sequence and an N-terminal highly hydrophobic putative signal peptide. This signal peptide showed a marked cytotoxic activity to *E. coli*. Deletion of the part of the gene that encodes this signal peptide enabled highly increased production of recombinant cellulase, yielding a mutant protein with threefold higher specific activity than that of the full-length enzyme. The mutant protein (Cel12A) is highly thermostable, has a half life of over 2 h at 90°C, and can function to 103°C (Wicher et al., 2001). Production of this cellulase in *E. coli* was optimized in substrate limited fed-batch cultures of *E. coli* BL21(DE3) (Ramchuran et al., 2002). The structure of the protein has now been resolved to 1.8 Å (Crennell et al., 2002).

Rhodothermus produces a thermostable amylase and a pullulanase (a debranching enzyme that hydrolyzes α-1,6 linkages in dextrins; see Table 3). Growth in the presence of maltose (1.5 g/liter) + yeast extract (8.3 g/liter) resulted in optimal induction of these enzymes (Gomes et al., 2003). A thermostable β-glucanase encoded by the gene *bglA* was discovered in a gene library of *R. marinus* in *E. coli*. Two transformants produced halos on lichenan plates after Congo-red staining. The gene encodes a 29.7-kDa β-glucanase with a 30-amino acid signal peptide. An 889-bp fragment containing the catalytic domain was overexpressed in *E. coli*. The enzyme showed activity on lichenan, β-glucan and laminarin, but not on carboxymethylcellulose or xylan (Spilliaert et al., 1994).

In addition, a laminarinase (transglycosylating 1,3[4]-β-glucanase; the LamR protein) was found that cleaves the 1,3-β-linkages of 3-O-substituted β-glucose in β-glucans such as laminarin and curdlan and also the 1,4-β-linkages of 3-O-substituted β-glucose in β-glucans such as lichenin and 1,3-1,4-β-glucan from the cell walls of barley endosperm (Petersen et al., 2000). The *lamR* gene has been cloned and expressed in *E. coli*, and site-directed mutagenesis studies have been performed to identify the residues involved in the active center to elucidate the catalytic mechanism (Krah et al., 1998).

When grown in a medium containing locust bean gum + yeast extract, *R. marinus* produces a thermostable endo-(1,4)-β-mannanase (Gomes and Steiner, 1998). Screening of expression libraries for mannanase-positive clones led to the identification of the gene encoding a 997-residue, 113-kDa protein. The enzyme contains an N-terminal domain of unknown function and a C-terminal mannanase domain of 550 amino acids with homology to known mannanases of glucosidase family 26. The enzyme effectively hydrolyzes carob-galactomannan (locust bean gum) and to a smaller extent guar gum. Truncated enzymes of 45 or 50 kDa containing the catalytic domain only have been produced in *E. coli*. These were heat-stable as well and retained more than 70 and 25% of their initial activity after 1 h at 70°C and 90°C, respectively (Politz et al., 2000).

The gene encoding a novel thermostable branching enzyme (transglucosidase) was isolated from "*R. obamensis*." The predicted 621-amino acid 72-kDa protein has no signal sequence, so it probably is an intracellular enzyme. The gene has been expressed in *E. coli* and in the fungus *Aspergillus oryzae*. The enzyme produces α-1,6 branching linkages by a transglycosylation reaction of α-1,4-glucan. Activity was higher toward amylose than toward amylopectin (Shinohara et al., 2001).

On media with galactooligo- or polysaccharides as single carbon source, *R. marinus* produces a thermostable α-galactosidase that hydrolyzes both high- and low molecular weight galactosaccharides (Blücher et al., 2000).

Some other enzymes of *Rhodothermus* have been investigated at the protein and/or at the gene level: 1) DNA helicase, a protein predicted to harbor a 428-amino acid intein, is a close homologue of the DnaB intein in *Synechocystis* PCC6803 (Liu and Hu, 1997). 2) The 5-aminolevulinic acid dehydratase encoded by the *hemB* gene is a predicted 340-amino acid 37.4-kDa protein that shows homology with eubacterial and eukaryotic 5-aminolevulinic acid dehydratases (Gudmundsdóttir et al., 1999). 3) Different restriction endonucleases. The most often encountered type, named *Rma*I, is a type II restriction endonuclease that is an isoschizomer of *Mae*I and recognizes the sequence 5' C/TAG 3'(Rönkä et al., 1991). And finally, 4) other type LI restriction endonucleases detected during screening of a large number of *Rhodothermus* strains are isoschizomers of *Bst*B1 and *Eco*RV (Hjörleifsdóttir et al., 1997a).

In addition to its thermophilic properties, *Rhodothermus* is also a moderate halophile, requiring salt for growth and tolerating NaCl concentrations up to 5–6%. Its mode of osmotic adaptation has been investigated, and the organism was found to contain two novel organic osmotic solutes: 2-*O*-α-mannosylglycerate and 2-*O*-α-mannosylglyceramide (Fig. 5). α-Mannosylglycerate has also been detected in many other themophilic and hyperthermophilic Bacteria and Archaea (*Thermus*, *Rubrobacter*, *Methanothermus*, *Archaeoglobus* and *Aeropyrum*), but the amide has thus far been found only in *Rhodothermus* (Santos and da Costa, 2002). The α-mannosylglyceramide was originally incorrectly identified as 2-*O*-β-mannosylglycerate (Nunes et al., 1995), but the structure has been revised later (Silva et al., 1999). "*Rhodothermus obamensis*" was found to contain α-mannosylglycerate only and to lack the amide form (Silva et al., 1999). The intracellular K^+ concentration, as measured by ^{39}K NMR, increases with salinity and is sufficiently high to balance the negative charges of the mannosylglycerate. Glutamate, trehalose and glucose were found as minor components that may participate in osmotic stabilization of *Rhodothermus* (Nunes et al., 1995).

At relatively low growth temperatures application of water stress to *R. marinus* led to accumulation of α-mannosylglycerate and its amide, in addition to low levels of trehalose, glutamate and glucose. At the highest growth temperatures α-mannosylglycerate was the major compatible

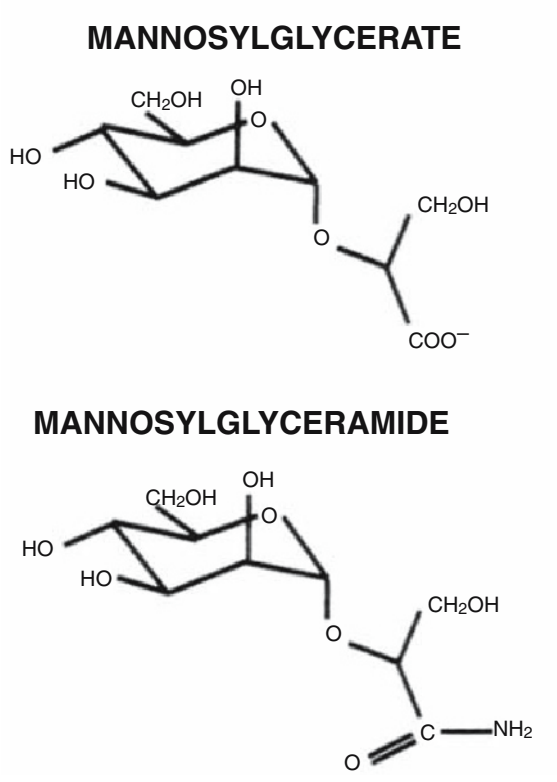

Fig. 5. Structures of mannosylglycerate and mannosylglyceramide. From Santos and da Costa (2002); reproduced with permission.

solute, and the amide was not detected (Silva et al., 1999). α-Mannosylglycerate accumulated in response to both supraoptimal temperatures and salinity, while α-mannosylglyceramide accumulated exclusively in response to salt stress (Nunes et al., 1995; Silva et al., 1999). The effectivity of α-mannosylglyceramide as a compatible solute, as judged by the stabilization of rabbit muscle lactate dehydrogenase against thermal inactivation, was less than that obtained with α-mannosylglycerate or hydroxyectoine, but similar to that of diglycerol phosphate (the compatible solute of *Archaeoglobus fulgidus*) and of trehalose (Borges et al., 2002).

On the basis of enzymatic activities detected and in vivo ^{13}C labeling experiments, the biosynthesis of α-mannosylglycerate in *Rhodothermus* was suggested to proceed via two alternative pathways (Fig. 6). In the first, guanosine-5′-diphosphate (GDP)-mannose is condensed with D-glycerate to produce α-mannosylglycerate in a single reaction catalyzed by mannosylglycerate synthase. In the other pathway, a mannosyl-3-phosphoglycerate synthase catalyzes the conversion of GDP-mannose and D-3-phosphoglycerate into a phosphorylated intermediate, which is then converted to mannosylglycerate by the action of a phosphatase. The activities of the enzymes of the pathway were not influenced by the NaCl concentration in the growth medium. However, the combined mannosyl-3-phosphoglycerate synthase/phosphatase system was optimally active only when NaCl or KCl had been added to the assay mixture (Martins et al., 1999).

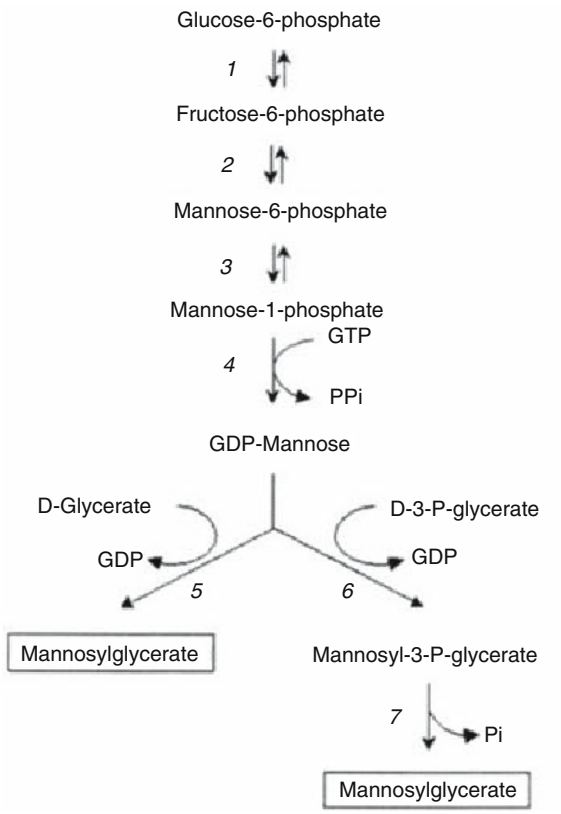

Fig. 6. Proposed pathways for the synthesis of mannosylglycerate in *Rhodothermus marinus*. 1, phosphoglucose isomerase; 2, phosphomannose isomerase; 3, phosphomannose mutase; 4, mannose-1-phosphate guanylyltransferase; 5, mannosylglycerate synthase; 6, mannosyl-3-phosphoglycerate synthase; and 7, mannosyl-3-phosphoglycerate phosphatase. From Martins et al. (1999); reproduced with permission.

The mannosylglycerate synthase of *Rhodothermus* has been purified, and the gene *mgs* that encodes it has been identified in a genome library. The gene, which codes for a 46.1-kDa protein, has been overexpressed in *E. coli*. The enzyme was optimally active at 85–90°C (see also Table 2) and has a broad pH optimum at 5.5–8.5 (Martins et al., 1999). Use of the *Rhodothermus msg* gene on a plasmid, combined with other genetic manipulations, enabled the production of high purity specifically labeled GDP-mannose and mannosylglycerate in *E. coli* (Sampaio et al., 2003).

The components of the respiratory chain of *Rhodothermus* have been investigated in-depth. Menaquinone MK-7 is the major lipoquinone. Minor amounts of MK6 (3% in the type strain of *R. marinus*, 10% in "*R. obamensis*") and MK-5 (7% in "*R. obamensis*") were also found (Tindall, 1991; Sako et al., 1996). A number of cytochrome-containing enzymes that make part of the electron transfer chain to oxygen have been characterized. Their properties are summarized in Table 4.

The respiratory chain contains a caa_3 terminal oxidase, a novel cytochrome *bc* complex, and a high potential iron sulfur protein that serves as an electron carrier between this complex and a terminal oxidase (Pereira et al., 1999a; Pereira et al., 1999b). The membrane-bound heme centers have been identified by spectroscopic methods such as electron paramagnetic resonance and by

Table 4. Cytochromes of the respiratory system of *Rhodothermus marinus*.

Activity	Cytochrome	E'_o, mV (heme center)
Electron carrier	*c*	+267 (C)
Succinate: menaquinone oxidoreductase	*b*	+75 (B), –65 (B)
Menaquinol:HiPIP oxidoreductase	*bc*	+235 (B), +235 (C), +80 (C), –45 (C+C)
Terminal oxidase	cbb_3	+195 (C), +120 (B), –50 ($C+B_3$)
Oxygen reductase, HiPIP oxidase	caa_3	+260 (C), +255 (A), +180 (A_3)

Abbreviation: HiPIP, high-potential iron-sulfur protein.
From Pereira et al. (2000a).

redox potentiometry. B- and C-type hemes are most abundant in the membrane, with small amounts of heme A being present as well. The heme centers have relatively low reduction potentials, ranging from +267 to –65 mV. No Rieske-type center could be detected, suggesting the absence of a canonical complex III (Pereira et al., 1999b; Pereira et al., 2000a).

The following components have been characterized: 1) An NADH:menaquinone oxidoreductase (Complex I), with at least four Fe-S centers (Fernandes et al., 2001a). The purified complex showed optimal activity at 50°C and pH 8.1. The purified enzyme contains 13.5 ± 3.5 iron atoms and about 3.7 menaquinone per flavin adenine mononucleotide (FMN). Electron paramagnetic resonance (EPR) spectroscopy showed the presence of at least five Fe-S centers: two $[2Fe\text{-}2S]^{2+/1+}$ and three $[4Fe\text{-}4S]^{2+/1+}$ centers (Fernandes et al., 2002).

2) A succinate:menaquinone oxidoreductase with three subunits of 70 kDa (the flavin containing subunit), 32 kDa (the iron-sulfur subunit), and 18 kDa (the anchor subunit). It is optimally active at 80°C and has a high affinity for succinate and fumarate. The complex contains two B-type hemes (Fernandes et al., 2001a; Fernandes et al., 2001b).

3) A membrane bound high potential iron sulfur protein (HiPIP) of about 10 kDa. It contains a single $[4Fe\text{-}4S]^{3+/2+}$ cluster with a high redox potential of +260 mV. It is part of the main membrane-bound electron transfer pathway. It is reduced by NADH or by succinate when further electron flow is blocked by addition of cyanide, and it serves as intermediate between the *bc* complex and the caa_3 terminal oxidase (Pereira et al., 1994; Pereira et al., 1999b).

4) Menaquinol:HiPIP oxidoreductase (complex III). This is a novel multihemic cytochrome *bc* that contains at least three subunits (43, 27 and 18 kDa) and five low-spin heme centers of the B and C types in a 1:4 ratio. All C-type hemes are found in the 27-kDa subunit. The complex shows three distinct redox transitions with midpoint potentials at +235, +80, and –45 mV. The first involves one B- and one C-type heme; the second and third transitions involve one and two C-type hemes, respectively. The complex couples the electron transfer between the quinols reduced by the dehydrogenases and a HiPIP, the final electron donor to the terminal oxidases (Pereira et al., 1999a).

5) A cbb_3-type oxidase as the major terminal oxidase. The complex was partially purified. Its five subunits have apparent molecular masses of 64 kDa, 57 kDa, 36 kDa, 26 kDa (the subunit carrying the C-type heme), and 13 kDa. There are two low-spin heme C centers and one high- and one low-spin heme B center. The reduction potentials are +195 mV (heme C), +120 mV (heme B), –50 mV (heme C), and –50 mV (heme B_3; Pereira et al., 2000a).

And lastly, 6) the caa_3 terminal oxidase, belonging to the superfamily of heme-copper oxidases, serves as an HiPIP: oxygen oxidoreductase. This complex was reconstituted in liposomes and was shown to function as a proton pump (Pereira et al., 2000b). It has three subunits with apparent molecular masses of 42 kDa, 19 kDa, and 15 kDa and a C-heme containing subunit of 35 kDa. The hemes show unusually low reduction potentials of +260 (heme C), +255 (heme A), and +180 mV (heme A_3; Pereira et al., 1999c). The complex acts as a proton pump (Pereira et al., 2000b). The kinetics of the different transformations and reactions within the complex have been characterized in-depth (Sigurdson et al., 2001). The genes for the subunits (*rcoxA – rcoxD*) are organized in an operon that contains at least five genes. These genes have been cloned and sequenced (Santana et al., 2001). It is interesting to note that the outcome of the cytochrome oxidase test has been reported differently by different authors. Alfredsson et al. (1988) and Sako et al. (1996) reported a positive oxidase reaction, but Kristjánsson and Alfredsson (1992) note a negative oxidase reaction for *R. marinus*. Nunes et al. (1992a) state that all their *Rhodothermus* isolates from the Azores were oxidase-negative.

Phospholipids are the main lipid components of *Rhodothermus*; glycolipids are absent or form minor components only. Five to seven phospholipids were detected, of which the following have been identified: phosphatidylethanolamine, diphosphatidylglycerol, and phosphatidylglycerol (Tindall, 1991; Nunes et al., 1992b).

The branched fatty acids *iso*-C15:0, *iso*-C17:0, *anteiso*-C15:0, and *anteiso*-C17:0 are the dominant fatty acids in *Rhodothermus* lipids. Straight unbranched fatty acids are present as well (Tindall, 1991; Chung et al., 1993; Moreira et al., 1996; Sako et al., 1996; Silva et al., 2000; Table 5). The relative amounts at which the different fatty acids are found in the lipids depend on the medium and the growth conditions. Thus, when grown on yeast extract + peptone or on yeast extract alone, the branched *iso*-C15:0 and *iso*-C17:0 were the major fatty acids. However, when glutamate was the sole source of carbon, *n*-C16 was the major component, and straight-chain fatty acids reached about 50% of the total fatty acids. Following growth on yeast extract + glutamate, the relative proportion of *iso*-C16:0 reached high levels (Chung et al., 1993).

Rhodothermus is colored red by carotenoids, and absorption spectra of cells in acetone show a peak at 476 nm and shoulders at 456 and 502 nm (Alfredsson et al., 1988). These carotenoids

Table 5. Fatty acid composition (% w/w) of two strains of *Rhodothermus marinus*.

	iso-C14:0	*iso*-C15:0	*anteiso*-C15:0	C15:0	*iso*-C16:0	C16:0	*iso*-C17:0	*anteiso*-C17:0	C17:0	*iso*-C18:0	C18:0
DSM 4252^T	3.70	4.56	8.50	trace	34.69	6.19	15.14	10.02	1.41	12.36	1.96
DSM 4253	4.60	5.29	12.40	trace	34.72	7.68	12.71	10.62	0.91	8.43	1.34

From Tindall (1991).

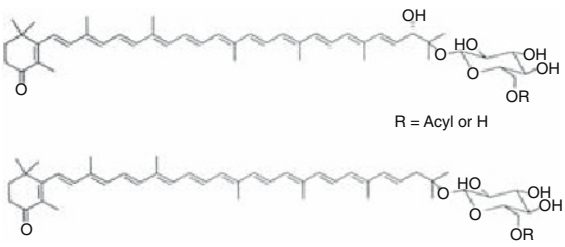

Fig. 7. Carotenoids of *Rhodothermus marinus*. From Lutnæs et al. (2004).

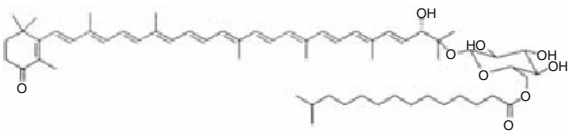

Fig. 8. The structure of salinixanthin, the major carotenoid pigment of *S. ruber*.

have recently been identified as (all-*E*, 2′*S*)-2′-hydroxy-1′-(β-D-glucopyranosyloxy)-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-4-one (3.3% of total carotenoid) and its C-6″ acyl derivative (55%) and (all-*E*)-1′-(β-D-glucopyranosyloxy)-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-4-one (2.5%) and its C-6″ acyl derivative (39%; Fig. 7). Both carotenoid acyl glucosides exhibited the same acyl profile, the major esterifying acids being the branched odd-carbon *iso*-C13:0, *anteiso*-C13:0, *iso*-C15:0 and *anteiso*-C15:0. A similar monocyclic carotenoid acyl glucoside is present in the phylogenetically related *Salinibacter ruber* (salinixanthin, Fig. 8).

The following polyamines have been detected in *Rhodothermus marinus*: spermidine [$NH_2(CH_2)_3NH(CH_2)_4NH_2$], spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$], thiopentamine [$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$], the tertiary tetra-amine N^4-aminopropylspermidine [$NH_2(CH_2)_3N((CH_2)_3NH_2)(CH_2)_4NH_2$], and the quaternary penta-amine N^4-*bis*(aminopropylspermidine) [$NH_2(CH_2)_3N^+((CH_2)_3NH_2)_2(CH_2)_4NH_2$] (Hamana et al., 1992; Hamana et al., 1998). "*Rhodothermus obamensis*" contains in addition two novel linear hexamines: $NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ and $NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH(CH_2)_3NH_2$ (Hamana et al., 1998).

Table 6. Medium for *Rhodothermus* (modified *Thermus* medium 162 of DSMZ).[a]

Yeast extract	2.5g
Tryptone	2.5g
Agar	28.0g
Nitrilotriacetic acid	100mg
$CaSO_4 \cdot 2H_2O$	40mg
$MgCl_2 \cdot 6H_2O$	200mg
0.01M Fe-citrate	0.5ml
Trace element solution[b]	0.5ml
Phosphate buffer[c]	100ml

[a]Combine all ingredients except phosphate buffer. Adjust pH to 7.2 with NaOH, autoclave, and add the sterile phosphate buffer.

[b]Trace element solution contains (per liter): nitrilotriacetic acid, 12.8g; $FeCl_2 \cdot 4H_2O$, 1.0g; $MnCl_2 \cdot 4H_2O$, 0.5g; $CoCl_2 \cdot 4H_2O$, 0.3g; $CuCl_2 \cdot 2H_2O$, 50mg; $Na_2MoO_4 \cdot 2H_2O$, 50mg; H_3BO_3, 20mg; and $NiCl_2 \cdot 6H_2O$, 20mg. Dissolve the nitrilotriacetic acid, adjust the pH to 7.0 with KOH. Dissolve the other salts separately, combine, and adjust the pH to 6.8 with NaOH or H_2SO_4.

[c]Phosphate buffer contains (per liter): 5.44g of KH_2PO_4 and 43.0g of $Na_2HPO_4 \cdot 12H_2O$, pH 7.2, and is autoclaved separately.

Isolation and Maintenance

Rhodothermus can be isolated from submarine hydrothermal environments worldwide. No selective medium for the isolation of *Rhodothermus* has been described yet. The species can be grown on a simple yeast extract-tryptone medium with the appropriate salt concentration, such as specified in Table 6. Red colonies developing on agar plates following aerobic incubation at 65°C may well be *Rhodothermus* strains. Final identification should be based on 16S rDNA sequencing and comparison with other properties described for the genus.

Rhodothermus is sensitive to penicillin, erythromycin, tetracycline, and chloramphenicol and resistant to the aminoglycoside antibiotics kanamycin, streptomycin, and gentamycin (Alfredsson et al., 1988; Nunes et al., 1992a).

Rhodothermus can be preserved by lyophilization.

Ecology

Rhodothermus lives around submarine hot springs (Alfredsson et al., 1988; Sako et al., 1996). Because of its narrow range of growth-

permissive temperature (54–77°C), it can only grow very close to the openings of these hot springs, as only a few cm from the openings the water would be too cold. Moreover, *Rhodothermus* is obligatory aerobic, and geothermic water is usually anaerobic until it emerges from the ground or the seabed and absorbs oxygen from the surroundings. *Rhodothermus* is a heterotroph, and suitable organic material must therefore be present as well in its habitat. The requirements for oxygen, salt, organic nutrients, and temperature thus define a very narrow niche for this genus in the hot spring environment (Alfredsson et al., 1988).

Material containing 16S rDNA sequences related to *Rhodothermus* (87% similarity to *R. marinus*) has been recovered from an orange-colored microbial mat in an alkaline hot spring in Nakabusa, Japan at 58–60°C (Nakagawa and Fukui, 2002).

Applications

The thermophilic nature of *Rhodothermus* makes the organism an interesting potential source for thermostable enzymes. Its ability to degrade xylans, cellulose, and other polysaccharides (see Table 3) makes the organism of special interest. As described above, the relevant enzymes have been cloned and in a number of cases optimized for biotechnological production by genetic engineering. To the knowledge of the author, *Rhodothermus* is not yet used in industrial applications, but a number of patents have been issued describing potential applications in the future (Dahlberg et al., 2002; Wicher et al., 2002).

Xylan is the most abundant hemicellulose in plant cell walls. Xylanases are therefore important in pulp and paper industry, as well as in the food industry. The thermostable *Rhodobacter* xylanase (both the native form of the enzyme and the truncated engineered form; Nordberg Karlsson et al., 1996; Nordberg Karlsson et al., 1998b; Nordberg Karlsson et al., 1999; Abou Hachem et al., 2000; Ramchuran et al., 2002) has successfully been used in small-scale experiments for the prebleaching of hardwood and softwood kraft pulps. The truncated enzyme performed very well, and the presence of the additional domains in the full-length enzyme, including carbohydrate-binding modules, did not improve the bleaching process (Pfabigan et al., 2002). The combined use of arabinofuranosidase-rich xylanase and mannanase from *R. marinus* in the prebleaching of softwood kraft pulp gave a significant increase in brightness of the pulp (Gomes et al., 2000).

No methods have yet been developed for the genetic manipulation of *Rhodothermus*. The finding of the 2935-bp cryptic plasmid pRM21 in seven out of the 41 strains investigated may open interesting possibilities regarding its genetic manipulation. The plasmid contains one major open reading frame with a deducted product similar to RepA proteins, with the highest similarity to RepA of *E. coli* plasmid pSa. The small size and the apparent stability of this first plasmid characterized from *Rhodothermus* make it an attractive candidate for future use as a cloning vector (Ernstsson et al., 2003).

The Genus *Thermonema*

In 1987, Schofield et al. described the isolation of five strains of very long, thin filamentous bacteria from hot springs in New Zealand. These isolates displayed terminal swellings (Fig. 9), showed gliding motility on agar plates, and grew optimally at 60°C. A formal description of the species as *Thermonema lapsum* followed two years later (Hudson et al., 1989). A second species of the genus, *T. rossianum*, was recovered from saline hot springs in the Bay of Naples, Italy (Tenreiro et al., 1997). The isolation of *Thermonema* strains from submarine smectite cones on the seafloor in northern Iceland (Marteinsson et al., 2001) suggests that such organisms can probably be found worldwide in hot spring areas.

Taxonomy and Phylogeny

Two species are presently recognized within the genus *Thermonema*: 1) *T. lapsum* (the type species of the genus), with type strain 23/9^T = ATCC 43542 = DSM 5718 (16S rDNA accession number: L11703). The G+C content of its DNA

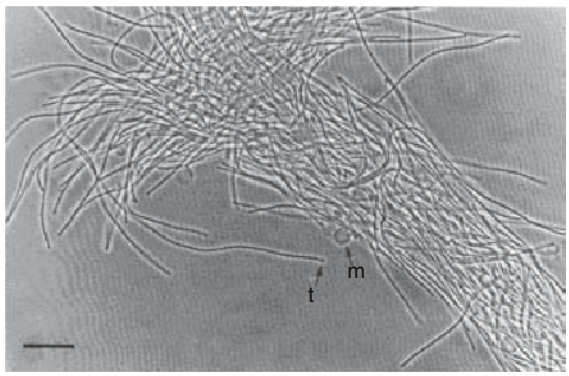

Fig. 9. Phase-contrast micrograph of *Thermonema lapsum* strain 23/9^T showing terminal (arrow t) and mid-filament (arrow m) swellings. The bar is 21 μm. From Schofield et al. (1987); reproduced with permission.

is 47 mol%. An additional strain is strain 19/15, deposited as ATCC 34543 (Hudson et al., 1989).

And 2) *T. rossianum*, with type strain NR-27^T = DSM 10300 (16S rDNA accession number: Y08956; Tenreiro et al., 1997). The G+C content of its DNA is 50.9 mol%.

Analysis of the 16S rRNA gene of *T. lapsum* showed the organism to form a deep branch affiliated with the *Cytophaga-Flavobacterium-Bacteroides* branch of the Bacteria (Andrésson and Friðjónsson, 1994; Patel et al., 1994; Fig. 1).

Morphological and Cultural Characteristics

Thermonema cells are very long slender filaments. The cell length may exceed 60 μm, but cells are only 0.25–0.3 μm (*T. lapsum*) to 0.7 μm wide (*T. rossianum*; Hudson et al., 1989; Tenreiro et al., 1997). As no septa can be seen within these thin filaments, each one probably represents a single cell. *Thermonema lapsum* is motile by gliding; no motility has been reported in *T. rossianum*.

Physiological, Biochemical and Chemotaxonomic Properties

The polar lipids of *T. rossianum* are dominated by lipids that show a positive ninhydrin reaction. Two of these are aminophospholipids. No information is available on the identity of the other three major fractions, except that they do not react with α-naphthol (detecting carbohydrates) and do not contain phosphate groups (Tenreiro et al., 1997). Dominant fatty acids in the lipids of *T. lapsum* are *iso*-C15:0, *anteiso*-C15:0, and C15:0. The lipopolysaccharide fraction of this organism is particularly rich in hydroxy fatty acids (Patel et al., 1994). The most abundant fatty acids in *T. rossianum* are *iso*-C15:0, *iso*-C17:0 3OH, *iso*-15:0 3OH and *anteiso* C15:0 (Tenreiro et al., 1997). Monounsaturated fatty acids were not detected. Table 7 presents some quantitative data on the fatty acid content of different lipid fractions of *Thermonema* isolates.

Both *T. lapsum* and *T. rossianum* form bright yellow colonies. Absorption spectra of *T. lapsum* acetone extracts show an absorbance maximum at 450 nm with a shoulder at 480 nm (Schofield et al., 1987; Hudson et al., 1989). The structure of the presumed carotenoid pigment(s) present remains to be elucidated.

Both species are catalase and oxidase-positive. The major respiratory quinone of *T. rossianum* is menaquinone-7 (Tenreiro et al., 1997).

Thermonema contains homospermidine [$NH_2(CH_2)_4NH(CH_2)_4NH_2$] as the major polyamine (also found as the major polyamine in mesophilic members of the *Flavobacterium* – *Cytophaga* – *Flexibacterium* complex), with minor amounts of homospermine [$NH_2(CH_2)_4NH(CH_2)_4NH(CH_2)_4NH_2$] (Hamana et al., 1992).

Isolation and Maintenance

No selective media have yet been described for the isolation of *Thermonema* species. The strains in culture have been obtained by spreading water samples on plates followed by incubation at 60–70°C (Schofield et al., 1987). All strains grow on media containing low concentrations of complex nutrients (tryptone and yeast extract) in the presence of 1–4% NaCl (see Table 8). *Thermonema* species give rise to the development of bright yellow colonies following incubation at 60°C. *Thermonema lapsum* grows at temperatures up to 70°C (a temperature too high for the growth of *T. rossianum*). The optimum pH is in the neutral range. *Thermonema rossianum* requires 1–3% NaCl for growth (Tenreiro et al., 1997). The minimum doubling time reported for *T. lapsum* was 11 h. In broth culture it grows in clumps and as pellicle on the surface of the medium (Schofield et al., 1987).

Table 7. The major fatty acids of *Thermonema* species.[a]

Fatty acid	*T. lapsum*		*T. rossianum*
	Phospholipids	Lipopolysaccharide	Total lipids
iso-C15:0	54.7	5.1	39.9
anteiso-C15:0	5.6	3.2	8.6
iso-C15:0 2OH	18.6	15.9	5.4
C15:0 2OH	2.5	4.6	0.6
iso-C15:O 3OH	0	28.4	8.3
C16:0 2OH	2.1	1.9	0
iso-C17:0 3OH	4.3	25.7	18.7
iso-C17:0 2OH	3.6	0.8	0.5
C15:0	3.7	0.4	1.8

[a]Values are percentages of total fatty acids.
Data were derived from Patel et al. (1994) and Tenreiro et al. (1997).

Table 8. Growth media for the cultivation of *Thermonema* species.

Species	*Thermonema lapsum* (DSMZ medium 86)[a]	*Thermonema rossianum* (DSMZ medium 878 = "*Thermus* 162 medium")[b]
Tryptone	1.0 g/liter	1.0 g/liter
Yeast extract	1.0 g/liter	1.0 g/liter
NaCl	8 mg/liter	0 or 10 g/liter
$MgSO_4 \cdot 7H_2O$	100 mg/liter	NA
$MgCl_2 \cdot 6H_2O$	NA	200 mg/liter
$CaSO_4 \cdot 2H_2O$	60 mg/liter	40 mg/liter
KNO_3	103 mg/liter	NA
$NaNO_3$	689 mg/liter	NA
$Na_2HPO_4 \cdot 2H_2O$	140 mg/liter	NA
Nitrilotriacetic acid	100 mg/liter	100 mg/liter
$FeCl_3 \cdot 6H_2O$	0.47 mg/liter	NA
$MnSO_4 \cdot 2H_2O$	2.2 mg/liter	NA
$ZnSO_4 \cdot 7H_2O$	0.5 mg/liter	NA
H_3BO_3	0.5 mg/liter	NA
$CuSO_4 \cdot 5H_2O$	25 µg/liter	NA
$Na_2MoO_4 \cdot 2H_2O$	25 µg/liter	NA
$CoCl_2 \cdot 6H_2O$	46 µg/liter	NA
Agar	NA	28.0 g
0.01 M Fe-citrate	NA	0.5 ml/liter
Trace element solution[c]	NA	0.5 ml/liter
Phosphate buffer[d]	NA	100 ml/liter

Abbreviation: NA, not added.
[a]Adjust pH to 8.2 with NaOH.
[b]Adjust pH to 7.2 with NaOH.
[c]Trace element solution contains (per liter): H_2SO_4, 0.5 ml; $MnSO_4 \cdot H_2O$, 2.28 g; $ZnSO_4 \cdot 7H_2O$, 0.5 g; H_3BO_3, 0.5 g; $CuSO_4 \cdot 5H_2O$, 25 mg; $Na_2MoO_4 \cdot 2H_2O$, 50 mg; and $CoCl_2 \cdot 6H_2O$, 45 mg.
[d]Phosphate buffer contains (per liter): 5.44 g of KH_2PO_4 and 43.0 g of $Na_2HPO_4 \cdot 12H_2O$; adjust pH to 7.2, and autoclave this solution separately from the solution of the other media components.

Thermonema species require complex mixtures of amino acids for growth (casamino acids, peptone and tryptone), and all strains are proteolytic; casein, elastin and gelatin are degraded by *T. rossianum*. No growth is obtained on carbohydrates, organic acids, or polyols. Starch and cellulose are not hydrolyzed (Hudson et al., 1989; Tenreiro et al., 1997). *Thermonema lapsum* was reported to show DNAse activity (Schofield et al., 1987). Growth is obligatory aerobic, and nitrate is not reduced.

Thermonema lapsum is susceptible to penicillin G and erythromycin and resistant to vancomycin, kanamycin, cycloserine and nalidixic acid (Hudson et al., 1989).

Thermonema can be preserved by lyophilization.

Ecology

Thermonema species are found in hot spring environments at temperatures around 60–70°C. They have been isolated from thermal springs in New Zealand (Schofield et al., 1987) and Italy (Tenreiro et al., 1997). Recently a number of isolates have been obtained from submarine smectite cones on the seafloor in northern Iceland, rising 25–45 m from the seafloor located at a depth 65 m. Hydrothermal fluid of 60–72°C rises here from small chimneys. The outer zone of these chimneys harbors *Thermonema* cells (Marteinsson et al., 2001). Surprisingly, no *Thermonema* strains have yet been isolated from terrestrial hot springs on Iceland.

The Genus *Hymenobacter*

The genus *Hymenobacter* consists of obligatory aerobic nonthermophilic, red-pigmented Gram-negative bacteria affiliated with the *Cytophaga-Flavobacterium-Bacteroides* branch of the *Bacteria*. The three species presently recognized were isolated from highly disparate environments: from soils and sandstone of the continental Antarctic (Hirsch et al., 1998; Fig. 10), from irradiated pork (Collins et al., 2000), and from air collected in the rooms of a museum in Venice (Buczolits et al., 2002). Phylogenetically affiliated and physiologically similar strains had earlier been isolated from antelope dung in South Africa and from soil on Crete. These isolates were at the time designated as members of the newly created genus "Taxeobacter" (Reichenbach, 1991; Reichenbach, 1992), but this genus name has never been validated. Although

reports of their isolation are few, organisms of this group are probably widely distributed and may be common members of the autochthonous soil flora (Reichenbach, 1992).

Taxonomy and Phylogeny

Three species of *Hymenobacter* have thus far been validly named: *H. roseosalivarius*, of which five isolates have been characterized (Hirsch et al., 1998), *H. actinosclerus* (Collins et al., 2000), and *H. aerophilus* (Buczolits et al., 2002; Table 9). *Hymenobacter roseosalivarius*, the type species, is a red to pink bacterium that was isolated from continental Antarctic soils and sandstone collected from the Antarctic dry valleys (Fig. 10).

The identification of isolates as strains belonging to the genus *Hymenobacter* is based on the color of the colonies, the shape of the cells, the range of substrates degraded, and the 16S rDNA sequence.

Morphological and Cultural Characteristics

Hymenobacter cells are rod-shaped and measure up to 4 μm in length (Table 9). *Hymenobacter roseosalivarius* has a tendency to become shorter to almost coccoid in the stationary growth phase (Fig. 10). Cells may show a tendency to aggregate. On agar plates cells may become arranged in palisades at the edge of young spreading colonies (Hirsch et al., 1998; Fig. 10 d). The same type of cell arrangement has also been noticed for "Taxeobacter" isolates (Reichenbach, 1992). *Hymenobacter roseosalivarius* cells often contain electron-dense polyphosphate granules near the cell poles, and they excrete large amounts of extracellular polymer.

No motility has been observed in *H. roseosalivarius*, *H. actinosclerus* and *H. aerophilus*. However, gliding motility has been reported in "Taxeobacter" strains (Reichenbach, 1991).

Physiological, Biochemical and Chemotaxonomic Properties

Meso-diaminopimelic acid has been identified in the peptidoglycan on *H. actinosclerus* and *H. aerophilus* (Collins et al., 2000; Buczolits et al., 2002).

Phosphatidylethanolamine was found as the major polar lipid in *H. roseosalivarius* and in *H. aerophilus*, together with several yet unidentified lipids. Bisphosphatidylglycerol was reported to occur in *H. roseosalivarius* but was not found in *H. aerophilus* (Hirsch et al., 1998; Buczolits et al., 2002). *iso*- and *anteiso*-methyl branched fatty acids and hydroxyl *iso*-methyl branched types are the major fatty acids (Table 10).

The major polyamine of *H. aerophilus* was identified as *sym*-homospermidine (Buczolits et al., 2002).

Hymenobacter cells are colored dark red by carotenoid pigments. A pigment extract of *H. aerophilus* in acetone has an absorbance maximum at 482 nm, with two slight inflection points at 453 and 505 nm (Buczolits et al., 2002). The major carotenoid pigment of "Taxeobacter" has been identified as (all-*E*,3S,2′*R*)-2′-hydroxyflexixanthin (3,1′,2′-trihydroxy-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-4-one, and (all-*E*,2′*R*)-3-deoxy-2′-hydroxyflexixanthin (1′,2′-dihydroxy-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-4-one) is present as a minor one (Fig. 11). The identification of these carotenoids was confirmed by their de novo synthesis and comparison with the natural compounds (Bircher and Pfander, 1997).

"Taxeobacter gelupurpurascens" was reported to show a very peculiar color change when grown at 2°C (Reichenbach, 1991), but no details were published of the nature of this color change.

All *Hymenobacter* isolates are strictly aerobic and catalase-positive. Menaquinone MK-7 has been identified as the major respiratory quinone in *H. roseosalivarius* (Hirsch et al., 1998), in *H. actinosclerus* (Collins et al., 2000), and in *H. aerophilus*. The latter was found to contain minor amounts of MK-6 as well (Buczolits et al., 2002).

The cytochrome oxidase reaction varies among different isolates: *H. roseosalivarius* and *H. actinosclerus* were reported as oxidase-positive (Hirsch et al., 1998; Collins et al., 2000), while *H. aerophilus* showed a negative or weakly positive oxidase reaction, depending on the strain and the method of detection (Buczolits et al., 2002).

Isolation and Maintenance

Hymenobacter strains and related isolates have been obtained from a wide variety of environments, including from soils in the Antarctic and the Mediterranean area, from air, and from irradiated meat products (see Table 9). They thus appear to be quite abundant, but no quantitative data are available on their distribution in different habitats.

No selective media have yet been developed for the enrichment and isolation of *Hymenobacter* species. Their isolation has generally been based on the color of their colonies on agar plates. Hirsch et al. (1998) obtained *H. roseosalivarius* from Antarctic soils and sandstone samples on low nutrient media incubated with illumination to encourage growth of algae, which might provide exudates for better growth of the bacteria. *Hymenobacter actinosclerus* was first

Table 9. The species of *Hymenobacter* and "Taxeobacter" and some of their properties.

	Hymenobacter			"Taxeobacter"		
Species	*roseosalivarius*	*actinosclerus*	*aerophilus*	"ocellatus"	"gelupurpurascens"	"chitinovorans"
Source of isolation	Soil and sandstone, Antarctica	Irradiated pork	Air, Museo Correr, Venice, Italy	Dung of antelope, South Africa	Soil	Soil, Crete
Culture collection accession numbers	Strain AA-718^T = DSM 11622 = CIP 106397; strain AA-688 = DSM 11621 = IFAM AA-495	CCUG 39621 = CIP 106628	I/26-Cor1 = DSM 13606 = LMG 19657. A second strain is I/27-Cor2 (DSM 13611 = LMG 19659)	Txo1 = DSM 11117 Myx2105	Txg1 = DSM 11116	Txc1 = DSM 11115
Cell size	1–1.25 × 2–2.7 μm	0.5–0.6 × 2–3.6 μm	0.4–0.75 × 1.3–5 μm		0.7–1.3 × 1.5–4 μm	
16S rDNA accession numbers	Y18833; Y18834	Y17356	AJ276901	Y18838 Y18835	Y18836	Y18837
G + C content in the DNA	55–61 (type strain: 56)	62	60–63 (type strain: 63.1)	55–65		
Reference	Hirsch et al., 1998	Collins et al., 2000	Buczolits et al., 2002	Reichenbach, 1991; Reichenbach, 1992		

Abbreviations: CCUG, Culture Collection of the University of Göteborg, Sweden; CIP, Collection de l'Institut Pasteur, Paris; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig; IFAM, Institut für Allgemeine Mikrobiologie, Universität Kiel; and LMG, Universiteit Gent, Laboratorium voor Mikrobiologie.

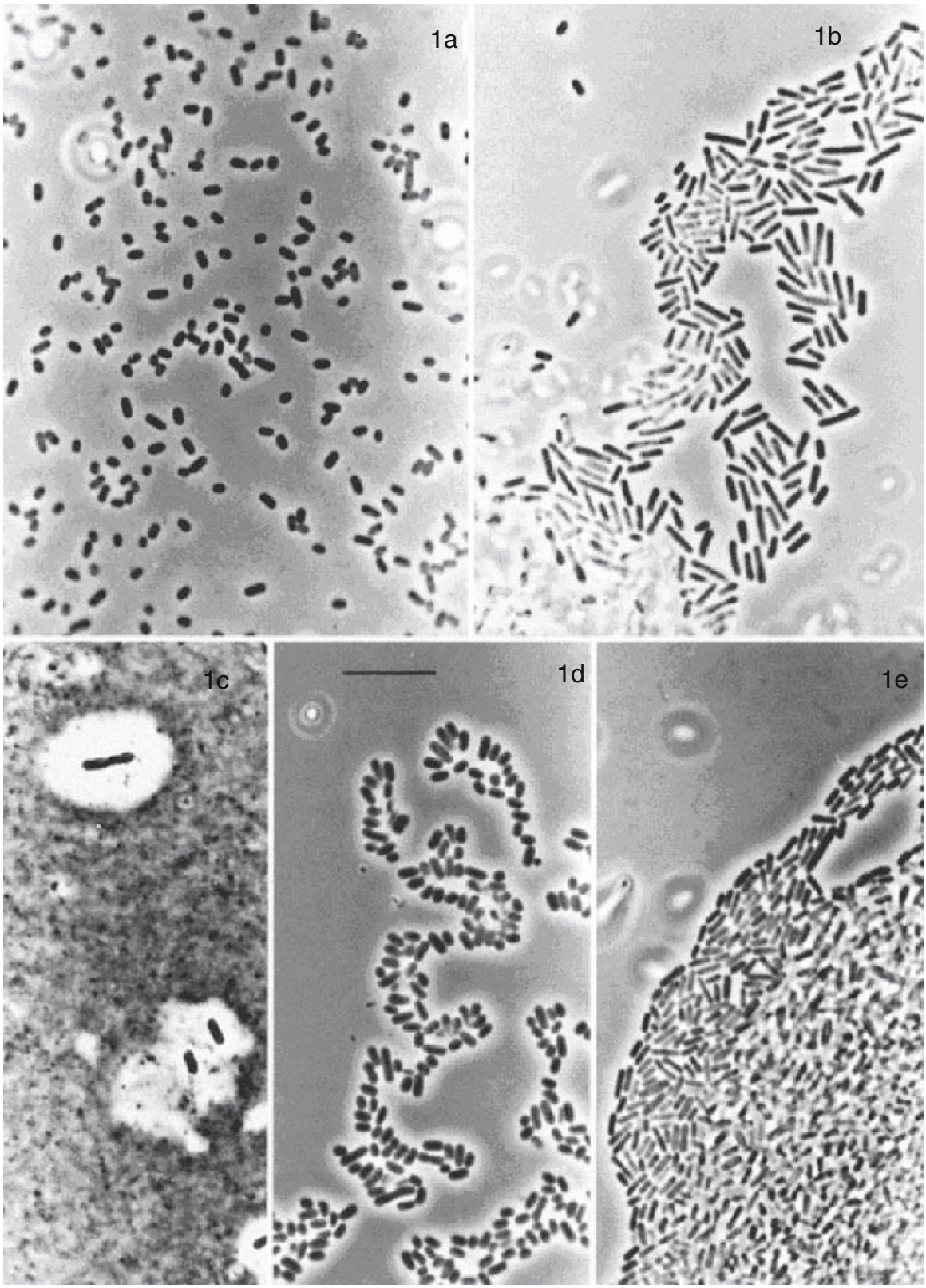

Fig. 10. *Hymenobacter roseosalivarius* strains isolated from the Antarctic. Phase contrast light micrographs. Magnification bar, 10 μm. a, strain AA-495 grown for four weeks on PYGV; b, strain AA-495 grown for eight days, colony edge; c, strain AA-495, the polymer layer was negatively stained with India ink; d, strain AA-1210, colony edge; and e, strain AA-718^T, colony edge. From Hirsch et al. (1998); reproduced with permission.

Table 10. The major fatty acid composition of *Hymenobacter* species.[a]

Fatty acid	*H. roseosalivarius* strain AA-718[T]	*H. actinosclerus*	*H. aerophilus* I/26-Corl[T]
iso-C15:0	8.3	22.3	10.8
anteiso-C15:0	None	25.8	22.3
C16:1 *ω5c*	23.3	3.7	7.9
C16:1 *ω7c* + *iso*-C15:0 2OH	29.8	13.1	21.4
iso-C17:0	1.7	1.8	4.5
iso-C17:0 3OH	5.8	3.1	3.5
anteiso-C17:1 B + *iso*-C17:1 I	18.5	19.9	17.7

[a]Values are percentages of total fatty acids.
From Buczolits et al. (2002).

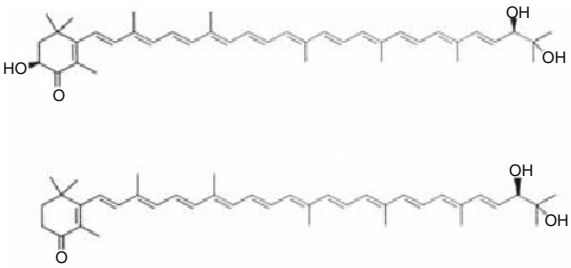

Fig. 11. 2′-Hydroxyflexixanthin and 3-deoxy-2′-hydroxyflexixanthin, the two carotenoid pigments of "Taxeobacter." After Bircher and Pfander (1997).

isolated from pork chops irradiated with 1.75 kGy. Its decimal reduction dose in buffer was found to be 3.45 kGy and in minced pork as high as 5.05 kGy (Collins et al., 2000). Whether other *Hymenobacter* species are as radiation-resistant and whether the property can be used in a positive selection procedure toward their selective isolation remain to be ascertained.

All *Hymenobacter* species grow aerobically with temperature optima of 10–30°C. The most thermotolerant species described ("Taxeobacter ocellatus") does not grow above 38°C (Reichenbach, 1992). The Antarctic isolates of *H. roseosalivarius* are adapted to relatively low temperatures, show optimal growth between 10 and 27°C, and grow within the range of minima of –0.5°C to <5°C and maxima of 25–32°C (Hirsch et al., 1998). All species can grow without salt, 0.5% NaCl is optimal for *H. roseosalivarius*, and no growth is obtained at salt concentrations exceeding 4–5%.

Hymenobacter species can be grown on aerobic agar plate media containing yeast extract and other commonly used substrates such as peptone, glucose and glutamate. Table 11 presents the composition of some media that have been recommended for the cultivation of members of the group. Anaerobic growth does not occur, and nitrate is not reduced.

Hymenobacter roseosalivarius can use glucose, sucrose, mannitol, sorbitol, aspartate, malate and acetate as growth substrates. Ribose, adonitol, alanine, glutamate, glycolate, lactate and succinate are not used. All isolates belonging to the species hydrolyze gelatin, starch, casein, xylan, Tween 80 and Tween 60, as well as dead or living yeast cells. Cellulose and pectin are not used (Hirsch et al., 1998). *Hymenobacter actinosclerus* also hydrolyzes starch (Collins et al., 2000). *Hymenobacter aerophilus* was reported to assimilate acetate, propionate, fructose, glucose, mannose and sucrose (Buczolits et al., 2002). Among the "Taxeobacter" strains are those that degrade chitin ("T. chitinovorans") or pectin.

Hymenobacter aerophilus and "Taxeobacter" have been successfully preserved by lyophilization.

Hymenobacter aerophilus is inhibited by bacitracin, chloramphenicol, colistin sulfate, erythromycin, fusidic acid, gentamycin, kanamycin, nitrofurantoin, penicillin G, polymyxin B, tetracycline and vancomycin (Buczolits et al., 2002). No information has been published on the sensitivity to antibiotics of the other species.

The Genus *Salinibacter*

The existence of extremely halophilic representatives of *Cytophaga-Flavobacterium-Bacteroides* branch was first suggested by the recovery of 16S rRNA genes affiliated with the group from NaCl-saturated saltern crystallizer ponds in Spain (Antón et al., 2000). Fluorescence in situ hybridization using probes designed to react with the novel phylotype showed that the bacteria harboring this 16S rRNA sequence have a slightly curved rod-shaped morphology and are abundant in the saltern environment: They may represent up to 5–25% of the total number of prokaryotic cells present in the Spanish crystallizer ponds (Antón et al., 1999). Enrichment of saltern brine samples with low concentrations of yeast extract led to an increase in the numbers of these rod-shaped cells, and multiplication was most rapid at salt concentrations between 20 and 25% (Antón et al., 2000). This new type of rod-

Table 11. Growth media for the cultivation of *Hymenobacter* and "Taxeobacter" species.

	Hymenobacter roseosalivarius	*Hymenobacter actinosclerus* and *H. aerophilus*	"Taxeobacter" media		
Medium designation	PYGV agar; DSMZ medium 621[a]	PYES medium; DSMZ medium 937[b]	MYX agar; DSMZ medium 729[c]	VY/2 agar; DSMZ medium 9[d]	CY-agar; DSMZ medium 67[c]
Peptone	0.25 g/liter	3.0 g/liter	NA	NA	NA
Casitone	NA	NA	NA	NA	3.0 g/liter
Na_2-glutamate	NA	NA	5.0 g/liter	NA	NA
Yeast extract	0.25 g/liter	3.0 g/liter	1.0 g/liter	NA	1.0 g/liter
Baker's yeast, heat-killed	NA	NA	NA	5.0 g/liter	NA
Na-succinate	NA	2.3 g/liter	NA	NA	NA
Glucose (autoclaved separately)	NA	NA	2.0 g/liter	NA	NA
$MgSO_4 \cdot 7H_2O$	NA	NA	1.0 g/liter	NA	NA
$CaCl_2 \cdot 2H_2O$	NA	NA	NA	NA	1.36 g/liter
Mineral salt solution[e]	20 ml/liter	NA	NA	NA	NA
Glucose solution (2.5%, filter-sterilized)	10 ml/liter	NA	NA	NA	NA
Vitamin solution[f]	5 ml/liter	NA	NA	NA	NA
Vitamin B_{12}[g]	NA	NA	NA	0.50 mg/liter	NA

Abbreviation: NA, not added.

[a]Adjust to pH 7.5 and solidify with agar (15 g/liter).

[b]Adjust to pH 7.2. May solidify with agar (15 g/liter).

[c]Adjust to pH 7.2 and solidify with agar (15 g/liter).

[d]Adjust to pH 7.2 with KOH and solidify with agar (15 g/liter).

[e]Mineral solution contains (per liter): nitrilotriacetic acid, 10 g; $MgSO_4 \cdot 7H_2O$, 29.7 g; $CaCl_2 \cdot 2H_2O$, 3.34 g; $Na_2MoO_4 \cdot 2H_2O$, 12.67 mg; $FeSO_4 \cdot 7H_2O$, 99 mg; and metal salt solution 44, 50 ml. Metal salt solution 44 contains (per liter): Na-EDTA, 2.5 g; $ZnSO_4 \cdot 7H_2O$, 10.95 g; $FeSO_4 \cdot 7H_2O$, 5.0 g; $MnSO_4 \cdot H_2O$, 1.54 g; $CuSO_4 \cdot 5H_2O$, 0.392 g; Co $(NO_3)_2 \cdot 6H_2O$, 0.248 g; $Na_2B_4O_7 \cdot 10H_2O$, 0.177 g; and a few drops of H_2SO_4 added to retard reprecipitation of the metal ions.

[f]Vitamin mixture contains (per liter): biotin, 4 mg; folic acid, 4 mg; pyridoxine. HCl, 20 mg; riboflavin, 10 mg; thiamine-$HCl \cdot 2H_2O$, 10 mg; nicotinamide, 10 mg; D-Ca-pantothenate, 10 mg; vitamin B_{12}, 0.2 mg; and *p*-aminobenzoic acid, 10 mg. This solution (which is stored in the dark and cold) is added after the mixture of other medium components has been autoclaved.

[g]Sterilized by filtration.

shaped extreme halophile was then designated "*Candidatus* Salinibacter" (Antón et al., 2000).

Isolation of the organism soon followed, and a formal description of *Salinibacter ruber* was published in 2002, based on the study of a number of strains obtained from saltern crystallizer ponds in Spain and on the Balearic Islands (Antón et al., 2002). Additional isolates of *Salinibacter* have recently been obtained from a salt crust near a highly saline pool in Death Valley National Park, California (Hollen et al., 2003).

Salinibacter isolates are orange-pigmented obligate aerobes that appear to prefer relatively low nutrient concentrations (yeast extract, amino acids and some sugars) for growth. All isolates are extremely halophilic, requiring at least 150 g of salt per liter for growth. Thus, they are among the most halophilic organisms within the domain Bacteria, and their salt requirement and tolerance are similar to those of the archaeal order Halobacteriales, which contains the most halophilic microorganisms known thus far (see the chapter on The Order Halobacteriales in Volume 3).

Taxonomy and Phylogeny

The 16S rDNA sequences of the *Cytophaga-Flavobacterium* group recovered from the saltern crystallizer ponds near Alicante, Spain, cluster in two groups, represented by sequences termed "Extremely Halophilic Bacteria" EHB-1 and EHB-2 (Antón et al., 2000; Fig. 3). The 16S rDNA sequences of the *Salinibacter* isolates obtained from these ponds are nearly identical and cluster with phylotype EHB-1 (Antón et al., 2002). No cultures have yet been isolated that carry the closely related phylotype EHB-2. New related 16S rDNA sequences were recovered from solar salterns on the Balearic islands Ibiza (EHB-3) and La Palma (EHB-4 and EHB-5; Fig. 3). Additional related 16S rDNA sequences have recently been amplified from a microbial mat within a salt crust developing around hypersaline pools at the Badwater site, Death Valley National Park, California (Hollen et al., 2003).

The closest relative of *Salinibacter* on the basis of 16S rDNA sequence is *Rhodothermus*, a genus of slightly halophilic (optimal growth at 0.5–2%

NaCl), thermophilic (optimum 65–70°C, maximum around 77°C) red bacteria isolated from marine hot springs. The properties of *Rhodothermus* were discussed in an earlier section (The Genus Rhodothermus). The 16S rDNA similarity between the *Salinibacter* strains and *Rhodothermus* is about 84–86%.

As specified in the sections below, *Salinibacter* shows many properties that are characteristic of the extremely halophilic Archaea of the order Halobacteriales (see the chapter on The Order Halobacteriales in Volume 3). Many of these common properties point to the possibility of convergent evolution of *Salinibacter* and the Halobacteriales toward adaptation to life at the highest salt concentrations (Oren, 2004a). It may be expected that a comparative genomic analysis of *Salinibacter* and of the halophilic Archaea may yield much relevant information on the nature of halophilism and the way halophilic properties are shared between organisms of disparate phylogenetic affiliation.

The five isolates from Spanish crystallizer ponds studied proved sufficiently similar to be classified in a single species, which was named *Salinibacter ruber* (Antón et al., 2002). The type strain is strain M31^T (DSM 13855; CECT 5946; 16S rDNA accession number: AF322499 [www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AF322499]), isolated from a saltern on Mallorca, Spain (Figs. 12 and 13). The DNA G+C content of the *S. ruber* isolates is 66.3–67.7 mol% (66.5 mol% for the type strain).

Salinibacter ruber can be identified by its cell morphology and by the color of its colonies. Sequence analysis of the 16S rDNA presents the most certain way of identification, enabling also a comparison with the 16S rDNA sequence of the type strain. A specific 16S rRNA-based phylogenetic probe ("EHB412," positions 412–429, 5′-ACACCCCUAUGGGGCGUA-3′) has been developed for fluorescent in situ hybridization and colony hybridization studies for the detection of *Salinibacter* (Antón et al., 2000; Antón et al., 2002).

Morphological and Cultural Characteristics

Salinibacter is a rod-shaped, often slightly curved bacterium measuring 2–6 × 0.4 μm (Figs. 12–14). The cells stain Gram-negative. In contrast to the non-coccoid representatives of the Halobacteriales, which lyse upon suspension in distilled water, maintenance of cell shape in *Salinibacter*

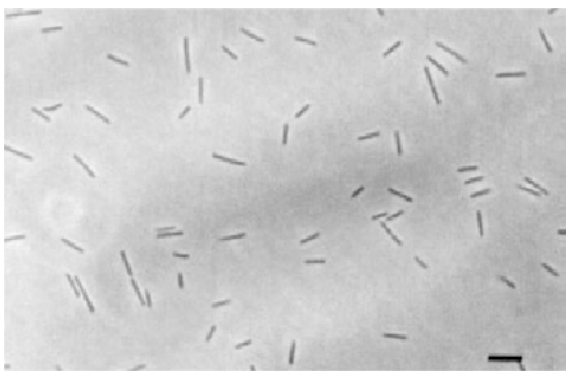

Fig. 12. Phase-contrast micrograph of *Salinibacter ruber* strain M31^T. Bar, 5 μm. From Antón et al. (2002); reproduced with permission.

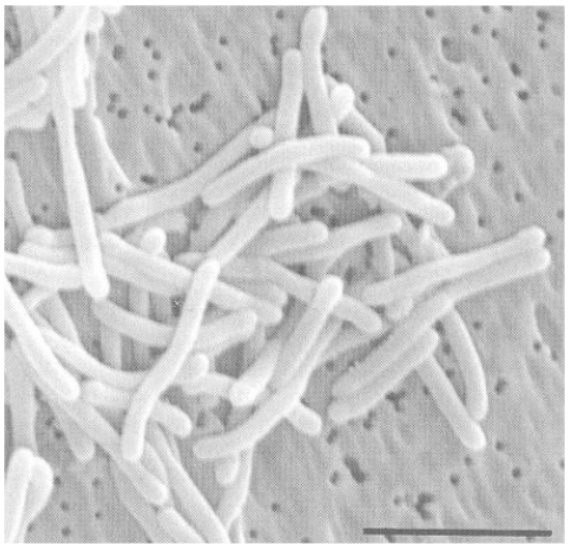

Fig. 13. Scanning electron micrograph of *Salinibacter ruber* strain M31^T. Bar, 2.5 μm. From Antón et al. (2002); reproduced with permission.

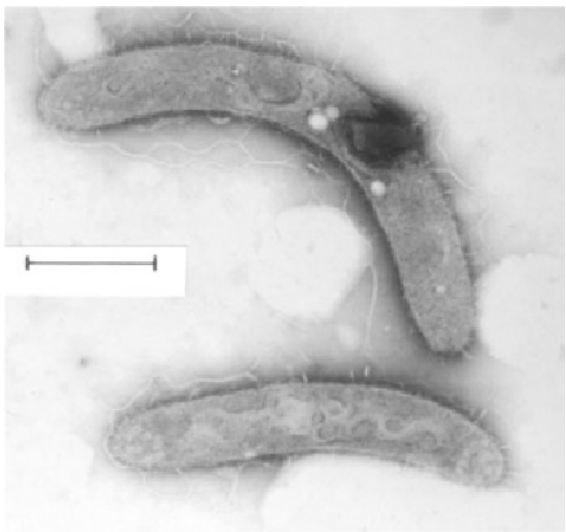

Fig. 14. Electron micrograph of *Salinibacter ruber* strain Pola-18 cells, stained with 1% phosphotungstic acid. Bar is 1 μm.

does not depend on the presence of high salt concentrations. *Salinibacter* is an obligate aerobe, shows positive oxidase and catalase reactions, and does not reduce nitrate.

Two *Salinibacter* isolates were recently obtained from the Badwater site, Death Valley National Park, California (Hollen et al., 2003). Their 16S rRNA sequence is identical and shows 95–96% similarity to that of the sequences directly amplified from that environment (F.A. Rainey, personal communication). The similarity of the 16S sequences of these isolates and the type strain of *S. ruber* is only 93–94%, suggesting that they should be classified in a separate species within the genus.

Physiological, Biochemical and Chemotaxonomic Properties

The major phospholipids detected in *S. ruber* are phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. These phospholipids contain a mixture of monounsaturated (mainly C16:1ω7 and C18:1ω7) and methyl-branched (mainly iso-branched C15:0) fatty acids (Oren et al., 2004b).

Colonies of *Salinibacter* have a bright red-orange color. The absorption spectrum of a cell extract in methanol/acetone (1:1, v/v) shows a maximum at 478 nm and a shoulder at 506–510 nm. The principal (>96 % of total) pigment of *S. ruber* has been identified as a novel acylated glucocarotenoid [(all-*E*, 2′*S*)-2′-hydroxy-1′-[6-*O*-(13-methyltetradecanoyl)-β-D-glycopyranosyloxy-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-4-one], now named "salinixanthin" (Lutnæs et al., 2002; Fig. 8). The esterifying fatty acid, the branched 13-methyltetradecanoic acid, is also the major fatty acid of the membrane lipids of *S. ruber*. The structure of salinixanthin resembles that of pigments of the phylogenetically related *Rhodothermus* (Alfredsson et al., 1988; Lutnæs et al., 2004; see also Fig. 7).

Salinibacter uses KCl rather than organic solvents to osmotically balance its cytoplasm with its highly saline medium. This mode of osmotic adaptation is rarely found in the domain Bacteria. Most halophilic and halotolerant representatives of the Bacteria use organic osmotic solutes instead (see the chapter on The Order Haloanaerobiales in Volume 4). This is true also for the marine, slightly halophilic *Rhodothermus marinus* (phylogenetically the closest known relative of *Salinibacter*), which uses organic solutes (α-mannosylglycerate and α-mannosylglyceramide) as osmotic solutes (Silva et al., 1999; see also the section The Genus Rhodothermus earlier in this Chapter). The only exception known previous to the discovery of *Salinibacter* was a group of anaerobic fermentative halophilic Bacteria of the order Halanaerobiales, affiliated with the low G+C branch of the Firmicutes (see the chapter The Order Halobacteriales in Volume 3). The use of KCl as osmotic solute has been well documented in the aerobic extremely halophilic Archaea of the order Halobacteriales (see the chapter on The Order Halobacteriales in Volume 3).

Intracellular potassium concentrations as high as 11–15 μmol K^+/mg of protein have been measured in *Salinibacter* strains (Antón et al., 2002). These values are in the same range as those detected in *Halobacterium salinarum* (Archaea) and correspond to 4–5 M intracellular K^+, assuming an intracellular volume of 2.75 μl/mg of protein. The presence of high intracellular K^+ concentrations in *S. ruber* was confirmed by X-ray microanalysis of single cells in the electron microscope, a technique that also confirmed the presence of high concentrations of Cl^- as the intracellular counterion, which approximately balances the sum of K^+ and Na^+ (Oren et al., 2002b). Very low concentrations of organic osmotic solutes were found in *Salinibacter* using techniques such as ^{13}C-NMR and high pressure liquid chromatography (HPLC). Compounds detected included glutamate, glycine betaine, and *N*-α-acetyl-lysine. *N*-α-acetyl-lysine was the only compound whose concentration increased with the medium salinity (Oren et al., 2002b). These organic solutes cannot be expected to significantly contribute to the osmotic balance of the cells in view of the low concentrations in which they were found.

If indeed KCl is the main osmotic stabilizer inside the cells, the intracellular enzymatic machinery should be fully adapted to the presence of high salt concentrations. In the halophilic Archaea of the order Halobacteriales such adaptation of the proteins to high salt has led to characteristic modifications of protein structure, including a high content of acidic amino acids, a low content of basic amino acids, a low content of hydrophobic amino acids, and a relatively high content of "borderline hydrophobic" amino acids serine and threonine (see the chapter on Life at High Salt Concentrations in Volume 2). Such salt-adapted proteins generally require high salt for stability and activity, and they often denature at low salt concentrations. Examination of the amino acid composition of the bulk protein of *S. ruber* showed a similar pattern as found in the Halobacteriales. The content of Asx and Glx of the *S. ruber* proteins is almost as high as that of the halophilic Archaea *Halobacterium salinarum* and *Haloarcula marismortui* (Asx + Glx = 29.2 mol%, as compared to 31.8 and 32.3 mol%, respectively), and a high apparent excess of acidic amino acids was found as well ([Asx + Glx] – [Lys + Arg] is 21.2 mol%, as compared to

25.4 and 26.2 mol% for the two halophilic Archaea). A low content of hydrophobic amino acids and a high content of serine were also observed in the *Salinibacter* bulk protein (Oren and Mana, 2002a).

The halophilic nature of the proteins of *S. ruber* was confirmed for a number of intracellular enzymatic activities. As shown in Table 12, most enzymes studied showed a marked halophilic character, most had their optimal activity at high salt concentrations, and many proved inactive in the absence of salt. The activities tested included enzymes of the central dissimilatory metabolism (isocitrate dehydrogenase and malate dehydrogenase), a central enzyme in amino acid metabolism (glutamate dehydrogenase), enzymes involved in sugar and glycerol dissimilation (hexokinase, glucose-6-phosphate dehydrogenase, glycerol dehydrogenase, and glycerol kinase), and the fatty acid synthetase complex, essential for lipid biosynthesis. Although the general trend of salt dependence of proteins is clear, significant differences occur in the relation to salt of the individual enzymes.

Isolation and Maintenance

The strains described in the literature (Antón et al., 2002) have all been isolated by plating dilutions of saltern crystallizer brine on agar plates (media described in Table 13). They were recognized as members of the genus *Salinibacter* either by colony hybridization with a probe designed to detect the *Salinibacter* 16S rRNA phylotype or by thin layer chromatography of polar lipids extracted from subcultures of selected orange colored colonies.

Table 12. The relation of several intracellular enzymatic activities of *S. ruber* to salt.

Enzyme	Salt requirement	Comments	References
Isocitrate dehydrogenase (NAD-dependent)	Optimum at 0.5–2M KCl, with rates of 60% of the optimum value at 3.3M; 70% of the optimum rates in KCl were found at 0.2–1.2M NaCl; and low activity above 3M NaCl	None	Oren and Mana, 2002a
Isocitrate dehydrogenase (NAD-dependent)	Activity approximately constant between 0 and 3.2M NaCl and increased with increasing KCl concentration	None	Oren and Mana, 2002a
Malate dehydrogenase (NAD-dependent)	Optimal activity in the absence of salt; approximately 25% of the maximum activity remained at NaCl and KCl concentrations above 3M	None	Oren and Mana, 2002a
Glutamate dehydrogenase (NAD-dependent)	Two enzymes were found (GDHI and GDHII); GDHI depends on high salt concentrations for stability, but GDHI is optimally active in the absence of salts; GDHII depends on high salt concentrations for both activity and stability; amination of 2-oxoglutarate is optimal in 3M KCl at pH 8; no activating effect was found when NaCl was replaced by KCl	Both enzymes catalyze reductive amination of α-ketoglutarate, but only GDHII displayed activity in the deamination reaction of glutamate; both enzymes were activated by certain amino acids and nucleotides; a low-molecular-weight cytoplasmic fraction activates GDHII in the presence of high NaCl concentrations	Oren and Mana, 2002a; Bonete et al., 2003
Hexokinase	Activity is inhibited by salt, and no activity was recorded in the presence of 2.1M KCl or 2.8M NaCl	None	Oren and Mana, 2003
Glucose-6-phosphate dehydrogenase (NADP-dependent)	Activity is optimal above 1.5–2M NaCl or KCl; no activity found below 0.8M salt	Constitutive	Oren and Mana, 2003
Glycerol kinase	Activity is similar from 0.6–2.8M KCl	Inducible	Sher et al., 2004
Fatty acid synthetase	Optimal activity found at 0.5–1.5M salt; little activity in the absence of salt	None	Oren et al., 2004a

Table 13. Media for the cultivation of *Salinibacter*.

Ingredient	DSMZ medium 936[b]	Modified R_2A medium[b]
NaCl	195.0 g/liter	200.0 g/liter
$MgCl_2 \cdot 6H_2O$	34.6 g/liter[a]	NA
$MgSO_4 \cdot 7H_2O$	49.5 g/liter[a]	20.0 g/liter
$CaCl_2 \cdot 2H_2O$	1.25 g/liter	NA
KCl	5.0 g/liter	0.1 g/liter
$NaHCO_3$	0.25 g/liter	NA
NaBr	0.625 g/liter	0.3 g/liter
KH_2PO_4	NA	NA
Yeast extract	1.0 g/liter	0.5 g/liter
Bacto-peptone	NA	0.5 g/liter
Casamino acids	NA	0.5 g/liter
Glucose	NA	0.5 g/liter
Starch	NA	0.5 g/liter
Na-pyruvate	NA	0.3 g/liter

Abbreviation: NA, not added.

[a]The author uses a version of this medium in which the concentrations of $MgCl_2 \cdot 6H_2O$ and $MgSO_4 \cdot 7H_2O$ are reduced to 25.0 and 16.3 g/liter, respectively.

[b]Adjust the final pH to 7.0. Liquid media may be solidified with agar (20 g/liter).

The fact that *Salinibacter* is insensitive to the antibiotics anisomycin and bacitracin, two antibiotics that are highly inhibitory to halophilic Archaea of the order Halobacteriales (see below), can be used to design selective enrichment and isolation protocols. Inoculation of saltern crystallizer brine from Eilat, Israel, into suitable growth medium amended with anisomycin (15 mg/liter) gave rise to a culture of *Salinibacter*. Similarly, when such a brine sample was inoculated on agar plates of modified R_2A medium (see Table 13) with bacitracin (25 mg/liter), most of the colonies that developed after 2–3 weeks of incubation at 35°C were orange colored *Salinibacter*-type cells.

Salinibacter can be grown in media with relatively low concentrations of yeast extract and other nutrients such as amino acids, in the presence of salt (200–250 g/liter). High nutrient levels are inhibitory for growth. Table 13 gives the composition of two media that proved suitable for its cultivation.

Salt concentrations of at least 150 g/liter are required, and all strains grow at NaCl concentrations up to saturation. The optimal pH range for growth is 6.5–8.0, and the temperature optimum is 35–45°C. Under optimal growth conditions doubling times of around 14–18 h are obtained.

Salinibacter is an obligate aerobe that does not reduce nitrate. Amino acids appear to be the preferred nutrients for growth. Most strains hydrolyze gelatin. According to the original description of *S. ruber* (Antón et al., 2002), growth is not stimulated by sugars at a concentration of 5 g/liter. However, it was later found that glucose, maltose, starch, and glycerol significantly increase the growth yield when added at a concentration of 0.5–1 g/liter (Oren and Mana, 2003; Oren et al., 2003). Glucose degradation starts only after more easily metabolizable substrates have been depleted from the medium. As a result, diauxic growth is obtained in medium containing both yeast extract and glucose as carbon and energy sources. No acidic products are produced during sugar metabolism. Enzymological studies suggest that glucose is metabolized by the Entner-Doudoroff pathway via hexokinase and NADP-dependent glucose-6-phosphate hydrogenase (Oren and Mana, 2003). No diauxic growth was obtained in media containing glycerol and yeast extract. Metabolism of glycerol was shown to involve an inducible glycerol kinase (Sher et al., 2004).

Salinibacter ruber is sensitive to penicillin G, ampicillin, chloramphenicol, streptomycin, novobiocin, rifampicin and ciprofloxacin. No growth inhibition was found by kanamycin, bacitracin, tetracycline and colistin. The cells are resistant to anisomycin and aphidicolin, two potent growth inhibitors of halophilic Archaea of the order Halobacteriales.

In microbiological studies of salterns and salt lakes the color of colonies on agar plates has often been used to discriminate between halophilic Archaea (red colonies) and halophilic or halotolerant representatives of the Bacteria (colorless colonies). The discovery of *Salinibacter* as a quantitatively important component of the prokaryotic community in certain hypersaline ecosystems shows that this approach may lead to incorrect identifications. It is quite possible that colonies of *Salinibacter* were observed long before the species was recognized, but were erroneously considered to be halophilic Archaea. Colony color alone is therefore insufficient to allow classification of extremely halophilic prokaryotes as members of the Archaea or the Bacteria. The color of *Salinibacter* colonies is more orange and less red-pink than that of colonies of most members of the Halobacteriales on high salt agar media. The absorption spectrum of a pigment extract in methanol-acetone (1:1, v/v) may help identify a culture as a member of the genus *Salinibacter* (salinixanthin; absorbance peak at 478 nm with a broad shoulder at 506–510 nm) or the halophilic Archaea (bacteriorubberin derivatives [see the chapter on The Order Halobacteriales in Volume 3] with absorbance maxima at 496 nm and 530 nm and a shoulder at 470 nm).

Salinibacter ruber can be preserved by lyophilization.

Ecology

Until recently there was little evidence that heterotrophic representatives of the domain Bacteria played a significant role in the microbial community inhabiting salt-saturated environments. However, now it has become clear that rod-shaped prokaryotes that carry the *Salinibacter* phylotype may be abundant in saltern crystallizer ponds, where they occur together with different types of halophilic Archaea of the order Halobacteriales (Antón et al., 1999; Antón et al., 2000). Fluorescent in situ hybridization experiments have shown that *Salinibacter* may represent up to 5–25% of the total prokaryote community in such environments (Antón et al., 2000). Moreover, pigment analysis has demonstrated that the salinixanthin of *Salinibacter* may contribute up to about 5% of the total prokaryotic carotenoids that color red-pink the crystallizer brines of the salterns near Alicante, Spain, and on Mallorca (Oren and Rodríguez-Valera, 2001). *Salinibacter* has now also been isolated from saltern crystallizer ponds in Israel (A. Oren and R. Elevi, unpublished results). The genus may thus occur widespread in hypersaline environments of near-neutral pH with salt concentrations between 150 g/liter and NaCl saturation, as was shown both by the finding of characteristic 16S rDNA sequences and by the direct isolation of new strains from the salt crust around saline pools in Death Valley, California (Hollen et al., 2003).

Salinibacter has nutritional requirements similar to those of the halophilic Archaea of the order Halobacteriales. The two groups may therefore be expected to compete for substrates such as amino acids and sugars in their natural environment. Glycerol is probably a readily available carbon and energy source in hypersaline water bodies, as it is produced in high quantities as osmotic solute by unicellular green algae of the genus *Dunaliella* (see the chapter on Life at High Salt Concentrations in Volume 2). It was shown that dissimilation of glycerol is an inducible property in *S. ruber* (Sher et al., 2004). Curiously, a recent study in which microautoradiography was combined with fluorescent in situ hybridization to obtain information about the organic substrates used by the microbial community in a saltern crystallizer pond on Mallorca suggested that glycerol was incorporated neither by *Salinibacter* nor by the members of the Halobacteriales present (Rosselló-Mora et al., 2003). The elucidation of the factors determining the community dynamics of *Salinibacter* and the halophilic Archaea in their shared habitat and the interrelationships between the two physiologically similar but phylogenetically distant groups will be a challenging topic for further study.

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Literature Cited

Abou Hachem, M., E. Nordberg Karlsson, E. Bartonek-Roxå, S. Raghothama, P. J. Simpson, H. J. Gilbert, M. P. Williamson, and O. Holst. 2000. Carbohydrate-binding modules from a thermostable Rhodothermus marinus xylanase: cloning, expression and binding studies. Biochem. J. 345:53–60.

Abou Hachem, M., E. Nordberg Karlsson, P. J. Simpson, S. Linse, P. Sellers, M. P. Williamson, S. J. Jamieson, H. J. Gilbert, D. N. Bolam, and O. Holst. 2002. Calcium binding and thermostability of carbohydrate binding module CBM4-2 of Xyn10A from Rhodothermus marinus. Biochemistry 41:5720–5729.

Alfredsson, G. A., J. K. Kristjansson, S. Hjörleifsdóttir, and K. O. Stetter. 1988. Rhodothermus marinus, gen. nov., sp. nov., a thermophilic, halophilic bacterium from submarine hot springs in Iceland. J. Gen. Microbiol. 134:299–306.

Andrésson, O. S., and Ó. H. Friðjónsson. 1994. The sequence of the single 16S rRNA gene of the thermophilic eubacterium Rhodothermus marinus reveals a distant relationship to the group containing Flexibacter, Bacteroides, and Cytophaga species. J. Bacteriol. 176: 6165–6169.

Antón, J., E. Llobet-Brossa, F. Rodríguez-Valera, and R. Amann. 1999. Fluorescence in situ hybridization analysis of the prokaryotic community inhabiting crystallizer ponds. Environ. Microbiol. 1:517–523.

Antón, J., R. Rosselló-Mora, F. Rodríguez-Valera, and R. Amann. 2000. Extremely halophilic Bacteria in crystallizer ponds from solar salterns. Appl. Environ. Microbiol. 66:3052–3057.

Antón, J., A. Oren, S. Benlloch, F. Rodríguez-Valera, R. Amann, and R. Rosselló-Mora. 2002. Salinibacter ruber gen. nov., sp. nov., a novel, extreme halophilic member of the Bacteria from saltern crystallizer ponds. Int. J. Syst. Evol. Microbiol. 52:485–491.

Bircher, C., and H. Pfander. 1997. Synthesis of carotenoids in the gliding bacteria Taxeobacter: (all-E,2′R)-3-deoxy-2′-hydroxyflexixanthin. Helv. Chim. Acta 80:832–837.

Blöndal, T., S. H. Thorbjarnardóttir, J. Kieleczawa, J. M. Einarsson, S. Hörleifsdóttir, J. K. Kristjánsson, and G. Eggertsson. 1999. Cloning, sequence analysis and overexpression of a Rhodothermus marinus gene encoding a thermostable thymidine kinase. FEMS Microbiol. Lett. 179:311–316.

Blöndal, T., S. H. Thorbjarnardóttir, J. Kieleczawa, S. Hörleifsdóttir, J. K. Kristjánsson, J. M. Einarsson, and G. Eggertsson. 2000. Cloning, sequence analysis and functional characterization of DNA polymerase I from the thermophilic eubacterium Rhodothermus marinus. Biotechnol. Appl. Biochem. 34:37–45.

Blücher, A., E. Nordberg Karlsson, and O. Holst. 2000. Substrate-dependent production and some properties of a thermostable, α-galactosidase from Rhodothermus marinus. Biotechnol. Lett. 22:663–669.

Bonete, M.-J., F. Pérez-Pomares, S. Díaz, J. Ferrer, and A. Oren. 2003. Occurrence of two different glutamate

dehydrogenase activities in the halophilic bacterium Salinibacter ruber. FEMS Microbiol. Lett. 226:181–186.

Borges, N., A. Ramos, N.D.H. Raven, R.J. Sharp, and H. Santos. 2002. Comparative study of the thermostabilizing properties of mannosylglycerate and other compatible solutes on model enzymes. Extremophiles 6:209–216.

Buczolits, S., E. B. M. Denner, D. Vybrial, M. Wieser, P. Kämpfer, and H.-J. Busse. 2002. Classification of three airborne bacteria and proposal of Hymenobacter aerophilus sp. nov. Int. J. Syst. Evol. Microbiol. 52:445–456.

Chung, A. P., O. C. Nunes, B. J. Tindall, and M. S. da Costa. 1993. The effect of the growth medium composition on the fatty acids of Rhodothermus marinus and "Thermus thermophilus" HB-8. FEMS Microbiol. Lett. 112:13–18.

Collins, M. D., R. A. Hutson, I. R. Grant, and M. F. Patterson. 2000. Phylogenetic characterization of a novel radiation-resistant bacterium from irradiated pork: description of Hymenobacter actinosclerus sp. nov. Int. J. Syst. Evol. Microbiol. 50:731–734.

Crennell, S. J., G. O. Hreggvidsson, and E. Nordberg Karlsson. 2002. The structure of Rhodothermus marinus Cel12A, a highly thermostable family 12 endoglucanase, at 1.8 Å resolution. J. Molec. Biol. 320:883–897.

Dahlberg, L., O. Holst, and J. K. Kristjansson. 1993. Thermostable xylanolytic enzymes from Rhodothermus marinus grown on xylan. Appl. Microbiol. Biotechnol. 40:63–68.

Dahlberg, L. A. L., O. P. Holst, and L. Anker. 2002. Thermostable Xylanase From A Strain of Rhodothermus marinus. Canadian Patent 2,119,912.

Ernstsson, S., S. H. Bjornsdottir, Z. O. Jónsson, S. H. Thorbjarnardóttir, G. Eggertsson, and A. Palsdottir. 2003. Identification and nucleotide sequence analysis of a cryptic plasmid, pRM21, from Rhodothermus marinus. Plasmid 49:188–191.

Fernandes, A. S., M. M. Pereira, and M. Teixeira. 2001a. Complexes I and II from the respiratory chain of the bacterium Rhodothermus marinus. J. Inorg. Biochem. 86:217.

Fernandes, A. S., M. M. Pereira, and M. Teixeira. 2001b. The succinate dehydrogenase from the thermohalophilic bacterium Rhodothermus marinus: redox-Bohr effect on heme b_L^1. J. Bioenerg. Biomembr. 33:343–352.

Fernandes, A. S., M. M. Pereira, and M. Teixeira. 2002. Purification and characterization of the complex I from the respiratory chain of Rhodothermus marinus. J. Bioenerg. Biomembr. 34:413–421.

Gomes, J., and W. Steiner. 1998. Production of a high activity of an extremely thermostable β-mannanase by the thermophilic eubacterium Rhodothermus marinus, grown on locust bean gum. Biotechnol. Lett. 20:729–733.

Gomes, J., I. Gomes, K. Terler, N. Gubala, G. Ditzelmüller, and W. Steiner. 2000. Optimisation of culture medium and conditions for α-L-arabinofuranosidase by the extreme thermophilic eubacterium Rhodothermus marinus. Enz. Microb. Technol. 27:414–422.

Gomes, I., J. Gomes, and W. Steiner. 2003. Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium Rhodothermus marinus: production and partial characterization. Biores. Technol. 90:207–214.

Gudmundsdóttir, K., E. Sigurdsson, S. I. Thorbjarnardóttir, and G. Eggertsson. 1999. Cloning and sequence analysis of the hemB gene of Rhodothermus marinus. Curr. Microbiol. 39:103–105.

Halldórsdóttir, S., E. T. Thórólfsdóttir, R. Spilliaert, M. Johansson, S. H. Thorbjarnardóttir, A. Palsdottir, G.Ó. Hreggvidsson, J. K. Kristjánsson, O. Holst, and G. Eggertsson. 1988. Cloning, sequencing and overexpression of a Rhodothermus marinus gene encoding a thermostable cellulose of glycosyl hydrolase family 12. Appl. Microbiol. Biotechnol. 49:277–284.

Hamana, K., H. Hamana, M. Niitsu, K. Samejima, and S. Matsuzaki. 1992. Distribution of unusual long and branched polyamines in thermophilic eubacteria belonging to "Rhodothermus" Thermus and Thermonema. J. Gen. Appl. Microbiol. 38:575–584.

Hamana, K., M. Niitsu, K. Samejima, T. Itoh, H. Hamana, and T. Shinozawa. 1998. Polyamines of the thermophilic eubacteria belonging to the genera Thermotoga, Thermodesulfovibrio, Thermoleophilum, Thermus, Rhodothermus and Meiothermus, and the thermophilic archaebacteria belonging to the genera Aeropyrum, Picrophilus, Methanobacterium and Methanococcus. Microbios 94:7–21.

Hirsch, P., W. Ludwig, C. Hethke, M. Sittig, B. Hoffmann, and C. A. Gallikowski. 1998. Hymenobacter roseosalivarius gen. nov., sp. nov. from continental Antarctic soils and sandstone: bacteria of the Cytophaga/Flavobacterium/Bacteroides line of phylogenetic descent. Syst. Appl. Microbiol. 21:374–383.

Hörleifsdóttir, S., S. K. Pétursdottir, J. Korpela, A. M. Torsti, P. Mattila, and J. K. Kristjánsson. 1997a. Screening for restriction endonucleases in aerobic, thermophilic eubacteria. Biotechnol. Tech. 10:13–18.

Hjörleifsdóttir, S., W. Ritterbusch, S. K. Petursdottir, and J. K. Kristjansson. 1997b. Thermostabilities of DNA ligases and DNA polymerases from four genera of thermophilic eubacteria. Biotechnol. Lett. 19:147–150.

Hollen, B. J., D. R. Bagaley, A. M. Small, A. Oren, C. P. McKay, and F. A. Rainey. 2003. Investigation of the microbial community of the salt surface layer at Badwater, Death Valley National Park. *In:* American Society for Microbiology Annual Meeting. Washington, DC.

Housby, J. N., S. H. Thorbjanardóttir, Z. O. Jónsson, and E. M. Southern. 2000. Optimised ligation of oligonucleotides by thermal ligases: comparison of Thermus scotoductus and Rhodothermus marinus DNA ligases to other thermophilic ligases. Nucl. Acids. Res. 28:E10.

Housby, J. N., and E. M. Southern. 2002. Thermus scotoductus and Rhodothermus marinus DNA ligases have higher ligation efficiencies than Thermus thermophilus DNA ligase. Analyt. Biochem. 302:88–94.

Hreggvidsson, G. O., E. Kaiste, O. Holst, G. Eggertsson, A. Palsdottir, and J. K. Kristjansson. 1996. An extremely thermostable cellulase from the thermophilic eubacterium Rhodothermus marinus. Appl. Environ. Microbiol. 62:3047–3049.

Hudson, J. A., K. M. Schofield, H. W. Morgan, and R. M. Daniel. 1989. Thermonema lapsum gen. nov., sp. nov., a thermophilic gliding bacterium. Int. J. Syst. Bacteriol. 39:485–487.

Jamieson, S. J., M. P. Williamson, M. Abou Hachem, E. Nordberg Karlsson, and P. J. Simpson. 2002. Letter to the editor: Virtually complete ^{1}H, ^{13}C and ^{15}N resonance assignments of the second family 4 xylan binding module of Rhodothermus marinus xylanase 10A. J. Biomol. NMR 22:187–188.

Krah, M., R. Misselwitz, O. Politz, K. K. Thomsen, H. Welfle, and R. Borriss. 1998. The laminariase from thermophilic eubacterium Rhodothermus marinus: Conformation, stability, and identification of active site carboxylic acid residues by site-directed mutagenesis. Eur. J. Biochem. 257:101–111.

Kristjánsson, J. K., and G. A. Alfredsson. 1992. The heterotrophic, thermophilic genera Thermomicrobium, Rhodothermus, Saccharococcus, Acidothermus and Scotothermus. *In:* J. K. Kristjánsson (Ed.) Thermophilic Bacteria. CRC Press. Boca Raton, FL. 63–76.

Liu, X.-Q., and Z. Hu. 1997. A DnaB intein in Rhodothermus marinus: indication of recent intein homing across remotely related organisms. Proc. Natl. Acad. Sci. USA 94:7851–7856.

Lutnæs, B. F., A. Oren, and S. Liaaen-Jensen. 2002. New C_{40}-carotenoid acyl glycoside as principal carotenoid in Salinibacter ruber, an extremely halophilic eubacterium. J. Nat. Prod. 65:1340–1343.

Lutnæs, B. F., Å. Strand, S. K. Pétursdóttir, and S. Liaaen-Jensen. 2004. Carotenoids of thermophilic bacteria: Rhodothermus marinus from submarine Icelandic hot springs. Biochem. Syst. Ecol. 32:445–468.

Manelius, Å., L. Dahlberg, and O. Holst. 1994. Some properties of a thermophilic β-xylosidase from Rhodothermus marinus. Appl. Biochem. Biotechnol. 44:39–48.

Marteinsson, V. T., J. K. Kristjánsson, H. Kristmannsdóttir, M. Dahlkvist, K. Sæmundsson, M. Hannington, S. K. Pétursdóttir, A. Geptner, and P. Stoffers. 2001. Discovery and description of giant submarine smectite cones on the seafloor in Eyjafjordur, northern Island, and a novel thermal microbial habitat. Appl. Environ. Microbiol. 67:827–833.

Martins, L. O., N. Empadinhas, J. D. Marugg, C. Miguel, C. Ferreira, M. S. da Costa, and H. Santos. 1999. Biosynthesis of mannosylglycerate in the thermophilic bacterium Rhodothermus marinus: Biochemical and genetic characterization of a mannosylglycerate synthase. J. Biol. Chem. 274:35407–35414.

Moreira, L., M. F. Nobre, I. Sá-Correira, and M. S. da Costa. 1996. Genomic typing and fatty acid composition of Rhodothermus marinus. Syst. Appl. Microbiol. 19:83–90.

Nakagawa, T., and M. Fukui. 2002. Phylogenetic characterization of microbial mats and streamers from a Japanese alkaline hot spring with a thermal gradient. J. Gen. Appl. Microbiol. 48:211–222.

Nordberg Karlsson, E., P. Linå Jorgensen, L. Dahlberg, and O. Holst. 1996. Cloning of xylanolytic activities from Rhodothermus marinus. *In:* E. Srebotnik and K. Messner (Eds.) Biotechnology in the Pulp and Paper Industry: Recent Advances in Applied and Fundamental Research. Facultas-Universitätsverlag. Vienna, Austria. 513–516.

Nordberg Karlsson, E., E. Bartonek-Roxå, and O. Holst. 1997. Cloning and sequence of a thermostable multidomain xylanase from the bacterium Rhodothermus marinus. Biochim. Biophys. Acta 1353:118–124.

Nordberg Karlsson, E., E. Bartonek-Roxå, and O. Holst. 1998a. Evidence for substrate binding of a recombinant thermostable xylanase originating from Rhodothermus marinus. FEMS Microbiol. Lett. 168:1–7.

Nordberg Karlsson, E., L. Dahlberg, N. Torto, L. Gorton, and O. Holst. 1998b. Enzymatic specificity and hydrolysis pattern of the catalytic domain of the xylanase Xyn1 from Rhodothermus marinus. J. Biotechnol. 60:23–35.

Nordberg Karlsson, E., O. Holst, and A. Tocaj. 1999. Efficient production of truncated thermostable xylanases from Rhodothermus marinus in Escherichia coli fed-batch cultures. J. Biosci. Bioengin. 87:598–606.

Nordberg Karlsson, E., M. Abou Hachem, O. Holst, M. J. Danson, and D. W. Hough. 2002. Rhodothermus marinus: a thermophilic bacterium producing dimeric and hexameric citrate synthase isoenzymes. Extremophiles 6:51–56.

Nunes, O. C., M. M. Donato, and M. S. da Costa. 1992a. Isolation and characterization of Rhodothermus strains from S. Miguel, Azores. Syst. Appl. Microbiol. 15:92–97.

Nunes, O. C., M. M. Donato, C. M. Manaia, and M. S. da Costa. 1992b. The polar lipid and fatty acid composition of Rhodothermus strains. Syst. Appl. Microbiol. 15:59–62.

Nunes, O. C., C. M. Manaia, M. S. da Costa, and H. Santos. 1995. Compatible solutes in the thermophilic bacteria Rhodothermus marinus and "Thermus thermophilus." Appl. Environ. Microbiol. 61:2351–2357.

Oren, A., and F. Rodríguez-Valera. 2001. The contribution of halophilic Bacteria to the red coloration of saltern crystallizer ponds. FEMS Microbiol. Ecol. 36:123–130.

Oren, A., and L. Mana. 2002a. Amino acid composition of bulk protein and salt relationships of selected enzymes of Salinibacter ruber, an extremely halophilic bacterium. Extremophiles 6:217–223.

Oren, A., M. Heldal, S. Norland, and E. A. Galinski. 2002b. Intracellular ion and organic solute concentrations of the extremely halophilic bacterium Salinibacter ruber. Extremophiles 6:491–498.

Oren, A., and L. Mana. 2003. Sugar metabolism in the extremely halophilic bacterium Salinibacter ruber. FEMS Microbiol. Lett. 223:83–87.

Oren, A. 2004a. Convergent evolution in extremely halophilic prokaryotes: A comparison between Salinibacter ruber (Bacteria) and the Halobacteriaceae (Archaea). *In:* S. P. Wasser (Ed.) Evolutionary Theory and Processes: Modern Horizons. Papers in Honour of Eviatar Nevo. Kluwer Academic Publishers. Dordrecht, The Netherlands. 43–64.

Oren, A., F. Rodríguez-Valera, J. Antón, S. Benlloch, R. Rosselló-Mora, R. Amann, J. Coleman, and N. J. Russell. 2004b. Red, extremely halophilic, but not archaeal: the physiology and ecology of Salinibacter ruber, a bacterium isolated from saltern crystallizer ponds. *In:* A. Ventosa (Ed.) Halophilic Microorganisms. Springer-Verlag. Berlin, Germany. 63–76.

Patel, B. K. C., D. S. Saul, R. A. Reeves, L. C. Williams, J.-E. Cavanagh, P. D. Nichols, and P. L. Bergquist. 1994. Phylogeny and lipid composition of Thermonema lapsum, a thermophilic gliding bacterium. FEMS Microbiol. Lett. 115:313–318.

Pereira, M. M., A. M. Antunes, O. C. Nunes, M. S. da Costa, and M. Teixeira. 1994. A membrane-bound HIPIP type center in the thermohalophile Rhodothermus marinus. FEBS Lett. 352:327–330.

Pereira, M. M., J. N. Carita, and M. Teixeira. 1999a. Membrane-bound electron transfer chain of the thermohalophilic bacterium Rhodothermus marinus: A novel multihemic cytochrome bc, a new complex III. Biochemistry 38:1268–1275.

Pereira, M. M., J. N. Carita, and M. Teixeira. 1999b. Membrane-bound electron transfer chain of the thermophilic bacterium Rhodothermus marinus: Characterization of the iron-sulfur centers from the dehydrogenases and

investigation of the high-potential iron-sulfur protein function by in vitro reconstitution of the respiratory chain. Biochemistry 38:1276–1283.

Pereira, M. M., M. Santana, C. M. Soares, J. Mendes, J. N. Carita, A. S. Fernandes, M. Saraste, M. A. Carrondo, and M. Teixeira. 1999c. The caa_3 terminal oxidase of the thermohalophilic bacterium Rhodothermus marinus: a HiPIP:oxygen oxidoreductase lacking the key glutamate of the D-channel. Biochim. Biophys. Acta 1413:1–13.

Pereira, M. M., J. N. Carita, R. Anglin, M. Saraste, and M. Teixeira. 2000a. Heme centers of Rhodothermus marinus respiratory chain: Characterization of its cbb3 oxidase. J. Bioenerg. Biomembr. 32:143–152.

Pereira, M. M., M. L. Verkhovskaya, M. Teixeira, and M. I. Verkhovsky. 2000b. The caa_3 terminal oxidase of Rhodothermus marinus lacking the key glutamate of the D-channel is a proton pump. Biochemistry 39:6336–6340.

Pereira, M. M., K. L. Jones, M. G. Campos, A. M. P. Melo, L. M. Saraiva, R. O. Louro, P. Wittung-Stafshede, and M. Teixeira. 2002. A ferredoxin from the thermohalophilic bacterium Rhodothermus marinus. Biochim. Biophys. Acta 1601:1–8.

Petersen, B. O., M. Krah, J. Ø. Duus, and K. K. Thomsen. 2000. A transglycosylating 1,3(4)-β-glucanase from Rhodothermus marinus: NMR analysis of enzyme reactions. Eur. J. Biochem. 267:361–369.

Pfabigan, N., E. Nordberg Karlsson, G. Ditzelmueller, and O. Holst. 2002. Prebleaching of kraft pulp with full-length and truncated forms of a thermostable modular xylanase from Rhodothermus marinus. Biotechnol. Lett. 24:1191–1197.

Politz, O., M. Krah, K. K. Thomsen, and R. Borriss. 2000. A highly thermostable endo-(1,4)-β-mannanase from the marine bacterium Rhodothermus marinus. Appl. Microbiol. Biotechnol. 53:715–721.

Ramchuran, S. O., E. Nordberg Karlsson, S. Velut, L. de Maré, P. Hagander, and O. Holst. 2002. Production of heterologous thermostable glycoside hydrolases and the presence of host-cell proteases in substrate limited fed-batch cultures of Escherichia coli BL21(DE3). Appl. Microbiol. Biotechnol. 60:408–416.

Reichenbach, H. 1991. Taxeobacter, a new genus of the Flavobacterium-Cytophaga group. Bioforum 14:35.

Reichenbach, H. 1992. Taxeobacter, a new genus of the Cytophagales with three new species. *In:* P. J. Joste (Ed.) Advances in the Taxonomy and Significance of Flavobacterium, Cytophaga, and Related Bacteria. University Press. Bloemfontein, South Africa. 182–185.

Rönkä, J., S. Hörleifsdóttir, T. Tenkanen, K. Pitkänen, P. Mattila, and J. K. Kristjansson. 1991. Rma1, a type II restriction endonuclease from Rhodothermus marinus which recognizes 5′ CTAG 3′. Nucl. Acids Res. 19:2789.

Rosselló-Mora, R., N. Lee, J. Antón, and M. Wagner. 2003. Substrate uptake in extremely halophilic microbial communities revealed by microautoradiography and fluorescence in situ hybridization. Extremophiles 7:409–413.

Sako, Y., K. Takai, Y. Ishida, A. Uchida, and Y. Katayama. 1996. Rhodothermus obamensis sp. nov., a modern lineage of extremely thermophilic marine bacteria. Int. J. Syst. Bacteriol. 46:1099–1104.

Sampaio, M.-M., H. Santos, and W. Boos. 2003. Synthesis of GDP-mannose and mannosylglycerate from labeled mannose by genetically engineered Escherichia coli without loss of specific isotopic enrichment. Appl. Environ. Microbiol. 69:233–240.

Santana, M., M. M. Pereira, N. P. Elias, C. M. Soares, and M. Teixeira. 2001. Gene cluster of Rhodothermus marinus high-potential iron-sulfur protein:oxygen oxidoreductase, a caa_3-type oxidase belonging to the superfamily of heme-copper oxidases. J. Bacteriol. 183:687–699.

Santos, H., and M. S. da Costa. 2002. Compatible solutes of organisms that live in hot saline environments. Environ. Microbiol. 4:501–509.

Schofield, K. M., J. A. Hudson, H. W. Morgan, and R. M. Daniel. 1987. A thermophilic gliding bacterium from New Zealand hot springs. FEMS Microbiol. Lett. 40:169–172.

Sher, J., R. Elevi, L. Mana, and A. Oren. 2004. Glycerol metabolism in the extremely halophilic bacterium Salinibacter ruber. FEMS Microbiol. Lett. 432:211–215.

Shinohara, M. L., M. Ihara, M. Abo, M. Hashida, S. Takagi, and T. C. Beck. 2001. A novel thermostable branching enzyme from an extremely thermophilic bacterial species, Rhodothermus obamensis. Appl. Microbiol. Biotechnol. 57:653–659.

Sigurdson, H., A. Namslauer, M. M. Pereira, M. Teixeira, and P. Brzezinski. 2001. Ligand binding and the catalytic reaction of cytochrome caa3 from the thermophilic bacterium Rhodothermus marinus. Biochemistry 40:10578–10585.

Silva, Z., N. Borges, L.O. Martins, R. Wait, M. S. da Costa, and H. Santos. 1999. Combined effect of the growth temperature and salinity of the medium on the accumulation of compatible solutes by Rhodothermus marinus and Rhodothermus obamensis. Extremophiles 3:163–172.

Silva, Z., C. Horta, M. S. da Costa, A. P. Chung, and F. A. Rainey. 2000. Polyphasic evidence for the reclassification of Rhodothermus obamensis Sako et al. 1996 as a member of the species Rhodothermus marinus Alfredsson et al. 1988. Int. J. Syst. Evol. Microbiol. 50:1457–1461.

Simpson, P. J., S. J. Jamieson, M. Abou Hachem, E. Nordberg Karlsson, H. J. Gilbert, O. Holst, and M. P. Williamson. 2002. The solution structure of the CBM4-2 carbohydrate binding module from a thermostable Rhodothermus marinus xylanase. Biochemistry 41:5712–5719.

Spilliaert, R., G. O. Hreggvidsson, J. K. Kristjansson, G. Eggertsson, and A. Palsdottir. 1994. Cloning and sequencing of a Rhodothermus marinus gene, bglA, coding for a thermostable β-glucanase and its expression in Escherichia coli. Eur. J. Biochem. 224:923–930.

Takai, K., Y. Sako, and A. Uchida. 1997a. Extrinsic thermostabilization factors and thermodenaturation mechanisms for phosphoenolpyruvate carboxylase (PEPC) from an extremely thermophilic bacterium Rhodothermus obamensis. J. Ferment. Bioengin. 84:291–299.

Takai, K., Y. Sako, A. Uchida, and Y. Ishida. 1997b. Extremely thermostable phosphoenolpyruvate carboxylase from an extreme thermophile, Rhodothermus obamensis. J. Biochem. 122:32–40.

Takai, K., T. Nunoura, Y. Sako, and A. Uchida. 1998a. Acquired thermotolerance and temperature-induced protein accumulation in the extremely thermophilic bacterium Rhodothermus obamensis. J. Bacteriol. 180:2770–2774.

Takai, K., Y. Sako, and A. Uchida. 1998b. ppc, the gene for phosphoenolpyruvate carboxylase from an extremely thermophilic bacterium, Rhodothermus obamensis:

cloning, sequencing and overexpression in Escherichia coli. Microbiology 144:1423–1434.

Tenreiro, S., M. F. Nobre, F. A. Rainey, C. Miguel, and M. S. da Costa. 1997. Thermonema rossianum sp. nov., a new thermophilic and slightly halophilic species from saline hot springs in Naples, Italy. Int. J. Syst. Bacteriol. 47:122–126.

Thorbjarnardóttir, S. H., Z. O. Jónsson, Ó. S. Andrésson, J. K. Kristjánsson, G. Eggertsson, and Á. Pálsdóttir. 1995. Cloning and sequence analysis of the DNA ligase-encoding gene of Rhodothermus marinus, and overproduction, purification and characterization of the two thermophilic DNA ligases. Gene 161:1–6.

Tindall, B. J. 1991. Lipid composition of Rhodothermus marinus. FEMS Microbiol. Lett. 80:65–68.

Wicher, K. B., M. Abou Hachem, S. Halldórsdóttir, S. H. Thorbjarnadóttir, G. Eggertsson, G. O. Hreggvidsson, E. Nordberg Karlsson, and O. Holst. 2001. Deletion of a cytotoxic, N-terminal putative signal peptide results in a significant increase in production yield in Escherichia coli and improved specific activity of Cel112A from Rhodothermus marinus. Appl. Microbiol. Biotechnol. 55:578–584.

Wicher, K. B., O. P. Holst, M. Y. Abou Hachem, E. M. Nordberg Karlsson, and G. O. Hreggvidsson. 2002. Thermostable Cellulase. US Patent Application 20020102699.

PART 7

Chlamydia

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CHAPTER 7.1

The Genus Chlamydia—Medical

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Introduction

A great deal has been written about *Chlamydia* during the past few years. Several excellent reviews are available as are recent symposia proceedings and a new volume dedicated to descriptions of these organisms (see Literature Cited). Members of the genus *Chlamydia* are among the "high profile" human pathogens of the 21st century (Peeling and Brunham, 1996). Millions of people are infected with *Chlamydia*, and these organisms are associated with a vast array of serious diseases. *Chlamydia trachomatis* is the most common sexually transmitted bacterial pathogen in the world. It can cause acute infections in both men and women but also can be insidious and result in serious long-term complications in women who have upper genital tract involvement. *Chlamydia pneumoniae* is a more recently identified human pathogen that has been implicated in a significant portion of community acquired pneumonia outbreaks in many parts of the world. Serologic evidence suggests that most adults have been exposed to *C. pneumoniae* by the middle stages of life. This nearly universal exposure has taken on added significance with the recognition that *C. pneumoniae* also is associated with a large proportion of cardiovascular disease patients.

The chlamydiae are a group of obligate intracellular prokaryotic pathogens that are known to be involved with human populations at least as far back as the earliest times when people settled together in large communities. Many of the great cities of North Africa and the Middle East were constructed with long, high-walled avenues without curves that led to a region of the city where those afflicted with blinding chlamydial ocular disease could go to live as beggars or, more likely, to die. These streets are called "straights" and they lead to the cities of the blind. The fact that ancient urban planners designed city layouts to accommodate citizens afflicted with a single infectious disease speaks significantly about the magnitude of human misery wrought by chlamydiae. Yet this is but a single example in the repertoire of major afflictions caused by these organisms that have impacted humankind.

Descriptions of trachoma, a blinding chlamydial ocular disease, are accurately depicted in the earliest written communications. A 5,000-year-old Egyptian document called "the Ebers Papyrus" states that eyes that suffer from a flow of matter be treated with a combination of castor oil tree leaves, goose grease, and milk of a woman who had borne a son. Interestingly, breast milk is now known to have anti-chlamydial activity according to a 1997 publication. Trichiasis is the abrasion of the in-turned eyelashes on the cornea. It is virtually pathognomonic for trachoma and its cure, as stated in the Ebers Papyrus, is to treat the afflicted individual with myrrh, lizard's blood and bat's blood, after tearing out the hairs. It is further stated that treatment with bat's blood, the rim of a new vessel, presumably rubbed along the conjunctiva, and honey may prevent exacerbation of trichiasis. These trachoma restoratives are possibly the first recorded chemotherapeutic regimens for an infectious disease. The degree of efficacy was not stated in these early writings, although there is no indication that the recommended treatment was a nostrum either. The remedies presented in the Ebers Papyrus do point out the fact that chlamydial infections have been with people for at least as long as people have been recording their activities.

There is no doubt that the disease described in the Egyptian writings was caused by chlamydiae. The problem of chlamydial diseases, however, is not just one of antiquity. It remains with us today, and chlamydial infection may be more significant now than when the Ebers Papyrus was written. Chlamydiae continue to be a worldwide infectious disease health threat in a variety of settings and conditions. These bacteria are arguably the most ubiquitously distributed pathogens on the face of the globe, causing infections in organisms ranging in complexity from mollusks to human beings. Their obligate intracellular requirements restrict the chlamydiae to life not only within the host, but also within the very cells of the host.

This limitation in niche exploitation has no doubt contributed to the phylogenetic and taxonomic separation of chlamydiae from other prokaryotes. The chlamydiae occupy a unique taxonomic position and have evolved a unique lifestyle that contributes to their peculiar pathogenic potential. The chlamydiae clearly can cause acute infectious diseases, but the hallmark of chlamydial infections is their tendency toward chronicity. Disease manifestations may become evident only years after initial exposure to these organisms. It is this feature of chlamydial infections that causes them to be so difficult to identify, recognize and effectively manage.

Taxonomy

The order Chlamydiales comprises a single family, Chlamydiaceae, and the single genus *Chlamydia*. Recently, it has been proposed, based on 16S rRNA and ribosomal intergenic spacer sequence analyses, that existing chlamydiae taxonomic schemes are inadequate and that the Chlamydiaceae are more properly grouped into two genera, the *Chlamydia* and the *Chlamydophila*. This new taxonomic scheme recently was updated to include phylogenetic analysis of a small subset of well-characterized proteins (Everett, 2000).

In this revised taxonomy, organisms clearly distinct from *Chlamydia* would form two new families. The Parachlamydiaceae would be a family comprising chlamydiae-like organisms found existing in protozoans and a variety of multicellular invertebrates, and the Simkaniaceae would be a family comprising a single strain of chlamydiae-like organisms originally isolated as a tissue culture contaminant. The genus *Chlamydia*, according to new taxonomic criteria, would comprise members of the classic *C. trachomatis* group, and the genus *Chlamydophila* would comprise most veterinary chlamydial pathogens, human-specific *C. pneumoniae* and other *C. pneumoniae*-like strains isolated from a variety of animal species. It is clear that existing taxonomic criteria are inadequate. This is especially true for classification of members of chlamydiae that cause infections in livestock and other veterinary based groups. It is also clear that there is less enthusiasm among the research community working in areas related to human diseases for splitting the genus *Chlamydia* into two genera, although there is wide agreement that some modification of existing species designations might be in order.

At the present time it is not clear if the genus *Chlamydophila* will be embraced as an alternative to *Chlamydia psittaci*, *C. pecorum* and especially *C. pneumoniae*. It is likely that a sensible compromise is needed as a way out of the current taxonomic morass. A scheme that might provide utility would be to maintain a single genus (*Chlamydia*) but increase the number of species from four to nine. Most changes would reflect a reorganization of the *C. psittaci* group, a taxonomic area that is badly in need of reorganization and was the impetus for initiating the more drastic changes at the genus level noted above. This compromise would serve to provide a meaningful working framework for the classification of veterinary chlamydial organisms and allow for *Chlamydia* to be recognized as a genus of obligate intracellular pathogens causing acute and chronic infectious diseases in people. This new classification scheme is outlined in Table 1.

General Basic Biology

The fundamental principles of chlamydial growth, development, interaction with the host, and basic biology center around the developmental cycle depicted in Fig. 1. This intracellular growth stratagem is unique to chlamydiae and the essential elements hold true for all chlamydial strains and species (Scidmore-Carlson and Hackstadt, 2000). Notable differences do exist in some of the developmental cycle details between species or strains, but this will be taken up later. In its most idealized condition, it involves the orderly alternation of two distinct developmental forms (sequence A–F of Fig. 1). One developmental form is specialized for invasion into susceptible host cells (elementary body [EB]), and the other is specialized for growth, replication and division within the host cell (reticulate body [RB]). The elementary body is a small (0.2–0.3 μm) spherical particle that is metabolically inactive but capable of initiating invasion into susceptible host cells by virtue of specialized adhesins that remain incompletely defined. It is probable that chlamydial invasion is a multistep process that requires a set of surface exposed chlamydial moieties interacting with unspecified host cell ligands. It has been suggested that chlamydial-derived, surface-exposed heparin sulfate polymers facilitate entry. Various surface-exposed chlamydial proteins also have been implicated in the entry process. The best current evidence implicates the chlamydial major outer-membrane protein as an adhesin. New information (http://www.stdgen.lanl.gov/), based initially on data provided by the complete genome sequence for several strains of chlamydiae (Stephens, 2000; Stagg, 1998), demonstrates that an entire family of chlamydial surface proteins (polymorphic membrane proteins or Pmps) also warrants study as putative host cell binding adhesins. Unfortunately, definitive statements

Table 1. The Compromise Approach to Chlamydial Taxonomy.

Traditional classification scheme	Proposed classification scheme
Human strains	Human strains
Chlamydia trachomatis L1–L3	*Chlamydia trachomatis*—similar serologic (as well as genotypic) groupings as before
Chlamydia trachomatis A, B, Ba, C	
Chlamydia trachomatis D–K	
Chlamydia pneumoniae	*Chlamydia pneumoniae*
Non-human strains	Non-human strains
Chlamydia trachomatis—mouse pneumonitis	*Chlamydia muridarum* (former mouse pneumonitis strains)
Chlamydia trachomatis—pig strains	*Chlamydia suis* (former *C. trachomatis* pig strains)
Chlamydia psittaci	*Chlamydia psittaci* (mainly bird strains) can cause serious respiratory and systemic zoonotic diseases in humans
Birds and mammals, associated with arthritis or abortion in livestock, especially sheep and cattle	*Chlamydia abortus* (former *C. psittaci*) associated with cattle and sheep abortion
Chlamydia pecorum found in various species including koalas and ruminants, causing arthritis and systemic infection	*Chlamydia caviae* (former *C. psittaci* guinea pig inclusion conjunctivitis strains)
Chlamydia pneumoniae strains found in horses and koalas	*Chlamydia felis* (former *C. psittaci* feline pneumonitis strains)
	Chlamydia pecorum (same as before)
	Uncertain taxonomic status
	Chlamydia pneumoniae—non-human strains

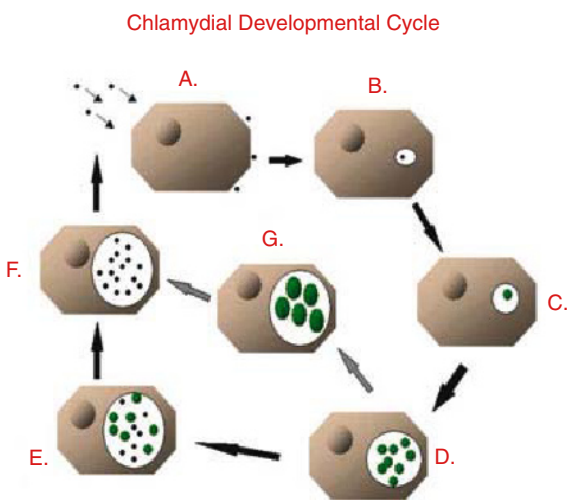

Fig. 1. Chlamydial life cycle. Chlamydiae adhere to the host cell and are endocytosed (A). The pathogen prevents phagosome-lysosome fusion (B), differentiates into the reticulate body (C) and begins replicating within the inclusion (D). Replicating reticulate bodies may re-differentiate back into elementary bodies (E, F) and lyse the host cell to begin a new round of infection. In addition, under conditions of immune-stress such as the presence of interferon-gamma, the pathogen may adopt a non-infectious, non-replicating "persistent" form (G); when the stress is removed, the pathogen can re-differentiate into infectious elementary bodies to begin a new cycle of infection.

about entry must await additional definitive studies in this area.

Chlamydial invasion resembles phagocytosis but occurs in both phagocytic (e.g., mononuclear phagocytes) and nonphagocytic (e.g., epithelial) cells and therefore has been referred to as pathogen-specified endocytosis. The internalized EB is contained within a vesicle that does not fuse with lysosomes, interrupting the normal acidification and intracellular degradation that are often the fate of less fortunate internalized organisms. It is not entirely clear how chlamydiae interfere with lysosomal fusion, but this inhibition is most likely a combination of specifying a fusion-deficient segment of the host cell membrane during entry, followed by active modification of the vesicle membrane as EB-to-RB differentiation begins. Differentiation of EB into RB occurs soon after entry. Genome sequence analysis reveals that chlamydiae have genes that code at least one and perhaps several two-component signaling systems. It is not known whether this regulatory mechanism is responsible for recognizing the new intracellular environment and initiating expression of a differentiation regulon, although a strategy such as this would be eminently reasonable.

The differentiation from EB to RB is accompanied by an entire repertoire of biochemical and morphologic changes. The RB is much larger than the EB. The small, dense EB is characterized by a rigid outer layer. The structural integrity of the EB is maintained via extensive interprotein disulfide bonds. Several chlamydial outer-membrane complex proteins are rich in cysteine, and there is evidence to suggest that cross-linking of these proteins provides EB structural integrity. The key outer-membrane complex proteins and their functions are shown in Table 2. The outer-membrane complex of RB is not extensively cross-linked, and this form of the organism is osmotically unstable once removed from the protection of the intracellular

Table 2. The chlamydial surface and outer membrane complex.

Surface structure	Properties	Function	Comments
A. Proteins			
MOMP	A 40-kDa protein, responsible for serovar specificity in *C. trachomatis*. Cysteine rich. Represents 50% of the protein in OMC of *C. trachomatis*. Some MOMP may be glycosylated.	Contributes to EB stability by disulfide cross-linkages. Has porin activity in RB. May have role in binding of EB to host cell and entry of EB. Glycosylated MOMP specifically implicated in entry process.	Surface-exposed in *C. trachomatis*. Four variable regions are surface accessible and can elicit serovar-specific antibody response and MIC test activity. Serovar specificity confirmed by genovar specificity.
OMC protein B (OmcB or Omp 2)	A 60-kDa, cysteine-rich outer membrane complex protein. May be periplasmic or surface exposed. Only present on EB. Isoelectric point varies between biovars (more disseminating strains have more basic OmcB).	Presumed to be involved in disulfide cross-linking of EB rigid OMC. Might be involved in attachment of EB to host cells.	Location (periplasm versus outer envelope) controversial as is role as heparin-sulfate-binding moiety thought to contribute to attachment and entry of EB.
OMC protein A (OmcA or Omp 3)	A 10-kDa cysteine-rich lipoprotein, periplasmic and anchored to inner leaflet of outer membrane by lipid. Only present on EB.	Presumed to be involved in disulfide cross-linking of EB rigid OMC.	Extremely cysteine-rich. Approximately 20% of the amino acids in this small (about 70 amino acids long) protein are cysteine.
POMPs	Variable in size (from 180 kDa to less than 30 kDa). Different species of chlamydiae have different numbers of *pomp* genes (from 6 in *C. psittaci* to 21 in *C. pneumoniae*) and the number of proteins expressed and surface exposed is not known and under investigation.	Not known, but could represent environment-dependent phase variation gene products. In *C. pneumoniae*, a subset of POMPs could be MIF antigen.	Regulation, expression, cellular location and function of POMPs in both *C. trachomatis* and *C. pneumoniae* is a very active area of research.
B. Other molecules			
Lipopolysaccharide	Genus specific antigen. Epitope in KDO structure. Has deep rough LPS features. Penta-acylated with fatty acids longer than C14.	Implicated as virulence factor as an inflammatory mediator with modest to low activity.	Poor activity due to penta-acylation (rather than hexa-acylation) and fatty acid composition longer than C14 (R1 = C14—C16, R2,3 = C20—O—C18–21, R4 = C14, R5 = C20—OH).
Glycosaminoglycan	Heparin-sulfate-like molecule, possibly linked to OmcB.	Suggested to be an attachment/entry ligand, at least for some strains of chlamydiae.	May be a chlamydial synthesized product or a chlamydial modified host cell product. It has certain characteristics of a capsule, which would be unusual for an intracellular organism.

Abbreviations: OMC, outer membrane complex; EB, elementary body; RB, reticulate body; MIC, micro-immunofluorescence; MOMP, major outer membrane protein; POMP, polymorphic outer membrane protein; MIF, macrophage inhibiting factor; and KDO, ketodeoxyoctonate.

vesicle. The chlamydial genome is compacted in EB. This is facilitated by the presence of histone-like proteins that bind to the DNA and cause it to form a tightly condensed, space-filling aggregate in the EB. One of the most noticeable morphologic changes that accompany EB-to-RB differentiation is the relaxation of the chlamydial DNA. This occurs in synchrony with the disappearance of the histone-like proteins, the appearance of mRNA transcripts, and a dramatic increase in the number of ribosomes present per cell. Fully differentiated RB are capable of genome replication, growth and division despite the apparent absence of at least one essential prokaryotic cell division gene (*ftsZ*) that is conserved in all other bacteria that have been examined thus far. Other oddities are associated with the basic elements of chlamydial growth, replication and metabolism. For example, peptidoglycan is not present in purified EB populations, yet genomic data indicate that the full complement of peptidoglycan biosynthetic genes are present in chlamydiae. Does this mean that a nonfunctional group of genes continues to be lugged around by these organisms? Is it possible that the peptidoglycan is present, but in such small quantities that it cannot be identified? Is peptidoglycan produced, but only during a restricted portion of intracellular development and then discarded somehow as RB mature into EB? How can all of this be explained in the context of the unusual effects that β-lactam antibiotics have on chlamydiae? It is known that sufficient penicillin penetrates into the infected host cell to affect chlamydiae in a way that interferes with cell division and results in the production of enlarged aberrant forms of the organism (Fig. 1 G). Radiolabeled penicillin, when added to chlamydial-infected host cells, binds to penicillin-binding proteins. These penicillin-binding proteins (PBP) presumably are transpeptidases, suggesting that alanyl-alanine peptide bonds are exchanged for peptide cross-linkages, but it is not clear what the putative peptides are bound to and how this relates to chlamydial cell wall structure. It is clear, however, that chlamydiae treated in this way are able to survive and resume normal growth after the penicillin is removed.

The growing population of intracellular RBs remains sequestered within the confines of a membrane-bound vesicle for their entire intracellular existence. This vesicle is termed "an inclusion," and as the number of chlamydiae within increases, a microcolony containing up to 103 organisms develops. The RBs do more than just replicate within the inclusion. There is evidence that they also influence host cell function in a variety of ways. First, fully differentiated RBs appear to line up against the luminal side of the inclusion membrane. Chlamydiae are known to have the genes responsible for the production and assembly of a type III secretion system, and it is tempting to speculate that this system may be active during this phase of intracellular development. It is clear that at least three chlamydial proteins (termed "IncA," "IncB" and "IncC") are secreted by chlamydiae and are found in the inclusion membrane. The identified Inc proteins may actually be representative of a larger family of potentially secreted/inclusion-specific proteins that share a common hydrophobic structural motif. It is not clear whether any Inc proteins are chaperoned from the chlamydial cytosol through a secretory apparatus, but it is known that when chlamydial IncA is cloned and expressed in *Shigella*, it is secreted via the *Shigella* type III secretion system in the absence of any chlamydial accessory proteins.

Chlamydia also is thought to secrete a proteosome-like apparatus that selectively degrades proteins in the host cell cytoplasm. For example, chlamydiae interfere with surface expression of both major histocompatibility complex (MHC) class I and MHC class II molecules. This inhibition is thought to be due to selective degradation of these molecules by the chlamydial proteosome. This may serve to render infected cells less visible to the immune system, although an undeniably robust immune response is generated as a result of a chlamydial infection. Details regarding the composition of the chlamydial proteosome are not known, nor is it known whether other host cell proteins are affected by the chlamydial proteosome or whether other secreted proteases are produced by chlamydiae. There is evidence that a group of unidentified host cell proteins are phosphorylated early in the course of the intracellular infectious cycle. Continued work in this area will reveal whether chlamydial gene products are involved in this interesting host cell alteration and what effect these modifications have on host cell function. Identification of a chlamydial type III secretion system has provided a stimulus for identifying and studying additional chlamydial proteins secreted directly into the host cell cytoplasm. Exploitation of this system should provide valuable new insight on subcellular interactions between chlamydiae and their host cells during the course of the intracellular growth cycle.

There are other avenues of communication between chlamydiae sequestered within the inclusion vesicle and the infected host cell. It is known that the chlamydial inclusion can fuse with secretory trafficking vesicles in ways that allow sphingomyelins to be modified to ceramides and incorporated into chlamydial membranes. There also is some evidence that chlamydial inclusions and mitochondria exhibit contact-type interactions. Ultrastructural evi-

dence suggests that this contact results in mitochondrial degradation with the suggestion that chlamydiae benefit energetically from this association.

Chlamydial Pathogenesis

In humans, three species account for the majority of chlamydial infections, and these infections lead to disease in a range of organ systems. *Chlamydia psittaci* is transmitted by contact with birds and causes psittacosis, a severe pneumonia often with systemic manifestations. *Chlamydia trachomatis* is transmitted by direct human-to-human contact to cause disease in the eye (trachoma and inclusion conjunctivitis), lung (neonatal pneumonia), or urogenital system (cervicitis, urethritis, salpingitis and pelvic inflammatory disease). *Chlamydia pneumoniae* is transmitted by airborne droplets to infect the respiratory tract (pharyngitis, bronchitis, community-acquired atypical pneumonia, and possibly adult-onset asthma); subsequently, it gains access to the vasculature and may infect the cardiovascular system, which contributes to atherosclerosis.

Many human diseases caused by bacteria result from tissue damage that occurs from replication of the organism or direct toxic effects of bacterial products. Chlamydiae also cause pathology through these means, but in addition, chlamydial pathogenesis involves tissue damage resulting from immune responses to chlamydial products (Ward, 1995). Chronic or repeated infection leads to increasingly severe chlamydial disease mediated by specific virulence determinants. In particular, the 57-kDa chlamydial heat shock protein, Hsp60, appears to be a key immunomodulator in chlamydial pathogenesis (LaVerda et al., 1999). Strong antibody responses to the protein have been associated with blinding trachoma, as well as tubal scarring and pelvic inflammatory disease, and it is possible that cross-reactivity of chlamydial Hsp60 to human heat shock proteins leads to immune-mediated pathology. In addition, chlamydial Hsp60 can induce inflammatory cascades to directly cause inflammation. For example, chlamydial Hsp60 can activate mononuclear phagocytes to secrete inflammatory cytokines, such as TNF-α, IL-1 and IL-6. Thus, Hsp60 may be involved in chlamydial pathogenesis in two ways: first by direct antigenic stimulation and second by activation of cells to induce local inflammatory reactions.

Another chlamydial virulence determinant likely involved in pathogenesis is the atypical genus-specific lipopolysaccharide (LPS). Chlamydial LPS is unique in that it has a pentaacyl lipid moiety rather than the hexaacyl moiety observed in typical lipopolysaccharides of Enterobacteriaceae (Kosma, 1999). Structure-function studies predict that this difference should cause chlamydial LPS to be only 1% as active as conventional lipopolysaccharides, although different chlamydial strains may produce distinct LPS molecules with greater or lesser amounts of activity and this is the case when TNF-α secretion is assessed from whole blood ex vivo. It has been proposed that the relatively weaker activity of chlamydial LPS results in maintenance of a low-grade, chronic inflammation characteristic of chlamydial infections. Recent work suggests that chlamydial Hsp60 and LPS share similar signaling pathways to trigger host cell activation, but further work is necessary to solidify this hypothesis.

Immunity to chlamydial infection remains incompletely understood. Natural infection confers little immunity to reinfection, and reinfection leads to increasing severity of disease. The protection that is conferred is short-lived, and vaccination studies have not shown long-lasting protection against productive infection. Both humoral and cell-mediated immune responses are elicited and likely necessary to control infection. Antibody response to the major outer-membrane protein (MOMP) can neutralize infection in animal models, but it is unclear whether humoral immunity is critical to control infection in humans. Cellular immunity is characterized by CD4 and CD8 responses. Available evidence supports an important role for CD4 cells in controlling infection, but both CD4 and CD8 T-cell restricted mechanisms likely play a role during specific stages of infection.

Chlamydiae as Human Pathogens

The following discussion focuses on the human diseases caused or associated with *C. psittaci*, *C. trachomatis* and *C. pneumoniae* (Table from Peeling et al.). The discussion is organized into organ systems to underscore the heterogeneity in chlamydial diseases. The intent of this discussion is not to replace excellent recent reviews (see Literature Cited) that detail chlamydial infections, but rather to emphasize putative pathogenic mechanisms common to all chlamydiae and outline differences between chlamydial diseases.

Chlamydia psittaci

Chlamydia psittaci is the causative agent of human psittacosis, an atypical pneumonia that leads to death in 30% of cases if left untreated and infection becomes systemic (Gregory et al.,

1997). Psittacosis is a zoonosis; 5–8% of birds are carriers and most humans are infected chiefly by respiring aerosol droplets from infected animals. *Chlamydia psittaci* has been shown to infect approximately 130 different bird species, but interestingly, psittacosis is particularly fulminant if acquired from turkeys and parrots. Accordingly, psittacosis remains a significant health threat mainly to certain professionals such as abattoir-workers and veterinarians.

The organism enters the respiratory tract within aerosol droplets and infects the respiratory epithelium, where it establishes a niche for one to three weeks before onset of symptoms. The infected individual often presents with symptoms of atypical pneumonia such as cough, fever, and chest soreness, and physical examination shows fever, rales and hepatomegaly in over 50% of patients. The latter sign highlights the capacity of *C. psittaci* to spread from the respiratory system to other organ systems. Indeed, the organism has been isolated from placentae following miscarriage, as well as detected from the liver and spleen in psittacosis models of infection. Although the exact mechanism of dissemination is not clear, mononuclear phagocytes may be the transport vehicle as evidenced by the ability of *C. psittaci* to infect and survive (but not multiply) within human, murine and pig alveolar macrophages. Also not clear is whether strains isolated from turkeys or parrots have a higher capacity to disseminate, given that psittacosis acquired from these birds tends to be more fulminant. Regardless of the involved mechanisms, disseminated psittacosis involves multiple organs, and neurological and gastrointestinal symptoms such as headache, malaise, vomiting, diarrhea or constipation, and nausea are common. If untreated, psitticosis may result in cardiac, hepatobiliary, neurologic and endocrine involvement with fatal consequences.

Diagnosis of psittacosis currently is most commonly based on clinical stigmata combined with the complement-fixation assay. However, antigen detection methods, such as enzyme immunoassay and molecular methods, such as the polymerase chain reaction (PCR), provide more sensitive and specific methods of diagnosis (Stephens et al., 1998; Shachter, 1997; Tuuminen, 2000; Mardh, 2000). Treatment with antichlamydial antibiotics, such as tetracycline or doxycycline, reduces mortality below 1%. Reinfection following recovery has been documented and there is no evidence of lasting immunity.

Chlamydia trachomatis

While *C. psittaci* establishes infection in the lung, *C. trachomatis* has the capacity to infect a range of organ systems. In the eye, *C. trachomatis* infection leads to multiple diseases including trachoma, the world's chief cause of infectious blindness (Mabey and Bailey, 1999). In the lung, the organism is responsible for pneumonia in both infants and adults. In the urogenital tract, the organism is the most common sexually transmitted bacterium that leads to salpingitis and pelvic inflammatory disease (PID), principal causes of infertility in the United States (Guaschino and De Seta, 2000; Robinson and Ridgway, 2000; Stamm, 1999). In addition, *C. trachomatis* can affect the rheumatological and lymphatic systems with diseases such as Reiter's syndrome and lymphogranuloma venereum (Inman et al., 2000; Schumacher, 2000).

Chlamydia trachomatis is grouped into three biovars, which in turn include multiple serovars based on serological reactivity to the organism's MOMP (Table). The trachoma biovar is associated with oculogenital disease and contains serovars A, B, Ba, C and D-K. Serovars A, B, Ba and C cause trachoma, and B, Ba, and D-K most often lead to sexually transmitted disease (STD) and inclusion conjunctivitis. However, serovars B and Ba have been isolated from the genital tract, suggesting that at least some serovars do not exhibit tissue specificity. The LGV biovar is composed of serovars L1, L2 and L3, which cause lymphogranuloma venereum. This sexually transmitted, lymphatic disease is very rare outside of endemic areas in Southeast Asia, South America, and Africa. The third *C. trachomatis* biovar is named "mouse pneumonitis" and is not associated with human disease.

Ocular Disease

Chlamydia trachomatis is the most common cause of chronic follicular conjunctivitis, defined as follicular conjunctival inflammation lasting for more than 16 days. *Chlamydia trachomatis* chronic follicular conjunctivitis is separated into three clinical syndromes: neonatal conjunctivitis (ophthalmia neonatorum), adult inclusion conjunctivitis (paratrachoma) and trachoma.

Neonatal Conjunctivitis. In the United States, *C. trachomatis* is the most common cause of neonatal conjunctivitis, defined as conjunctivitis that occurs within the first four weeks of life. The newborn acquires the organism from the female genital tract during delivery. It is estimated that infants born to untreated, infected mothers have a 40% chance of developing neonatal conjunctivitis and 20% chance of developing neonatal pneumonia. It therefore is imperative to screen high-risk pregnant populations for *C. trachomatis* genital tract infection and treat high-risk newborns with topical antichlamydial antibiotics shortly after delivery.

Typically, the neonate develops a watery discharge within two weeks of delivery. The dis-

charge may become mucopurulent and a pseudomembrane over the conjunctiva may form, but often the infection is mild and self-limited. However, if the infection is severe and untreated, conjunctival and corneal scarring may ensue with vision-threatening sequelae. The disease can be diagnosed with a variety of tests, the most useful being an immunofluorescent monoclonal antibody stain of conjunctival smears (100% sensitivity, 94% specificity for *Chlamydia*). Treatment often can precede diagnosis if the mother is a high-risk individual, considering topical tetracycline or erythromycin ointment applied within one hour of delivery essentially prevents chlamydial conjunctivitis. However, if the disease manifests, treatment with topical antibiotics alone may not be sufficient to eradicate the organism; furthermore, *C. trachomatis* that may have colonized the respiratory tract must be eliminated to prevent neonatal pneumonia. Therefore, in addition to topical antibiotics, a two-week course of oral erythromycin syrup is recommended in infants with chlamydial conjunctivitis, with re-treatment if symptoms and signs have not resolved.

Adult Inclusion Conjunctivitis. The route of transmission for neonatal conjunctivitis underscores the capacity of *C. trachomatis* to infect the genital tract as well as conjunctiva. Therefore, when the organism is transmitted from the adult genital tract to the adult eye, it is not surprising that a chronic follicular conjunctivitis results with similar characteristics to neonatal conjunctivitis. This form of conjunctivitis is termed "adult follicular conjunctivitis," previously known as "paratrachoma." It is estimated that half of all individuals with adult inclusion conjunctivitis also have *C. trachomatis* infection of their genital tract, but that <1% of those with genital tract infection develop conjunctivitis.

The disease manifests within two weeks of exposure. Patients often present mucopurulent discharge, hyperemia, photophobia, and foreign body sensation in one eye. If infection does not resolve, a chronic follicular conjunctivitis ensues, characterized by lymphoid follicle formation. Such follicles are rich in mononuclear phagocytes, as well as lymphocytes, and characterize initiation of chronic inflammation. If untreated, the disease progresses to resemble early trachoma, with pannus formation, papillary hypertrophy, and diffuse punctate keratitis. Corneal scarring may ensue and threaten vision; however, unlike trachoma, adult inclusion conjunctivitis often resolves without severe sequelae.

Diagnosis can be made from conjunctival swabs by a variety of methods, including Giemsa staining, immunofluorescent staining, or isolation. Direct immunofluorescent staining with monoclonal antibody has a high sensitivity and specificity for diagnosis. Treatment is with oral antibiotics, preferably with macrolides or derivatives.

Trachoma. The leading infectious blindness, this disease is also the third most common blinding disease in the world (figure from the World Health Organization). Worldwide, approximately 6 million people are blind secondary to trachoma, 150 million people exhibit active disease, and 500 million are infected. Blinding trachoma is endemic in many third world countries within Africa, the Middle East, Southeast Asia, and South America and was widely prevalent elsewhere but regressed in Europe and North America in part owing to improved living standards. The disease is well responsive to antibiotic treatment, and a World Health Organization (WHO) initiative aims to combine antibiotic treatment with surgery, hygiene education, and environmental improvements to eradicate trachoma by the year 2020.

Trachoma is a chronic, infectious, follicular keratoconjunctivitis that initially only involves the conjunctiva, but progresses to affect the eyelashes, lids and ultimately the cornea. Involvement of the latter eventually leads to corneal scarring and vision loss. Children are the primary reservoir of infection. *Chlamydia trachomatis* is transmitted chiefly by hand-to-eye contact, although vector transmission by flies also is possible. The organism establishes infection in the conjunctiva and initiates a chronic inflammation. This is characterized by lymphoid follicles, germinal centers of lymphocytes, and mononuclear phagocytes, and underscores the inflammatory nature of disease. The lymphoid follicles are surrounded by hyperemic tissue and appear grossly as papillary hypertrophy. If infection is treated at this point, the inflammation may subside with resolution of disease. However, continued conjunctival inflammation leads to conjunctival scarring with subsequent entropion (inward distortion of lids) with or without trichiasis (inwardly turned eyelashes). The latter irritates the cornea, and chronic corneal insult leads to ulceration and corneal opacity. Corneal damage leads to decrease in visual acuity, and enough damage may ensue to cause complete blindness. A simplified grading scheme has been adopted by the WHO and highlights progression of the disease. This grading scheme replaces an old classification system created by MacCallan to obtain accurate documentation during treatment trials.

Community risk factors for the development of trachoma include poverty, crowding, and unavailability of safe water for household use. Treatment for trachoma is based on the "SAFE" protocol that considers many of these important risk factors. Adopted by the WHO to eradicate trachoma by 2020, SAFE is composed of four

arms of treatment. They are: 1) Surgery to treat entropion and trichiasis; 2) Antibiotic treatment of entire communities with azithromycin or topical oxytetracycline to treat infection, as well as to lower transmission rates; 3) Face washing to improve personal hygiene; and 4) Environmental measures to improve community hygiene, such as providing safe water and adequate disposal of feces. Antibiotic treatment is perhaps the critical arm of the SAFE protocol because treatment of entire communities reduces prevalence of infection as well as re-emergent infection. However, antibiotic treatment is not sufficient for long-term control of trachomatous blindness, and surgery (where indicated), personal hygiene, and environmental improvements also must be available to eradicate the disease by 2020.

Respiratory Disease *Chlamydia trachomatis* can infect the respiratory tract and cause pneumonia in both the adult and infant. However, *C. trachomatis* adult pneumonia is exceedingly rare and documented cases only have been reported in laboratory workers inhaling large doses of the pathogen and in severely immunocompromised individuals. In contrast, *C. trachomatis* infant pneumonia is relatively common when the pathogen is transmitted from the genital tract of the infected mother to the newborn during delivery. The latter pneumonia often is mild and associated with neonatal conjunctivitis.

Chlamydia trachomatis infant pneumonia was first identified by Beem and Saxon, who detected the pathogen in approximately 90% of infants with mild pneumonia. These individuals remained afebrile but had chronic, diffuse lung involvement associated with high serum anti-*C. trachomatis* IgG and IgM. Prospective studies that followed show that 16–28% of infants born to infected mothers develop pneumonia, with a 1–5% prevalence of disease in a range of communities. The pathogen can infect a range of tissues in the neonate including the conjunctiva, respiratory tract, gastrointestinal tract, and vagina. Therefore, neonatal disease may manifest first in the conjunctiva and then in the respiratory tract because half of infants with *C. trachomatis* pneumonia also have had neonatal conjunctivitis. As with neonatal conjunctivitis, it is possible to prevent neonatal pneumonia by adequately identifying and then treating mothers with the STD prior to delivery.

Incubation time for infant pneumonia is approximately eight weeks, slightly longer than for neonatal conjunctivitis. Infants often present with a staccato cough, tachypnea and nasal discharge. However, patients usually are afebrile. Radiology is nonspecific with interstitial infiltrates and bilateral hyperexpansion; there is no pleural effusion and lobar consolidation. Most *C. trachomatis* infant pneumonia is mild and resolves even if not treated, but very young infants or immunocompromised children may develop significant disease. Complications are rare and most cases resolve spontaneously. Interestingly, an association exists between *C. trachomatis* infant pneumonia and obstructive airway disease later in life, but causality has not been determined. Disease can be diagnosed by culture, immunofluorescent staining or nucleic acid detection of sputum specimens. Macrolides are the mainstay of therapy.

Urogenital Disease The hallmark of *C. trachomatis* genital tract infections is their chronicity and persistence. These characteristics lead to human infections that often are asymptomatic or only mildly symptomatic, which may lead to severe scarring and infertility in women before the patient seeks medical attention. In addition, *Chlamydia trachomatis* is the most common sexually transmitted bacterial pathogen in the United States, with approximately 4–5 million new cases occurring annually. The combination of silent disease and frequency of infection makes chlamydial disease a serious health concern in the United States. Indeed, *C. trachomatis* genital tract disease leads to over 25,000 cases of involuntary infertility in females each year. In addition, sequelae of infection are the second most costly outcome of any STD, costing an estimated 4 billion dollars each year in health care costs.

Chlamydia trachomatis STD is transmitted from humans to humans by direct genital contact. The probability of transmission from an individual encounter is between 30 and 70%, where the higher estimates are based on sensitive nucleic acid amplification tests. Risk factors for *C. trachomatis* STD vary among study populations but often include younger age, unmarried status, low socioeconomic use, and use of oral contraceptives. Similar to other STDs, *C. trachomatis* STD is most prevalent for individuals in their late teens and early twenties.

In men, the pathogen enters the distal urogenital tract and infects urethral epithelium. A large portion of infected men remain asymptomatic, but those who do manifest disease present with symptoms within 1–2 weeks. Patients complain of dysuria and often a nonpurulent, clear-to-white penile discharge. Effective treatment for symptomatic men is available with a variety of antibiotics; however, mildly symptomatic or asymptomatic men may not seek medical attention and therefore develop epididymitis, proctitis, sexually reactive arthritis, or unknowingly transmit the organism to women.

In women, the bacterium causes a continuum of inflammatory diseases as it ascends the genital tract. *Chlamydia trachomatis* first establishes infection within the transitional epithelium of the cervix. Those individuals who become symptomatic often present with vaginal discharge and bleeding, mild abdominal pain, and dysuria. Treatment of these patients with antichlamydial antibiotics is effective and prevents sequelae. However, 70–90% of endocervical infections are asymptomatic or only mildly symptomatic, and most infected women do not present for medical treatment. The result is persistent infection that ascends by intraluminal spread up the genital tract. Thus, untreated endocervical infection leads to pelvic inflammatory disease (PID), defined as ascending infection progressing to endometritis (inflammation of the uterine endometrium), salpingitis (inflammation of the fallopian tubes), and pelvic peritonitis. *Chlamydia trachomatis* and *Neisseria gonorrhoeae* account for 65–85% of PID cases in the Unites States. In general, *N. gonorrhoeae* is recovered twice as frequently as *C. trachomatis* from PID patients, and detection of concurrent infection also is common. Furthermore, compared with *N. gonorrhoeae*, *C. trachomatis* urogenital infection generally follows a more indolent course.

Approximately half of all untreated *C. trachomatis* cervical infection leads to endometritis. Elementary bodies released from lysed cervical cells initiate infection of healthy epithelium proximal to the cervix, allowing the organisms to ascend the genital tract by repetitive cycles of epithelial cell infection and lysis. Patients with endometritis may present with abnormal menstrual bleeding and be found to have uterine tenderness and leukocytosis. Often, however, other symptoms and signs of endometritis including abdominal pain, fever and adnexal tenderness are not present, consistent with silent disease caused by *C. trachomatis*. The subclinical or minimally symptomatic disease persists, causing low-grade inflammation and tissue damage over months.

Untreated endometrial *C. trachomatis* infection ascends by intracanalicular spread up the genital tract to infect and inflame the fallopian tubes. The resulting salpingitis may manifest as bilateral lower abdominal and pelvic pain, but subclinical disease is more common with *C. trachomatis* PID. The fallopian tube infection may persist for months and cause peritubular scarring. Experimental data from lower primates suggest that women who become repeatedly infected develop more intense tubular scarring. Salpingitis, if untreated, leads to severe damage to the fallopian tubes with subsequent infertility or ectopic pregnancy.

Untreated *C. trachomatis* salpingitis may further ascend into the peritoneal cavity and cause perihepatitis and periappendicitis. The former, also known as "Fitz-Hugh-Curtis syndrome," is characterized by inflammation of the liver capsule. *Chlamydia trachomatis* perihepatitis usually is asymptomatic, but 3–10% of PID patients have cholecystitis-type symptoms such as fever and right, upper-quadrant abdominal pain. Such patients undergo laparoscopy and laparoscopic cholecystectomy, where advanced perihepatitis is seen as impressive "violin-string" adhesions between the liver capsule and visceral peritoneum. Periappendicitis, or inflammation of the appendiceal serosa and intestinal mucosa, is a less likely complication but also has been associated with chlamydial PID.

Advances in nucleic acid amplification have made available highly sensitive and specific tests for the diagnosis of chlamydial STD. The ligase chain reaction (LCR) and polymerase chain reaction (PCR) have become widely available and are more sensitive, yet still highly specific, when compared with culture. These technologies can be used to detect chlamydial nucleic acid from urine or vaginal swabs and are preferable to older methods (culture, direct fluorescent antibody, and EIA) for the diagnosis of *C. trachomatis* STD.

Treatment with a 7–14 day course of tetracyclines and macrolides is highly effective, and resistance to anti-chlamydial antibiotics has not developed in a clinical setting. It is important to treat the infection with at least 7 days of the aforementioned antibiotics to eradicate the organism. This prolonged treatment is explained in part by the biphasic life cycle of *Chlamydia*, where the extracellular EBs are metabolically inert and intracellular RBs are relatively sequestered within inclusions in host cells. Noncompliance or shorter duration of treatment may result in ineffective clearance, persistence of the organism, and development of sequelae. An alternative to tetracyclines and older macrolides is the newer azolide azithromycin, which remains within the intracellular environment considerably longer than traditional antichlamydial antibiotics. Thus, a single 1-g dose of azithromycin is equally efficacious as a 7-day course of doxycycline in the treatment of chlamydial cervicitis. Cost-effectiveness analyses demonstrate that azithromycin therapy leads to an overall reduction in morbidity and cost despite higher procurement prices for the medication.

Prevention of chlamydial STD especially is important given that most infections are asymptomatic and yet lead to silent disease and infertility. Universal screening is prohibitively costly in low-prevalence populations. Practical prevention strategies are aimed at screening of high-risk populations. Such groups often are defined as adolescent individuals with multiple, new, or

symptomatic partners that rarely or inconsistently use barrier contraception. Both universal and selective screening methods are effective in reducing the prevalence of *C. trachomatis* infection. For example, in the Pacific Northwest, prevalence of *C. trachomatis* infection declined from 10–12% to 3–5% within 8 years after screening young women attending all family planning clinics in the region. In addition, selective screening of women demonstrated a significant reduction in subsequent symptomatic PID (odds ratio 0.44, 95% confidence interval 0.2–0.9). These data clearly suggest that wide implementation of selective screening will curb chlamydial STD in the United States and reduce severe sequelae such as PID, infectious infertility, and ectopic pregnancy.

Rheumatological Disease Reactive arthritis is a systemic inflammatory condition that develops after infection of a distant site. The disease is commonly referred to as Reiter's syndrome after Hans Reiter (1881–1969), who in 1916 described a patient who presented with arthritis, urethritis and conjunctivitis. This condition is an uncommon, systemic complication of certain sexually or enterically transmitted infections, including *C. trachomatis* STD.

Approximately 1–3% of patients with urethritis develop Reiter's, and *C. trachomatis* can be isolated from the urethra of two-thirds of patients who present with Reiter's syndrome in the absence of diarrhea. The disease is much more common in men compared with women. In addition, there is a strong association between HLA-B27 and Reiter's syndrome. Four-fifths of Reiter's patients are HLA-B27 positive, suggesting that chlamydial antigens cross-react with self-antigens to induce an exaggerated immune response distant from the site of infection. It also is conceivable that such molecular mimicry prevents control of infection and leads to dissemination of the pathogen and joint seeding.

Reiter's syndrome is a spondylarthropathy characterized primarily by lower extremity oligoarthritis after urethritis or cervicitis. Patients present one to three weeks following an episode of urethritis or cervicitis. Musculoskeletal diseases include arthritis, enthesopathy (inflammation of the tendons and ligaments), and sacroiliitis. Ocular disease manifests as conjunctivitis in self-limited Reiter's or anterior uveitis in established, chronic disease. Mucocutaneous diseases are characterized by painless oral ulcers or erythematous erosion of the glans penis. Cardiovascular disease is rare and may manifest early as pericardial rubs or late as aortitis.

Diagnosis is difficult because symptoms occur serially over a long period rather than simultaneously. *Chlamydia trachomatis* most likely establishes persistent infection at the inflammatory site inasmuch as chlamydial protein and DNA can be identified at inflamed joints from Reiter's patients. In addition, chlamydial mRNA is detected in synovial biopsies, suggesting active chlamydial infection. These mRNA transcripts are specific for the inflammatory heat shock protein 60 (chsp60) but not the major outer-membrane protein (omp1), consistent with persistent chlamydial infection. Treatment is aimed at reducing inflammation with nonsteroidal anti-inflammatory drugs (NSAIDs) and eradicating infection with antibiotics. Sixty to 80% of first-time Reiter's patients develop chronic disease, and as many as a quarter of patients become functionally disabled as a result of their illness.

Lymphatic Disease The LGV biovar of *C. trachomatis* causes lymphogranuloma venerum, a sexually transmitted disease that affects the lymphatic system. The disease is endemic only in limited regions in India, Southeast Asia, and South America, occurring sporadically elsewhere. The infected individual presents with symptoms within one month of infection, and the disease progresses in three stages. In the first stage, the patient develops a painless, asymptomatic papule on the rectal or genital mucosa. If untreated, the infection gains access to the lymphatic system within weeks. Patients develop inguinal or femoral lymphadenopathy, which are distinct and painful. Infection may spread to adjacent tissue and form a bubo, which then may burst spontaneously to form an abscess or sinus tract. Spread of infection is associated with development of systemic symptoms and signs including fever and myalgias; the organism may gain access to cerebrospinal fluid and cause stigmata of meningitis. The disease then progresses to the final stage, where the granulomatous inflammation leads to ulceration of genital mucosa and obstruction of the lymphatics. The latter sequela is characterized by elephantiasis of genitalia. Diagnosis can be made clinically, serologically and by detecting the pathogen in diseased tissue. Treatment is with antichlamydial antibiotics and draining abscesses to eradicate the site of infection.

Chlamydia pneumoniae

Isolated first in 1965, *C. pneumoniae* was thought to be an unusual *C. psittaci* strain. Saikku and Grayston made the first association between the pathogen and respiratory disease in 1986, and the organism was speciated in 1989 as a leading cause of community-acquired atypical pneumonia (Saikku et al., 1988). Twelve years after speciation, *C. pneumoniae* now stands associated with a spectrum of chronic diseases ranging from atherosclerosis (Grayston, 2000) to

adult-onset asthma (Hahn et al., 1991). Its broad association with diseases in multiple organ-systems underscores the pathogen's capacity to infect a variety of host cell types. For example, whereas *C. trachomatis* is limited to productive infection within epithelial cells and fibroblasts, *C. pneumoniae* is capable of infecting and multiplying within epithelial, endothelial and smooth muscle cells in addition to fibroblasts and macrophages.

Chlamydia pneumoniae probably is a strictly human pathogen that is transmitted by respiratory droplets. It therefore initially accesses the body through the respiratory system. Upper respiratory infection leads to subclinical disease or pharyngitis, sinusitis or bronchitis. Transmission into the lower respiratory tract is followed by productive infection of alveolar macrophages and subsequent development of community-acquired atypical pneumonia. It is thought that the inflammatory response that ensues leads to microvascular damage, which enables the pathogen to escape into the vasculature. Monocytes or lymphocytes may serve as the vehicle of transport for the obligate intracellular bacterium. The pathogen thus may seed multiple sites within the vasculature, including the coronary arteries, abdominal aortic aneurysms or carotid bifurcations to induce or contribute to atherosclerotic heart and vessel disease (Muhlestein et al., 1998; Campbell et al., 2000).

Respiratory Disease The epidemiology of pneumonias is different between hospitalized patients and immunocompetent adults in the outpatient setting. While *Streptococcus pneumoniae* remains the leading cause of pneumonia in hospitals, atypical agents such as *C. pneumoniae* and *Mycoplasma pneumoniae* are very frequent causes of community-acquired pneumonias. Indeed, *C. pneumoniae* is identified in 11–36% of ambulatory patients diagnosed with pneumonia and may be the most commonly identified pathogen in community-acquired atypical pneumonias. In addition, the organism is encountered in 28% of ambulatory pediatric patients diagnosed with atypical pneumonia.

The clinical course of *C. pneumoniae* pneumonia is difficult to distinguish from other atypical pneumonias. It often follows a milder clinical course than *S. pneumoniae* pneumonia, and therefore infected patients may not seek medical attention as frequently. The organism initially is delivered into the upper respiratory tract through aerosol droplets. Upon infecting the epithelium, the pathogen incubates for up to 4 weeks and then initiates a biphasic set of symptoms. The first phase of symptoms includes stigmata of upper respiratory tract infection and includes sinusitis, pharyngitis and bronchitis. It is likely that these symptoms are the result of *C. pneumoniae*-epithelial cell interactions that trigger an inflammatory cascade, evidenced by expression of IL-8, ICAM-1 and PGE-2 by human epithelial cells and subsequent neutrophil migration following *C. pneumoniae* infection. In addition, infection of respiratory epithelium induces ciliostasis, which may aid transmission of the pathogen from the upper- to the lower-respiratory tract. The latter triggers the second phase of symptoms. These patients present with stigmata of atypical pneumonia, characterized by cough, low-grade fever and pleuritic pain. A physical examination may reveal rales and chest X-rays may show pneumonitis, but neither is specific for *C. pneumoniae* pneumonia. Onset of disease is gradual and the course chronic, with reinfection resulting in fewer cases of pneumonia. Most *C. pneumoniae* pneumonias resolve without treatment, although severe cases do occur, especially in patients with chronic obstructive pulmonary disease and in immunocompromised individuals. An important finding is the high rate of coinfection with other organisms, which suggests that the pathogen may predispose to pyogenic infections that may increase severity of disease.

Diagnosis of *C. pneumoniae* respiratory infection can be made by a variety of methods. Most diagnostic studies make use of the microimmunofluorescence (MIF) test, which has been useful in showing high prevalence of *C. pneumoniae* in community-acquired atypical pneumonia. However, cross-reactivity of *C. pneumoniae* and *C. trachomatis* by MIF makes this test suboptimal in diagnosing *C. pneumoniae* pneumonias in certain populations. In addition, different laboratories define arbitrary criteria for positive tests. Alternative diagnostic methods that detect chlamydial nucleic acid in sputum samples may be preferable but less widely available. Clinically, however, a diagnosis is not always necessary before effective treatment is begun. Patients that present with stigmata of community-acquired atypical pneumonia are treated effectively with macrolide antibiotics such as azithromycin.

Pulmonary Associations

Viral and bacterial infections, including *C. pneumoniae*, may exacerbate or trigger acute asthmatic events in patients with long-standing asthma. However, much more controversial is the hypothesis that *C. pneumoniae* may cause asthma in certain adults with no previous history of asthma. Hahn et al. proposed this hypothesis in 1991 when they reported an association between acute *C. pneumoniae* infection and adult-onset wheezing, asthmatic bronchitis and asthma (Hahn et al., 1991). Various small-scale

studies since have examined this hypothesis with equivocal results, and a large-scale prospective study will be necessary to establish a causal link between infection and adult-onset asthma.

Cardiovascular Associations

Excellent recent reviews are available on evidence linking *C. pneumoniae* infection and atherosclerotic heart and vessel disease (see Literature Cited). *Chlamydia pneumoniae*, capable of infecting all cell types within the atherosclerotic plaque, has been associated with coronary artery disease by seroepidemiological and pathological studies, experiments performed with appropriate animal models, and cell culture techniques exploring pathogenic mechanisms. Seroepidemiological evidence comes from more frequent detection of antibodies to the organism in patients with coronary artery disease compared with a control population. Pathological evidence is based on detection of *C. pneumoniae* within atherosclerotic tissue but not normal vasculature. Indeed, the organism is present within macrophage foam cells by electron microscopy and within endothelial cells, smooth muscle cells and macrophages by immunohistochemistry. In addition, *C. pneumoniae* nucleic acid is frequently recovered from atheroma, and in some instances the pathogen has been isolated from plaques. Animal model evidence is highlighted by experiments by Muhlestein et al., who showed that intranasal infection of non-hyperlipidemic rabbits with *C. pneumoniae* led to the development of atherosclerotic changes, whereas mock-infection of controls did not. Furthermore, the infected rabbits were treated with azithromycin and found to undergo regression of atherosclerosis. Cell culture studies reveal that *C. pneumoniae* is capable of altering cellular physiology in ways that may contribute to atherosclerosis. For example, infected cells release chemokines and an inflammatory cytokine cascade, induce lipoprotein oxidation, and accumulate excess levels of lipids, all events strongly linked to the pathogenesis of atherosclerosis.

It is likely that *C. pneumoniae* at least contributes to atherosclerosis and is an emerging risk factor, but does the pathogen cause the disease? The high prevalence of infection and lack of widely available, affordable and specific diagnostic methods in part make it difficult to answer this question. Well-designed experiments are necessary in seroepidemiological, pathological, animal-based, cell culture-based, and treatment-based studies. Importantly, this bacterial infection potentially is another treatable risk factor for atherosclerosis. Indeed, large scale, multicenter, randomized, placebo-controlled treatment trials of patients with coronary artery disease are currently underway to elucidate the role of antibiotics in atherosclerosis.

Neurological Associations

Chlamydia pneumoniae has been associated with diseases of the neurological system such as Alzheimer's disease and multiple sclerosis, but evidence for these associations remains highly controversial. Further work is necessary to strengthen these associations and establish *C. pneumoniae* as a risk factor, or etiologic agent, in Alzheimer's disease and multiple sclerosis.

Literature Cited

Campbell, L. A., M. Rosenfeld, and C. C. Kuo. 2000. The role of Chlamydia pneumoniae in atherosclerosis: recent evidence from animal models. Trends Microbiol. 8:255–257.

Everett, K. D. 2000. Chlamydia and Chlamydiales: More than meets the eye. Vet. Microbiol. 75:109–126.

Grayston, J. T. 2000. What is needed to prove that Chlamydia pneumoniae does, or does not, play an etiologic role in atherosclerosis? J. Infect. Dis. 181 (Suppl. 3):S585–S586.

Gregory, D. W., W. Schaffner, S. R. Hyde, K. Benirschke, J. T. Kirchner, J. R. Papp, and P. E. Shewen. 1997. Psittacosis. Semin. Respir. Infect. 12:7–11.

Guaschino, S., and F. De Seta. 2000. Update on Chlamydia trachomatis. Ann. NY Acad. Sci. 900:286–292.

Hahn, D. L., R. W. Dodge, and R. Golubjatnikov. 1991. Association of Chlamydia pneumoniae (strain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. JAMA 266:225–230.

Inman, R. D., J. A. Whittum-Hudson, H. R. Schumacher, and A. P. Hudson. 2000. Chlamydia and associated arthritis. Curr. Opin. Rheum. 12:254–262.

Kosma, P. 1999. Chlamydial lipopolysaccharide. Biochim. Biophys. Acta 1455:387–402.

LaVerda, D., M. V. Kalayoglu, and G. L. Byrne. 1999. Chlamydial heat shock proteins and disease pathology: New paradigms for old problems? Infect. Dis. Obstet. Gynecol. 7:64–71.

Mabey, D., and R. Bailey. 1999. Eradication of trachoma worldwide. Br. J. Ophthalmol. 83:1261–1263.

Mardh, P. A. 2000. Chlamydia screening—yes, but of whom, when by whom, and with what? Ann. NY Acad. Sci. 900:286–292.

Muhlestein, J. B., J. L. Anderson, E. H. Hammond, L. Zhao, S. Trehan, E. P. Schwobe, and J. F. Carlquist. 1998. Infection with Chlamydia pneumoniae accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. Circulation 97:633–636.

Peeling, R. W., and R. C. Brunham. 1996. Chlamydiae as pathogens: New species and new issues. Emerging Infect. Dis. 2:307–319.

Robinson, A. J., and G. L. Ridgway. 2000. Concurrent gonococcal and chlamydial infection: How best to treat. Drugs 59:801–813.

Saikku, P., M. Leinonen, K. Mattila, M. R. Ekman, M. S. Nieminen, P. H. Makela, J. K. Huttunen, and V. Valtonen. 1988. Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet 2:983–986.

Shachter, J. 1997. DFA, EIA, PCR, LCR and other technologies: What tests should be used for diagnosis of chlamydia infections? Immunol. Invest. 26:157–161.

Schumacher, H. R. 2000. Chlamydia-associated reactive arthritis. Isr. Med. Assoc. J. 2:532–535.

Scidmore-Carlson, M., and T. Hackstadt. 2000. Chlamydia internalization and intracellular fate. Subcell. Biochem. 33:459–478.

Stagg, A. J. 1998. Vaccines against Chlamydia: Approaches and progress. Molec. Med. Today 4:166–173.

Stamm, W. E. 1999. Chlamydia trachomatis infections: Progress and problems. J. Infect. Dis. 179 (Suppl.):S380–S383.

Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, Q. Zhao, E. V. Koonin, and R. W. Davis. 1998. Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. Science 282:754–759.

Stephens, R. S. 2000. Chlamydial genomics and vaccine antigen discovery. J. Infect. Dis. 181 (Suppl.):S521–S523.

Tuuminen, T., P. Palomaki, and J. Paavonen. 2000. The use of serologic tests for the diagnosis of chlamydial infections. J. Microbiol. Methods 42:265–279.

Ward, M. 1995. The immunobiology and immunopathology of chlamydial infections. Acta Pathologica Microbiologica et Immunologica Scandinavica 103:769–796.

PART 8

Planctomyces and Related Bacteria

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CHAPTER 8.1

The Order Planctomycetales, Including the Genera *Planctomyces*, *Pirellula*, *Gemmata* and *Isosphaera* and the Candidatus Genera *Brocadia*, *Kuenenia* and *Scalindua*

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Introduction

The order Planctomycetales comprises a remarkable group of budding bacteria. They and their nearest relatives, the chlamydiae (Weisburg et al., 1986; see The Genus Chlamydia–Medical in this Volume), are the only known cell-wall containing bacteria that lack peptidoglycan. Furthermore, the planctomycetes are morphologically distinctive because of their budding division, their spherical to ovoid cells with crateriform pits (Figs. 1 and 2), and the nonprosthecate appendages (stalks) produced by some members of the group (Fig. 3). Multicellular aggregates or rosettes are formed by some species that produce polar holdfasts (Fig. 3). One genus, *Isosphaera*, is a multicellular filamentous bacterium that moves by gliding. Other motile members of the group produce flagella. A relatively recent addition to the morphological oddity of the planctomycetes is the discovery of cellular compartmentalization, posing a challenge to the traditionally held view of the prokaryote:eukaryote dichotomy (Fuerst and Webb, 1991; Lindsay et al., 1997; Lindsay et al., 2001). Knowledge of the order is limited owing to the relatively few species that have been obtained in pure culture and characterized. However, through the application of molecular microbial ecology techniques over the last 10 years, it has become apparent that planctomycetes are ubiquitous in a wide range of terrestrial and aquatic environments; the physiological diversity underlying this geographic ubiquity has not yet been fully explored. The availability of genome sequence data should provide a valuable resource for the future investigation of planctomycete biology and promises to reveal previously unknown aspects of these unique organisms.

General Planctomycete Phylogeny

The planctomycetes have a typical and unique ribosomal RNA composition. Early analysis of their 16S rRNA by oligonucleotide cataloging and sequence analyses originally placed this order as a deep branch within the Bacteria (Stackebrandt et al., 1984) and/or as a group that is undergoing rapid evolution (Woese, 1987; Liesack et al., 1992b). Thus, the planctomycetes differed markedly from the heterotrophic budding and prosthecate bacteria that fall within the alpha-proteobacteria. This was supported by studies of their 5S rRNA (Bomar et al., 1988), which indicated that its length is significantly shorter than that of most bacteria, ranging from 109 to 111 nucleotides rather than the "minimal" length of 118 bases of other bacteria and archaea (Erdmann and Wolters, 1986). In addition, position 66 lacks an insertion, and numerous transversions were noted in the secondary structure, features previously unknown for other bacteria.

Since the original 16S rRNA gene sequence analyses of described planctomycete species, application of novel culturing techniques has led to the isolation of new planctomycete strains (Fuerst et al., 1991; Schlesner, 1994; Wang et al., 2002). Phylogenetic analysis of these strains using 16S rDNA sequences (Fuerst et al., 1991; Ward et al., 1995; Griepenburg et al., 1999; Wang et al., 2002) has supported the distinct phylogenetic position of the planctomycete lineage and demonstrated the great phylogenetic diversity of the group (Fig. 4). For some of these strains, analysis of sequences encoding 23S rRNA (Ward et al., 2000), the 70-kDa heat shock protein (HSP70; Ward-Rainey et al., 1997), and the β-subunit of ATP synthase (Rönner et al., 1991) supports these findings. These analyses also indicate that while the planctomycete and Verrucomicrobia lineages tend to cluster together, the grouping is not statistically significant. The relationship between the planctomycetes and the chlamydiae, first proposed as sister taxa by Weisburg et al. (1986), is better supported by bootstrapping and other resampling methods. However, an analysis of elongation factor-Tu proteins from planctomycetes suggested long-branch attraction effects as a cause for the incon-

sistent branching position of the planctomycetes and did not support a close relationship between the planctomycetes and the chlamydiae (Jenkins and Fuerst, 2001). Despite the use of alternative phylogenetic markers, the position of the planctomycetes relative to other phyla is still unclear. A recent 16S rRNA-based phylogenetic analysis of the Bacteria using the most slowly evolving sequence positions suggests that the planctomycetes may emerge at the base of the Bacterial tree—reasonable bootstrap values support early

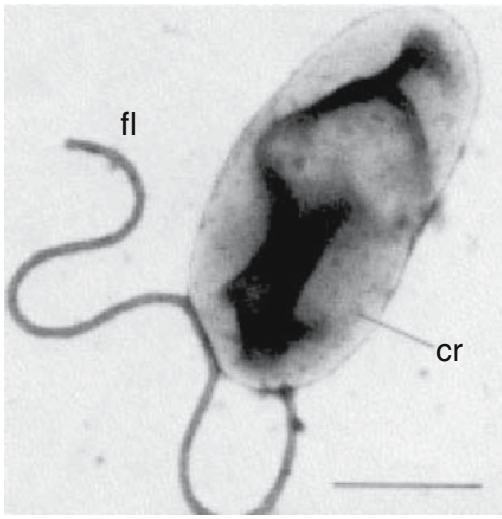


Fig. 1. An electron micrograph of a motile cell of *Pirellula marina* showing flagellum (fl) and numerous circular surface structures referred to as "crateriform structures" (cr). More distinctive crateriform structures are evidenced on some species (see Fig. 2 below). Bar = 1.0 mm. Courtesy of Heinz Schlesner.

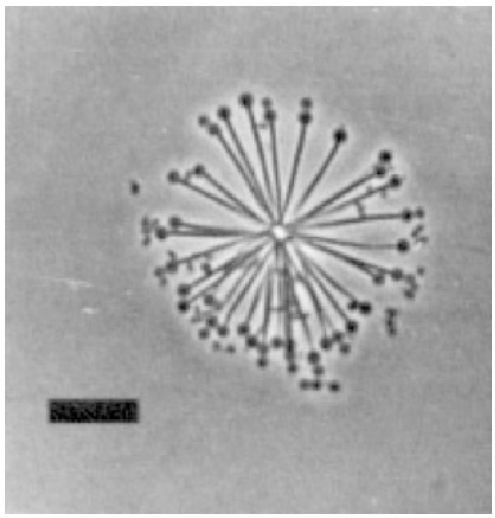


Fig. 3. A phase photomicrograph of a rosette of *Planctomyces bekefii* from University Lake, St. Lucia, Australia. The rosette consists of many spherical cells joined together at the distal tips of their stalks. Bar = 10.0 mm.

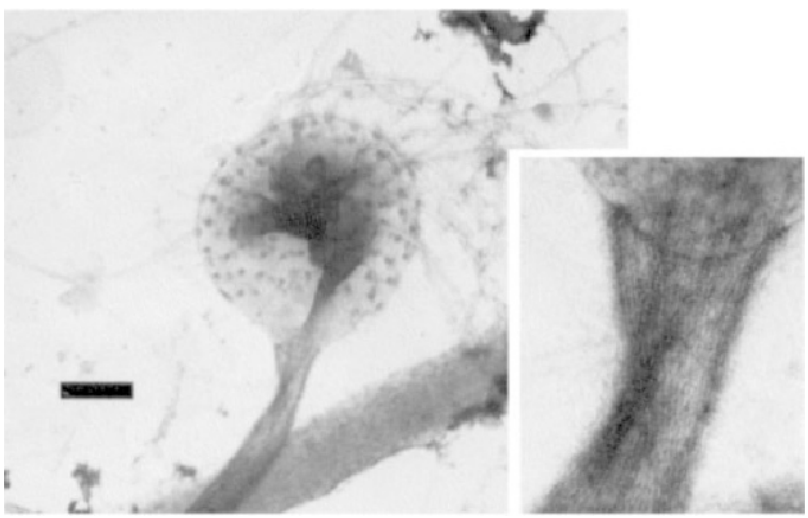
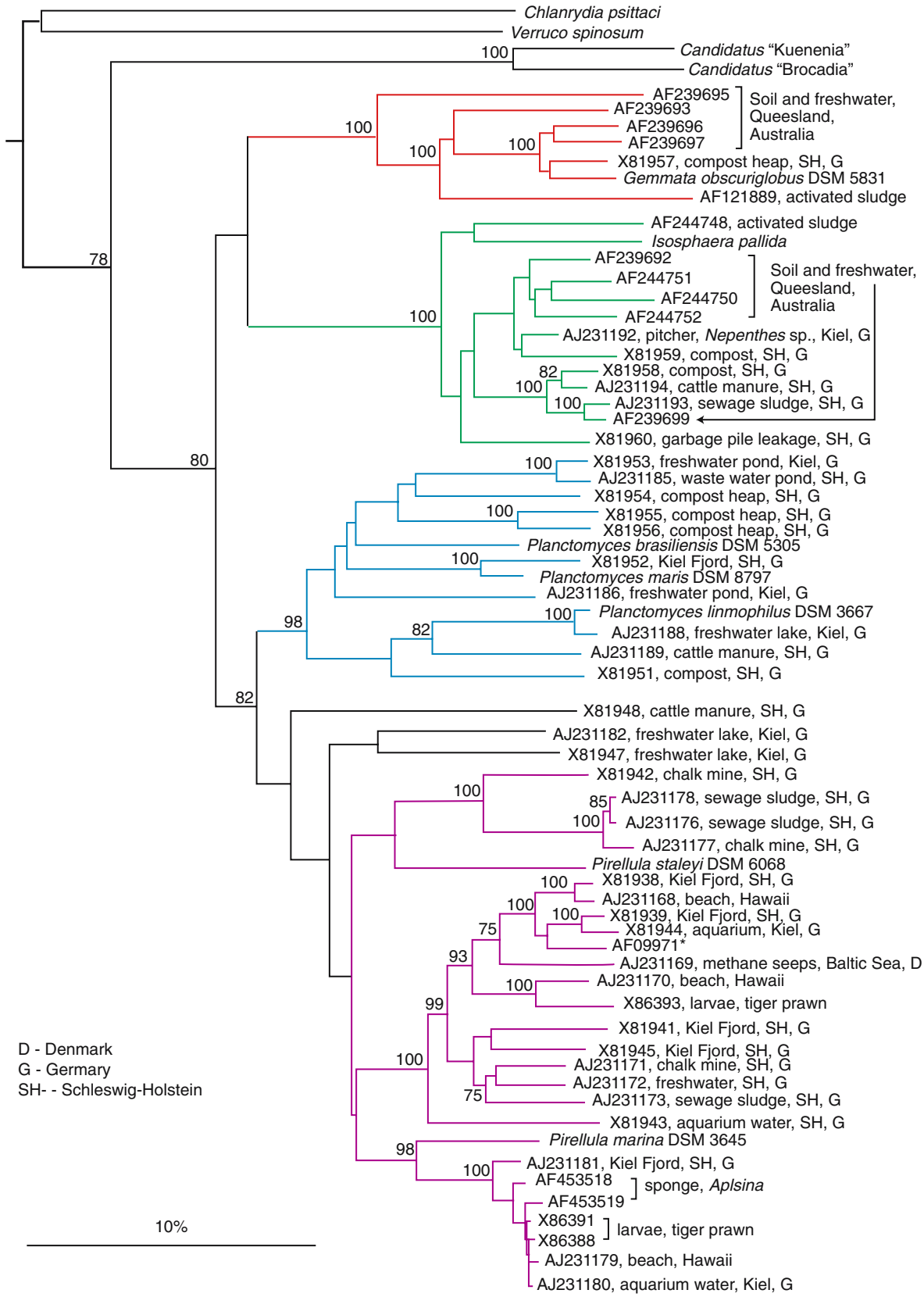


Fig. 2. An electron micrograph of a whole cell of *Planctomyces bekefii* showing many crateriform structures. The inset is an enlargement of the stalk-cell junction, showing the fibrillar nature of the stalk. Sample was collected from University Lake, St. Lucia, Australia, and stained with 1% uranyl acetate in 0.4% sucrose. Bar = 0.5 mm. Courtesy of John A. Fuerst.

Fig. 4. 16S rDNA dendrogram indicating the phylogenetic relationships between described species of the family Planctomycetaceae and isolates from various sites. Letters indicate the geographic origin of the majority of isolates (D = Denmark, G = Germany, and SH = Schleswig-Holstein). Analysis was based on 1170 unambiguous nucleotides (gaps removed). The sequence X86393 was added to the tree manually using the phylogenetic position determined on the basis of 750 nucleotides. The root was determined by using the 16S rDNA sequence of *Escherichia coli* K12 as an outgroup reference. The bar represents 10% sequence divergence, as determined by measuring the lengths of the horizontal lines connecting any two organisms. Numbers refer to bootstrap values; only values above 70% are shown.


Chlanrydia psittaci
Verruco spinosum
Candidatus "Kuenenia"
Candidatus "Brocadia"
AF239695
AF239693
AF239696
AF239697
Soil and freshwater, Queesland, Australia
X81957, compost heap, SH, G
Gemmata obscuriglobus DSM 5831
AF121889, activated sludge
AF244748, activated sludge
Isosphaera pallida
AF239692
AF244751
AF244750
AF244752
Soil and freshwater, Queesland, Australia
AJ231192, pitcher, Nepenthes sp., Kiel, G
X81959, compost, SH, G
X81958, compost, SH, G
AJ231194, cattle manure, SH, G
AJ231193, sewage sludge, SH, G
AF239699
X81960, garbage pile leakage, SH, G
X81953, freshwater pond, Kiel, G
AJ231185, waste water pond, SH, G
X81954, compost heap, SH, G
X81955, compost heap, SH, G
X81956, compost heap, SH, G
Planctomyces brasiliensis DSM 5305
X81952, Kiel Fjord, SH, G
Planctomyces maris DSM 8797
AJ231186, freshwater pond, Kiel, G
Planctomyces linmophilus DSM 3667
AJ231188, freshwater lake, Kiel, G
AJ231189, cattle manure, SH, G
X81951, compost, SH, G
X81948, cattle manure, SH, G
AJ231182, freshwater lake, Kiel, G
X81947, freshwater lake, Kiel, G
X81942, chalk mine, SH, G
AJ231178, sewage sludge, SH, G
AJ231176, sewage sludge, SH, G
AJ231177, chalk mine, SH, G
Pirellula staleyi DSM 6068
X81938, Kiel Fjord, SH, G
AJ231168, beach, Hawaii
X81939, Kiel Fjord, SH, G
X81944, aquarium, Kiel, G
AF09971*
AJ231169, methane seeps, Baltic Sea, D
AJ231170, beach, Hawaii
X86393, larvae, tiger prawn
X81941, Kiel Fjord, SH, G
X81945, Kiel Fjord, SH, G
AJ231171, chalk mine, SH, G
AJ231172, freshwater, SH, G
AJ231173, sewage sludge, SH, G
X81943, aquarium water, SH, G
Pirellula marina DSM 3645
AJ231181, Kiel Fjord, SH, G
AF453518
AF453519
sponge, Aplsina
X86391
X86388
larvae, tiger prawn
AJ231179, beach, Hawaii
AJ231180, aquarium water, Kiel, G
D - Denmark
G - Germary
SH- - Schleswig-Holstein
10%

emergence of the group, even as the first branching one in place of hyperthermophiles (Brochier and Philippe, 2002). If confirmed, such a position would place planctomycetes at the center of evolutionary microbiology and the search for the last common ancestor of the domains of life.

General Planctomycete Taxonomy

Because of their unique position within the Bacteria, a new order, Planctomycetales, was proposed to accommodate members of this group (Schlesner and Stackebrandt, 1986b). The order is named for the species *Planctomyces bekefii*, which was first described early in the century by Gimesi (1924), who observed this rosette-forming microorganism in a pond in Hungary. He regarded it as a planktonic fungus, thereby explaining the root "*myces*" in the genus name. Organisms of this group were also reported by Henrici and Johnson (1935), who observed them in lakes in the northern United States but assigned them to the bacteria as *Blastocaulis sphaerica*. Neither Gimesi nor Henrici and Johnson obtained pure cultures. The two taxonomic entities were united in 1972 when a careful comparison of both type material (archival drawings) and water samples from the original environments showed the organisms to be identical; *Pl. bekefii* was given priority (Hirsch, 1972). Subsequently several other planktonic, rosette-forming colonial species, including *Pl. crassus*, *Pl. stranskae*, *Pl. gracilis* and *Pl. guttaeformis*, were named but not isolated (see Starr and Schmidt, 1989). Some, such as *Pl. crassus*, were designated fungi, whereas others were described as bacteria. The issue was not finally resolved until 1980, when Schmidt and Starr (1980a, b) demonstrated by electron microscopy that *Pl. bekefii* from both the Hungarian and North American type localities had the cell ultrastructure of a prokaryote. Perhaps in the light of recent findings on cell compartmentalization in planctomycetes, the case from structure alone was not entirely simple and may have been due largely to the use only of chemical fixation in these early studies.

The first reports of pure cultures of this group were published in the 1970s (Staley, 1973; Bauld and Staley, 1976; Schmidt, 1978a). The organisms grew as slow-growing heterotrophs on dilute organic media. Nevertheless, many of the large rosette forms have not yet been isolated, including the type species of the genus, *Planctomyces bekefii*. All cultivated named species, and all named species described only on the basis of microscopic observation, are aquatic, occurring in freshwater lakes, marine habitats, and salt ponds. However, as described below, the presence of planctomycetes in soil and other nonaquatic habitats has been detected using culture-independent 16S rRNA-based methods. This wider habitat range is also reflected in the isolate collections obtained by Schlesner (1994) and Wang et al. (2002), who used cell wall synthesis-inhibiting antibiotics and novel carbon sources to isolate strains from soil, water, compost, manure, etc.

In addition to the unicellular and rosette-forming colonial forms, the order contains filamentous organisms. The filamentous types were initially thought to be cyanobacteria and were named "*Isocystis pallida*" (Woronichin, 1927). However, strains that have been isolated do not contain chlorophyll *a* or other photosynthetic pigments (Giovannoni et al., 1987b). Isolated strains that were obtained from neutral and alkaline hot springs at temperatures between 35 and 55°C were placed in a new genus as *Isosphaera pallida*. Table 1 can be used to distinguish the genera of the order Planctomycetales.

Chemical Properties and Chemotaxonomy

Cell Wall Structure

With the exception of the planctomycetes and *Chlamydia* spp., all other bacteria that possess a cell wall contain peptidoglycan as the main cell

Table 1. Characteristics differentiating the genera of the order Planctomycetales.

Genus	Cell arrangement	Stalk	Flagellar type	Crateriform structure	G + C content (mol%)
Pirellula	Rosettes in pure culture	–[a]	Single, polar	Reproductive pole	54–57
Gemmata	Single cells	–	Polar bundle	Over entire cell surface	64
Planctomyces	Single cells or rosettes	+	Single, polar	Over entire cell surface or reproductive pole	50–58
Isosphaera	Filamentous	–	–[b]	Over entire cell surface	62

Symbols: +, property present; and –, property absent.

[a]A rudimentary "stalk" has been reported in ATCC strain 27377 (Starr et al., 1983).

[b]Moves by gliding.

wall structural compound. The absence of peptidoglycan and the occurrence of a proteinaceous cell wall in the planctomycetes were first reported for several strains of *Planctomyces* and *Pirellula* (then *Pasteuria*; König et al., 1984). This finding explained the considerable resistance of these strains to antibiotics known to target the synthesis of peptidoglycan, including not only the ß-lactams but also non-ß-lactams such as D-cycloserine (Schmidt and Starr, 1982a; König et al., 1984); β-lactamase activity (a possible explanation for resistance) was tested in *Pi. marina* and *Pl. maris* and found to be absent (Claus et al., 2000).

The inclusion of *Gemmata obscuriglobus* and *I. pallida* in the order Planctomycetales was supported by the presence of a proteinaceous cell wall in these organisms (Liesack et al., 1986; Stackebrandt et al., 1986b; Giovannoni et al., 1987a). A more detailed analysis of the cell wall amino acid composition of members of *Planctomyces* and *Pirellula* revealed proteins rich in glutamate and cystine/cysteine, the disulfide bonds of which may contribute to the sodium dodecylsulfate (SDS)-resistance of the cell envelope (Liesack et al., 1986). The cell wall of *G. obscuriglobus*, on the other hand, is low in cystine/cysteine (Stackebrandt et al., 1986b).

Quinone Composition

Another chemotaxonomic marker that confirms the phylogenetic coherence of the order Planctomycetales is the presence of the isoprenoid quinone MK6, a menaquinone (Sittig and Schlesner, 1993) in all species investigated (a strain of *I. pallida* was not included in the study). The main isoprenoid quinone among Gram-negative bacteria is ubiquinone, but menaquinone does occur in members of lines of descent other than the planctomycetes, e.g., the *Bacteroides*, *Cytophaga*, *Flavobacterium* phylum (Collins and Jones, 1981) and certain archaeal families.

DNA Base Composition

The genomic DNA G+C content of members of the order Planctomycetales ranges between 51 and 70 mol%, with values above 60% found in strains of *G. obscuriglobus* and *I. pallida* and their relatives and in two strains that are phylogenetically related to *Pl. limnophilus* and *Pl. brasiliensis* (Griepenburg et al., 1999). The base composition of the 16S rRNA gene for most planctomycetes ranges between 53 and 57 mol%. *Isosphaera pallida* and related strains exhibit 16S rRNA values ranging between 60 and 62%; it has been suggested that these values indicate the 16S rRNA genes of these organisms have been subjected to a higher-than-normal evolutionary rate.

Lipid Composition

Members of the genera *Planctomyces* and *Pirellula* (the latter formerly called "*Pirella*") have characteristic Bacteria-type ester-linked polar lipids rather than the ether-linked lipids of the Archaea (Kerger et al., 1988). However, this may not be a universal phenomenon in planctomycetes as a whole (see below).

Early investigations of planctomycete cellular fatty acids revealed some unusual features. The presence of palmitic, palmitoleic, and oleic acids in *Pirellula* spp. and *Planctomyces* spp. was interpreted as a pattern more typical of microeukaryotes than of bacteria (Kerger et al., 1988). However, these fatty acids are not exclusive to planctomycetes but have subsequently been found widely distributed in other prokaryotic taxa. Similarly, the 18-carbon monounsaturated 18:1ω9c and 18:1ω11c fatty acids detected in members of *Planctomyces* and *Pirellula* (Kerger et al., 1988) are widely distributed among other bacteria. *Pirellula* strains possess hydroxy fatty acids of the 3-hydroxy-octadecanoic acid (3-OH-*n*-18:0) type, but lack 3-hydroxyeicosanoic acid (3-OH-*n*-20:0), whereas in *Planctomyces* strains the distribution of these compounds is reversed (Sittig and Schlesner, 1993). Evidence for the presence of 3-hydroxy acids in *I. pallida* has also been obtained (Giovannoni et al., 1987a), but attempts to obtain a more detailed composition of hydroxy fatty acids in *G. obscuriglobus* were not successful (Sittig and Schlesner, 1993). Claims of lipopolysacharide production by planctomycetes (Kerger et al., 1988) must thus await data derived by methods other than detection of hydroxy fatty acids, since lipopolysaccharide (LPS) itself has not been directly detected (Fuerst, 1995).

Data on phospholipid composition are available for only a few planctomycete strains; phospholipid pattern has been shown to be similar in phylogenetically closely related strains, but different among species (Sittig and Schlesner, 1993; Table 2).

A remarkable novel "ladderane" lipid has been found in an anaerobic chemolithotrophic ammonium-oxidizing planctomycete *Candidatus* "Brocadia anammoxidans." This lipid is a series of linearly concatenated cyclobutane rings and occurs in the membrane surrounding the internal compartment (the anammoxosome) that contains enzymes involved in ammonium oxidation. These lipids contribute to the exceptional density of this membrane and may act to limit diffusion, protecting the rest of the cell from toxic anammox intermediates (Sinninghe Damste et al.,

Table 2. Phospholipids of *Pirellula* and *Planctomyces* strains.

Strains	Phospholipids							
	PG	PE	MPE	DPE	PC	BPG	UP	Glyl
Pirellula								
Group 1[a]	+	–	–	+	(+)	(+)	–	+
Group 2	+	–	–	+	+	+	–	–
Group 3	+	–	–	–	–	(+)	(+)	–
Pi. staleyi (ATCC 27377^T)	+	–	–	+	–	(+)	–	–
Planctomyces								
Group 1	+	–	–	–	–	–	(+)	+
Group 2	+	–	–	–	–	+	(+)	+
Group 3	(+)	–	+	+	(+)	(+)	(+)	+

Symbols: +, main component; (+), present in less amounts; –, not present.
Abbreviations: PG, phosphatidylglycerol; PE, phosphatidylethanolamine; MPE, phosphatidylmono-methylethanolamine; DPE, phosphatidyldimethylethanolamine; PC, phosphatidylcholine; BPG, biphosphatidylglycerol; UP, unknown phospholipids; and Glyl, glycolipid.
[a]Groups are defined by 16S rDNA-based phylogenetic analyses as follows. *Pirellula* group 1: strain 1 (IFAM 1310), strain 140 (IFAM 1319), strain 198 (IFAM 1735); group 2: strain 143 (IFAM 1358), strain 158 (IFAM 1452); group 3: *Pi. marina* (IFAM 1313^T), strain 217 (IFAM 1945).
Planctomyces group 1: strain 203 (IFAM 1999), strain 244 (IFAM 2075), strain 237 (IFAM 2079); group 2: *Pl. limnophilus* (IFAM 1008^T), *Pl. brasiliensis* (IFAM 1448^T), strain 240 (IFAM 3001); group 3: *Pl. maris* (ATCC 29201^T), strain 130 (IFAM 1317), strain 219 (IFAM 1951).

2002). Also significant is their finding that ladderane-containing lipids included dialkyl glycerol diethers, ether-linked lipids typical of Archaea and atypical of Bacteria (i.e., found only in a few deep-branching thermophiles and some sulfate-reducing mesophiles).

Polyamine Distribution

Like phospolipid composition, polyamine distribution has been shown to be a useful chemotaxonomic marker at and below the species level (Griepenburg et al., 1999). All analyzed members of the order Planctomycetales contained significant amounts of polyamines and *sym*-homospermidine, except *Pl. limnophilus* and related strains, which had high amounts of putrescine as the dominant polyamine component. The distribution of putrescine, *sym*-homospermidine and spermidine reflects the phylogenetic diversity within the order Planctomycetales, as closely related representatives of the phylogenetic groups defined by described species and novel isolates exhibit similar polyamine patterns.

Planctomycete Ecology

Habitats and Trophic States

Planctomycetes were first observed in freshwater environments but are now known to be present in other aquatic habitats, including marine and hypersaline waters, and also in nonaquatic environments such as soil and sewage sludge. Many observations provide a record of planctomycete distribution. *Planctomyces bekefii* morphotypes have been reported in freshwater in geographically diverse locations: Hungary, Denmark, other European sites, Vietnam, Venezuela, Israel, Australia and the United States. Isolates have been obtained from environments existing in all trophic (i.e., oligotrophic to highly polluted) states. Some reports have suggested that higher numbers of planctomycetes are found in eutrophic and polluted environments than in eutrophic samples. High pH values (6.8–9.4), high conductivity, and temperate summer temperatures have also been suggested to be associated with increases in planctomycete numbers. However, in a study of Australian freshwater samples, Staley et al. (1980) suggested that planctomycetes could be found in all trophic states and that the proportion of planctomycetes to total viable heterotrophs was not dependent on trophic state. Fuerst (1995) has suggested *Pl. bekefii* as an environmental indicator for eutrophic conditions.

Mineral Encrustation

Many of the rosette-forming planctomycetes have been observed to accumulate iron and/or manganese on the surface of the stalk in their natural freshwater habitats (Schmidt et al., 1981b; Schmidt et al., 1982b). These deposits appear as phase-bright regions of the rosette structure under phase microscopy. The accumulation can be observed either at the central point where the stalks join to each other via holdfasts or all along the length of the stalks. Energy-

dispersive X-ray spectroscopy has been used to demonstrate that manganese is accumulated by members of morphotype 1a (i.e., *Pl. bekefii*), while both iron and manganese are deposited on the stalks of morphotype 1b species (*Pl. crassus*; Schmidt et al., 1981b; Schmidt et al., 1982b). Iron encrustation may be due to passive accumulation of oxidized iron, but oxidation of reduced manganese may be biologically catalyzed by the planctomycetes.

Association with Algal Blooms

A considerable amount of evidence suggests that there is an association between the occurrence of algal or cyanobacterial blooms and increases in the numbers of freshwater planctomycetes. There have been several reports that the numbers of the rosette-forming planctomycetes (*Pl. bekefii*, *Pl. guttaeformis* and *Pl. stranskae*) have been found to increase in freshwater habitats after algal or cyanobacterial blooms. Granberg (1969) observed that increases in numbers of *Pl. bekefii* and *Pl. condensatus* correlated with a seasonal increase in heterotrophic phytoplankton in a reservoir. Organisms resembling *Pl. bekefii* and *Pl. guttaeformis* have been observed in a Louisiana lake during and subsequent to a bloom of a *Pithophora* species (N. Ward, unpublished observation). Further evidence for this hypothesis is provided by the fact that laboratory enrichments of planctomycetes are favored by the presence of ambient light and consequent phototroph development (Starr and Schmidt, 1989) and that freshwater *Pirellula* occur as epiphytes of ensheathed cyanobacteria (The Order Planctomycetales and the Genera *Planctomyces*, *Pirellula*, *Gemmata*, and *Isosphaera* in the second edition). It has been suggested (Kristiansen, 1971) that decomposition of phytoplankton produces hydrogen sulfide (H_2S) and results in rising iron and manganese concentrations that may favor *Pl. bekefii* blooms.

The evidence garnered so far for the interrelationship of planctomycete and algal/cyanobacterial population dynamics in freshwater systems is mostly anecdotal, a situation that could be remedied by a systematic investigation of a freshwater body in which planctomycete numbers have been reported to fluctuate on a seasonal basis. Such a study would include detailed measurements of physicochemical parameters such as temperature, pH, dissolved oxygen, carbon and nitrogen, as well as quantification and identification of planctomycete species by microscopy and molecular (16S rDNA based) techniques. An alternative approach could involve the use of microcosms in the laboratory, if cultures of the rosette-forming species were available.

Association with Crustaceans

There have been several reports of possible association of planctomycetes with crustacea. The first of these was Metchnikoff's report of pear-shaped parasitic organisms associated with cladoceran water fleas of *Daphnia* spp. (Metchnikoff, 1888). This organism was later shown to be the non-planctomycete endospore-forming *Pasteuria ramosa* (related to *P. penetrans* and *P. thornei*, pathogens of plant-parasitic nematodes; Starr et al., 1983).

However, genuine planctomycetes have also been found in association with crustacea, including putative *Pirellula* species attached to the carapace of *Daphnia pulex* (Staley, 1973) and isolated from the hepatopancreas or whole post-larvae of the giant tiger prawn *Penaeus monodon* (Fuerst et al., 1991; Fuerst et al., 1997). The prawn isolates have been shown to be phylogenetically related to *Pirellula* or *Planctomyces* species, in the latter case very closely to *Pl. brasiliensis*, on the basis of 16S rRNA gene sequence analysis (Fuerst et al., 1997). These species may inhabit prawn hemolymph as well as the gut and may be selected for, especially in baculovirus-infected prawns. The exact nature of the association has not yet been determined.

Association with Sponges

Recently, the association of planctomycetes with sponges has been reported. Hosts were the Mediterranean sponges *Aplysina cavernicola* (Friedrich et al., 1999) and *A. aerophoba* (Friedrich et al., 2001). Also the sponge *Rhopaloeides odorabile* from the Great Barrier Reef, Australia, was found to host planctomycetes (Webster et al., 2001).

Ecology and Physiology

Despite the reported widespread distribution of planctomycete bacteria, little is known of their role in the environment. The physiology and metabolism of planctomycetes are not well understood, and most of the available physiological data were obtained to allow taxonomic description of new species and genera. All planctomycete strains currently held in pure culture are chemoheterotrophs, but representatives in mixed laboratory culture include the chemoautotrophs performing anaerobic oxidation of ammonium (see below). Carbohydrates serve as the major carbon source, but *Pl. limnophilus*, *Pl. brasiliensis* and *Pi. marina* can also utilize gelatin, while starch is used by *Pl. brasiliensis*, *Pi. marina* and *G. obscuriglobus* (Franzmann and Skerman, 1984; Hirsch and Müller, 1985; Schlesner, 1986a; Schlesner, 1989). The plancto-

mycetes are relatively slow growing; the shortest recorded generation time is 13 h for *Pl. maris* and *G. obscuriglobus* (Bauld and Staley, 1976; Franzmann and Skerman, 1984). Isolation and maintenance of many planctomycete strains require rather dilute media, and rich media have been shown to be inhibitory to growth in some cases (Schlesner, 1994).

Some species are obligate aerobes (*Pl. maris*, *G. obscuriglobus* and *I. pallida*; Bauld and Staley, 1976; Franzmann and Skerman, 1984; Giovannoni et al., 1987b), while others (*Pi. marina*, *Pl. limnophilus*; Hirsch and Müller, 1985; Schlesner, 1986a) are facultative anaerobes, in the sense that they can ferment carbohydrates. The presence of cytochrome *c* oxidase and the ability of some strains—*Pi. marina* and *Pl. brasiliensis* (Schlesner, 1986a; Schlesner, 1989)—to reduce nitrate suggest that they possess electron transport chains resembling those found in other facultative aerobes.

Isosphaera pallida exhibits a number of physiological properties not seen in other members of the order Planctomycetales. Most of these characteristics bear relation to the hot spring environments from which *I. pallida* has been isolated or in which organisms morphologically similar to *I. pallida* have been observed. The most obvious of these is the moderate thermophily (maximum temperature for growth of 55°C with an optimum of 41°C; Giovannoni et al., 1987b) of *I. pallida*, but also its phototactic ability (unique among heterotrophs) and formation of gas vesicles, which would allow vertical positioning in the water column (Giovannoni et al., 1987b). It has been suggested (Fuerst, 1995) that the phototaxis of *I. pallida* in the absence of phototrophy may make it a good model organism for studying the mechanisms of phototaxis. *Isosphaera pallida* can be grown in coculture with a hot spring phototroph (*Heliothrix oregonensis*) under aerobic conditions in the light; the phototroph cannot be grown in culture in the absence of *I. pallida* (Pierson et al., 1985). Ward et al. (1990) report detection of *I. pallida* by microscopy and 16S rRNA gene sequence-based molecular studies in the top layers of the Octopus Spring cyanobacterial mat, suggesting that this organism may be widely distributed in hot springs other than its type locality. Other physiological traits of *I. pallida* include obligate oligotrophy (growth is inhibited by glucose concentrations greater than 0.025%) and gliding motility, never previously reported in a budding bacterium.

Recent reports have identified deep-branching planctomycetes as the "missing lithotrophs" carrying out anaerobic ammonia oxidation ("anammox"; Strous et al., 1999; Schmid et al., 2000; Van Dongen et al., 2001). The anammox process removes ammonia nitrogen from wastewater, combining ammonia and nitrite into dinitrogen gas, and is important for the reduction of ammonia emissions due to agricultural use of chemical fertilizers. The planctomycetes carrying out this process have been identified by electron microscopy showing the presence of characteristic cellular compartmentalization and crateriform structures and by 16S rDNA sequencing and fluorescent in situ hybridization (FISH) with specific oligonucleotide probes directly on anammox bioreactor biomass or concentrated fractions from such biomass. This finding extends the known physiological range of the planctomycetes to include anaerobic autotrophs and has resulted in the proposition of two new *Candidatus* genera, "Brocadia" and "Kuenenia" (Strous et al., 1999; Schmid et al., 2000). Studies attempting to elucidate the mechanism of the anammox process have begun (Jetten et al., 2001).

Recent studies of sediments from the Baltic-North Sea area indicated that anaerobic ammonium oxidation is occurring (Dalsgaard and Thamdrup, 2002; Thamdrup and Dalsgaard, 2002). Ammonium-oxidizing anammox planctomycetes and their activities have also been detected in the Black Sea, the world's largest anoxic basin (Kuypers et al., 2003), and in anammox activity in an anoxic bay off the coast of Costa Rica (Dalsgaard et al., 2003), indicating a significant role for anammox planctomycetes in the global nitrogen cycle, as biological agents for molecular nitrogen regeneration in anoxic conditions.

It is worth noting that the phylogenetic diversity and depth of the planctomycete lineage suggest that physiological types other than those already reported for this group may be detected. So too, full physiological potential of existing cultured strains may not be appreciated until genome sequence annotations are interpreted and the "metabolome" deduced (see Whole-Genome Sequencing Studies).

Molecular Ecology Studies

Planctomycete sequences were detected in the very first 16S rDNA library to be constructed from a soil (Mt. Coot-tha, Australia; Liesack and Stackebrandt, 1992a; Stackebrandt et al., 1993). Since then, they have been found in multiple libraries generated from terrestrial, marine, freshwater, industrial, and organism-associated environments around the world (Table 3). This wide distribution suggests a ubiquitous distribution and diverse ecological niches for the planctomycetes. It has also stimulated renewed attempts, several of them successful, to recover some of these organisms in pure culture (see

Table 3. Selected environmental clone sequences (published) identified in public databases as members of the order Planctomycetales.

Source	Sequence accession nos.	References
Terrestrial		
Australian forest soil	X64375-X64379, X64383, and X64384	Liesack and Stackebrandt, 1992a
Soil, eastern Amazonia	U68622	Borneman and Triplett, 1997
Deltaic muds, Papua New Guinea	AF193570	Todorov et al., 2000
Geothermally heated soil	AF465649 and AF465657	Norris et al., 2002
Ikaite tufa columns, Greenland	AJ431346 and AJ431347	Stougaard et al., 2002
Indonesian forest soil	AF256037	Krave et al., 2002
Wisconsin soil	a214620, a214638, a214657, and a214668	Liles et al., 2003
Marine		
Marine aggregate	L10942	DeLong et al., 1993
Marine sediment	UEU43644 and UEU43645	Gray and Herwig, 1996
Coastal picoplankton, North Carolina	U70681 and U70712	Rappe et al., 1997
Marine	AF029076–AF029079	Vergin et al., 1998
Cold marine sediments	AJ297459 and AJ297460	Ravenschlag et al, 1999
Antarctic deep sea	AF257295	Lopez-Garcia et al., 2001
Antarctic coastal shelf sediment	AF424457–AF424476, AF424478–AF424484, and AF424486–AF424503	Bowman and McCuaig, 2003
Marine sediment (ammonia oxidizing)	AJ301578	Freitag and Prosser, 2003
Freshwater		
Lake Baikal	X99985	Bel'kova et al., 1996
Lake Baikal picoplankton	AJ011147	Semenova and Kuznedelov, 1998
Hot spring, Yellowstone	AF027057–AF027059	Hugenholtz et al., 1998
Mono Lake (soda lake)	AF507874	Humayoun et al., 2003
Organism-associated		
Human colon	UPU58225	Wilson and Blitchington, 1996
Algae	AF259633	Meusnier et al., 2001
Chewing lice	AF467389	Reed and Hafner, 2002
Diseased coral	AF441983, a038393, a038488, a038494, a038496, a038561, a038579, AF473941, and AF544881	Frias-Lopez et al., 2002 Cooney et al., 2002 Pantos et al., 2003
Industrial		
Activated sludge	UNC225334, UNC225371, UNC225359, and UNC225363	Christensson et al., 1998
Ammonium-oxidizing biofilm	AB057453	Fujii et al., 2002
Uranium mining waste (ammonium-oxidizing)	AJ296618–AJ296620, AJ296629, and AJ301578	Radeva and Selenska-Pobell, 1999

Application of New Isolation Strategies) and to detect them using specific 16S rRNA-targeted oligonucleotide probes (e.g., Neef et al., 1998). The latter study confirmed widespread distribution of planctomycetes in different ecosystems and demonstrated that the "universal" bacterial probe EUB338 does not generally hybridize to planctomycete cells. A separate study (Vergin et al., 1998) indicated a consistent mismatch between planctomycetes and another commonly used "universal" primer (27F) and, importantly, reported the first environmental, genomics data for planctomycetes; four fosmid clones containing planctomycete genomic DNA (as indicated by 16S rRNA signature) were obtained from a marine environment. In a recent study of agricultural soils, planctomycetes were found to comprise 3–12% of the ribosomal RNA abundance (Buckley and Schmidt, 2003). As the number of environmental genomics projects, both terrestrial and aquatic, continues to rapidly increase, we can look forward to a wealth of genomic sequence data from planctomycete constituents of the microbial communities that are sampled and a better understanding of their ecological roles in these communities. Comparison of these data with those obtained from ongoing genome sequence projects for cultivated planctomycete strains (see Whole-Genome Sequencing Studies) may allow us to pinpoint differences between cultivated and as-yet-uncultured strains.

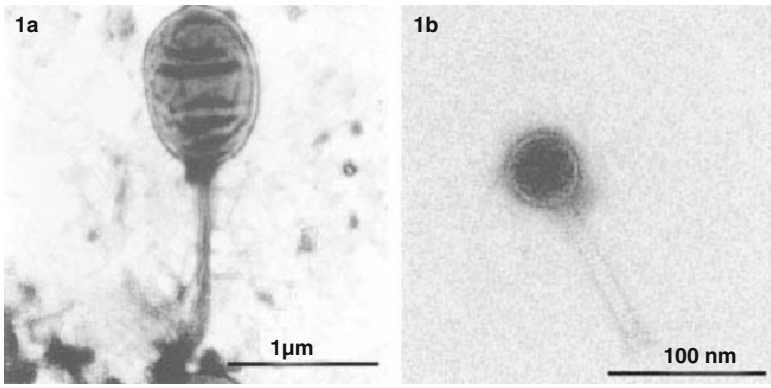


Fig. 5. Electron micrographs of a) *Planctomyces limnophilus* (DSM 1358) and b) its phage. With permission from C. Gliesche.

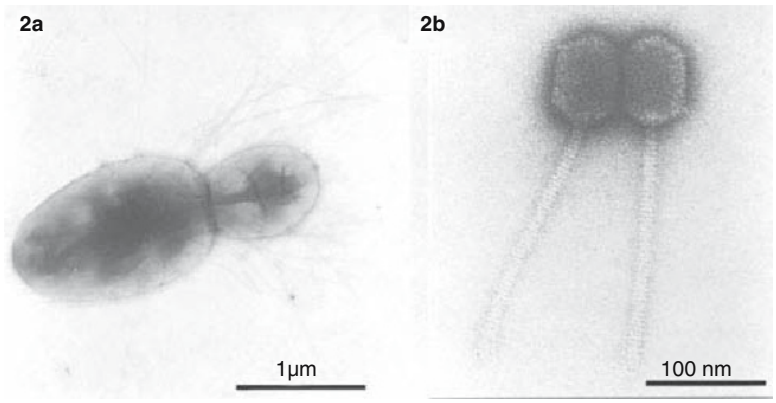


Fig. 6. Electron micrographs of a) *Pirellula* sp IFAM 1358 and b) its phage. With permission from C. Gliesche.

Bacteriophages of Planctomycetes

The only reports of the occurrence of bacteriophages in planctomycete strains originate from the laboratory of Peter Hirsch, Kiel, Germany (Gliesche, 1980; Majewski, 1985). Phage Pl-89 was enriched by polyethylene glycol (PEG)-6000 precipitation of a mass culture of *Planctomyces limnophilus* IFAM 1008 (DSM 3776^T). This phage has been classified as a member of morphogroup B_1 (Akkerman and Eisenstark, 1974), family Styloviridae (Fig. 5). The linear double-stranded (ds)DNA has a genome size of 24.4 (±0.7) MDa (electron microscopic determination) or 28.13 MDa (*Bgl*II digest). The mol% G+C content of the DNA is 57.8%. The host strain of Phage Pi-57 is *Pirellula* sp. IFAM 1358. This phage is also a member of the Styloviridae and belongs to morphogroup B_2 (Fig. 6). The linear dsDNA has a genome size of 30.34 (± 2.19) MDa (electron microscopic determination) or 31.80 MDa (*Bgl*II digest). The mol% G+C content of the DNA is 58.7%. The DNA conformation of these tailed bacteriophages of planctomycete strains is in accordance with the description of these properties in bacteriophages of other budding bacteria such as *Hyphomicrobium*, *Caulobacter*, *Asticcacaulis* and *Ancalomicrobium* (Reanney and Akkermann, 1982).

Application of New Isolation Strategies

The most successful attempt at isolation of members of the order included the use of media containing ampicillin, cycloheximide, and a range of nutrients, including very selective carbon and nitrogen sources such as *N*-acetylglucosamine, and enrichment in dilute media (Schlesner, 1994). Many of these planctomycete isolates did not grow on nutrient-rich media; rapidly growing organisms were previously shown to overgrow planctomycetes on rich media (Hirsch and Müller, 1985). The strategy used by Schlesner (1994) resulted in the isolation of 257 putative planctomycete strains variously resembling members of the genera *Planctomyces* and *Pirellula*, but also including spherical and prosthecate cell morphologies. These strains were subsequently shown by 16S rRNA gene sequence analysis to be a set of phylogenetically novel isolates (Ward et al., 1995; Griepenburg et al., 1999). Some isolates were closely related to described planctomycete species, but the

majority of strains, isolated from a geographically rather small area (Northern Germany), were as unrelated to each other (as are the type species of the four planctomycete genera that were isolated from different continents), demonstrating that planctomycete strains were more diverse than strains held in pure culture up to that point (Ward et al., 1995; Griepenburg et al., 1999; see General Planctomycete Phylogeny). The strains were isolated from a wide range of environmental samples, extending the known habitats of the planctomycetes.

Future Directions for Isolation Strategies

Considering the enormous phylogenetic depth of the order Planctomycetales, comparable to that within the class Proteobacteria or the phylum of Gram-positive bacteria (Fig. 4), the full range of planctomycete diversity has most likely not been fully explored. The cumulative impact of recent isolation studies such as that of Schlesner (1994) and the results of 16S rRNA gene sequence based molecular ecology studies (see Molecular Ecology) suggest that the planctomycetes are a diverse and possibly ecologically very important group of bacteria. The difficulty experienced in their cultivation may be explained by the almost complete lack of knowledge of their metabolic processes. Our current attempts to isolate planctomycetes will undoubtedly be superseded by more focused and directed strategies once this physiological information becomes available and is supplemented by genomic and proteomic data.

New Approaches to Identification

Our lack of knowledge about biochemical and physiological properties that are routinely used in the identification of other prokaryotic species means that planctomycete species have often been described and identified on morphological grounds, as they were 75 years ago (Gimesi, 1924). It is only recently that comparative 16S rDNA sequence analyses and the recognition of planctomycete-specific oligonucleotides (Woese et al., 1985) and other molecular signatures (Liesack and Stackebrandt, 1992a; Liesack et al., 1992b) have provided microbiologists with a reliable and rapid tool to unambiguously affiliate strains to the order Planctomycetales and gave evidence that the genus-specific morphological properties are of phylogenetic significance (Liesack et al., 1992b). Table 4 compiles a set of 16S rDNA signatures that define the majority of strains of the four genera of the family Planctomycetaceae. This information is derived from the sequences of cultured species type strains and from the sequences of isolates found to be closely to moderately related to these type strains. Novel species can be predicted when isolates share lower than 97–98% sequence similarity with the sequence of one of the type strains.

This approach has been applied to isolates from a variety of habitats and the occurrence of many culturable potentially new species can be predicted from the molecular data. The molecular approach complements the phenotype-based classification including cell morphology and other properties such as crateriform structures and multifibrillar appendages.

Table 4. 16S rDNA signature nucleotide pairs (in bold) defining the four genera of the family Planctomycetaceae.[a]

Nucleotide position[b]	*Planctomyces* (3 species, 14 isolates)	*Pirellula* (2 species, 38 isolates)	*Gemmata* (1 species, 8 isolates)	*Isosphaera* (1 species, 13 isolates)
115–312	**U-A**	**G-C**	**C-G**	**G-C**
125–236	U-A	U-R	U-A	**A-U**
340–349	A-U	A-U	A-U	**U-A**
408–434	G-C	G-C	G-C	**A-U**
409–433	G-C	G-C	**C-G**	G-C
501–544	C-G	**G-C**	C-G	C-G
578–763	**U-A**	**U-A**	**C-G**	**C-G**
680–710	**C-G**	**A-U**	**U-A**	**C-G**
822–878	A-U	A-U	**G-C**	G-C, A-U
1028–1033	Y-R	C-G	**G-C**	C-G
1133–1141	A-U	G-C	C-G	G-C[c]
1308–1329	U-A	U-A	**C-G**	U-A[d]

Abbreviations: Y, pyrimidine; and R, purine.

[a]Only organisms available in pure culture are considered. A base pair is considered a signature when present in >95% of strains of a genus.

[b]*E. coli* position (Brosius et al., 1978).

[c]*Isosphaera pallida* has A-U.

[d]*Isosphaera pallidai* has C-G.

It can be predicted that the few phenotypic and physiological differences observed in members of the Planctomycetaceae will not be sufficient to describe a significantly larger number of novel species than the ones described so far. As judged from the phylogenetic distance to the two described species of *Pirellula*, many of the strains with a *Pirellula*-type morphology represent novel species (Ward et al., 1995; Griepenburg et al., 1999). The finding of novel planctomycete organisms in different environments makes their increased isolation in the future even more likely. Certainly the spectrum of chemotaxonomic and physiological properties has not yet been fully explored in these organisms, and considering the large phylogenetic interstrain distance of many of the isolates, one would expect these phenotypic differences to be present among the planctomycete strains. However, it may not be taxonomically wise to describe these new species exclusively on the basis of 16S rDNA sequence properties, such as secondary structure idiosyncrasies or the presence of unique signature nucleotides. On the basis of the argument that each individual nucleotide position represents a genomically stable and conserved single state phenotypic character, the accumulation of discriminating molecular properties should have at least the same taxonomic weight as many of those physiological, cultural and nutritional properties traditionally used in the description of novel species. This approach has been included to describe a new species of the genus *Sulfitobacter* (*S. mediterraneus*), for which hardly any discriminating phenotypic properties have been detected when compared to the type strain of *S. pontiacus* (Pukall et al., 1999). The past 20 years have shown the potential of 16S rDNA sequence analysis to reliably indicate the presence of new species and phenotypic properties yet to be discovered; these can then be used to emend the description. However, the failure of probes and primers described as "universal" for the Bacteria to detect planctomycetes (Neef et al., 1998; Vergin et al., 1998) means that overreliance on 16S rDNA sequence analysis as a tool for systematics may pose problems. With the availability of genomic sequence from representative planctomycetes, it should be possible to target genes that are not subject to these limitations or to use a combinatorial approach that targets multiple genes.

Cell Compartmentalization in Planctomycetes

Both *Pirellula marina* and *Pirellula staleyi* possess a characteristic type of cell compartmentalization, in which a single membrane (the intracytoplasmic membrane or ICM) divides the cell into two separate regions, the pirellulosome and a polar cap region, later termed "the paryphoplasm" (Lindsay et al., 1997). The pirellulosome region contains the nucleoid and most of the RNA. A structure analogous to the pirellulosome has also been reported in *Planctomyces maris*, *Isosphaera pallida*, *Gemmata obscuriglobus* and "*Candidatus* Brocadia anammoxidans" (Lindsay et al., 2001). Additional compartments are seen within *G. obscuriglobus* (see Gemmata), i.e., a double-layered membrane encloses the nucleoid, and within "*Candidatus* Brocadia anammoxidans," i.e., a single membrane encloses each anammoxosome. This shared cellular organization has not been previously reported in other bacteria and appears to be both a taxonomically and a phylogenetically significant character common to all planctomycetes so far examined (see Figs. 7–11). The anammoxosome (Fig. 10) is correlated with production of the unique ladderane lipid discussed above; the ladderane structure is suitable for creating a membrane barrier sealing off this compartment, which is postulated to contain toxic intermediates of ammonium oxidation metabolism like hydrazine, from the remainder of the cell including the DNA and ribosomes.

Genomic Organization

Genome Size

Genome size in the planctomycetes, according to physical analysis methods, appears to be in the upper range for bacteria (i.e., 5.2 Mb in *P. limnophilus* to greater than 9 Mb in *G. obscuriglobus*; Ward-Rainey et al., 1996b). DNA-DNA reassociation techniques have also been used to investigate genome size in a number of strains (Kölbel-Boelke et al., 1985). The apparent large genome size of planctomycetes is not surprising, given that the majority of planctomycetes, both cultured and uncultured, have been found in terrestrial and aquatic samples and are therefore assumed to be free-living. Free-living organisms require greater nutritional versatility and other types of adaptability than their relatives engaged in parasitic and mutualistic relationships and therefore greater coding capacity and larger genomes (Peterson and Fraser, 2001). A relatively large genome has been suggested to benefit bacteria living in fluctuating environments, giving them the genetic flexibility to adapt to changing conditions (Roussel et al., 1994). The apparent association between the occurrence of blooms of some *Planctomyces* species and the eutrophication process raises the possibility that

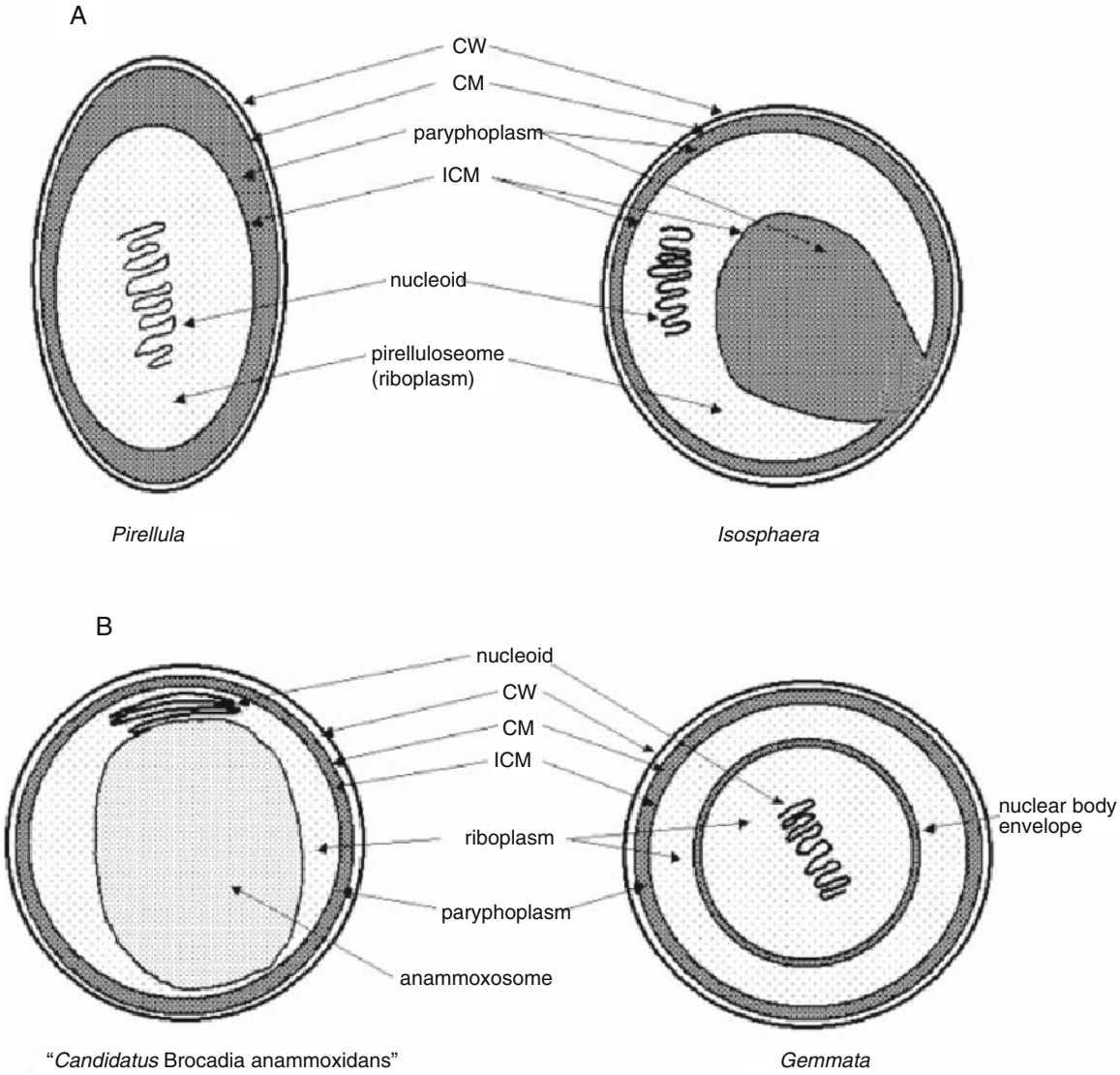


Fig. 7. Diagrams of cell organization and compartmentalization in A) *Pirellula marina* and *Isosphaera pallida* (also applies to *Planctomyces maris*) and B) "*Candidatus* Brocadia anammoxidans" and *Gemmata obscuriglobus*. The varieties of cell compartmentalization found in different planctomycetes are shown, as well as the underlying similarities in topology of their internal organization (i.e., possession of paryphoplasm compartment and ICM). ICM, intracytoplasmic membrane; CW, cell wall; and CM, cytoplasmic membrane. From Lindsay et al. (2001).

in some strains part of the genome may be devoted to the exploitation of changing nutritional conditions during eutrophication. It should be noted, however, that these species have not yet been confirmed as planctomycetes using phylogenetic/molecular approaches, although it seems likely that they will be at some point.

The morphological complexity and lifecycle of planctomycetes may also necessitate a large genome. Large genomes are associated with the ability to undergo complex differentiation, e.g., in *Streptomyces* species (8000 kb; Kieser et al., 1992; Leblond et al., 1993) and *Myxococcus xanthus* (9500 kb; Chen et al., 1991). The majority of planctomycetes possess some form of cellular appendage and have a dimorphic lifecycle. It is conceivable that a significant genetic burden would be imposed by the synthesis of stalks, holdfast material, fimbrae and flagella. An alternative explanation is that extensive genome duplication and rearrangement, such as has been found in the large genomes of members of the genus *Streptomyces* (Kieser et al., 1992; Leblond

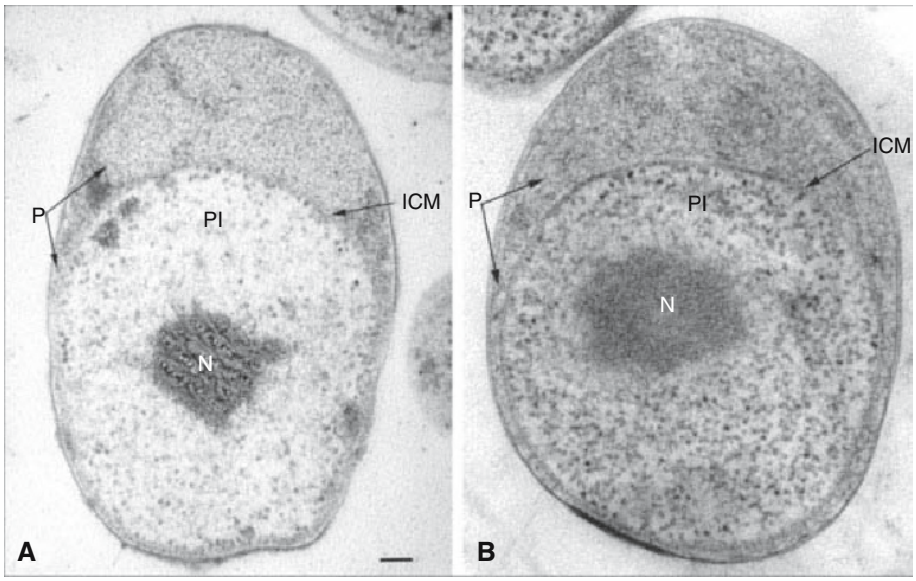


Fig. 8. Transmission electron micrograph of a thin section of a cryosubstituted cell of A) *Pirellula marina* and B) *Pirellula staleyi* displaying compartmentalization into pirellulosome (PI) and paryphoplasm (P) separated by the intracytoplasmic membrane (ICM). The nucleoid (N) is contained within the pirellulosome and thus compartmentalized and surrounded by the single ICM. Bar is 0.1 mm. From Lindsay et al. (1997).

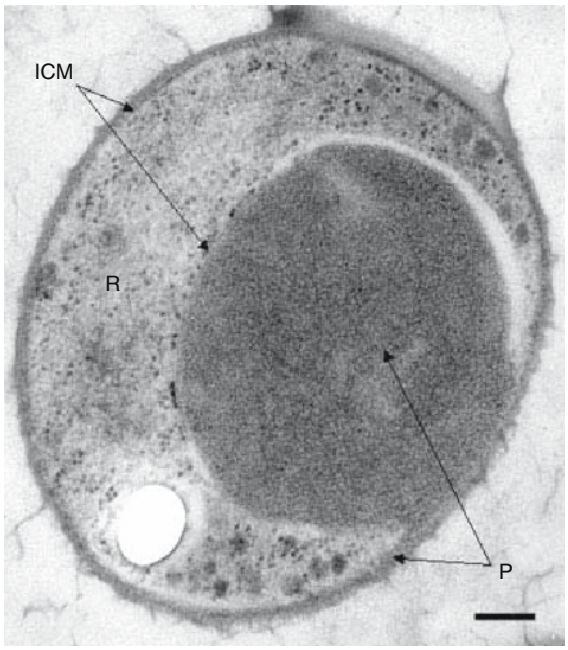


Fig. 9. Transmission electron micrograph of a thin section of a cryosubstituted cell of *Isosphaera pallida* displaying a central single intracytoplasmic membrane (ICM)-bounded invagination of the relatively electron-dense paryphoplasm (P); the other cell compartment, equivalent to a pirellulosome, contains ribosome-like particles (R). Compare with Fig. 7, diagram of planctomycete cell plans. Bar is 0.2 mm. From Lindsay et al. (2001).

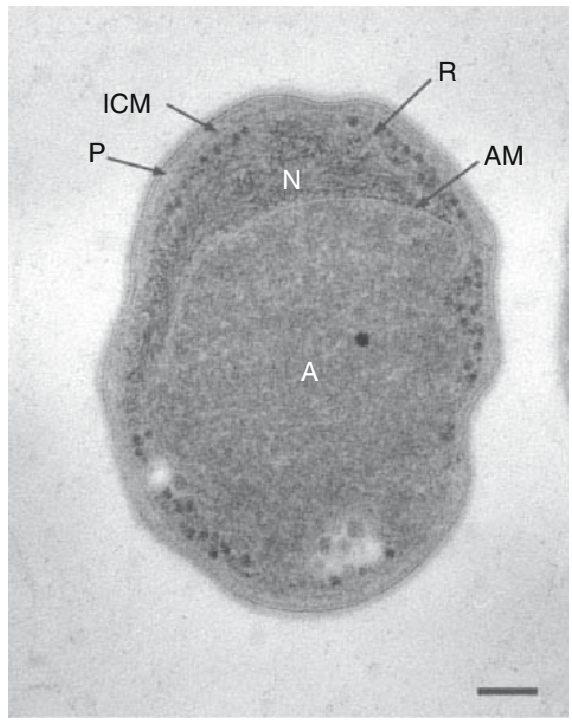


Fig. 10. Transmission electron micrograph of a thin section of a cryosubstituted cell of "*Candidatus* Brocadia anammoxidans" from a bioreactor performing anaerobic ammonium oxidation. A central anammoxosome (A) is surrounded by a single membrane (AM). The cytoplasm external to the AM contains the nucleoid (N) and ribosome-like particles and is surrounded by the intracytoplasmic membrane (ICM). A paryphoplasm (P) lies at the rim of the cell between the ICM and the cytoplasmic membrane closely apposed to the cell wall. Tubule structures are visible inside the anammoxosome. Bar is 0.1 mm. Courtesy of M. Strous, R.I. Webb, and J.A. Fuerst.

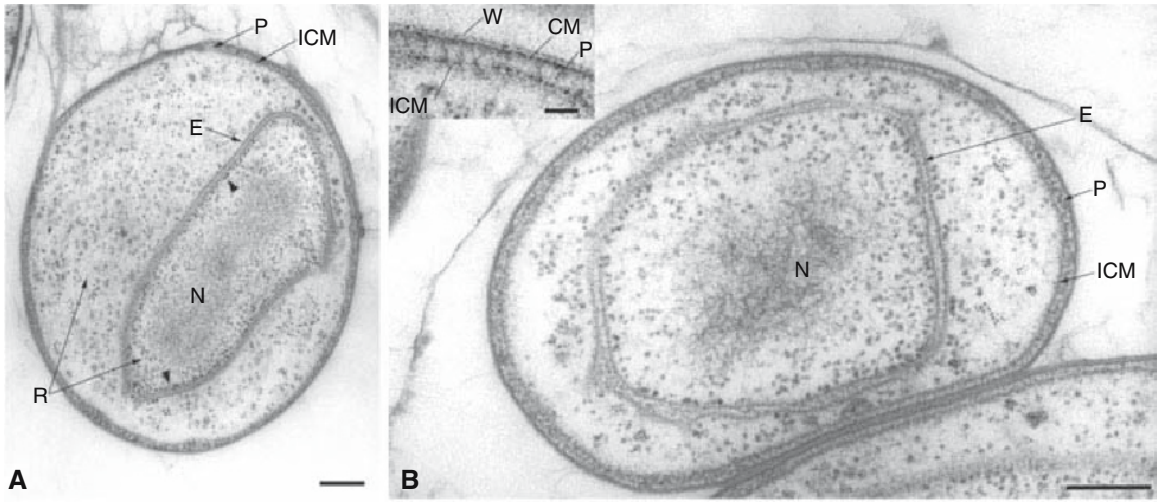


Fig. 11. A) Transmission electron micrograph of thin section of cryosubstituted *Gemmata obscuriglobus*. A nuclear body containing the fibrillar nucleoid (N) and surrounded by a nuclear envelope (E) consisting of two membranes is visible at the right side of the cell. Ribosome-like particles are visible lining the inner side of the nuclear envelope (arrowheads). Riboplasm (R) occurs in two regions, one surrounding the nucleoid within the nuclear body and the other in a larger region surrounding the nuclear body. The cell is rimmed by an electron-dense paryphoplasm (P) and the intracytoplasmic membrane (ICM). Bar is 0.2 mm. From Lindsay et al. (2001). B) Transmission electron micrograph of a thin section of a cryosubstituted *Gemmata obscuriglobus* bud cell with large nuclear body containing an extensive fibrillar nucleoid (N) bounded by nuclear envelope (E) consisting of two membranes between which is a clear electron-transparent space. The nuclear body contains ribosome-like particles as well as the nucleoid. It is surrounded by cytoplasm which contains superficially similar ribosome-like particles and is bounded by a single ICM. The cell external to this ICM is rimmed by a paryphoplasm containing relatively electron-dense cytoplasm (P). Bar is 0.2 mm. Inset: enlarged view of envelope of bud cell showing a bilayered cytoplasmic membrane (CM) underneath the cell wall (W) but closely apposed to that wall. Paryphoplasm (P) is bounded peripherally by the cytoplasmic membrane (CM) and internally by the ICM. Bar is 20 nm. From Lindsay et al. (2001). Bar is 0.5 mm. From Fuerst and Webb (1991).

et al., 1993), have also occurred in the planctomycetes. Data indicating multiple chromosomal loci for some key housekeeping genes in three planctomycete strains (see below) lend credence to this hypothesis.

It is to be expected that the ongoing planctomycete genome sequencing projects (see Whole-Genome Sequencing Studies) will allow us to better understand the relationship between the free-living lifestyle of the planctomycetes and their genome size and complexity.

Genome Topology

The chromosome of *Pl. limnophilus* was shown to be a circular molecule (Ward-Rainey et al., 1996b), as are the vast majority of bacterial strains investigated so far (Cole and Saint Girons, 1994; Fonstein and Haselkorn, 1995; see also completed genome sequencing projects). However, the phylogenetic depth within the order Planctomycetales is as great as that within the class Proteobacteria and the order Spirochetales (groups which exhibit considerable diversity in chromosomal topology); therefore, the presence of a single, circular chromosome in the planctomycete strains studied so far does not imply that all planctomycete genomes are circular.

Ward-Rainey et al. (1996b) reported the presence of an extrachromosomal element in *Pl. limnophilus* detected by pulsed field gel electrophoresis (PFGE) of undigested chromosomal DNA, and application of the same technique to *Pi. marina* also revealed a plasmid species in this strain (Ward-Rainey, 1996a). The presence of extrachromosomal DNA in some planctomycete strains suggests that plasmid transfer or plasmid-mediated conjugal transfer of chromosomal DNA may occur, but as yet there are no reports in the literature of genetic mechanisms in planctomycetes, although genomic evidence for conjugation (*tra* genes) has been reported in *G. obscuriglobus* (Jenkins et al., 2002).

Gene Distribution

Physical mapping of planctomycete genomes (*Pl. limnophilus*, *Pi. marina*, and *Pirellula* strain 140)

using probes for conserved housekeeping genes suggested that: 1) the chromosomal location of these genes varied greatly between strains, 2) the genes coding for 16S rRNA (*rrs*) and 23S rRNA (*rrl*) are separated in the genomes of all three strains investigated, and 3) more than one chromosomal locus exists for several of the genetic markers (Ward-Rainey, 1996a; Ward-Rainey et al., 1996b). Unlinked *rrn* organization had been previously reported for the planctomycete species *Pirellula marina* (Liesack and Stackebrandt, 1989) and *Planctomyces limnophilus* (Menke et al., 1991). The organization of the five sets of *rrn* genes in *Gemmata obscuriglobus* could not be unambiguously determined either by the ribotyping approach (Menke et al., 1991) or by using PFGE techniques (Ward-Rainey, 1996a). In contrast, *Isosphaera pallida* and related bacteria appear to possess the more typical rRNA gene linkage (Ward-Rainey, 1996a). The disjointed *rrn* organization has also been reported in phylogenetically unrelated lineages such as *Thermus* and *Thermotoga* (Menke et al., 1991). The lack of phylogenetic relationship between these taxa suggests that this feature has arisen independently in multiple lineages within the Bacteria and is not a phylogenetically significant marker.

Whole-genome Sequencing

Partial genome sequence data became available for *Gemmata obscuriglobus* and *Pirellula marina* (Jenkins et al., 2002), providing the first glimpse into the planctomycete genome. Many of the genes discovered by this sequence tag approach (including those for metabolism, transport, regulation, translation, and DNA replication) are consistent with the known biology of the strains. Interestingly, there were also significant matches to nuclear genes from members of the domain Eukarya that may have arisen by horizontal gene transfer events.

Whole-genome sequencing projects have been initiated for the type strain of *G. obscuriglobus* (TIGR website) and *Gemmata* sp. Strain Wa1-1. A complete genome sequence is available for *Pirellula* sp. strain 1 (Glockner et al., 2003; Real Environmental Genomics website). These studies will almost certainly shed further light on the genomic basis of planctomycete biology. Genomic and proteomic studies of *Pirellula* sp. strain 1 have already identified genes involved in *N*-acetylglucosamine metabolism (Rabus et al., 2002), which may be a key process in marine systems where *Pirellula* species are abundant (Llobet-Brossa et al., 1998; Glockner et al., 1999).

The Infancy of Planctomycete Genetics

As discussed above, the late 1990s was a period of relatively intense activity in the field of planctomycete biology. One of the welcome by-products of these research efforts is that planctomycetes are becoming more widely known amongst the general community of microbiologists, resulting in renewed interest in the organisms from various perspectives. A recent study by Leary et al. (1998) provides an example of the application of the tools of molecular biology to begin to determine mechanisms of genetic control in the planctomycetes. The *rpoN* gene, which encodes the alternative sigma factor σ^{54}, was cloned from *Pl. limnophilus* and shown to complement the Ntr^- phenotype of a *Salmonella typhimurium rpoN* mutant strain and to share significant homology with other members of the σ^{54} family. This alternate sigma factor has been found in several phylogenetic lineages within the domain Bacteria and is involved in diverse metabolic functions such as nitrogen fixation, hydrogen metabolism, and degradation of aromatic compounds (Kustu et al., 1989). Thus the detection of σ^{54} in a member of the planctomycetes suggests many further avenues to explore, including determination of the promoters in *Pl. limnophilus* with which σ^{54} participates in transcription and identification of structure-function relationships in the σ^{54} group as a whole.

An experimental system for gene transfer to model planctomycetes will be needed for substantial future progress in cell biology of planctomycetes, to take full advantage of established and emerging technologies such as fusions with green fluorescent protein (GFP) to localize proteins likely to be involved in compartmentalization. So far foreign genes or plasmids have been introduced only once into a planctomycete. The GFP-encoding plasmid with the *gfp* gene under control of a *lac* promoter was transferred via conjugation from *Pseudomonas putida* into *Planctomyces maris*, with successful expression of the fluorescent GFP protein (Dahlberg et al., 1998). Extension of such gene transfer to other planctomycetes will open new areas of planctomycete cell biology to experimental investigation.

The Described Genera

The order Planctomycetales comprises four genera, containing only 10 species. Of these species, only seven have been cultured. Three of the six species of *Planctomyces*, including the so far uncultured type species *Pl. bekefii* (Gimesi, 1924), are validly described on the basis of their morphological properties (Starr and Schmidt,

1984), a procedure allowed for by rule 18a of the International Code of Nomenclature of Bacteria (Lapage et al., 1992). Two *Candidatus* status genera, "Brocadia" and "Kuenenia," have been proposed for the anaerobic ammonium oxidizers (Strous et al., 1999; Schmid et al., 2000), and a third genus, "Scalindua," is in the process of being proposed (Schmid et al., 2003; see Ecology and Physiology).

Planctomyces

Since the first report of *Pl. bekefii* by Gimesi (1924), limnologists and bacteriologists have periodically reported similar organisms and ascribed several new species and generic names to these bacteria. Isolates of *Planctomyces* spp. were not obtained until the late 1970s (Bauld and Staley, 1976; Hirsch et al., 1977; Schmidt, 1978a). Even today, *Pl. bekefii*, *Pl. guttaeformis* and *Pl. stranskae*, as well as several other unrecognized species, remain unisolated although they are common residents of freshwater habitats. Their phylogenetic relationships to planctomycetes have also not yet been confirmed by molecular sequencing or oligonucleotide probing.

HABITAT. These bacteria have been isolated from (or observed microscopically in) freshwater, marine and saline habitats. Unicellular forms are frequently found attached to other organisms in the environment (Fig. 12). They attach by a holdfast structure located at the tip of the stalk. Species with the most striking morphology, such as *Pl. bekefii*, occur in the plankton of freshwater lakes as microcolonies of free-floating rosettes (Fig. 13). It is not known how they can specifically associate with one another to form these rosettes in the natural habitat. The colonial forms (perhaps because of their peculiar cell envelope structure) do not appear to occur in soft-water (low osmotic pressure) habitats. They are most common during the summer and early fall,

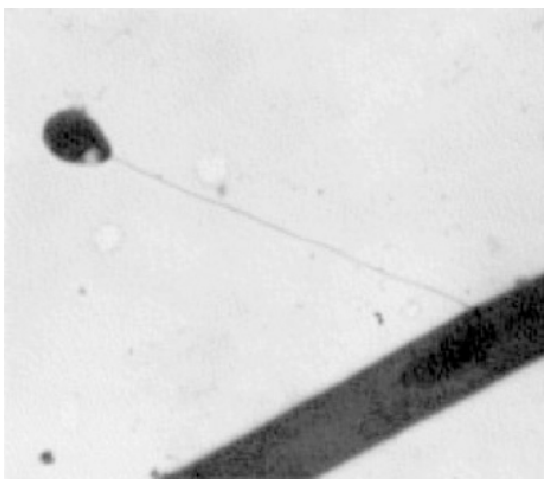


Fig. 12. A lone *Planctomyces* cell attached by its stalk to a sheathed cyanobacterial filament in a sample collected from Lake Washington. Presumably the flexible stalk acts as a tether, allowing the cell to be moved by water currents while the holdfast anchors the cell to the cyanobacterial filament. Cell diameter is about 1 mm. Electron micrograph.

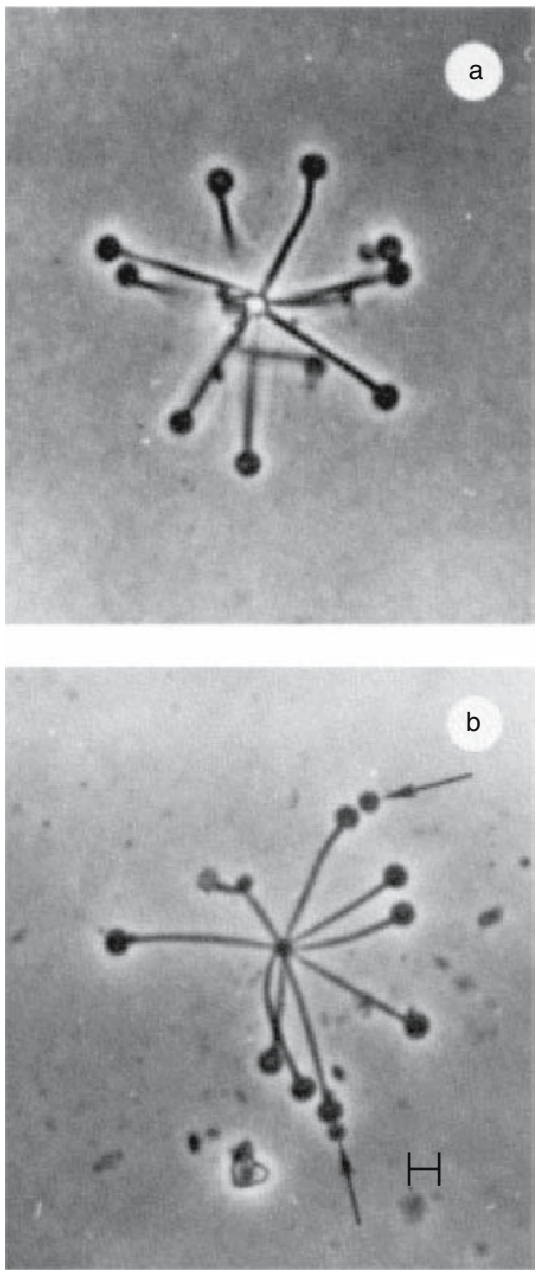


Fig. 13. Phase photomicrographs of a rosette-forming species of *Planctomyces* from University Lake, Chapel Hill, North Carolina. a) Note the encrustation in the central holdfast region of the rosette owing to deposition of iron and/or manganese oxides. b) Arrows point to buds. Bar is 2.0 mm.

particularly in eutrophic lakes. For example, in North America they are found in eutrophic lakes in the Midwest (Wintergreen Lake near Kalamazoo, Michigan) and Southeast (University Lake near Chapel Hill, North Carolina, as well as most other lakes of the Carolina and Virginia Piedmont; J. Staley, unpublished observations). These colonial forms have also been reported to occur in eutrophic ponds in Arizona (Starr and Schmidt, 1984). In contrast, rosette-forming species have not been reported in the mesotrophic to eutrophic soft-water lakes of the Pacific Northwest such as Lake Washington in Seattle, where unicellular forms are found (J. Staley, unpublished observations; see Fig. 12).

Planctomyces spp. have been enumerated in a variety of Australian freshwater habitats using Most-Probable-Number (MPN) techniques with a dilute peptone broth medium (0.01%). They were found in habitats of all trophic states and their proportion to total viable heterotrophs by this procedure (about 0.025%) was essentially the same, regardless of the trophic state of the habitat (Staley et al., 1980). Thus, higher concentrations were found in eutrophic habitats that contained correspondingly higher concentrations of heterotrophic bacteria than did the less nutrient-rich habitats.

ISOLATION: SAMPLE COLLECTION. Water samples (200 ml or more) should be collected aseptically from the environment. If enrichments are to be prepared at the day of sampling, samples can be kept at the collection temperature. For periods longer than 12–24 h, it is advisable to refrigerate. In situ temperature and salinity should be known since these parameters will determine the appropriate conditions for enrichment and isolation.

DIRECT ISOLATION AND ENRICHMENT METHODS. Because these organisms occur in low numbers in most natural environments, they usually have to be enriched. Although they are very distinctive morphologically, they have few physiological features that can be used for the development of enrichment and isolation procedures. The addition of penicillin G (1,000–2,000 U/ml) or other antibiotics that inhibit peptidoglycan synthesis may be used with some success (Schmidt and Starr, 1981a; Schlesner, 1994; Wang et al., 2002), at least at the time of plating, since all isolated strains lack peptidoglycan. Use of protein synthesis inhibitors and antifungal agents may also aid isolation of planctomycetes (Schlesner, 1994; Wang et al., 2002).

In some cases, it may be possible to isolate strains from natural samples by direct streaking of the sample onto an appropriate agar medium. For example, Hirsch et al. (1977) describe a water agar plating procedure used successfully for the isolation of *Planctomyces limnophilus*. Likewise, *P. brasiliensis* was isolated directly from a salt pit sample from Brazil (Schlesner, 1989) by streaking on a saline medium "M13" (described in Pirellula).

A variety of techniques have been applied for the enrichment of *Planctomyces* when numbers from the environment are low. A common procedure is to use the dilute peptone enrichment procedure described for *Prosthecomicrobium* and *Ancalomicrobium* (see The Genera *Prosthecomicrobium*, *Ancalomicrobium*, and *Prosthecobacter* in the second edition). Peptone (Bauld and Staley, 1976) is added to a freshly collected natural sample to a final concentration of 0.01% (or peptone and yeast extract at 0.005% each) and the enrichment incubated at room temperature (or the temperature of the environment from which it was collected). Schmidt (1978a) used somewhat lower concentrations of peptone ranging from 0.002 to 0.005% in her enrichments.

Hirsch and Müller (1985) have described other procedures that have been used successfully in the enrichment of *Planctomyces* spp. 1) In addition to using media with low nutrient concentrations, they have incubated water samples directly with no nutrient addition in either light or dark for periods of 4–16 weeks. 2) Another procedure entails hanging sterile glass slides into fresh or stored water samples and incubating in the light at 20°C. This is reminiscent of the original Henrici procedure in which submerged slides were used to detect periphytic bacteria in lakes (Henrici and Johnson, 1935). When interesting forms appear, material is scraped from the slide with a sterile scalpel and streaked onto an isolation medium. 3) In a different procedure, sterile glass coverslips, some coated with 2% water agar, were inserted vertically into a sterile water agar layer (2 cm deep) in Petri dishes to which was added sufficient sample water to cover the coverslips. Coverslips were examined periodically by phase microscopy for the attachment of budding bacteria. When coverslips contained organisms of interest, replicate ones were removed and material streaked for isolation (Hirsch et al., 1977).

ISOLATION PROCEDURES. Enrichments are examined periodically by phase microscopy to identify types characteristic of the genus: spherical to ovoid cells (oftentimes large [i.e., ≥1.0 μm in diameter]) that attach to detritus or other cells (especially sheathed organisms) and that produce polar to subpolar buds and stalks. The stalks may not be sufficiently thick to be seen by phase microscopy, so to identify these it is advisable to examine samples from prospective enrichments with the transmission electron microscope, by preparing negative stains of enrichment culture material (Fig. 14). Positive

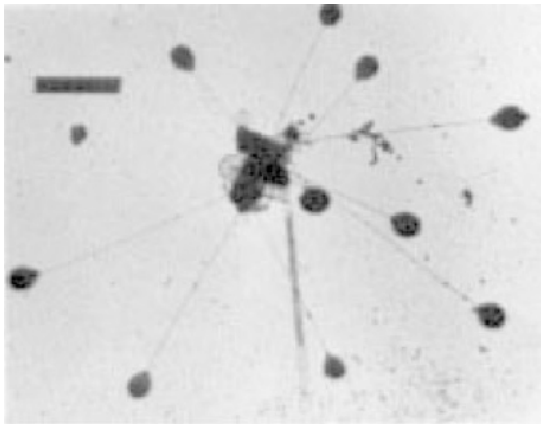


Fig. 14. An unidentified rosette-forming species of *Planctomyces* from a 0.01% peptone enrichment culture of water collected from the Mississippi River in Minneapolis. Bar is 5.0 mm.

enrichment samples are streaked or dilutions spread (spread 0.1 ml samples from 10^{-2} to 10^{-4} dilutions) on agar media. Media that have been used successfully for this purpose include PYGV medium or PYG medium (same as PYGV, but without vitamins; Staley, 1968).

PYGV Medium

Peptone	0.025%
Yeast extract	0.025%
Glucose	0.025%
Hutner's salts solution (see The Genera *Prosthecomicrobium, Ancalomicrobium*, and *Prosthecobacter* in the second edition)	20 ml/liter
Vitamin solution (see Pirellula)	10 ml/liter
Agar	1.5%

For freshwater strains, add distilled water; for marine strains, add aged seawater or artificial seawater solution at half- or full-strength (see Pirellula); for strains from other saline or brackish sources, use an appropriate salt solution mimicking that of the environment. Adjust the pH to 7.0 before autoclaving.

Schmidt and Starr (1981a) recommend using one of three other media for streaking from enrichment culture material. One medium contains 0.02% peptone, 0.01% yeast extract, 0.1% filter-sterilized glucose or galactose, 10 ml of Hutner's mineral base (Cohen-Bazire et al., 1957), 5 mM $MgSO_4 \cdot 7H_2O$, and 1.0 or 1.5% agar. Their second medium contains 0.04% peptone, 0.02% yeast extract, 5 or 10 mM magnesium sulfate, and 1.0 or 1.5% agar made up in either tap water or distilled water to which 10 ml of the Hutner's salts solution is added. The pH of this medium can be adjusted above 7.0 for alkaline samples using 0.005 M tris [hydroxymethyl] aminomethane (Trizma) buffer (Sigma Chemical Co., St. Louis, MO). Usually a pH of 7.8 or 8.0 is used. Their final medium contains 0.2% peptone, 0.1% yeast extract, Hutner's salts solution (10 ml/liter), and 5 mM magnesium sulfate, with 1.0 or 1.5% agar made up with distilled or tap water. Autolysis of osmotically unstable strains can occur in this latter medium.

Colonies of *Planctomyces* spp. develop slowly. Cultures from primary plates need to be incubated for at least one week, and often more than a month is required before they fully develop. One of the most striking characteristics of the colonies is that they continue to develop after most other colony types have stopped growing and are beginning to recede. Typical colonies of the *Planctomyces* types as well as *Pirellula* persist and form impressive mounds about a month after streaking when incubating at room temperature. Colonies may be pigmented (light rose, bright red, or yellow to ochre) or they may be unpigmented.

Repeated restreaking may be necessary to purify some strains. Indeed, some strains cannot be readily isolated even after several restreaking attempts, but some of these can be maintained in monoxenic culture with another heterotrophic bacterium (J. Staley, unpublished observations).

IDENTIFICATION. Members of the genus *Planctomyces* are budding organisms in which the daughter cells are motile by means of a subpolar flagellum. Subsequently, in their transition to the reproductive stage, these cells form noncellular, fibrillar, tubular, ribbon-like or ropelike stalks; their presence in the three cultured species (*Pl. limnophilus*, *Pl. brasiliensis* and *Pl. maris*) can only be observed by electron microscopy (Bauld and Staley, 1976). Thus, the most direct means to verify the identity of putative *Planctomyces* isolates is by transmission electron microscopy (see Figs. 10 and 12). Whole cell preparations that are negatively stained should show the typical spherical to ovoid cell types with polar stalks. Stalks are the primary characteristic used to distinguish *Planctomyces* from *Gemmata* and *Pirellula*. Although stalks have been infrequently reported from these latter two genera, they are quite rare and often nondistinctive. In contrast, stalks are very common in *Planctomyces* spp. Indeed, in a typical growing preparation of *Planctomyces* spp., all mature, budding cells would be expected to bear a stalk.

Classification of species in the genus has been based largely upon morphological attributes, especially for types that have not yet been isolated. Schmidt and Starr (Schmidt and Starr, 1978b; Schmidt and Starr, 1979) and Starr and Schmidt (1989) classified this genus into five morphotypes, I–V. Cells of morphotype I are spherical, joined together in rosettes, as represented by *Planctomyces bekefii* (Figs. 13 and 15). Cells of morphotype III are ellipsoidal and are

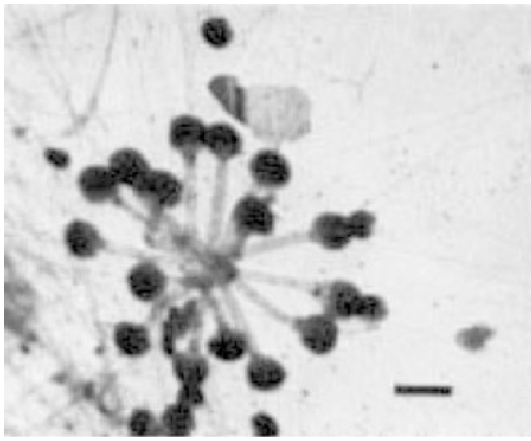


Fig. 15. An electron micrograph of an organism that resembles *Planctomyces bekefii*. Though this organism is similar to *Pl. bekefii*, note that its cells have two to four or more very long prominent filiform appendages extending outward from each cell of the rosette near the reproductive pole rather than several shorter ones characteristic of *P. bekefii* (see Fig. 2). This type of Planctomyces is common in lakes in the southeastern United States. From University Lake, Chapel Hill, North Carolina. Bar is 2.0 mm.

represented by the isolated species, *Pl. maris*, *Pl. limnophilus* and *Pl. brasiliensis*. Cells of morphotype V are bulbiform-shaped and form rosettes (Figs. 16 and 17). *Pl. guttaeformis* and *Pl. stranskae* are members of this group. No species names have been proposed for morphotypes II and IV, cells of which are ovoid. However, one member of morphotype IV is now classified as *Pirellula staleyi*. One possible future application of the morphotype scheme would be in combination with taxon-specific labeled oligonucleotide probe-based FISH. The physical distinctiveness of the individual morphotypes could thus allow determination of their phylogenetic position in relation to other planctomycete species, in the absence of cultured isolates. Fuerst (1995) suggested cloning of 16S rDNA amplified from micromanipulated cells and FISH using planctomycete-specific fluorescent oligo probes.

Ultrastructure of cultured *Planctomyces* species (e.g., *Pl. maris*) conforms to the cell plan shared by other planctomycetes (Lindsay et al., 2001).

Pure cultures are available of three described species, all of morphotype III, including *Pl. maris* (Fig. 18), *Planctomyces limnophilus* and *Pl. brasiliensis*. The stalks on these species are so fine they cannot be discerned by observation with the phase contrast microscope. When observed by the electron microscope, the stalk can be seen to contain a number of fine fibrils bundled together to form a fascicle that emanates at one pole of the ellipsoidal cells. This stalk develops at the pole near the site of the subpolar flagellum.

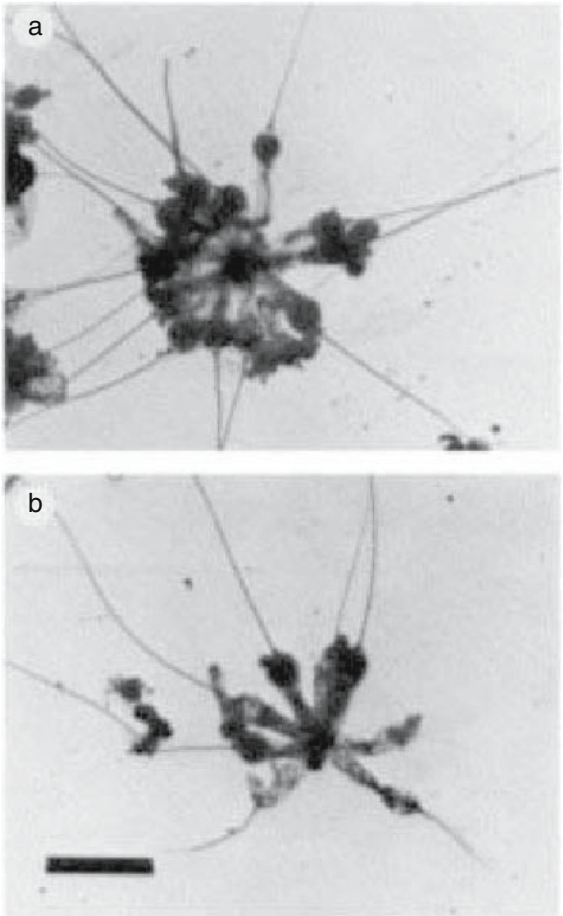


Fig. 16. Rosettes of *Planctomyces guttaeformis*. a) Note the bulbiform cells that are joined together at their narrow poles. Also note the long spike appendage extending away from the bulbous reproductive pole of mature cells in the rosette. b) Another rosette from same locale contains budding cells at different stages of development. Buds begin as spherical protuberances but then become bulbiform. Several cells in this rosette are lysed. From University Lake, Chapel Hill, North Carolina. Bar is 5.0 mm.

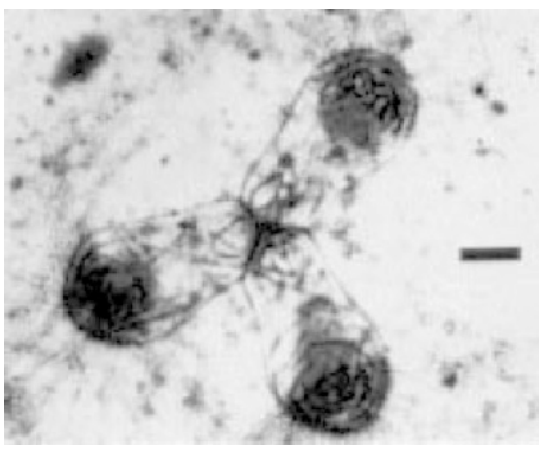


Fig. 17. *Planctomyces stranskae* collected from Kiwanis Park in Tempe, Arizona. Cells of this species do not develop terminal spikes. Bar is 1.0 mm. Courtesy of Jean Schmidt.

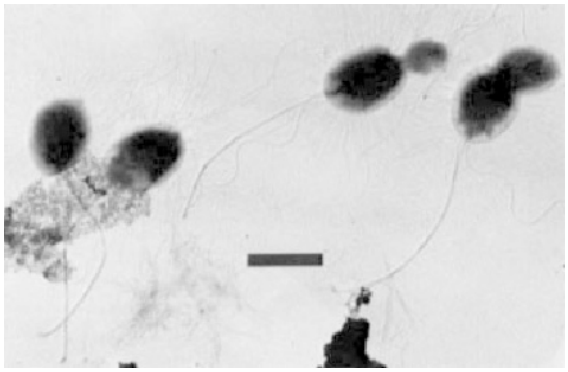


Fig. 18. Shadowed electron micrograph of cells of *Planctomyces maris*. Note the polar to subpolar buds, fibrillar stalks, subpolar flagella, and peritrichous fimbriae. Bar is 1.0 mm.

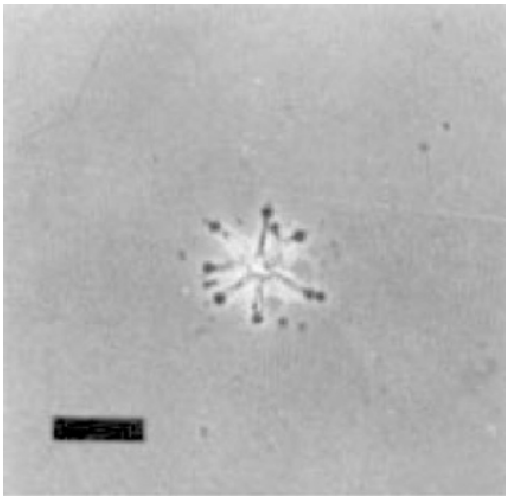


Fig. 19. A phase photomicrograph of a small rosette-forming species of *Planctomyces* from University Lake, St. Lucia, Australia, showing heavy deposition of iron and or manganese oxides on the stalks. Bar is 10 mm.

Table 5. Differentiation among isolated species of *Planctomyces*.

Character	*P. maris*	*P. limnophilus*	*P. brasiliensis*
Cell shape; maximum cell diameter	Ovoid; 1.5µm	Ovoid; 1.5µm	Spheroid; 1.8µm
Source	Marine	Fresh water	Salt pit
Colony pigmentation	Colorless to light rose	Red	Yellow
Salinity growth range (%)	1.5–4.0	<1.0	0.7–10.0
NaCl growth range (mM)	100–>300	ND	100–170
Temperature growth range (°C)	6–38	17–39	ND
G+C content (mol%)	51	53	55–58
Genome size ($\times 10^9$Da)	3.62	2.67	2.81

Abbreviation: ND, not determined.

These species are differentiated by 16S rRNA gene sequence, salt requirements for growth, and various other morphological and physiological characteristics (Table 5).

All cultured isolates have dimorphic life cycles. Reproduction occurs by budding, in which the cell envelope of the daughter bud is formed de novo (Staley, 1973; Tekniepe et al., 1981). Daughter cells are motile by a polar to subpolar flagellum. Though they lack stalks initially, these are formed as part of their maturation process in a fashion that is analogous to that of *Caulobacter* (although the stalks of *Planctomyces* spp. are not prosthecae).

All three of these species are heterotrophic organisms that grow aerobically. Various mono- and dissacharides are used, including glucose, galactose, cellobiose, maltose and *N*-acetylglucosamine. *Planctomyces maris* and *Pl. brasiliensis* also use glucuronic acid. Ammonium salts can be used as the sole nitrogen source by *Pl. maris* and *Pl. limnophilus*. Vitamins are not required by any of the isolates.

The characteristics of the other unisolated but currently recognized species are given in Table 6. All of these species produce large, conspicuous rosettes in the natural freshwater habitats in which they occur. Each cell of *Pl. bekefii* produces a distinctive thick stalk that terminates in a holdfast. The stalk is a tubular structure comprising many fibrils (Schmidt and Starr, 1980a, b). The holdfasts enable cells to join together in the natural habitat to form rosettes (Figs. 13 and 15). Some colonial forms such as *Pl. bekefii* can develop stalk encrustations (Fig. 19) containing iron and/or manganese oxides (Schmidt et al., 1981b; see Ecology and Physiology).

The most characteristic feature of *Pl. guttaeformis* and *Pl. stranskae* is their bulbiform cells (Starr and Schmidt, 1984), which are joined together at their narrow poles to form rosettes (Figs. 14 and 15). In addition, *Pl. guttaeformis* produces a long terminal spike from its large pole, a feature lacking in *Pl. stranskae*.

The use of 16S rRNA gene sequences for identification and classification of cultured isolates,

Table 6. Differentiation among recognized species of *Planctomyces* that have not yet been isolated in pure culture.[a]

Characteristic	*P. bekefii*	*P. stranskae*	*P. guttaeformis*
Cell shape	Spherical	Bulbiform	Bulbiform
Stalk	Tubular	Absent	Absent
Other appendages	Fimbriae and larger spikes[a]	Fimbriae only	Fimbriae and a single, long tapering spike

[a]At least two variants (species?) exist. One type has several shorter spikes (see Fig. 2) whereas another type has fewer, very long spikes (see Fig. 3).

now so widespread as to be almost universal, allows strains tentatively identified as *Planctomyces* spp. on the basis of light microscopy to be assigned to this genus, to other planctomycete genera, or to novel groupings (Ward et al., 1995; see also New Approaches to Identification).

Pirellula

HABITAT. *Pirellula* spp. and morphologically similar bacteria have been observed in and isolated from a variety of aquatic environments: freshwater (Staley, 1973; Tekniepe et al., 1981; Schlesner, 1994), brackish water (Gebers et al., 1985; Kölbel-Boelke et al., 1985; Schlesner, 1986a), hypersaline water of a salt pit (Gebers et al., 1985), hot springs of Tiberias in Israel (Kahan, 1961), groundwater (P. Hirsch, personal communication), alkaline water from ponds in a lime pit near Lägerdorf, Schleswig-Holstein, Germany (Schlesner, 1994), lakes rich in Na_2SO_4, Na_2CO_3 and $NaHCO_3$ ("Lacken") in Burgenland, Austria, wastewater aeration lagoons of a sugar factory, marine and brackish water basins in an aquarium (Institut für Meereskunde, Universität Kiel), water basins in a greenhouse in the botany garden of the University of Kiel, and even the pitcher trap of the insectivorous plant *Nepenthes* sp. (Schlesner, 1994).

In spite of their widespread distribution, *Pirellula*-like bacteria are only occasionally observed in water samples. After storage at room temperature (a few days to some weeks), however, such organisms are often found in high numbers, especially on the bottom or at the wall of the vessel. Obviously, the organisms live attached to surfaces and only the swarmer cells are free in the water column. When the population is very dense, however, *Pirellula*-like organisms may be found in high numbers in the free water.

ISOLATION. Various methods have been successfully applied for the enrichment of *Pirellula* spp.: 1) Storage of samples in the laboratory; 2) addition of small amounts (0.005–0.01%) of peptone, yeast extract, or glucose to the water sample; 3) addition of 0.1% chitin to the sample; and 4) addition of 0.1% KNO_3 and anaerobic incubation.

Isolation procedures take advantage of resistance to antibiotics that affect the biosynthesis of peptidoglycan and, furthermore, of the ability to utilize *N*-acetylglucosamine as sole carbon and nitrogen source (Schlesner, 1994). The salinity of the medium should not differ much from that of the natural environment, since many strains show a rather narrow salinity tolerance (H. Schlesner, unpublished observation). The following media have successfully been applied for isolation of strains of *Pirellula* spp.:

Medium M 1 + A

Solution 1

$CaCO_3$	5.0 g
$Na_2HPO_4 \cdot 2H_2O$	0.1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Hutner's basal salts (see below)	20 ml
Gellan gum (Gelrite)	9.0 g
Vitamin solution no. 6	10 ml
Distilled water	920 ml

Adjust pH to 9.0; autoclave 121°C for 20 min.

Solution 2

N-Acetylglucosamine	2.0 g
Ampicillin sodium salt	0.2 g
Distilled water	50 ml

Adjust to pH 9.0, filter sterilize, and add to solution 1.

Hutner's Basal Salts (Cohen-Bazire et al., 1957)

Nitrilotriacetate (NTA)	10.00 g
$MgSO_4 \cdot 7H_2O$	29.70 g
$CaCl_2 \cdot 2H_2O$	3.34 g
$NaMoO_4 \cdot 2H_2O$	12.67 mg
$FeSO_4 \cdot 7H_2O$	99 mg
Metal salt solution "44" (see below)	50 ml
Double-distilled water	900 ml

First dissolve the NTA by neutralization with KOH. Add the other salts. Adjust pH to 7.2 with KOH or H_2SO_4. Adjust volume to 1000 ml with double distilled water. Store cold (5°C) and clean. The solution is clear.

Metal Salts Solution "44"

Ethylene diamino tetraacetate (EDTA)	250.0 mg
$ZnSO_4 \cdot 7H_2O$	1095.0 mg
$FeSO_4 \cdot 7H_2O$	500.0 mg
$MnSO_4 \cdot H_2O$	154.0 mg
$CuSO_4 \cdot 5H_2O$	39.2 mg
$CoCl_2 \cdot 6H_2O$	20.3 mg
$Na_2B_4O_7 \cdot 10H_2O$	17.7 mg
Double-distilled water	1 liter

To retard precipitation, add a few drops of H_2SO_4 before making to volume. Store cold (5°C).

Vitamin Solution No. 6 (Staley, 1968)

Biotin	4.0 mg
Pyridoxine hydrochloride	20.0 mg
Thiamine hydrochloride	10.0 mg
Ca pantothenate	10.0 mg
p-Aminobenzoic acid	10.0 mg
Folic acid	4.0 mg
Riboflavin	10.0 mg
Nicotinamide or nicotinic acid	10.0 mg
Vitamin B_{12}	0.2 mg
Double-distilled water	1 liter

Stirring of the mixture improves dissolution. Sterilize by filtration only. Store dark and cold (5°C).

Medium M 30 + A

Solution 1	
Hutner's basal salts	20 ml
Artificial seawater	250 ml
Buffer: 0.1 M Tris/HCl, pH 7.5	50 ml
Agar	18 g
Distilled water	630 ml

Autoclave at 121°C for 20 min.

Solution 2

N-Acetylglucosamine	2.0 g
$Na_2HPO_4 \cdot H_2O$	0.1 g
Ampicillin sodium salt	0.2 g
Vitamin solution no. 6	10 ml
Distilled water	40 ml

Add filter-sterilized to solution 1.

Artificial Seawater (Lyman and Fleming, 1940)

NaCl	23.477 g
$MgCl_2$	4.981 g
Na_2SO_4	3.917 g
$CaCl_2$	1.102 g
KCl	0.664 g
$NaHCO_3$	0.192 g
KBr	0.096 g
H_3BO_3	0.026 g
$SrCl_2$	0.024 g
NaF	0.003 g

Medium 31 + A

Solution 1	
$CaCl_2 \cdot 2H_2O$	0.1 g
$MgCl_2 \cdot 6H_2O$	0.1 g
Hutner's basal salts	20 ml
Buffer: 0.1 M Tris/HCl, pH 7.5	50 ml
Agar	18.0 g
Distilled water	880 ml

Adjust pH to 7.5; autoclave at 121°C for 20 min.

Solution 2

N-acetylglucosamine	2.0 g
$Na_2HPO_4 \cdot 2H_2O$	0.1 g
Ampicillin sodium salt	0.2 g
Vitamin solution no. 6	10 ml
Distilled water	40 ml

Adjust pH to 7.5, filter-sterilize, and add to solution 1.

To minimize growth of fungi, cycloheximide (0.2 g/liter) may be added to solution 2 of each respective medium. For culturing or long-term storage of pure cultures, the above media should be enriched by adding 0.25 g of peptone and 0.25 g of yeast extract to solution 1. Good results are also obtained with medium M 13.

Medium M 13 (Schlesner, 1986a)

Peptone	0.2 g
Yeast extract	0.2 g
Glucose	0.2 g
Hutner's basal salts	20 ml
Vitamin solution no. 6	10 ml
Buffer: 0.1 M tris/HCl, pH 7.5	50 ml
Artificial seawater	250 ml
Distilled water	670 ml

IDENTIFICATION. *Pirellula* spp. are easily recognized by their morphology. The cells are ovoid, sometimes elliptical, or nearly spherical. They are polarly organized. From the smaller cell pole, holdfast substance is excreted which allows the cells to attach to surfaces or to each other and form rosettes (Fig. 20). Buds develop at the broader pole as a minor mirror image of the mother cell (Fig. 21). Immunoferritin labeling experiments have shown that at least the surface components of the new buds are synthesized de novo (Tekniepe et al., 1982). The buds are motile by means of a 20-nm thick single flagellum that is inserted polarly to subpolarly (Schlesner, 1986a) at the proximate (reproductive) pole (see Fig. 1).

Crateriform structures are scattered over the whole cell surface of the buds in the early developmental stage, while in adult cells their distribution is restricted to the reproductive pole only (Fig. 22A). Adult cells also have fimbriae, originating from the crateriform structures (Fig. 22A). Another type of surface structure,

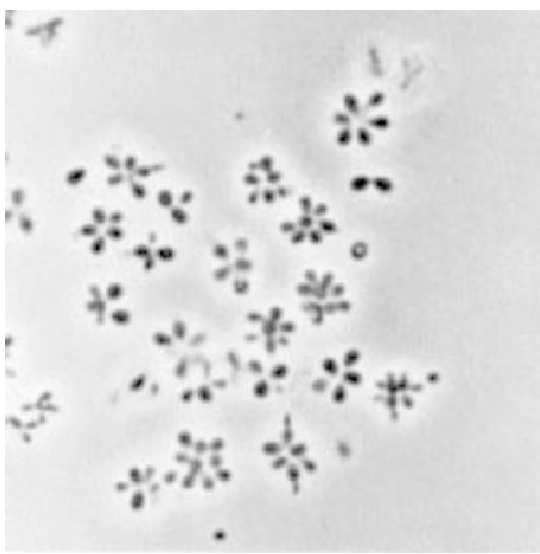


Fig. 20. Phase photomicrograph illustrating rosette formation by *Pirellula marina*.

described as "stacked disks" (Schmidt and Starr, 1979), is shown in thin sections through the reproductive cell pole (Liesack et al., 1986).

Ultrastructure of *Pirellula marina* and *Pirellula staleyi* in thin section conforms to the planctomycete cell plan, with the nucleoid enclosed within a membrane-bounded pirellulosome compartment (Fig. 8). An unusual feature found on cells of two strains of *Pirellula staleyi* are hump-like prosthecate protrusions observed on either side of the cell (Butler et al., 2002; Fig. 22B).

Colonies are red, pink or colorless. Pigmentation seems to be of taxonomic significance, as homology groups derived from DNA/DNA hybridization experiments with 54 strains contained either pigmented or unpigmented strains (Bartels and H. Schlesner, unpublished observations).

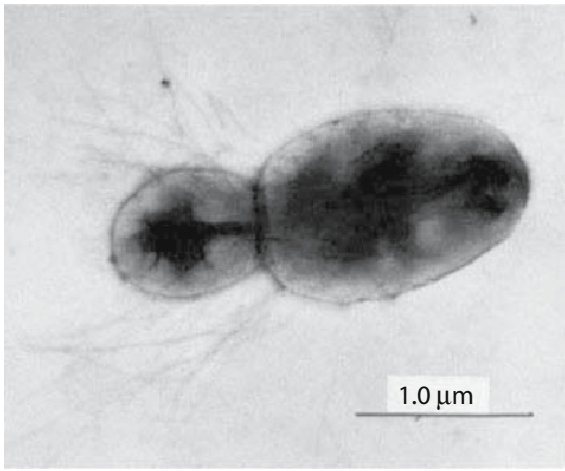


Fig. 21. An electron micrograph of *Pirellula marina* showing a single polar bud.

Phylogenetic clusters of Australian isolates from the prawn *Penaeus monodon* grouping loosely with *Pirellula marina* also were either pink-pigmented or nonpigmented (Fuerst et al., 1997), with the pink strains most closely related to pink or red strains from Europe and forming a cluster distinct from *Pi. marina*.

The phylogenetic position of seven strains of *Pirellula* spp. (including *Pi. staleyi* and *Pi. marina*), analyzed by 16S rRNA cataloging, showed a great phylogenetic diversity among these strains (Stackebrandt et al., 1986a). These findings were supported by analysis of 5S rRNA (Bomar et al., 1988) and subsequent 16S rRNA gene sequencing studies (Ward et al., 1995; Griepenburg et al., 1999). An isolate (ATCC 35122), originally identified as *Pirellula staleyi* on the basis of morphology, has been confirmed by 16S rRNA sequencing as *Pirellula staleyi* (Butler et al., 2002).

Despite their phylogenetic diversity, the strains investigated are phenotypically very similar. As carbon sources, mainly sugars and sugar derivatives are utilized; *N*-acetylglucosamine serves as both carbon and nitrogen source. Sugar alcohols may or may not be utilized. Some strains are able to reduce nitrate anaerobically. All strains tested so far hydrolyze gelatin; casein, DNA or starch is hydrolyzed by some strains, but cellulose is not hydrolyzed at all. Additional methods will have to be applied to find more discriminating taxonomic characteristics.

Two species are described. The type species is from freshwater: *Pirellula staleyi* (Schlesner and Hirsch, 1987), syn. *Pirella staleyi* (Schlesner and Hirsch, 1984), syn. *Planctomyces staleyi* (Starr et al., 1983), syn. *Pasteuria ramosa* (Staley, 1973). The other species is marine: *Pirellula marina* syn. *Pirella marina* (Schlesner, 1986a). The strain

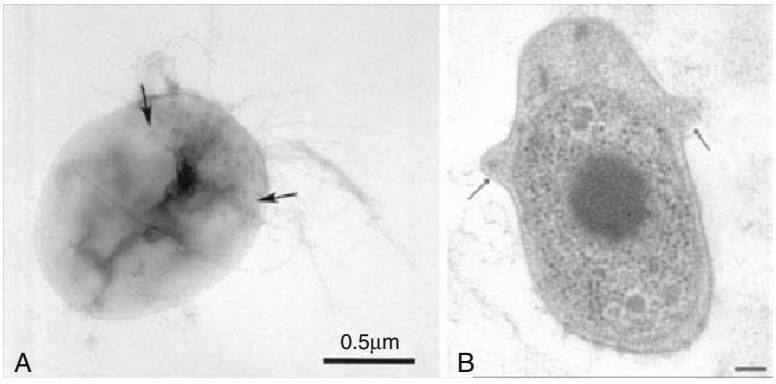


Fig. 22. A) Transmission electron micrograph of negatively stained cell of *Pirellula staleyi* (ATCC 35122) showing polar crateriform structures on cell surface (arrows) and polar flagella and fimbriae appendages. Micrograph courtesy of M. K. Butler and J. A. Fuerst. B) Transmission electron micrograph of thin section of cryosubstituted *Pirellula staleyi* ATCC 35122, showing hump-like protrusions (arrows) on either side of the cell. The typical *Pirellula* cell plan including the pirellulosome compartment is also illustrated here. Bar is 0.1 mm. From Butler et al. (2002).

ATCC 35122 was recently confirmed as a strain of *Pi. staleyi* on the basis of its molecular and ultrastructural characteristics (Butler et al., 2002).

Gemmata

HABITAT. The genus *Gemmata* is at present represented by one species, *G. obscuriglobus*. The single strain UQM 2246 representing the single species was isolated from a single source, the surface waters of Maroon Dam, during a study of the microflora of freshwaters in southeast Queensland, Australia (Franzmann and Skerman, 1984). Additional, as yet undescribed, *Gemmata*-like bacteria have been isolated from leakage water from a compost heap (Schlesner, 1994; Ward et al., 1995) and, more recently, from Australian soil (Wang et al., 2002). The latter is the first report of cultivated soil-dwelling *Gemmata* strains; previously the only evidence for soil harboring planctomycetes was from molecular ecology studies (see Molecular Ecology).

TAXONOMY. The discoverers of the strain recognized the similarities of this coccoid budding bacterium to a strain of great significance in the history of Planctomycetales systematics, strain ATCC 27377, which at one time was proposed as the neotype of *Pasteuria ramosa*, but later transferred to the genus *Planctomyces* (Starr et al., 1983) and to the genus *Pirella* (Schlesner and Hirsch, 1984), a genus name later rejected in favor of *Pirellula* (Schlesner and Hirsch, 1987; see Pirellula for a detailed account of these changes). Franzmann and Skerman (1984) distinguished *Gemmata obscuriglobus* (UQM 2246) from ATCC 27377 by DNA base content, number of flagella on swarmer cells, lack of a discrete holdfast, the possession of a phase-dark inclusion in *Gemmata* postulated to contain DNA, and several other phenotypic characters outlined in Table 7. Later molecular systematics investigation of the 16S rRNA and 5S rRNA of *G. obscuriglobus* confirmed its status as a distinct genus and as a member of the phylogenetically distinct bacterial order Planctomycetales (Stackebrandt et al., 1986a; Bomar et al., 1988). *Gemmata* occupies a distinct position within the Planctomycetales phylogenetically; both 16S rRNA and 5S rRNA sequence similarities with other Planctomycetales genera indicate that the root of the evolutionary tree derived for members of the order lies close to the point at which the ancestor of *G. obscuriglobus* branched off. Thus, this species is the deepest branching organism within the order Planctomycetales (Stackebrandt et al., 1986a; Bomar et al., 1988; see Fig. 4).

ISOLATION. The only existing strain of *G. obscuriglobus*, UQM 2246, was isolated from

Table 7. Characteristics of *Gemmata obscuriglobus* UQM 2246 and strain ATCC 27377.

Character	UQM2246[a]	ATCC 27377[b]
G+C content (mol%)	6.4 ± 1.0	5.7
Cell shape	"Puckered" spherical to ovoid	Ovoid
Cell size (μm)	(1.4–3.0) × (1.4–3.0)	(0.5–3.0) × (1.0–5.0)
Crateriform structure distribution	Uniform	Polar
Reproduction	Budding	Budding
Gram reaction	–	+
Fimbriae	+	+
Motile swarmer cells	+	+
Flagellation	Multitrichous	Monotrichous
Colony color	Rose	Yellow
Catalase	+	+
Oxidase	–	+
Anaerobic growth	–	–
Generation time (h)	13.1 ± 2.8	ca. 13
Growth temperature (°C)	16.0–35.0	17.7–29.6
OF test	Oxidative	Oxidative
Carbon source utilization		
Glucose	+	+
Fructose	–	+
Pyruvate	–	+
Attachment to glass	+	+
Habitat	Freshwater	Freshwater
Rosettes formed	–	+
Phase-dark inclusion	+	–

Symbols and abbreviation:
+, property present; –, property absent; and OF test, oxidative-fermentative test.
[a]Data from Franzmann and Skerman (1984) supplemented by unpublished observation (J. A. Fuerst).
[b]Data from Staley (1973) and Schlesner and Hirsch (1984).

the surface waters of the littoral zone of a freshwater dam in subtropical Queensland, Australia. Micromanipulation using the equipment and methods of Skerman (1968) as applied by Franzmann (1983) was employed in the isolation. This method employs a micromanipulator consisting of a glass microtool attached to a magnet, which is in turn carried on a metal slide that is part of a lens "collar" screwed onto the microscope objective mounting. A Leitz 32× Phaco phase objective is used on a phase contrast microscope (preferably an Olympus CHA microscope equipped with a Reichert phase contrast condenser or any system compatible with a Leitz 32× Phaco objective). The microtool is focused by moving the lens collar up and down on the objective mounting; once focused, it can be used to transfer cells on an agar surface across the surface and away from other cells by trapping cells in the water film around the microtool and moving the mechanical stage controls, the agar plate being mounted on the stage. The whole assembly can be placed in a UV-sterilizable Perspex chamber or in a plastic bag with eyepiece holes to facilitate aseptic handling of plates. Nine-cm plastic Petri dishes containing 10 ml of lakewater agar (filtered lakewater solidified with 1.5% Noble agar for optical clarity; see below for formula) are inoculated by running an approximately 0.5-ml drop of sample water down the center of a plate which is surface-dried in advance. Storage of samples before inoculation should be avoided. Inoculated plates are allowed to remain horizontal until free moisture is absorbed. Plates are examined after 8 h incubation at 28°C or until microcolony formation is apparent. Cells from microcolonies displaying cell morphology and size consistent with *Gemmata* are manipulated away from the central inoculum line to a position on the surface of the same plate, well separated from the inoculation line. Position of a single cell can be marked using a microtool and an agar block circumscribing that area is excised with a sterile scalpel and transferred to a fresh lakewater agar plate or to a fresh lakewater peptone yeast extract agar plate (see formula below). Purity can be checked microscopically at each stage of the isolation. Strains can be maintained on soil extract agar supplemented with 0.1% glucose or on casitone yeast extract agar: *Gemmata obscuriglobus* and a strain closely related to *Gemmata* from soil (Wang et al., 2002) grow well on M1 agar of Schlesner based on *N*-acetylglucosamine as the carbon source (Schlesner, 1994). Strains of planctomycetes isolated more recently than *G. obscuriglobus* have been isolated using this or a similar medium combined with peptidoglycan synthesis-inhibiting ampicillin alone (Schlesner, 1994) or ampicillin combined with the protein synthesis inhibitor streptomycin (Wang et al., 2002).

Lakewater Noble Agar (Franzmann and Skerman, 1981)

Filter water from a eutrophic lake or pond through a 2-mm membrane filter and add Bacto Noble agar to 1.5%. Sterilize by autoclaving at 121°C for 20 min.

Lakewater Peptone Yeast Extract Agar (Franzmann and Skerman, 1981)

Bacto peptone	0.1 g
Bacto yeast extract	0.1 g
Bacto agar	15 g
Filtered eutrophic lakewater	1 liter

Filter the eutrophic lakewater through a 0.2-m membrane. Sterilize by autoclaving at 121˚C for 20 min.

Casitone Yeast Extract Agar

Bacto casitone	5 g
Bacto yeast extract	3 g
$MgSO_4 \cdot 7H_2O$	2 g
Bacto agar	15 g
Distilled water	1 liter

Adjust pH to 7.2, add the agar, and sterilize the medium by autoclaving at 121˚C for 20 min.

Soil Extract Agar with 0.1% Glucose

Soil	1000 g
Distilled water	1 liter

Mix soil with water and autoclave at 121°C for 20 min. Add 10 g of $CaCO_3$ and mix. Decant and filter liquid. Autoclave filtrate at 121°C for 20 min. and store as stock solution. To the stock solution, add agar to 1.5% and autoclave at 121°C for 20 min. After autoclaving, add filter-sterilized glucose aseptically to the agar medium to give a final concentration of 0.1%.

Additional *Gemmata*-like strains have been isolated from soil, freshwater, and the leakage water from a compost heap, using media formulated with antibiotics inhibiting cell-wall synthesis (combinations of ampicillin [200 μg/ml], streptomycin [1000 μg/ml] and cycloheximide [20–100 μg/ml] or penicillin G [500 μg/ml], streptomycin [1000 μg/ml] and amphotericin B [Fungizone; 0.25–0.5 μg/ml]; Wang et al., 2002). The media used were freshwater media M1 or M31 (Schlesner, 1994; see Pirellula).

IDENTIFICATION. *Gemmata obscuriglobus* is a budding, nonprosthecate aerobic organism with spherical to ovoid cells. Initial appearances from negative-stain transmission electron microscopy and phase contrast light microscopy (Fig. 23) suggest a spherical or ovoid shape.

Consistent with the position of the genus within the order Planctomycetales, cells observed by negative staining possess crateriform structures—circular areas on the cell that accumulate negative stain (Fig. 24). These are distributed all over the cell surface, similar to the

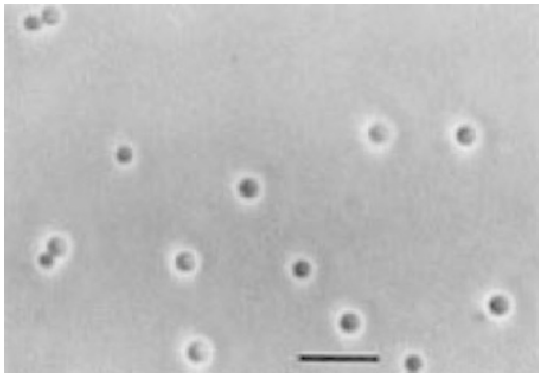


Fig. 23. Phase contrast photomicrograph of cells of *Gemmata obscuriglobus* (UQM 2246). Budding is displayed in addition to the superficially spherical cell shape. Bar is 10 mm.

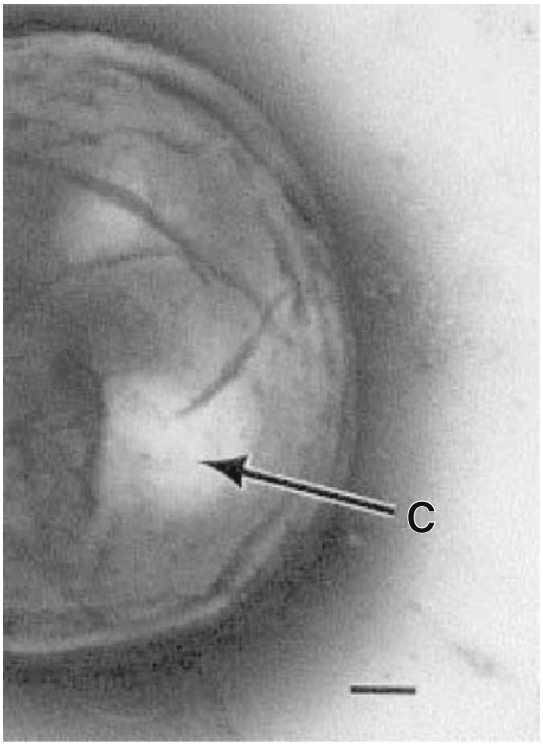


Fig. 24. Electron micrograph of a portion of a negatively stained cell of *Gemmata obscuriglobus* (UQM 2246), displaying crateriform structures (C), which are electron-dense pits distributed uniformly over the cell surface. The arrow points to one example of such a structure; many others are visible on the cell surface displayed. Fimbriae are visible outside the cell perimeter. Negatively stained with 1% uranyl acetate supplemented with 0.4% sucrose. Bar is 0.2 mm.

distribution found in the genus *Planctomyces*, but unlike that in *Pirellula* species (Schlesner and Hirsch, 1984). Such crateriform structures have also been observed in metal-shadowed cell walls of *G. obscuriglobus* (Stackebrandt et al., 1986b). The cell walls of *G. obscuriglobus*, like those of other Planctomycetales, lack peptidoglycan and diaminopimelic acid and are also similar to those of other members of the order in the predominately proteinaceous composition of the wall (Stackebrandt et al., 1986b). In thin sections, the cell wall has the typical appearance expected for the peptidoglycan-less walls of Planctomycetales (Fig. 11), with inner and outer electron-dense layers separated by an electron-transparent layer, the inner layer being more electron-dense, as found for morphotype IV planctomycete ultrastructure by Tekniepe et al. (1981). *Gemmata* thus shares budding reproduction, crateriform structures, and cell-wall composition with other genera of the Planctomycetales, and this relationship is confirmed by the high number of Planctomycetales-specific signature oligonucleotides present in the 16S rRNA oligonucleotide catalog of the genus (Stackebrandt et al., 1986a) and by the presence of the unique Planctomycetales-specific "short" 5S rRNA with lack of insertion at position 66 and numerous characteristic base-pair inversions (Bomar et al., 1988).

In the initial study of *G. obscuriglobus*, Franzmann (1983) noted that in some thin-sectioned cells nuclear material was packaged in a discrete body within the cell and that this might be correlated with the appearance of phase-dark inclusions in light microscopy preparations. Intracellular membrane material was commonly seen in thin sections, and although in some cases an appearance of membrane involvement with nuclear packages suggested that they may be membrane-bound structures, it was thought that this may be artifactual and further studies were required to resolve the nature of the nuclear packages. Such packages often consist of an appearance of fibrillar material within a shell of electron-dense amorphous material separating the material from the rest of the cell. Several such packages can occur within a single cell. A cell inclusion in *G. obscuriglobus* was indeed later confirmed as a DNA-containing body, surrounded by a double membrane, by transmission electron microscopy. This region was termed "the membrane-bounded nuclear body" (Fuerst and Webb, 1991, and Fig. 11A–C), although recent DAPI (4′,6′-diamidino-2-phenylindole hydrochloride) studies suggest that it is the phase-light rather than phase-dark interior cell region in phase contrast microscopy which contains the DNA. *Gemmata obscuriglobus* structure (Fig. 11A, B) including the membrane-bounded nuclear body is particularly well-preserved by cryosubstitution (a technique that avoids some of the artifacts of chemical fixation, which in *G. obscuriglobus* can result in crescent-shaped cells due to osmotic shrinkage effects)

and can also be demonstrated by freeze-fracture techniques (Fuerst and Webb, 1991; Fig. 11C). This was the first example of cell compartmentalization in planctomycetes, which was also later demonstrated in other planctomycete species (Lindsay et al., 1997; Lindsay et al., 2001). In addition to the membrane-bound nucleoid, the cell of *G. obscuriglobus* displays the common features of all planctomycete compartmentalization—an intracytoplasmic membrane and a paryphoplasm region, in this case at the outer rim of the cell (Fig. 11A, B). The intracytoplasmic membrane is distinct from and not connected with the cytoplasmic membrane, which is closely apposed to the cell wall. Both the membrane-bounded nucleoid and the region between the nuclear envelope and the intracytoplasmic membrane contain electron-dense ribosome-like particles. The membrane-bounded nuclear body has also been found in new isolates phylogenetically most closely related to *Gemmata* (Wang et al., 2002) so that this character appears to be an ultrastructural feature of potential taxonomic significance for delineating members of the *Gemmata* genus.

The recently isolated strains of Wang et al. (2002) conform to the general morphological properties of the genus *Gemmata* by having spherical cells bearing uniformly distributed crateriform structures, containing a membrane-bounded nuclear body and a distinct ribosome-containing compartment, and reproducing by budding. They also share most of the characteristic *Gemmata*-specific 16S rRNA signature nucleotides and, like *G. obscuriglobus*, contain a 10-base sequence insertion at *E. coli* position 998 (Fuerst et al., 1991). On the basis of their 16S rDNA sequence similarity values, these strains probably represent novel species.

All isolated *Gemmata* strains are pink or red in colony color (Franzmann and Skerman, 1984; Wang et al., 2002).

As shown in Table 7, differentiation of *Gemmata obscuriglobus* from the morphologically similar ATCC 27377 is based on the higher G+C content of *Gemmata*, occurrence of phase-dark inclusions, flagellation, oxidase test, utilization of fructose and pyruvate, temperature range for growth, and colony color. The G+C content for ATCC 27377 has been the subject of several determinations, with slightly variable results; thus, Starr et al. (1983) reported a high value of 59.00 mol% by buoyant density and Schlesner has noted a lowest value of 56.40 mol% by thermal denaturation (Schlesner and Hirsch, 1984), while for their comparison, Franzmann and Skerman (1981) used the figure of 57.1 mol% found by Staley (1973) by thermal denaturation. In all cases, however, the G+C content of *G. obscuriglobus* is significantly higher.

Isosphaera

Isosphaera pallida (an aerobic, heterotrophic budding bacterium) is unusual because it is phototactic and motile by gliding. With other members of the Planctomycetales, *Isosphaera* shares characteristics that are of potential evolutionary significance—the presence of proteinaceous cell walls and phospholipids containing β-hydroxylated fatty acids (Stackebrandt et al., 1984; Giovannoni et al., 1987a; Kerger et al., 1988). These traits, and the budding mode of reproduction, first led to the suggestion that *I. pallida* was a planctomycete (Giovannoni et al., 1987a); molecular phylogenetic comparisons of 5S rRNA supported this classification (Bomar et al., 1988) but showed that *I. pallida* was only distantly related to members of the genera *Gemmata*, *Pirellula* and *Planctomyces*. Subsequent analyses of 16S rRNA gene sequences have confirmed the results obtained with the 5S molecule.

HABITAT. *Isosphaera pallida* commonly occurs in North American and European hot springs at temperatures from 35 to 55°C. It is found either as a component of microbial mats or planktonically, in which case it may be suspended in the water column by gas vacuoles. The co-occurrence of *I. pallida* with cyanobacteria may be a coincidence. *Isosphaera pallida* is an obligate aerobe and, like cyanobacteria, requires neutral or alkaline conditions for growth.

The first axenic cultures of *Isosphaera pallida* (strain IS1B) were obtained from hot springs located on the Warm Springs Indian Reservation, Oregon, United States. Subsequently, five additional strains were isolated from hot springs in Yellowstone National Park, United States, and Big Spring, Thermopolis, Wyoming, United States. The reports of Geitler (Geitler, 1955; Geitler, 1963) had suggested that mesophilic strains of *Isosphaera* may inhabit environments other than hot springs, and these recent results confirm this. Recently isolated *Isosphaera*-like strains have been obtained from leakage water from a compost heap (Schlesner, 1994; Ward et al., 1995) and from lakewater and a laboratory ampicillin solution (Wang et al., 2002). Studies on filamentous isolates of the "Nostocoida limicola III" morphotype from activated sludge have shown them to be close relatives of *Isosphaera*. These strains are nonthermophilic and do not display gliding motility (Liu et al., 2001).

TAXONOMY. *Isosphaera pallida*, the only recognized species of the genus *Isosphaera*, inspired a debate in the taxonomic literature long before it was first isolated in pure culture in 1979 (Giovannoni et al., 1987b). The organism attracted attention because of its conspicuous habitat and morphology. The distinctive chains of spherical, budding cells formed by *Isosphaera*

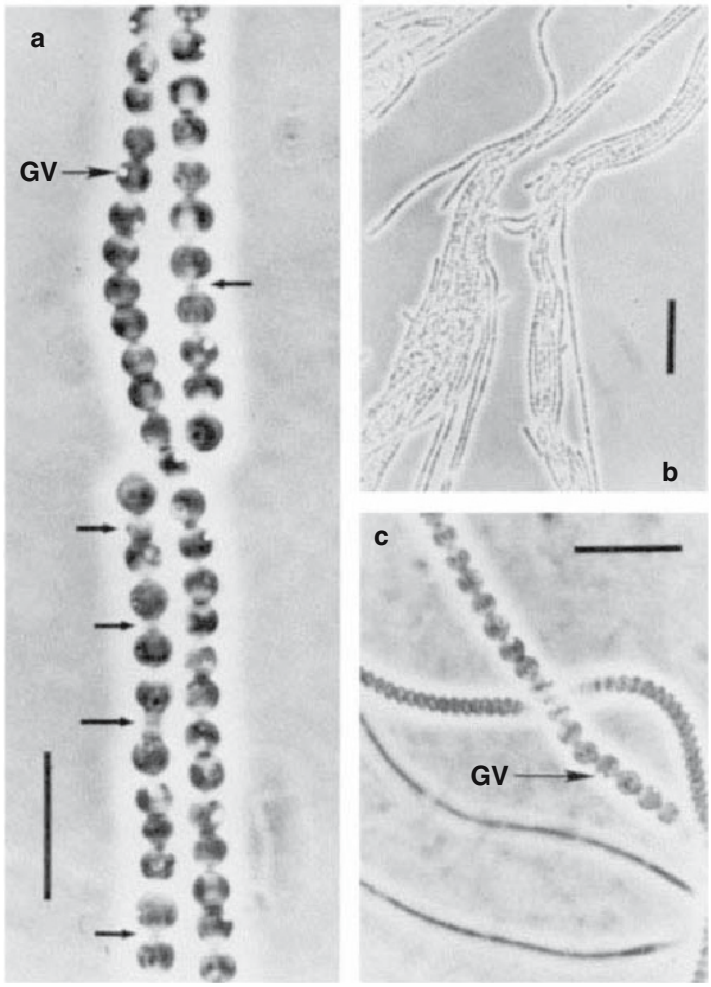


Fig. 25. Phase contrast micrographs of *Isosphaera pallida*. a) Exponential phase culture (strain IS1). Bar is 10 mm. b) Exponential phase culture (strain IS1). Bar is 50 mm. c) *Isosphaera* from a natural population, Mammoth Hot Springs, Yellowstone National Park, United States, showing *Isosphaera pallida* and *Spirulina*. Bar is 10 mm. Arrows indicate buds. GV, gas vacuoles. Note that the frequency of buds in the exponentially growing culture (b) is low, suggesting that the process of bud development is relatively short compared to the cell cycle.

(Fig. 25) are readily distinguishable from morphologically similar organisms, such as cyanobacteria of the genus *Pseudanabaena*. However, because of this similarity, *Isosphaera pallida* was originally described by Woronichin (1927) as a cyanobacterium, *Isocystis pallida*, only on the basis of observations of collected field specimens. This early case of mistaken identity was understandable, considering that *I. pallida* is invariably found in the euphotic zone of hot springs in association with cyanobacteria.

Geitler (1955) obtained the first enrichment cultures of an organism resembling *Isosphaera*. These were taken from a water-filled depression in a rotting spruce stump. He concluded that his isolate was a yeast and assigned it to a novel genus and species, *Torulopsidosira filamentosa*. After examining collected specimens of *Isocystis pallida*, he assigned it also to the genus *Torulopsidosira* (Geitler, 1963). This decision was based on two observations. The fact that the organisms divided by budding suggested that they were yeasts, since Geitler was dubious that budding occurred in bacteria. Secondly, cytological staining indicated that the nuclear material was sequestered within a small region of the cell—indicating a structure similar to a eukaryotic nucleus. Although it is now clear that *Isosphaera* is a prokaryote, modern staining techniques (e.g., 4×, 6-diamidino-2-phenylindole) confirm Geitler's early observation (Geitler, 1963) that the nuclear material is located in a distinct region of the cell.

The micrographs of *Torulopsidosira filamentosa* provided by Geitler show an organism strongly resembling *I. pallida* in size, shape, and the distinctive formation of intercalary buds within filaments. This suggests that species similar to *Isosphaera* may occur in habitats outside of hot springs. Indeed, in the original definition of the cyanobacterial genus *Isocystis* by Borzi, eight species were named, not including *I. pallida*. One of these, *I. salina* was described from saline springs and mineral waters. We obtained mixed enrichment cultures of cyanobacteria and *Isosphaera* from a saline (13% NaCl) hot spring in Utah, United States. Attempts to obtain pure cultures failed, even when the *Isos-*

phaera medium was supplemented with 0.2 M NaCl.

The conclusions of Geitler were later disputed by Rathsack-Kunzenbach, who was familiar with *Isocystis pallida* from hot spring collections in Greece. Rathsack-Kunzenbach's extensive description and discussion indicated that *Isocystis pallida* from their cultures and *I. pallida* are the same. They detected a weak red fluorescence attributed to cyanobacterial pigments in their cultures. However, we have been unable to confirm this fluorescence with North American collections or cultured material.

Following the first isolation, it immediately became apparent that *Isosphaera* was neither a yeast nor a cyanobacterium, but instead, an aerobic, heterotrophic bacterium. It is a salmon color in culture owing to the presence of carotenoids, but absorbance spectra reveal no maxima corresponding to chlorophylls or phycobiliproteins. rRNA sequence analysis and a number of phenotypic characters, including the presence of a proteinaceous cell wall and the possession of phospholipids with β-hydroxylated fatty acids, revealed the *Isosphaera* strains to be planctomycetes.

ISOLATION. *Isosphaera pallida* can be isolated on plates of medium IM incubated in an atmosphere of 5% CO_2/95% air. Medium IM is a dilute mineral medium containing no added carbon sources and containing 50 mM bicarbonate. The pH of the medium (7.9) is determined by the bicarbonate/CO_2 buffering system.

Medium IM (Pfennig and Trüper, 1981)

Solution A

$CaCl_2 \cdot 2H_2O$	0.32 g
$MgSO_4 \cdot 7H_2O$	0.4 g
KCl	0.5 g
NaCl	1.0 g
$(NH_4)_2SO_4$	0.5 g
KH_2PO_4	0.3 g
$FeCl_3$	0.292 mg
Trace element solution SL-7a	10 ml
Vitamin B_{12}	5 mg
Water	1 liter

Adjust to pH 7.6 with NaOH, remove precipitate by filtration through Whatman no. 1 filter paper, and store at 4°C.

Solution B

$NaHCO_3$	42.0 g
Water	1 liter

Autoclave, then bubble vigorously with sterile CO_2 for 1 h.

Final Medium

Solution A	250 ml
Solution B	100 ml
H_2O	650 ml

After combining solution A + H_2O and autoclaving, add sterile solution B.

In the dark, or in the light with 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (an inhibitor of photosystem II) present, medium IM is selective for *Isosphaera*. Plates of medium IM streaked with microbial mat material from a hot spring and incubated in the dark at 45°C usually develop small (1–2 mm) colonies of *Isosphaera* after two weeks. The colonies are firm to the touch of an inoculating loop. In part, the basis for the selectivity of medium IM seems to be the absence of added carbon other than the contaminating organic compounds present in Bacto-agar. However, *Isosphaera* is not agarolytic.

The *Isosphaera*-like strains of Schlesner (1994) and Wang et al. (2002) were isolated using media containing the antibiotics ampicillin (inhibiting cell wall synthesis in peptidoglycan-synthesizing bacteria) and streptomycin (inhibiting protein synthesis in many bacteria; see above for concentrations used). D-Arabinose Supernatant R2A Medium employing filtered effluent from secondary clarifier of an activated sludge plate was found to be essential for isolation of *Isosphaera*-like isolates from activated sludge (Liu et al., 2001).

CULTIVATION. *Isosphaera* can be routinely cultivated in liquid batch culture on medium IMC sparged with 5% CO_2/95% air.

Medium IMC

To Medium IM, add 0.025% D-glucose, 0.025% casamino acids, and vitamin solution (0.5 ml/liter; added after autoclaving). Vitamin solution contains: nicotinic acid, 2 mg/ml; thiamine HCl, 1 mg/ml; p-aminobenzoic acid, 0.2 mg/ml; and biotin, 0.02 mg/ml.

The minimum doubling time observed (18 hours) occurs at 42°C, but substantial growth is seen at temperatures from 37 to 55°C. A high concentration of CO_2 is probably not required for growth.

Although visible growth occurs on Bacto-agar without organic supplements, the addition of carbon sources at low concentration enhances growth on plates. Only a few compounds, including glucose, ribose, and lactate, are utilized as sole sources of carbon for growth. Growth is more robust in the presence of casamino acids, suggesting that they are assimilated but not utilizable as sole carbon sources. D-Glucose concentrations of 0.025% are routinely employed because higher concentrations (0.05% D-glucose) inhibit growth. Ribose, which serves as a sole source of carbon for growth in three of four strains investigated, is also inhibitory at concentrations above 0.25%.

The optimal growth temperature of strain IS1B is 42°C. Growth of this strain occurs at temperatures up to 55°C and as low as 37°C.

IDENTIFICATION. The moderately thermophilic aerobic and heterotrophic *I. pallida* is unambiguously identifiable. *Isosphaera pallida* cells are spherical and typically vary in size from 2.0 to 2.5 μm, although some cells are larger in stationary phase cultures. In wild-type strains, the cells are arranged in unbranched filaments of indefinite length. Repeated subcloning results in selection for short chain mutants, such as the type strain, IS1B (S. Giovannoni, unpublished observations). Cells are budding, but the frequency of buds within the unbranched filaments of indefinite length is low. Ultrastructural observations have revealed that the cell surface is uniformly and densely covered with pili. However, there is no evidence for flagella. The mechanism of motility remains unknown. As with other planctomycetes, surface crateriform structures are present. In *I. pallida* they appear to be uniformly distributed over the cell. Electron-transparent "holes" of similar size and density are found in the isolated SDS-insoluble cell wall fraction.

Freshly isolated strains produce gas vacuoles containing gas vesicles with typical conical ultrastructure (Fig. 26). The gas vacuoles are small

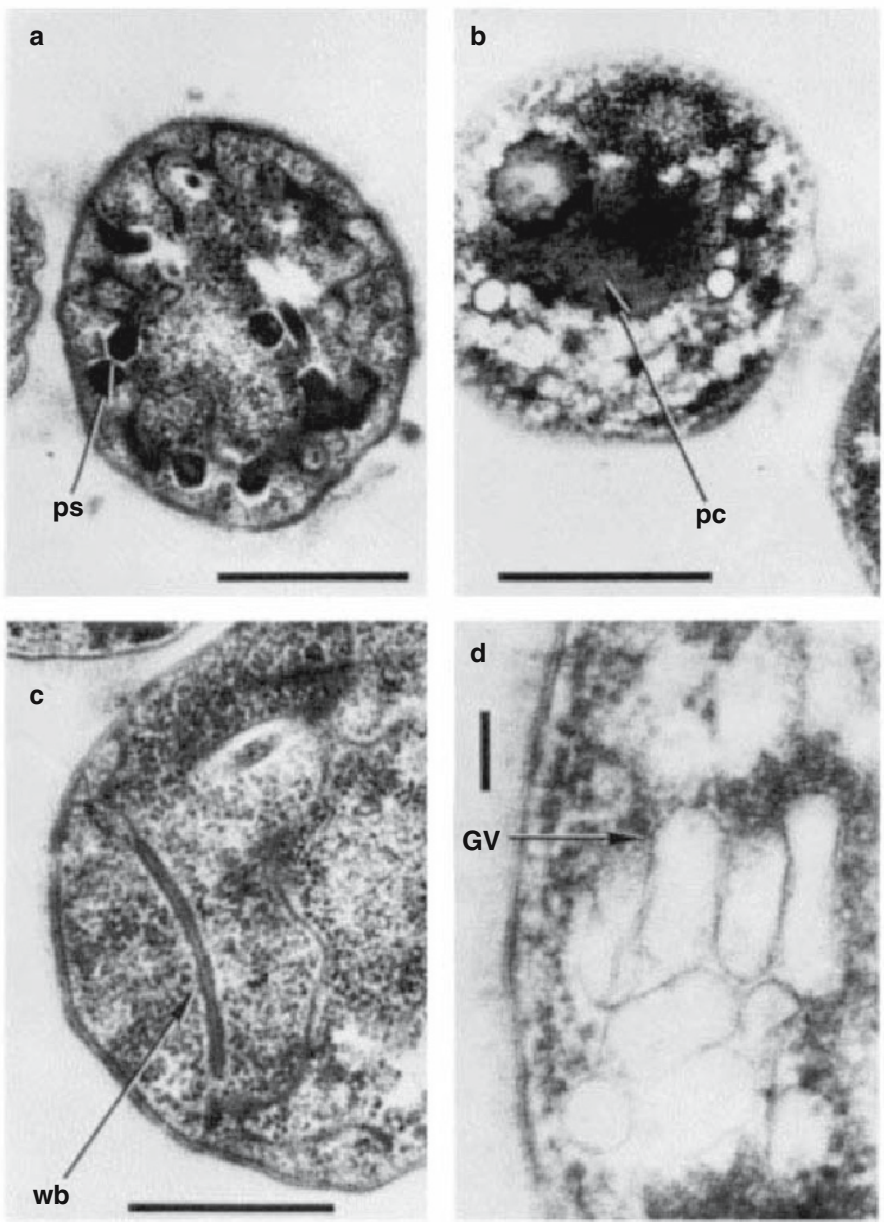


Fig. 26. Thin-sections showing cytoplasmic inclusions of *Isosphaera pallida*. a) Polyhedral structures (ps). Bar is 1 mm. b) Paracrystalline inclusion (pc). Bar is 1 mm. c) Wall body (wb). Bar is 1 mm. d) Gas vesicles (GV). Bar is 0.1 mm.

and usually occur singly in cells. Polyhedral structures, similar in appearance to carboxysomes, are often seen. However, there is no evidence for autotrophic metabolism. Also seen are paracrystalline inclusions and "wall bodies." No functions are associated with these structures. Electron microscopy of thin sections of cells prepared by cryosubstitution have confirmed that the cell plan of *Isosphaera* conforms to the general plan shared by all planctomycetes (Lindsay et al., 2001), but in *I. pallida* the paryphoplasm is invaginated to form a large central region (Fig. 9).

The salmon-colored colonies of *I. pallida* are motile. Motility occurs most readily on Gelrite surfaces, though migration is observable to a lesser degree on agar, particularly if it has excess surface moisture. When suspensions of concentrated cells are dropped on plates, they form multiple aggregates (called "comets") of variable size. These move randomly at about an equal rate in darkness or in uniform light. In the presence of a directional light source, the aggregates are phototactic.

Wang et al. (2002) isolated *Isosphaera*-like strains that conformed to the general properties of the genus *Isosphaera* as currently described (spherical cells, reproducing by budding; uniformly distributed crateriform structures; cell compartmentalization, with a single membrane containing the nucleoid; and characteristic 16S rRNA signature nucleotides). However, the filaments seen in *I. pallida* were not observed in these strains.

Preservation of Planctomycete Strains

Planctomycete strains have been successfully stored by lyophilization and under liquid nitrogen; they have been revived after liquid nitrogen storage for several years. Also, when reviving stored ampoules of *G. obscuriglobus* from liquid nitrogen, soil extract agar supplemented with 0.1% glucose is superior to lakewater agar. Planctomycetes including *G. obscuriglobus* can also be successfully stored frozen in 10% glycerol in deionized water at –70°C, with revival onto Schlesners M1 agar (J. A. Fuerst, unpublished results).

Literature Cited

Akkerman, H. W., and A. Eisenstark. 1974. The present state of phage taxonomy. Intervirol. 3:201–219.

Bauld, J., and J. T. Staley. 1976. Planctomyces maris sp. nov.: A marine isolate of the Planctomyces-Blastocaulis group of budding bacteria. J. Gen. Microbiol. 97:45–55.

Bel'kova, N. L., L. Denisova, E. N. Manakova, E. F. Zaichikov, and M. A. Grachev. 1996. Species diversity of deep water microorganisms in Lake Baikal, detected by 16S rRNA sequences [in Russian]. Dokl. Akad. Nauk. 348(5):692–695.

Bomar, D., S. Giovannoni, and E. Stackebrandt. 1988. A unique type of eubacterial 5S rRNA in members of the order Planctomycetales. J. Molec. Evol. 27(2):121–125.

Borneman, J., and E. W. Triplett. 1997. Molecular microbial diversity in soils from eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol. 63(7):2647–2653.

Bowman, J. P., and R. D. McCuaig. 2003. Biodiversity, community structural shifts, and biogeography of prokaryotes within Antarctic continental shelf sediment. Appl. Environ. Microbiol. 69(5):2463–2483.

Brochier, C., and H. Philippe. 2002. Phylogeny: A non-hyperthermophilic ancestor for bacteria. Nature 417 (6886): 244.

Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli. Proc. Natl. Acad. Sci. USA 75(10):4801–4805.

Buckley, D. H., and T. M. Schmidt. 2003. Diversity and dynamics of microbial communities in soils from agro-ecosystems. Environ. Microbiol. 5(6):441–452.

Butler, M. K., J. Wang, R. I. Webb, and J. A. Fuerst. 2002. Molecular and ultrastructural confirmation of classification of ATCC 35122 as a strain of Pirellula staleyi. Int. J. Syst. Evol. Microbiol. 52(Part 5):1663–1667.

Chen, H. W., A. Kuspa, I. M. Keseler, and L. J. Shimkets. 1991. Physical map of the Myxococcus xanthus chromosome. J. Bacteriol. 173(6):2109–2115.

Christensson, M., L. L. Blackall, and T. Welander. 1998. Metabolic transformations and characterisation of the sludge: Community in an enhanced biological phosphorus removal system. Appl. Microbiol. Biotechnol. 49:226–234.

Claus, H., H. H. Martin, C. A. Jantos, and H. Konig. 2000. A search for beta-lactamase in chlamydiae, mycoplasmas, planctomycetes, and cyanelles: Bacteria and bacterial descendants at different phylogenetic positions and stages of cell wall development. Microbiol. Res. 155(1):1–6.

Cohen-Bazire, G., W. R. Sistrom, and R. Y. Stanier. 1957. Kinetic studies of pigment synthesis by non-sulphur purple bacteria. J. Cell. Comp. Physiol. 49:25–68.

Cole, S. T., and I. Saint Girons. 1994. Bacterial genomics. FEMS Microbiol. Rev. 14(2):139–160.

Collins, M. D., and D. Jones. 1981. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. Microbiol. Rev. 45(2):316–354.

Cooney, R. P., O. Pantos, M. D. Le Tissier, M. R. Barer, A. G. O'Donnell, and J. C. Bythell. 2002. Characterization of the bacterial consortium associated with black band disease in coral using molecular microbiological techniques. Environ. Microbiol. 4(7):401–413.

Dahlberg, C., M. Bergstrom, and M. Hermansson. 1998. In situ detection of high levels of horizontal plasmid transfer in marine bacterial communities. Appl. Environ. Microbiol. 64(7):2670–2675.

Dalsgaard, T., and B. Thamdrup. 2002. Factors controlling anaerobic ammonium oxidation with nitrite in marine sediments. Appl. Environ. Microbiol. 68(8):3802–3808.

Dalsgaard, T., D. E. Canfield, J. Petersen, B. Thamdrup, and J. Acuna-Gonzalez. 2003. N_2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. Nature 422(6932):606–608.

DeLong, E. F., D. G. Franks and A. I. Alldredge. 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. 38:924–934.

Erdmann, V. A., and J. Wolters. 1986. Collection of published 5S, 5.8S and 4.5S ribosomal RNA sequences. Nucleic Acids Res. 14 (Suppl.):r1–r59.

Fonstein, M., and R. Haselkorn. 1995. Physical mapping of bacterial genomes. J. Bacteriol. 177(12):3361–3369.

Franzmann, P. D., and V. B. D. Skerman. 1981. Agitococcus lubricus gen. nov. sp. nov., a lipolytic, twitching coccus from freshwater. Int. J. Syst. Bacteriol. 31:177–183.

Franzmann, P. 1983. The Aerobic, Heterotrophic, Nongliding Bacteria of the Fresh Waters of South-east Queensland [thesis]. University of Queensland. Brisbane, Australia.

Franzmann, P. D., and V. B. Skerman. 1984. Gemmata obscuriglobus, a new genus and species of the budding bacteria. Ant. v. Leeuwenhoek 50(3):261–268.

Freitag, T. E., and J. I. Prosser. 2003. Community structure of ammonia-oxidizing bacteria within anoxic marine sediments. Appl. Environ. Microbiol. 69(3):1359–1371.

Frias-Lopez, J., A. L. Zerkle, G. T. Bonheyo and B. W. Fouke. 2002. Partitioning of bacterial communities between seawater and healthy, black band diseased, and dead coral surfaces. Appl. Environ. Microbiol. 68(5):2214–2228.

Friedrich, A. B., H. Merkert, T. Fendert, J. Hacker and U. Hentschel. 1999. Microbial diversity in the marine sponge Aplysina cavernicola (formerly Verongia cavernicola) analyzed by fluorescence in situ hybridization (FISH). Mar. Biol. 134:461–470.

Friedrich, A. B., J. Hacker, I. Fischer, P. Proksch and U. Hentschel. 2001. Temporal variations of the microbial community associated with the mediterranean sponge Aplysina aerophoba. FEMS Microbiol. Ecol. 38:105–113.

Fuerst, J. A., S. K. Sambhi, J. L. Paynter, J. A. Hawkins and J. G. Atherton. 1991. Isolation of a bacterium resembling Pirellula species from primary tissue culture of the giant tiger prawn (Penaeus monodon). Appl. Environ. Microbiol. 57(11):3127–3134.

Fuerst, J. A., and R. I. Webb. 1991. Membrane-bounded Nucleoid in the Eubacterium Gemmatata obscuriglobus. Proc. Natl. Acad. Sci. USA 88(18):8184–8188.

Fuerst, J. A. 1995. The planctomycetes: Emerging models for microbial ecology, evolution and cell biology. Microbiology 141 (Part 7):1493–1506.

Fuerst, J. A., H. G. Gwilliam, M. Lindsay, A. Lichanska, C. Belcher, J. E. Vickers, and P. Hugenholtz. 1997. Isolation and molecular identification of planctomycete bacteria from postlarvae of the giant tiger prawn, Penaeus monodon. Appl. Environ. Microbiol. 63(1):254–262.

Fujii, T., H. Sugino, J. D. Rouse, and K. Furukawa. 2002. Characterization of the microbial community in an anaerobic ammonium-oxidizing biofilm cultured on a nonwoven biomass carrier. J. Biosci. Bioengin 194:412–418.

Gebers, R., U. Wehmeyer, T. Roggentin, H. Schlesner, J. Koelbel-Boelke, and P. Hirsch. 1985. Deoxyribonucleic acid base compositions and nucleotide distributions of 65 strains of budding bacteria. Int. J. Syst. Bacteriol. 35(3):260–269.

Geitler, L. 1955. Torulopsidosira n. gen., ein neuer hefeartiger Pilz, und andere knospende Mikroorganismen. Arch. Mikrobiol. 22:324–334.

Geitler, L. 1963. Die angebliche Cyanophyceae Isosphaera pallida is ein hefeartiger Pilz. Arch. Mikrobiol. 46:238–242.

Gimesi, N. 1924. Hydrobiologiai Tanulmanyok (Hydrobiologische Studien). I: Planctomyces bekefii Gim. nov. gen. et sp. [in Hungarian, with German translation]. Kiadja a Magyar Ciszterci Rend. Budapest, Hungary. 1–8.

Giovannoni, S. J., W. Godchaux, 3rd, E. Schabtach, and R. W. Castenholz. 1987a. Cell wall and lipid composition of Isosphaera pallida, a budding eubacterium from hot springs. J. Bacteriol. 169(6):2702–2707.

Giovannoni, S. J., E. Schabtach, and R. W. Castenholz. 1987b. Isosphaera pallida, gen., and comb. nov., a gliding, budding eubacterium from hot springs. Arch. Microbiol. 147:276–284.

Gliesche, C. 1980. *In:* Isolierung und Charakterisierung von Bakteriophagen knospender Bakterien [Isolation and Characterization of Bacteriophages of Budding Bacteria]. Institute for General Microbiology, Christian-Albrechts-Universität. Kiel, Germany.

Glöckner, F. O., B. M. Fuchs and R. Amann. 1999. Bacterioplankton compositions of lakes and oceans: A first comparison based on fluorescence in situ hybridization. Appl. Environ. Microbiol. 65(8):3721–3726.

Glöckner, F. O., M. Kube, M. Bauer, H. Teeling, T. Lombardot, W. Ludwig, D. Gade, A. Beck, K. Borzym, K. Heitmann, R. Rabus, H. Schlesner, R. Amann and R. Reinhardt. 2003. Complete genome sequence of the marine planctomycete Pirellula sp. strain 1. Proc. Natl. Acad. Sci. USA 100(14):8298–8303.

Granberg, K. 1969. Kasviplanktonin merkityksesta vesilaitoksen raakvaveden tarkkailussa. Limnol. Foren. I Finland Limnol. Symp. 1968:34–43.

Gray, J. P., and R. P. Herwig. 1996. Phylogenetic analysis of the bacterial communities in marine sediments. Appl. Environ. Microbiol. 62(11):4049–4059.

Griepenburg, U., N. Ward-Rainey, S. Mohamed, H. Schlesner, H. Marxsen, F. A. Rainey, E. Stackebrandt, and G. Auling. 1999. Phylogenetic diversity, polyamine pattern and DNA base composition of members of the order Planctomycetales. Int. J. Syst. Bacteriol. 49 (Part 2):689–696.

Henrici, A. T., and D. E. Johnson. 1935. Studies of freshwater bacteria. II. Stalked bacteria, a new order of schizomycetes. J. Bacteriol. 30:61–93.

Hirsch, P. 1972. Two identical genera of budding and stalked bacteria: Planctomyces Gimesi 1924 and Blastocaulis Henrici and Johnson 1935. Int. J. Syst. Bacteriol. 22(2):107–111.

Hirsch, P., M. Müller and H. Schlesner. 1977. New aquatic budding and prosthecate bacteria and their taxonomic position. Proceedings of the Symposium on Aquatic Microbiology, Lancaster, UK. Academic Press. London, UK. 107–133.

Hirsch, P., and M. Müller. 1985. Planctomyces limnophilus sp. nov., a stalked and budding bacterium from freshwater. Syst. Appl. Microbiol. 6:276–280.

Hugenholtz, P., C. Pitulle, K. L. Hershberger and N. R. Pace. 1998. Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. 180(2):366–376.

Humayoun, S. B., N. Bano, and J. T. Hollibaugh. 2003. Depth distribution of microbial diversity in Mono Lake, a

meromictic soda lake in California. Appl. Environ. Microbiol. 69(2):1030–1042.

Jenkins, C., and J. A. Fuerst. 2001. Phylogenetic analysis of evolutionary relationships of the planctomycete division of the domain bacteria based on amino acid sequences of elongation factor Tu. J. Molec. Evol. 52(5):405–418.

Jenkins, C., V. Kedar, and J. A. Fuerst. 2002. Gene discovery within the planctomycete division of the domain Bacteria using sequence tags from genomic DNA libraries. Genome Biol. 3:6.

Jetten, M. S., M. Wagner, J. Fuerst, M. van Loosdrecht, G. Kuenen, and M. Strous. 2001. Microbiology and application of the anaerobic ammonium oxidation ("anammox") process. Curr. Opin. Biotechnol. 12(3):283–288.

Kahan, D. 1961. Thermophilic microorganism of uncertain taxonomic status from the hot springs of Tiberias (Israel). Nature 192(4808):1212–1213.

Kerger, B. D., C. A. Mancuso, P. D. Nichols, D. C. White, T. Langworthy, M. Sittig, H. Schlesner, and P. Hirsch. 1988. The budding bacteria, Pirellula and Planctomyces, with atypical 16S rRNA and absence of peptidoglycan, show eubacterial phospholipids and uniquely high proportions of long chain beta-hydroxy fatty acids in the lipopolysaccharide lipid A. Arch. Microbiol. 149:255–260.

Kieser, H. M., T. Kieser, and D. A. Hopwood. 1992. A combined genetic and physical map of the Streptomyces coelicolor A3(2) chromosome. J. Bacteriol. 174(17): 5496–5507.

Kölbel-Boelke, J., R. Gebers, and P. Hirsch. 1985. Genome size determinations for 33 strains of budding bacteria. Int. J. Syst. Bacteriol. 35(3):270–273.

König, E., H. Schlesner, and P. Hirsch. 1984. Cell wall studies on budding bacteria of the Planctomyces/Pasteuria group and on a Prosthecomicrobium sp. Arch. Microbiol. 138:200–205.

Krave, A. S., B. Lin, M. Braster, A. M. Laverman, N. M. van Straalen, W. F. Roling, and H. W. van Verseveld. 2002. Stratification and seasonal stability of diverse bacterial communities in a Pinus merkusii (pine) forest soil in central Java, Indonesia. Environ. Microbiol. 4(6):361–373.

Kristiansen, J. 1971. On Planctomyces bekefii and its occurrence in Danish lakes and ponds. Bot. Tidsskr. 66:293–392.

Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss. 1989. Expression of sigma 54 (ntrA)-dependent genes is probably united by a common mechanism. Microbiol. Rev. 53(3):367–376.

Kuypers, M. M., A. O. Sliekers, G. Lavik, M. Schmid, B. B. Jorgensen, J. G. Kuenen, J. S. Sinninghe Damste, M. Strous, and M. S. Jetten. 2003. Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Nature 422(6932):608–611.

Lapage, S. P., P. H. A. Sneath, E. F. Lessel, V. B. D. Skerman, H. P. R. Seeliger, and W. A. Clark. 1992. International Code of Nomenclature of Bacteria. American Society for Microbiology. Washington, DC.

Leary, B. A., N. Ward-Rainey, and T. R. Hoover. 1998. Cloning and characterization of Planctomyces limnophilus rpoN: Complementation of a Salmonella typhimurium rpoN mutant strain. Gene 221(1):151–157.

Leblond, P., M. Redenbach, and J. Cullum. 1993. Physical map of the Streptomyces lividans 66 genome and comparison with that of the related strain Streptomyces coelicolor A3(2). J. Bacteriol. 175(11):3422–3429.

Liesack, W., H. Koenig, H. Schlesner, and P. Hirsch. 1986. Chemical composition of the peptidoglycan-free cell envelopes of budding bacteria of the Pirella/Planctomyces group. Arch. Microbiol. 145:361–366.

Liesack, W., and E. Stackebrandt. 1989. Evidence for unlinked rrn operons in the Planctomycete Pirellula marina. J. Bacteriol. 171(9):5025–5030.

Liesack, W., and E. Stackebrandt. 1992a. Occurrence of novel groups of the domain Bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. Appl. Environ. Microbiol. 174:5072–5078.

Liesack, W., R. Soeller, T. Stewart, H. Haas, S. Giovannoni, and E. Stackebrandt. 1992b. The influence of tachytelically (rapidly) evolving sequences on the topology of phylogenetic trees: Intrafamily relationships and the phylogenetic position of Planctomycetaceae as revealed by comparative analysis of 16S ribosomal RNA sequences. Syst. Appl. Microbiol. 15:357–362.

Liles, M. R., B. F. Manske, S. B. Bintrim, J. Handelsman, and R. M. Goodman. 2003. A census of rRNA genes and linked genomic sequences within a soil metagenomic library. Appl. Environ. Microbiol. 69(5):2684–2691.

Lindsay, M. R., R. I. Webb, and J. A. Fuerst. 1997. Pirellulosomes: A new type of membrane-bounded cell compartment in planctomycete bacteria of the genus Pirellula. Microbiology 143:739–748.

Lindsay, M. R., R. I. Webb, M. Strous, M. S. Jetten, M. K. Butler, R. J. Forde, and J. A. Fuerst. 2001. Cell compartmentalisation in planctomycetes: Novel types of structural organisation for the bacterial cell. Arch. Microbiol. 175(6):413–429.

Liu, J. R., C. A. McKenzie, E. M. Seviour, R. I. Webb, L. L. Blackall, C. P. Saint, and R. J. Seviour. 2001. Phylogeny of the filamentous bacterium "Nostocoida limicola" III from activated sludge. Int. J. Syst. Evol. Microbiol. 51 (Part 1):195–202.

Llobet-Brossa, E., R. Rossello-Mora, and R. Amann. 1998. Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Appl. Environ. Microbiol. 64(7):2691–2696.

Lopez-Garcia, P., A. Lopez-Lopez, D. Moreira, and F. Rodriguez-Valera. 2001. Diversity of free-living prokaryotes from a deep-sea site at the Antarctic Polar Front. FEMS Microbiol. Ecol. 36(2–3):193–202.

Lyman, J., and R. H. Fleming. 1940. Composition of sea water. J. Mar. Res. (Sears Foundation) 3:134–146.

Majewski, D. M. 1985. Molekularbiologische Charakterisierung von Bakteriophagen knospender Bakterien [Molecular Biological Characterisation of Bacteriophages of Budding Bacteria]. Institute for General Microbiology, Christian-Albrechts-Universität. Kiel, Germany.

Menke, M. A. O. H., W. Liesack, and E. Stackebrandt. 1991. Ribotyping of 16S and 23S rRNA genes and organization of rrn operonsin members of the bacterial genera Gemmata, Planctomyces, Thermotoga, Thermus and Verrucomicrobium. Arch. Microbiol. 155:263–271.

Metchnikoff, M. E. 1888. Pasteuria ramosa, un representant des bacteries a division longitudinale. Ann. Inst. Pasteur (Paris) 2:165–170.

Meusnier, I., J. L. Olsen, W. T. Stam, C. Destombe and M. Valero. 2001. Phylogenetic analyses of Caulerpa taxifolia (Chlorophyta) and of its associated bacterial microflora provide clues to the origin of the Mediterranean introduction. Molec. Ecol. 10(4):931–946.

Neef, A., R. Amann, H. Schlesner, and K.-H. Schleifer. 1998. Monitoring a widespread bacterial group: In situ detection of planctomycetes with 16S rRNA-targeted probes. Microbiology 144 (Part 12):3257–3266.

Norris, T. B., J. M. Wraith, R. W. Castenholz, and T. R. McDermott. 2002. Soil microbial community structure across a thermal gradient following a geothermal heating event. Appl. Environ. Microbiol. 68(12):6300–6309.

Pantos, O., R. P. Cooney, M. D. Le Tissier, M. R. Barer, A. G. O'Donnell, and J. C. Bythell. 2003. The bacterial ecology of a plague-like disease affecting the Caribbean coral Montastrea annularis. Environ. Microbiol. 5(5):370–382.

Peterson, S. N., and C. M. Fraser. 2001. The complexity of simplicity. Genome Biol. 2:2.

Pfennig, N., and H.-G. Trüper. 1981. Isolation of members of the families Chromatiaceae and Chlorobiaceae. *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel. The Prokaryotes. Springer-Verlag. New York, NY. 279–289.

Pierson, B. K., S. J. Giovannoni, D. A. Stahl, and R. W. Castenholz. 1985. Heliothrix oregonensis, gen. nov., sp. nov., a phototrophic filamentous gliding bacterium containing bacteriochlorophyll a. Arch. Microbiol. 142(2):164–167.

Pukall, R., D. Buntefuss, A. Fruhling, M. Rohde, R. M. Kroppenstedt, J. Burghardt, P. Lebaron, L. Bernard, and E. Stackebrandt. 1999. Sulfitobacter mediterraneus sp. nov., a new sulfite-oxidizing member of the alpha-Proteobacteria. Int. J. Syst. Bacteriol. 49 (Part 2):513–519.

Rabus, R., D. Gade, R. Helbig, M. Bauer, F. O. Glockner, M. Kube, H. Schlesner, R. Reinhardt, and R. Amann. 2002. Analysis of N-acetylglucosamine metabolism in the marine bacterium Pirellula sp. strain 1 by a proteomic approach. Proteomics 2(6):649–655.

Rappe, M. S., P. F. Kemp, and S. J. Giovannoni. 1997. Phylogenetic diversity of marine coastal picoplankton 16S rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. Limnol. Oceanogr. 42:811–826.

Ravenschlag, K., K. Sahm, J. Pernthaler, and R. Amann. 1999. High bacterial diversity in permanently cold marine sediments. Appl. Environ. Microbiol. 65(9): 3982–3989.

Reanney, D. C., and H. W. Akkermann. 1982. Comparative biology and evolution of bacteriophages. Adv. Vir. Res. 27:205–280.

Reed, D. L., and M. S. Hafner. 2002. Phylogenetic analysis of bacterial communities associated with ectoparasitic chewing lice of pocket gophers: A culture-independent approach. Microb. Ecol. 44(1):78–93.

Rönner, S., W. Liesack, J. Wolters, and E. Stackebrandt. 1991. Cloning and sequencing of a large fragment of the atpD-gene of Pirellula marina: A contribution to the phylogeny of Planctomycetales. Endocytol. Cell. Res. 7:219–229.

Roussel, Y., M. Pebay, G. Guedon, J. M. Simonet, and B. Decaris. 1994. Physical and genetic map of Streptococcus thermophilus A054. J. Bacteriol. 176(24):7413–7422.

Schlesner, H., and P. Hirsch. 1984. Assignment of ATCC 27377 to Pirella gen. nov. as Pirella staleyicomb. nov. Int. J. Syst. Bacteriol. 34(4):492–495.

Schlesner, H. 1986a. Pirella marina sp. nov., a budding, peptidoglycan-less bacterium from brackish water. Syst. Appl. Microbiol. 8:177–180.

Schlesner, H., and E. Stackebrandt. 1986b. Assignment of the genera Planctomyces and Pirella to a new family Planctomycetaceae fam. nov., and description of the order Planctomycetalesord. nov. Syst. Appl. Microbiol. 8:174–176.

Schlesner, H., and P. Hirsch. 1987. Rejection of the genus name Pirella for pear-shaped budding bacteria and proposal to create the genus Pirellula gen. nov. Int. J. Syst. Bacteriol. 37(4):441.

Schlesner, H. 1989. Planctomyces brasiliensis sp. nov., a halotolerant bacterium from a salt pit. Syst. Appl. Microbiol. 12:159–161.

Schlesner, H. 1994. The development of media suitable for the microorganisms morphologically resembling Planctomyces spp., Pirellula spp., and other Planctomycetales from various aquatic habitats using dilute media. Syst. Appl. Microbiol. 17:135–145.

Schmid, M., U. Twachtmann, M. Klein, M. Strous, S. Juretschko, M. Jetten, J. W. Metzger, K. H. Schleifer, and M. Wagner. 2000. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst. Appl. Microbiol. 23(1):93–106.

Schmid, M., K. Walsh, R. Webb, W. I. C. Rijpstra, K. van de Pas Schoonen, M. J. Verbruggen, T. Hill, B. Moffett, J. A. Fuerst, S. Schouten, J. S. S. Damsté, J. Harris , P. Shaw, M. Jetten, and M. Strous. 2003. Candidatus "Scalindua brodae", spec. nov., Candidatus "Scalindua wagneri", spec. nov., two new species of anaerobic ammonium oxidizing bacteria. Syst. Appl. Microbiol. 26:529–538.

Schmidt, J. M. 1978a. Isolation and ultrastructure of freshwater strains of Planctomyces. Curr. Microbiol. 1:65–70.

Schmidt, J. M., and M. P. Starr. 1978b. Morphological diversity of freshwater bacteria belonging to the Blastocaulis-Planctomyces group as observed in natural populations and enrichments. Curr. Microbiol. 1:325–330.

Schmidt, J. M., and M. P. Starr. 1979. Morphotype V of the Blastocaulis-Planctomyces group of budding and appendaged bacteria Planctomyces guttaeformis Hortobagyi (sensu Hajdu). Curr. Microbiol. 2:195–200.

Schmidt, J. M., and M. P. Starr. 1980a. Current sightings, at the respective type localities and elsewhere, of Planctomyces bekefii Gimesi 1924 and Blastocaulis sphaerica Henrici and Johnson 1935. Curr. Microbiol. 4:183–188.

Schmidt, J. M., and M. P. Starr. 1980b. Some ultrastructural features of Planctomyces bekefii, morphotype I of the Blastocaulis-Planctomyces group of budding and appendaged bacteria. Curr. Microbiol. 4:189–194.

Schmidt, J. M., and M. P. Starr. 1981a. The Blastocaulis-Planctomyces group of the budding and appendaged bacteria. *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel. The Prokaryotes. Springer-Verlag. New York, NY. 496–504.

Schmidt, J. M., W. P. Sharp, and M. P. Starr. 1981b. Manganese and iron encrustations and other features of Planctomyces crassus Hortobagyi 1965, morphotype Ib of the Blastocaulis-Planctomyces group of budding and appendaged bacteria, examined by electron microscopy and X-ray micro-analysis. Curr. Microbiol. 5:241–246.

Schmidt, J. M., and M. P. Starr. 1982a. Ultrastructural features of budding cells in a prokaryote belonging to mor-

photype IV of the Blastocaulis-Planctomyces group. Curr. Microbiol. 7:7–11.

Schmidt, J. M., W. P. Sharp, and M. P. Starr. 1982b. Metallic-oxide encrustations of the nonprosthecate stalks of naturally occurring populations of Planctomyces bekefii. Curr. Microbiol. 7:389–394.

Selenska-Pobell S., G. Kampf, K. Hemming, G. Radeva, G. Satchanska. 2001. Bacterial diversity in soil samples from two uranium waste piles as determined by rep-APD, RISA and 16S rDNA retrieval. Antonie Van Leeuwenhoek 79(2):149–161.

Semenova, E. A., and K. D. Kuznedelov. 1998. Species diversity of picoplankton from Lake Baikal by comparative analysis of 5'-terminal segments of 16S rRNA genes [in Russian]. Mol. Biol. (Mosow) 32(5):895–901.

Sinninghe Damste, J. S., M. Strous, W. I. Rijpstra, E. C. Hopmans, J. A. Geenevasen, A. C. van Duin, L. A. van Niftrik, and M. S. Jetten. 2002. Linearly concatenated cyclobutane lipids form a dense bacterial membrane. Nature 419(6908):708–712.

Sittig, M., and H. Schlesner. 1993. Chemotaxonomic investigation of various prosthecate and/or budding bacteria. Syst. Appl. Microbiol. 16:92–103.

Skerman, V. B. 1968. A new type of micromanipulator and microforge. J. Gen. Microbiol. 54(2):287–297.

Stackebrandt, E., W. Ludwig, W. Schubert, F. Klink, H. Schlesner, T. Roggentin, and P. Hirsch. 1984. Molecular genetic evidence for early evolutionary origin of budding peptidoglycan-less eubacteria. Nature 307 (5953):735–737.

Stackebrandt, E., A. Fischer, P. Hirsch, T. Roggentin, and H. Schlesner. 1986a. The phylogeny of an ancient group of budding peptidoglycan-less eubacteria: The genera Planctomyces and Pirella. Endocyt. Cell. Res. 3:29–40.

Stackebrandt, E., U. Wehmeyer, and W. Liesack. 1986b. 16S ribosomal RNA- and cell wall analysis of Gemmata obscuriglobus, a new member of the order Planctomycetales. FEMS Microbiol. Lett. 37:289–292.

Stackebrandt, E., W. Liesack and B. M. Goebel. 1993. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. FASEB J. 7(1):232–236.

Staley, J. T. 1968. Prosthecomicrobium and Ancalomicrobium: New prosthecate freshwater bacteria. J. Bacteriol. 95(5):1921–1942.

Staley, J. T. 1973b. Budding bacteria of the Pasteuria-Blastobacter group. Can. J. Microbiol. 19(5):609–614.

Staley, J. T., K. C. Marshall, and V. B. D. Skerman. 1980. Budding and prosthecate bacteria from fresh water habitats of various trophic states. Microb. Ecol. 5:245–251.

Starr, M. P., R. M. Sayre, and J. M. Schmidt. 1983. Assignment of ATCC 27377 to Planctomyces staleyisp. nov., and conservation of Pasteuria ramosa Metchnikoff 1888 on the basis of type descriptive material; request for an opinion. Int. J. Syst. Bacteriol. 33(3):666–671.

Starr, M. P., and J. M. Schmidt. 1984. Planctomyces stranskae (ex Wawrik 1952) sp. nov., nom. rev., and Planctomyces guttaeformis (ex Hortobagyi 1965) sp. nov., nom. rev. Int. J. Syst. Bacteriol. 34(4):470–477.

Starr, M. P., and J. M. Schmidt. 1989. Genus Planctomyces-Gimesi 1924. *In:* J. T. Staley, M. P. Bryant, N. Pfennig and J. G. Holt. Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 3:1946–1958.

Stougaard, P., F. Jorgensen, M. G. Johnsen, and O. C. Hansen. 2002. Microbial diversity in ikaite tufa columns: An alkaline, cold ecological niche in Greenland. Environ. Microbiol. 4(8):487–493.

Strous, M., J. A. Fuerst, E. H. Kramer, S. Logemann, G. Muyzer, K. T. van de Pas-Schoonen, R. Webb, J. G. Kuenen, and M. S. Jetten. 1999. Missing lithotroph identified as new planctomycete. Nature 400(6743):446–449.

Tekniepe, B. L., J. M. Schmidt, and M. P. Starr. 1981. Life cycle of a budding and appendaged bacterium belonging to morphotype IV of the Blastocaulis-Planctomyces group. Curr. Microbiol. 5:1–6.

Tekniepe, B. L., J. M. Schmidt, and M. P. Starr. 1982. Immunoferritin labeling shows de novo synthesis of surface components in buds of a prokaryote beloning to morphotype IV of the Blastocaulis-Planctomyces group. Curr. Microbiol. 7:1–6.

Thamdrup, B., and T. Dalsgaard. 2002. Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Appl. Environ. Microbiol. 68(3):1312–1318.

Todorov, J. R., A. Y. Chistoserdov, and J. Y. Aller. 2000. Molecular analysis of microbial communities in mobile deltaic muds of Southeastern Papua New Guinea. FEMS Microbiol. Ecol. 33(2):147–155.

Van Dongen, U., M. S. Jetten, and M. C. van Loosdrecht. 2001. The SHARON-Anammox process for treatment of ammonium rich wastewater. Water Sci. Technol. 44(1):153–160.

Vergin, K. L., E. Urbach, J. L. Stein, E. F. DeLong, B. D. Lanoil, and S. J. Giovannoni. 1998. Screening of a fosmid library of marine environmental genomic DNA fragments reveals four clones related to members of the order Planctomycetales. Appl. Environ. Microbiol. 64(8):3075–3078.

Wang, J., C. Jenkins, R. I. Webb, and J. A. Fuerst. 2002. Isolation of Gemmata-like and Isosphaera-like planctomycete bacteria from soil and freshwater. Appl. Environ. Microbiol. 68(1):417–422.

Ward, D. M., R. Weller, and M. M. Bateson. 1990. 16S rRNA sequences reveal numerous uncultured inhabitants in a natural community. Nature 345:63–65.

Ward, N., F. A. Rainey, E. Stackebrandt, and H. Schlesner. 1995. Unraveling the extent of diversity within the order Planctomycetales. Appl. Environ. Microbiol. 61(6): 2270–2275.

Ward, N. L., F. A. Rainey, B. P. Hedlund, J. T. Staley, W. Ludwig, and E. Stackebrandt. 2000. Comparative phylogenetic analyses of members of the order Planctomycetales and the division Verrucomicrobia: 23S rRNA gene sequence analysis supports the 16S rRNA gene sequence-derived phylogeny. Int. J. Syst. Evol. Microbiol. 50 (Part 6):1965–1972.

Ward-Rainey, N. 1996a. Genetic diversity in members of the order Planctomycetales. Biological Sciences. University of Warwick. Coventry, UK. 344.

Ward-Rainey, N., F. A. Rainey, E. M. Wellington, and E. Stackebrandt. 1996b. Physical map of the genome of Planctomyces limnophilus, a representative of the phylogenetically distinct planctomycete lineage. J. Bacteriol. 178(7):1908–1913.

Ward-Rainey, N., F. A. Rainey, and E. Stackebrandt. 1997. The presence of a dnaK (HSP70) multigene family in members of the orders Planctomycetales and Verrucomicrobiales. J. Bacteriol. 179(20):6360–6366.

Webster, N. S., K. J. Wilson, L. L. Blackall, and R. T. Hill. 2001. Phylogenetic diversity of bacteria associated with the marine sponge Rhopaloeides odorabile. Appl. Environ. Microbiol. 67(1):434–444.

Weisburg, W. G., T. P. Hatch, and C. R. Woese. 1986. Eubacterial origin of chlamydiae. J. Bacteriol. 167(2):570–574.

Wilson, K. H., and R. B. Blitchington. 1996. Human colonic biota studied by ribosomal DNA sequence analysis. Appl. Environ. Microbiol. 62(7):2273–2278.

Woese, C. R., E. Stackebrandt, T. J. Macke, and G. E. Fox. 1985. A phylogenetic definition of the major eubacterial taxa. Syst. Appl. Microbiol. 6:143–151.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51(2):221–271.

Woronichin, N. N. 1927. Materiali k agologitscheskoj flore i rastitjelnosti mineralnich istotchnikov gruppie Kaukaskich mineralnich wod. Travaux de l'Institut Balnéologique aux Eaux Minerales du Caucase 5:90–121.

PART 9

Thermus

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CHAPTER 9.1

The Genus *Thermus* and Relatives

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Introduction

In 1969, Brock and Freeze described a thermophilic organism that they named *Thermus aquaticus*. Since then the bacteria of the genus *Thermus* have become the archetypal thermophilic bacteria even though other organisms have been described that grow at much higher temperatures. Not only are these organisms easy to grow, but some strains are transformable and are rapidly becoming the mainstays of molecular biology of thermophilic bacteria. The importance of these organisms to our knowledge of thermophilic lifestyles was recently reaffirmed by the complete genome sequencing of *Thermus thermophilus* HB27 (Henne et al., 2004).

This chapter should probably be entitled the "Family Thermaceae" or the "Order Thermales" as defined by us (da Costa and Rainey, 2001a; Rainey and da Costa, 2001), but we have decided to retain the title of the original chapter in *The Prokaryotes* as an indication of continuity and because the species of the genus *Thermus* continue to be the most studied organisms of this family. Moreover, the four new genera of the family Thermaceae have only recently been proposed to accommodate thermophilic or slightly thermophilic bacteria that are distinct but closely related to the species of the genus *Thermus*. These recently proposed genera are *Meiothermus* (Nobre et al., 1996b), *Marinithermus* (Sako et al., 2003), *Oceanithermus* (Miroshnichenko et al., 2003a; Mori et al., 2004) and *Vulcanithermus* (Miroshnichenko et al., 2003b). The organisms of these genera constitute the family Thermaceae, which together with the species of the genus *Deinococcus* constitute a deep branching lineage designated the "Phylum *Deinococcus*/*Thermus*" (Rainey et al. 1997; Battista and Rainey, 2001).

The Genera *Thermus* and *Meiothermus*

Taxonomy and Phylogeny

The genus *Thermus* currently comprises eight validly described species, namely *T. aquaticus* (Brock and Freeze, 1969), *T. thermophilus* (T. Oshima and Imahori, 1974; Manaia et al., 1994), *T. filiformis* (Hudson et al., 1987b), *T. brockianus* (Williams et al., 1995), *T. oshimai* (Williams et al., 1996), *T. scotoductus* (Kristjánsson et al., 1994), *T. antranikianii* and *T. igniterrae* (Chung et al., 2000).

Comparison of the 16S rDNA sequences of the type strains of each of the eight validly described species of the genus *Thermus* shows the 16S rDNA sequence similarities to be in the range 91.2–96.4% (Fig. 1). *Thermus oshimai* is the most unrelated of the species of the genus *Thermus* as reflected by the 16S rDNA sequence similarity values. The other species of the genus *Thermus* have 16S rDNA sequence similarities in the range 94–96%. Within each species the 16S rDNA similarity values are in the range of 98.9–99.7% for *T. aquaticus*, 99.9–100% for *T. brockianus*, 99.2–99.9% for *T. filiformis*, 99.8–100% for *T. oshimai*, 98.7–99.9% for *T. scotoductus*, 99.4–100% for *T. thermophilus*, and 99.9–100% for *T. igniterrae* and *T. antranikianii* (Chung et al., 2000). These values are based on the comparison of all published strains and a large number of unpublished full 16S rDNA sequences for strains of the species of the genus *Thermus* (F. A Rainey, unpublished results). Such data are however of little use in the differentiation of strains within a species. In these organisms the phenotypic characteristics of most species overlap and it is becoming increasingly difficult to differentiate new species from those that have been described (Table 1). Diversity within groups of strains considered to belong to the same species is sometimes extreme and easily verified by the variation in the fatty acid composition (da Costa et al., 2001b). The species *T. thermophilus*, for example, has extremely variable fatty acid composition even though some strains, namely HB8, HB27 and AT-62, share extremely high DNA-DNA hybridization values and 16S rDNA sequence similarity (Manaia et al., 1994; Nobre et al., 1996a). This species has been easily distinguished from the strains of other species by the ability of the strains to grow at temperatures above 80°C and in media containing 3% NaCl. However, one unpublished strain does not grow in NaCl-

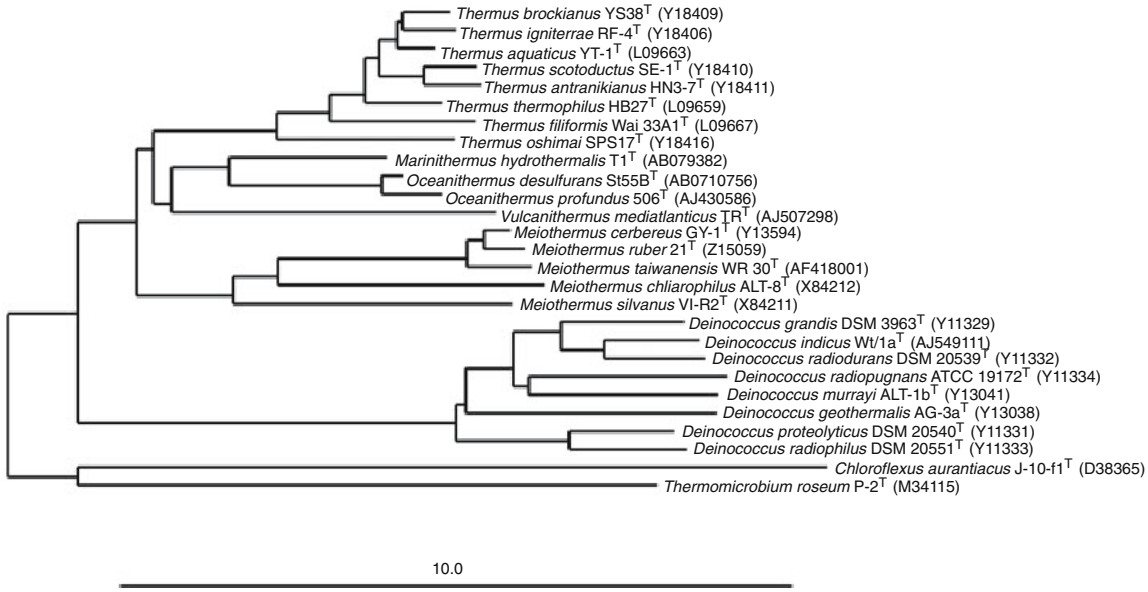

Fig. 1. Phylogenetic dendogram based on 16S rRNA gene sequence comparisons of the validly described type species of the Phylum *Deinococcus-Thermus*. *Thermomicrobium roseum* and *Chloroflexus aurantiacus* are outgroups. The dendrogram was constructed from evolutionary distances using the neighbor-joining method. The scale bar represents 10 inferred nucleotide changes per 100 nucleotides.

containing media because it lacks the genes leading to the synthesis of the compatible solute mannosylglycerate, necessary for growth in media containing more than 1% NaCl (da Costa et al., 2001b; S. A. Alarico et al., unpublished results).

In addition to the type strain of *Thermus aquaticus* (YT-1, = ATCC 25104), another of the original isolates of Brock and Freeze (1969), designated "Y-VII-51B" (=ATCC 25105), isolated from Pacheteaus Calistoga in California, has recently been found to be very closely related to the type strain of *T. antranikianii* (Chung et al., 2000). At the time, however, it was perfectly acceptable that these strains were classified in the same species for the lack of more precise methods of classification of prokaryotes. Moreover, this species is only easily distinguished from all other species on the basis of fatty acid composition (Table 2). The species *T. filiformis* was described on the basis of one strain from New Zealand with a stable filamentous morphology (Hudson et al., 1987b). Other strains from New Zealand belong to this species on the basis of DNA-DNA hybridization values but are not filamentous (Georganta et al., 1993). Moreover, the type strain of *T. filiformis* possesses very high levels of *anteiso*-fatty acids as well as 3-OH fatty acids, while the other strains have high levels of *iso*-fatty acids and lack 3-OH fatty acids (Ferraz et al., 1994; Nobre et al., 1996a). The biochemical and physiological diversity of *T. filiformis* strains that share high DNA-DNA hybridization values makes it difficult, if not impossible, to define a distinct phenotype for this species (Hudson et al., 1987b; Hudson et al., 1989; Georganta et al., 1993).

The description of the species *T. scotoductus* was based on strains isolated from hydrothermally fed hot water taps in Iceland, as well as strain X-1 isolated from a thermally polluted stream in the United States (Ramaley and Hixson, 1970; Kristjánsson et al., 1994). The lack of pigmentation was one of the characteristics used to discriminate *T. scotoductus* from other species of the genus which rarely comprise nonpigmented strains. Two other nonpigmented strains NH and DI, isolated from hot tap water in London, were later found to be practically identical to the type strain from Iceland (Pask-Hughes and Williams, 1975; Tenreiro et al., 1995b). Strains from a hot spring at the end of a nonilluminated tunnel at Vizela, Portugal, were also found to be closely related to the type strain of *Thermus scotoductus* (Tenreiro et al., 1995b) and were, not surprisingly, nonpigmented. However, yellow-pigmented strains from hot springs in the Azores exposed to sunlight were also found to be very closely related to *T. scotoductus* (Santos et al., 1989; Tenreiro et al., 1995b; Williams et al., 1996; F. A. Rainey et al., unpublished observation). Moreover, only the strains from Iceland and from London have identical fatty acid compositions (Nobre et al., 1996a).

Table 1. Biochemical characteristics that distinguish the type strains of the species of the genus *Thermus*.[a]

Charactheristic	*T. aquaticus* (YT-1^T)	*T. thermophilus* (HB8^T)	*T. filiformis* (Wai33-A1^T)	*T. scotoductus* (ITI-252^T)	*T. brockianus* (YS038^T)	*T. oshimai* (SPS-17^T)	*T. igniterrae* (RF-4^T)	*T. antranikianii* (HN3-7^T)
Pigmentation	Yellow	Yellow	Yellow	White	Yellow	Yellow	Yellow	Yellow
Presence of:								
α-Galactosidase	−	+	+	+	+	+	−	+
β-Galactosidase	−	+	+	−	+	+	+	+
Degradation of *p*-nitrophenyl substrates:								
β-Glucopyranoside	+	+	+	−	+	+	+	+
Degradation of:								
Arbutin	w	+	+	w	+	+	+	+
Esculin	−	+	+	w	+	+	+	+
Hydrolysis of:								
Elastin	+	−	−	−	−	+	+	−
Starch	+	+	+	+	−	+	+	+
Fibrin	+	−	−	+	+	+	+	−
Gelatin	+	+	+	+	−	+	+	−
Casein	+	+	+	+	−	+	+	+
Tween 80	−	+	−	−	+	+	+	+
Reduction of nitrate	−	−	−	+	+	+	+	+
Growth at/in:								
80°C	−	+	−	−	−	−	−	+
82°C	−	+	−	−	−	−	−	−
2% NaCl	−	+	−	−	−	w	−	−
3% NaCl	−	+	−	−	−	−	−	−
4% NaCl	−	+	−	−	−	−	−	−

Table 2. Percent fatty acid composition of the type strains of the species of the genus *Thermus* after growth at 70°C.[a]

Fatty acid	*T. aquaticus* (YT-1^T)	*T. thermophilus* (HB8^T)	*T. filiformis* (Wai33-A1^T)	*T. scotoductus* (ITI-252^T)	*T. brockianus* (YS038^T)	*T. oshimai* (SPS-17^T)	*T. igniterrae* (RF-4^T)	*T. antranikianii* (HN3-7^T)
iso-C13:0	—[b]	—	—	—	0.7	0.7	1.1	—
iso-C14:0	1.0	0.7	0.9	—	1.3	—	—	—
C14:0	1.4	—	—	—	0.7	—	—	—
iso-C15:0	19.3	32.4	4.0	17.9	31.8	36.2	50.7	10.8
anteiso-C15:0	2.1	4.3	17.9	13.8	2.5	2.9	2.9	1.7
C15:0	—	—	—	1.0	0.8	3.1	1.3	1.9
iso-C16:0	13.4	5.3	8.7	1.6	11.0	2.5	1.0	9.6
C16:0	16.2	10.0	4.1	8.6	12.3	8.8	9.0	11.9
Un[c]	—	0.7	4.0	2.4	—	—	—	—
iso-C15:03-OH	3.2	—	—	—	—	—	—	—
iso-C17:0	24.9	41.4	6.3	30.3	35.2	38.3	31.1	51.0
anteiso-C17:0	2.6	5.1	35.5	22.1	2.8	3.2	1.9	6.2
C17:0	—	—	—	1.3	—	2.1	—	3.1
iso-C16:03-OH	2.6	—	0.9	—	—	—	—	—
C16:03-OH	2.3	—	—	—	—	—	—	—
iso-C18:0	0.6	—	1.0	—	0.6	—	—	1.4
iso-C17:03-OH	7.6	—	2.3	—	—	—	—	—
C17:03-OH	0.8	—	8.6	—	—	—	—	—
C19:0	—	—	1.0	0.6	—	—	—	—

Symbol and abbreviations: —, none present; ^{T}, indicates type strain; w, weak growth; 13[DM2]:0*i*,; 14:0*i*,; 14:0, tetradecanoic acid; 15:0*i*,; 15:0*a*,; 15:0,; 16:0*i*,; 15:0*i*,; 3-OH,; 17:0*i*,; 17:0*a*,; 17:0,; 16:0*i* 3-OH,; 16:0 3-OH,; 18:0*i*,; 17:0*i* 3-OH,; 17:0 3-OH; and 19:0.

[a]The method used did not allow the quantification of long-chain diols.

[b]Undetected fatty acids or those whose values are less than 0.5% in all strains are not shown.

[c]Unknown fatty acid or alcohol with an equivalent chain length (ECL) of 16.090.

These results also appear to leave the species *T. scotoductus* without distinct phenotypic characteristics. The species *T. aquaticus* can be easily distinguished from *T. brockianus*, but all of the extensively characterized strains of the former species originate from Yellowstone National Park (Munster et al., 1986; Williams et al., 1995). However, the strains of *T. brockianus* and *T. aquaticus* are also very difficult to distinguish from strains of other species of the genus *Thermus*.

Phylogenetic analysis based on 16S rDNA sequence analysis shows that the species of the genus *Meiothermus* form a separate line of descent from the species of the genus *Thermus* with which they share about 86% 16S rDNA sequence similarity (Nobre et al., 1996b). It was suspected for some time that the red-pigmented "low-temperature" species designated "*Thermus ruber*" formed a separate line of descent from the "high-temperature" species of the genus *Thermus* (Weisburg et al., 1989; Bateson et al., 1990; Embley et al., 1993), but aside from the different growth temperature ranges and the red pigmentation, there were no other characteristics that allowed the proposal of a new genus. The description of two new, slightly thermophilic species that, based on 16S rRNA sequence analysis and chemotaxonomic parameters, were more closely related to *Thermus ruber* than to the other species of the genus *Thermus* led to the proposal of the genus *Meiothermus* for the species with low growth temperatures (Tenreiro et al., 1995a; Nobre et al., 1996b).

The species *M. ruber*, *M. silvanus*, *M. chliarophilus*, *M. cerbereus* and *M. taiwanensis* have been validly described (Loginova et al., 1984; Tenreiro et al., 1995a; Nobre et al., 1996b; Chung et al., 1997; M. Y. Chen et al., 2002). The phylogenetic analysis of the species of the genus *Meiothermus* shows that *M. ruber*, *M. cerbereus* and *M. taiwanensis* are closely related to each other, sharing about 97–98% 16S rDNA sequence similarity (Chung et al., 1997; M. Y. Chen et al., 2002). *Meiothermus silvanus* and *M. chliarophilus* are more distantly related to the *M. ruber*/*M. cerbereus*/*M. taiwanensis* clade, sharing no more than about 90% sequence similarity with each other or with the other three species of *Meiothermus* (Tenreiro et al., 1995a; Chung et al., 1997; M. Y. Chen et al., 2002). It could be argued that *M. chliarophilus* and *M. silvanus* should be placed in two separate genera, but the lack of physiological and biochemical distinctiveness of these two species does not support such a proposal (Fig. 1).

One strain, named "*M. rosaceus*," which shares about 99% 16S rDNA sequence similarity with *M. taiwanensis*, has not been validly described (C. Chen et al., 2002b). This organism was isolated from the Tengchong hot springs in Yunnan Province, China, and may represent a new species.

The validly described species of the genus *Meiothermus* are still very easy to distinguish from each other primarily because the number of species is small and some of the species (such as the yellow-pigmented species *M. chliarophilus*) have been isolated from only one site, which limits diversity of characteristics within this species (Tables 3 and 4). All the species can at present be distinguished from other species of the genus by their distinct fatty acid compositions, namely the strains of *M. ruber*, which have been isolated from hydrothermal areas throughout the world (Nobre et. al., 2001). An additional species of this genus named "*Meiothermus timidus*" has been characterized recently. The strains of this species, isolated from hot springs in Continental Portugal and the Island of S. Miguel in the Azores, are yellow-pigmented and most closely related to *M. chliarophilus*. These organisms show that yellow-pigmentation is more common than previously recognized in this genus (A. L. Pires et al., unpublished observations).

Habitats

The strains of the genus *Thermus* are generally isolated from hydrothermal areas where the range of water temperature is 55–70°C and that of pH is 5.0–10.5 (Kristjánsson and Alfredsson, 1983; Munster et al., 1986; Hudson et al., 1989; Santos et al., 1989). The recovery of strains of the genus *Thermus* from geothermal sites with very high temperatures and low pH, as well as from cold water sources, is believed to be due to the dispersal of organisms from thermal environments that the organisms actually colonize (Williams and da Costa, 1992).

The first isolates of the genus *Thermus* were obtained from hydrothermal areas in Yellowstone National Park and Pacheteaus Calistoga in California (Brock and Freeze, 1969). Isolates were then recovered from several hydrothermal areas in Japan (Yoshida and Oshima, 1971; Saiki et al., 1972; Taguchi et al., 1982), followed by isolations from Iceland (Pask-Hughes and Williams, 1977; Kristjánsson and Alfredsson, 1983; Hudson et al., 1987a), New Zealand (Hudson et al., 1986), New Mexico (Hudson et al., 1989), continental Portugal, the Island of São Miguel in the Azores (Santos et al., 1989; Manaia and da Costa, 1991), the Australian Artesian Basin (Denman et al., 1991), and the Kamchakta Peninsula (R. Sharp, unpublished results). Strains of the genus *Thermus* have also been isolated from shallow marine hot springs in Ice-

Table 3. Biochemical characteristics that distinguish the type strains of the species of the genus *Meiothermus*.[a]

Characteristics	*M. ruber*	*M. silvanus*	*M. chliarophilus*	*M. cerbereus*	*M. taiwanensis*[b]
Pigmentation	Red	Red	Yellow	Red	Red
Presence of:					
Catalase	+	–	–	–	+
α-Galactosidase	+	–	+	+	+
Hydrolysis of:					
Starch	–	–	+	–	–
Esculin	+	–	+	+	+
Utilization of:					
D-Cellobiose	+	–	+	+	+
D-Mannitol	+	–	+	–	+
D-Raffinose	+	–	+	–	+
D-Sorbitol	+	–	+	–	+
D-Trehalose	+	–	+	+	–
D-Xylose	+	+	+	–	+
Glycerol	+	–	+	–	–
L-Arabinose	–	–	–	–	+
L-Rhamnose	–	–	–	–	+
Malate	+	–	–	–	–
myo-Inositol	+	–	+	–	+
Pyruvate	–	+	+	+	+
Ribitol	–	–	–	–	+
Succinate	+	–	–	–	+
L-Asparagine	+	+	+	–	+
L-Glutamine	+	+	+	–	+
L-Serine	+	+	+	–	–
L-Arginine	+	+	+	–	+

Symbol and abbreviations: +, positive result or growth; –, negative result or growth.
[a]*M. Chliarophilus* was grown at 50°C; the other strains were grown at 55°C.
[b]Results from Chen et al., 2002a.

land (Kristjánsson et al., 1986), the islands of Fiji (Hudson et al., 1989), and the island of São Miguel in the Azores (Manaia and da Costa, 1991). Recently, isolates of the genus *Thermus* have also been obtained from abyssal geothermal areas in the Mid-Atlantic Ridge and in the Guaymas Basin, Gulf of California, at depths of 3500 and 2000 m, respectively (Marteinsson et al., 1995; Marteinsson et al., 1999). Some shallow marine hydrothermal areas contain less salt than the surrounding seawater (Kristjánsson et al., 1986), but others have salt concentrations similar to those of seawater. To our knowledge, all *Thermus* isolates from these springs are halotolerant (Manaia and da Costa, 1991; Tenreiro et al., 1997). Most of these isolates have higher growth rates in media without added NaCl but grow in media containing 3–6% NaCl. All the *Thermus* strains isolated from saline thermal environments appear to belong to the species *T. thermophilus* (da Costa et al., 2001b). On the other hand the vast majority of the strains of *Thermus* isolated from inland hydrothermal areas do not grow at salinities above 1% NaCl (Kristjánsson et al., 1986; Hudson et al., 1989; Santos et al., 1989; Manaia and da Costa, 1991; Manaia et al., 1994). However, some inland geothermal areas in Japan and Iceland also vent saline water and yield halotolerant *Thermus* strains, many of which also belong to *T. thermophilus* (Waring, 1965; Yoshida and Imahori, 1971; Alfredsson and Kristjánsson, 1995).

Strains of the species of the genus *Thermus* are also commonly isolated from man-made thermal environments. The isolation of strains from man-made environments was, in fact, simultaneous with the description *Thermus aquaticus* (Brock and Freeze, 1969). Later, nonpigmented *Thermus* strains were isolated from hot water taps, thermally polluted streams (Ramaley and Hixson, 1970; Pask-Hughes and Williams, 1975; Degryse et al., 1978; Stramer and Starzik, 1981), and hot water systems (Brock and Boylen, 1973). Strains of the genus *Thermus* have also been isolated from self-heating compost piles in Switzerland and Germany (Beffa et al., 1996). One strain SA-01 and classified as *T. scotoductus* was isolated from a rock surface with a temperature of about 60°C from a gold mine, at a depth of 3200 meters, in South Africa (Kieft et al., 1999).

The strains of the genus *Meiothermus* have been isolated from a large number of geothermal areas ranging from Yellowstone National Park, the Island of São Miguel in the Azores, several hot springs in continental Portugal, the Hveragerthi and Geysir areas of Iceland, and the Kamchatka Peninsula to the North Island of New Zealand and China (Loginova et al., 1984;

Table 4. Fatty acid composition of the type strains of the species of the genus *Meiothermus* grown at 50°C.

	Fatty acid composition (% of total)				
Fatty acids[a]	*M. ruber*	*M. silvanus*	*M. chliarophilus*	*M. cerbereus*	*M. taiwanensis*
iso-C13:0	0.4	0.4	1.6	1.5	0.7
iso-C14:0	1.3	0.6	1.9	2.6	0.7
iso-C13:0 3-OH	0.4	1.0	—	—	1.1
C15:1 ω9*c*	1.3	—	—	4.1	0.3
iso-C15:0	30.9	25.9	39.0	35.5	38.4
anteiso-C15:0	6.5	22.5	8.9	6.2	2.9
C15:0	3.3	0.2	1.8	2.0	2.0
C16:1 ω7 alcohol	0.7	—	—	2.0	—
iso-C16:0	4.8	1.6	2.2	4.1	2.6
iso-C15:0 2-OH	0.5	0.4	0.5	0.4	0.7
C16:0	4.9	5.5	8.2	5.1	6.1
Unknown 16.090[b]	0.5	1.6	0.7	0.6	0.4
iso-C15:0 3-OH	0.2	—	1.1	0.6	—
C15:0 2-OH	0.9	—	0.4	0.4	0.3
iso-C17:1 ω9*c*	3.4	—	—	5.2	1.1
iso-C17:0	16.5	12.7	13.4	6.0	17.4
anteiso-C17:0	4.4	6.9	2.5	1.6	2.4
C17:1 ω8*c*	0.6	—	—	0.7	—
C17:1 ω6*c*	0.9	1.0	0.9	1.0	0.3
C17:0	2.1	0.3	1.1	0.4	1.7
C16:0 2-OH	0.6	0.4	0.7	0.7	1.0
iso-C17:0 2-OH	6.8	9.6	10.9	5.7	12.0
anteiso-C17:0 2-OH	0.3	3.0	0.8	—	0.2
iso-C17:0 3-OH	1.5	—	0.2	4.7	—
C17:0 2-OH	—	—	0.3	0.8	0.7
anteiso-C17:0 3-OH	1.0	—	—	—	—
iso-C19:0	—	1.8	0.2	—	0.3
anteiso-C19:0	—	1.1	—	—	—
iso-C18:0 diol	0.7	2.2	—	—	4.5
anteiso-C18:0 diol	—	0.4	—	—	—

Symbol and abbreviations: —, less than 0.5%; 13:0 *iso*,; 14:0 *iso*,; 13:0 *iso* 3OH,; 15:1 *iso* F,; 15:0 *iso*,; 15:0 *anteiso*,; 15:0,; 16:1 ω7*t* alcohol,; 15:0 *iso* 2OH,; 15:0 2OH,; 16:0,; Unknown 16.090,; 15:0 *iso* 3OH,; 15:0 2OH,; 17:1 *iso* ω9*c*,; 17:0 *iso*,; 17:0 *anteiso*,; 17:1 ω8c,; 17:1 ω6*c*,; 17:0,; 16:0 2OH,; 17:0 *iso* 2OH,; 17:0 *anteiso* 2OH,; 17:0 *iso* 3OH,; 17:0 2OH,; 17:0 *anteiso* 3OH,; 19:0 *iso*,; 19:0 *anteiso*,; 18:0 *iso* diol,; and 18:0 *anteiso* diol.

[a]Undetected fatty acids or those whose values are less than 0.5% in all strains are not shown.

[b]Unknown fatty acid or alcohol with an equivalent chain length (ECL) of 16.090.

Sharp and Williams, 1988; Donato et al., 1991; Tenreiro et al., 1995a; Chung et al., 1997; M. Y. Chen et al., 2002; C. Chen et al., 2002b). Strains of the species *M. ruber* were also recovered from fermentors fed with yeast factory wastewater and maintained at 60°C (Hensel et al., 1986). In natural environments, these organisms are generally isolated from areas where the range of water temperature is 40–70°C and the pH is neutral to alkaline. Strains of *Meiothermus*, unlike strains of *T. thermophlus*, have not been isolated from marine hot springs and no halotolerant or slightly halophilic strains are known.

The strains of *M. silvanus* have been isolated from hot springs in northern Portugal and from the Geysir area of Iceland (Tenreiro et al., 1995a; Nobre and da Costa., 2001). Strains of *M. cerbereus* have been isolated from the Geysir area of Iceland, and closely related but not extensively characterized strains have also been isolated from Yellowstone National Park (Nold and Ward, 1995; Chung et al., 1997). Strains of *M. chliarophilus* have only been isolated from the hot spring at Alcafache in central Portugal with a vent temperature of 50.5°C and a pH of 8.1 (Tenreiro et al., 1995a). The strains of *M. taiwanensis* have been isolated from hot springs in Taiwan (Chen et al., 2002a). However, strains of *M. ruber* have been isolated worldwide (Nobre and da Costa, 2001). One factor leading to the frequent isolation of *M. ruber* strains from several geothermal areas around the world may be the high temperature used for enrichment of these organisms from water and sediment samples. Enrichments performed in liquid media at 60°C or higher invariably lead to the isolation of strains with optimum growth temperatures of about 60–65°C and closely related to *M. ruber*. Other species with optimum growth temperatures of about 50–55°C and maximum growth temperatures of about 60°C are not isolated under these enrichment conditions.

Genome Sequence of *T. thermophilus* HB27

Strain HB27 is not the type strain of the species *Thermus thermophilus* but is probably the most investigated strain of the genus *Thermus*. Strain HB27, like other strains of *T. thermophilus*, is amenable to genetic manipulation and is now a model organism for genetic studies in *Thermus* spp. For these reasons the genome was completely sequenced (Henne et al., 2004). The genome consists of a chromosome with 1,894,877 base pairs and a megaplasmid with 232,605 base pairs. The complete genome sequence confirms the results of several physiological, metabolic and biochemical studies. The organism, for example, possesses genes for the Emden-Meyerhof pathway, a complete tricarboxylic acid cycle, and gluconeogenesis. The respiratory chain contains reduced nicotinamide adenine dinucleotide (NADH)-dehydrogenase, a cytochrome-dependent succinate dehydrogenase, two sets of genes for terminal cytochrome *c* oxidases, and cytochromes *a*, *caa3* and *ba3* among others. ATP synthesis is accomplished via a V/A type synthase. It was known that strain HB27 did not use nitrate as an electron acceptor for anaerobic respiration, and the putative genes for dissimilatory nitrate reductase are not present in the genome (da Costa et al., 2001b). Also interesting is that a gene cluster with homology to *sox* genes of sulfur-oxidizing organisms is present in this organism. Moreover, another gene cluster has homology to the periplasmic thiosulfate/cytochrome oxidoreductase complex of *Rhodopseudomonas palutris*. The implication, of course, is that energy could be derived from oxidation of reduced sulfur compounds, but this has not been shown in strain HB27, although it has been detected in strains of *T. scotoductus* (Skirnisdottir et al., 2001). Motility has never been observed in any strain of *Thermus*; this observation was confirmed from the complete genome sequence of strain HB27, which does not possess genes for flagellum synthesis.

Isolation Procedures

A low concentration of organic constituents and an incubation temperature of 65–75°C were stressed by Brock (1984) as important factors for the isolation of *T. aquaticus*. *Therrnus* strains are easily isolated by inoculating samples of water, biofilms or mud in a medium containing Castenholz D basal salts medium (Castenholz, 1969) supplemented with yeast extract (1.0 g · liter^{-1}) and tryptone (1.0 g · liter^{-1}). This medium has been used in many studies to grow strains of the genus *Thermus* and has been called simply *Thermus* medium (Brock and Freeze, 1969; Munster et al., 1986; Williams and da Costa, 1992). Sample volumes of 0.5–1.0 ml are generally sufficient, but the medium can be made up at twice the concentration to accommodate large inocula. Turbid cultures are streaked on the same medium solidified with 2–3% agar. Plates are sealed or placed in containers to reduce evaporation and incubated aerobically for several days at the appropriate temperature. Streaking samples directly onto solidified *Thermus* medium has also been successful (Hudson et al., 1986). Large sample volumes have been filtered using membrane filtration (0.20 or 0.45 μm pore size), after which the filters have been placed on the plates (Chung et al., 2000).

Basal mineral medium 162 (Degryse et al., 1978) with 0.25% tryptone and 0.25% yeast extract, with or without solidifying agents, has also been extensively used to grow *Thermus* and *Meiothermus* strains. Many strains will grow on 0.4% yeast extract, 0.8% polypeptone, and 0.3% NaCl (T. Oshima and Imahori, 1974), but this is not true of *T. aquaticus*. The growth of the majority of the strains of *Thermus* is inhibited by levels of organic nutrients higher than about 1.0%. Hexoses are particularly inhibitory, apparently because of acidification of the medium. Some strains, particularly those closely related to *T. thermophilus* HB8, are more resistant to organic nutrients in the culture medium and are frequently grown in a medium containing (per liter of water) trypticase or polypeptone, 8.0 g; yeast extract, 4.0 g; and NaCl, 2.0 g (M. Oshima and Yamakawa, 1974). The review by Sharp et al. (1995) gives an extensive list of media, and their formulae, used to grow the strains of this genus for several purposes and should be consulted. Defined media have also been used with some success for experimental protocols but not for enrichment of samples (Silva et al., 2003).

Preservation of Strains

Most strains of *Thermus* and *Meiothermus* can be stored frozen at –80°C in *Thermus* medium containing 10–15% glycerol, or in liquid nitrogen, for years without loss of viability. Lyophilized strains have been maintained for many years. Strains grown densely on plates of *Thermus* medium survive for about 1 month at 4°C.

Morphology, Metabolism, Physiology and Biochemical Characteristics

The strains of the genus *Thermus* form rod-shaped or filamentous cells that stain Gram negative. The type strain of *Thermus filiformis* has, in contrast to other strains, a stable filamentous morphology and does not form short rod-shaped cells on solid or in liquid media. Transmission electron microscopy shows that the strains of the

genus *Thermus* have an envelope consisting of a cytoplasmic membrane with a simple outline, a cell wall with an inner, electron-dense thin layer representing the peptidoglycan connected to an outer corrugated "cobble-stone" layer by irregularly spaced invaginations (Brock and Edwards, 1970). Unusual morphological structures, called "rotund bodies," are occasionally seen in many strains by phase-contrast and transmission electron microscopy. These structures consist of several cells bound by a common external layer of the cell envelope enclosing a large space between the cells (Brock and Edwards, 1970; Becker and Starzyk, 1984). The strains of the closely related genus *Meiothermus* have cells with similar morphology. Rotund bodies have, however, not been seen in any of the strains of *Meiothermus*. The type strain of *M. taiwanensis* also forms long stable filaments (M. Y. Chen et al., 2002). A crystalline surface protein layer (S-layer) has been identified in *T. thermophilus* strains $HB8^T$ and HB-27. The S-layer of strain HB8 is composed of a major protein with a molecular weight of 100 kDa, designated P100 (Caston et al., 1988; Faraldo et al., 1992; Olabarría et al., 1996; Fernández-Herrero et al., 1997).

The growth temperature range of the species of the genus *Thermus* is between about 45°C and 82–83°C. Only a few strains, all belonging to *T. thermophilus*, are capable of growth at 80°C or above (Manaia et al., 1994). The majority of the strains of the genus *Thermus* have a maximum growth temperature slightly below 80°C (Brock and Freeze, 1969; Chung et al., 2000; da Costa et al., 2001b). The optimum growth temperature of these organisms is about 70°C, but the optimum growth temperature of some strains can be as high as 75°C. The species of the genus *Meiothermus* have lower growth temperature ranges than those of *Thermus*; *M. ruber* has an optimum growth temperature of about 60–65°C, a maximum growth temperature just below 70°C, and a minimum growth temperature of about 35–40°C. The species *M. silvanus*, *M. cerbereus* and *M. taiwanensis* have lower growth temperature ranges with an optimum of about 55°C, whereas *M. chliarophilus* is the least thermophilic of the known species of this genus, with an optimum growth temperature of about 50°C (Loginova et al., 1984; Tenreiro et al., 1995a; Chung et al., 1997; M. Y. Chen et al., 2002).

The strains of this genus use carbohydrates, organic acids, and amino acids as sources of carbon and energy. None of the strains of the genus *Thermus* appears, however, to be able to carry out fermentation. The strains of the species of the genus *Thermus* have a respiratory metabolism and many are strictly aerobic. Some strains are also capable of growth under anaerobic conditions using nitrate as electron acceptor; some strains also reduce nitrite (Hudson et al., 1989; Santos et al., 1989; Manaia et al., 1994; Chung et al., 2000). A few strains (among them SA-01 and NMX2 A1, classified as *T. scotoductus*), use NO_3^{-1}, Fe(III) and S° as terminal electron acceptors coupled to growth (Kieft et al., 1999). These strains also reduce Mn(IV), Co(III), Cr(VI) and U(VI). Another strain of *T. scotoductus*, designated IT-7254, oxidizes thiosulfate to sulfate in the presence of organic carbon sources (Skirnisdottir et al., 2001). The enhancement of growth by thiosulfate in medium containing organic carbon substrates, such as acetate, indicates that the organism is facultatively chemolithoheterotrophic. *Thermus thermophilus* HB27 may also be able to obtain energy from reduced sulfur compounds, since several gene homologues related to sulfur oxidation are present in the genome of this organism (Henne et al., 2004).

The strains of the species of the genus *Meiothermus* also have a respiratory metabolism, and most strains seem to be strictly aerobic. However, some strains are also capable of growth using nitrate as electron acceptor, but none of the strains appears to be capable of carrying out fermentation. All strains are chemoorganotrophic, growing on amino acids, peptides and proteins and simple and complex carbohydrates. The strains of *M. cerbereus* and some strains of *M. silvanus* require reduced sulfur compounds, such as thiosulfate, cysteine, or thioglycolate, for growth in liquid media but not in the corresponding medium solidified with agar. Under the conditions examined, sulfate is not produced during growth on thiosulfate, and the requirement for reduced sulfur compounds during growth in liquid medium cannot be easily explained. The type strain of *M. ruber* oxidizes thiosulfate to sulfate, although this strain shows no requirement for reduced sulfur compounds and growth is not improved by their addition to the growth medium (Chung et al., 1997). The oxidation of thiosulfate to sulfate by the type strain of *M. ruber* may be analogous to the so-called "gratuitous oxidation of sulfur compounds" found in other heterotrophic bacteria (Das et al., 1996).

The majority of the isolates of the species of the genus *Thermus* form yellow-pigmented colonies, although the color varies considerably from deep yellow to very pale yellow. Many strains, isolated primarily from man-made environments that are maintained dark, are nonpigmented, although yellow-pigmented strains can also be isolated in low numbers in these environments. Nonpigmented *Thermus* strains have been isolated from abyssal hot springs, although some isolates were also yellow-pigmented (Marteinsson et al., 1995). In some strains, pigmentation appears to be an unstable characteristic because spontaneous nonpigmented mutants are fre-

quently produced that never revert to yellow-pigmentation. Moreover, the consistent isolation of nonpigmented strains from dark environments leads to the hypothesis that the yellow pigmentation of *Thermus* is favored in natural thermal areas exposed to sunlight where carotenoids would protect the cells from sunlight, while nonpigmented strains would have a selective advantage in nonilluminated environments because the production of carotenoids is energetically expensive and serves no useful function. A gene cluster involved in latter part of the synthesis of carotenoids in strain HB27 is located in the megaplasmid designated TT27 (Henne et al., 2004). Carotenoid-overproducing mutants are more resistant to ultraviolet irradiation than the wild-type strain and carotenoid-underproducing mutants are (Hoshino et al., 1994; Tabata et al., 1994). Nevertheless, the carotenoid-overproducing mutants grow slower than the wild-type strain at supraoptimal temperatures. These results could explain why nonpigmented strains predominate in dark environments over pigmented strains. The curing of carotenogenic gene-containing plasmids from plasmid-bearing strains under laboratory conditions could also explain the high frequency of pigmentation loss by some strains in the laboratory.

The strains of four of the five species of the genus *Meiothermus* form reddish-pigmented colonies, although the color varies considerably from orange-red to deep red. The differences in pigmentation appear unrelated to the site of isolation or to the taxonomic status of these strains, because isolates recovered from the same geothermal area and belonging to the same species can have different pigmentation (Hensel et al., 1986; Sharp and Williams, 1988; Chung et al., 1997). The strains of *M. chliarophilus* form bright yellow-pigmented colonies (Tenreiro et al., 1995a). This species has been recovered only from one hot spring in central Portugal, and the isolates may belong to one clone. It is possible, therefore, that strains of *M. chliarophilus* originating from other geothermal areas could also produce red-pigmented cells. However, recent results show that isolates of "Meiothermus timidus" from the Azores and from Continental Portugal produce yellow-pigmented cells, leading us to envision that this new species has a stable yellow pigmentation.

The peptidoglycan of the strains of the genus *Thermus* contains L-ornithine as the diamino acid and glycylglycine as the interpeptide bridge (Merkel et al., 1978; Pask-Hughes and Williams, 1978), consistent with the A3β murein type of Schleifer and Kandler (1972). This peptidoglycan type is also found in species of the genera *Meiothermus* and *Deinococcus* (Hensel et al., 1986; Embley et al., 1987; Sharp and Williams, 1988). The major respiratory quinone of the strains of all the genera of the family *Thermaceae* is menaquinone 8 (MK-8; Collins and Jones, 1981; da Costa et al., 2001b; Miroshnichenko et al., 2003a; Miroshnichenko et al., 2003b; Sako et al., 2003). The presence of ornithine and MK-8 corroborates the phylogenetic interpretation that the genera of the Thermaceae and Deinococcus are related to each other, although they share few other phenotypic characteristics.

The polar lipid composition of the species of *Thermus* consists of one major phospholipid, designated phospholipid 2 (PL-2), and one major glycolipid, designated glycolipid 1 (GL-1), which comprise 80–95% of the total polar lipid phosphorus and carbohydrate. Other minor polar lipids, namely phospholipid 1 (PL-1) and glycolipid 2 (GL-2), are also detected by thin-layer chromatography in most strains of the genus *Thermus* (Prado et al., 1988; Donato et al., 1990). The major glycolipid of several strains has been identified as a diglycosyl-(*N*-acyl)glycosaminylglucosyldiacylglycerol, which contains three hexose residues and one *N*-acylated hexosamine. The polar head group of GL-1 may contain *N*-acylglucosamine or *N*-acylgalactosamine, three glucose residues, two glucose residues plus one galactose, or one glucose plus two galactose residues (M. Oshima and Yamakawa, 1974; Prado et al., 1988; Wait et al., 1997). The terminal galactose, present in GL-1 of several strains, such as *T. thermophilus* strain HB8, is in the rare furanose configuration instead of the more common pyranose configuration (Wait et al., 1997). The polar lipid composition of the species of *Meiothermus* consists of one major phospholipid that is probably very similar to PL-1 of the strains of *Thermus* spp. and two prominent glycolipids, designated "glycolipid 1a" (GL-1a) and "glycolipid 1b" (GL-1b), that migrate very close to each other (Donato et al., 1991; Tenreiro et al., 1995a; Chung et al., 1997). The formation of two separate glycolipids by *Meiothermus* spp. is due to the differential binding of fatty acids to the hexosamine of the polar head group; 2-hydroxy fatty acids are exclusively amide-linked to the hexosamine of GL-1a, whereas 3-hydroxy and saturated branched-chain fatty acids are amide-linked to the hexosamine of GL-1b (Wait et al., 1997; Ferreira et al., 1999).

Unexpectedly, terminally and subterminally branched long chain diols, identified as 16-methylheptadecane-1,2-diol and 15-methylheptadecane-1,2-diol, were detected as major components of GL-1 and GL-2 of *T. scotoductus* X-1 and *T. filiformis* Tok A4 (Wait et al., 1997). Similar diols were also recently detected as major glycolipids of other *T. scotoductus* strains (Balkwill et al., 2004). Long chain diols had only been detected in *Thermomicrobium roseum*

(which apparently lacks glycerol-based lipids), where they appeared to be the exclusive backbone structure of the polar lipids (Pond et al., 1986; Pond and Langworthy, 1987). In the species of *Thermus*, long chain diol-based lipids never completely replace the normal glycerolipids. The levels of diols vary with the strain, and some strains have only vestigial amounts. Long chain diols have also been identified in the glycolipids of *Meiothermus* spp. (Ferreira et al., 1999; M. Y. Chen et al., 2002). The presence of long chain diols in *Thermomicrobium roseum*, *Thermus* and *Meiothermus* species, in conjunction with 16S rDNA phylogenies, leads to the hypothesis that these lipid backbones reflect a distant but definite relationship between the Deinococcus–Thermus phylum and the phylum Thermomicrobi (van de Peer et al., 1994).

Iso- and *anteiso*-branched C15:0 and C17:0 fatty acids are the predominant acyl chains of the strains of the genera *Thermus* and *Meiothermus*. Straight chain saturated fatty acids and unsaturated branched chain fatty acids are minor components at the optimum growth temperature in the vast majority of the strains (Donato et al., 1990; Nobre et al., 1996a); nevertheless, straight chain C16:0 reaches levels of about 20% of the total fatty acids in *T. thermophilus* AT-62 (Nobre et al., 1996a). *Iso*-branched fatty acids predominate over *anteiso*-branched fatty acids in the vast majority of the strains at the optimum growth temperature (Nobre et al., 1996a) except in the type strain of *T. filiformis,* where *anteiso*-branched fatty acids account for 60–70% of the fatty acids (Table 3). Some strains of the genus *Thermus*, namely most strains of *T. aquaticus* and the type strain of *T. filiformis*, also contain moderate levels of branched chain 3-hydroxy fatty acids. 3-Hydroxy fatty acids are exclusively amide-linked to the galactosamine present in the glycolipids but are never present in the strains where glucosamine replaces galactosamine (Carreto et al., 1996). Branched-chain 2-hydroxy fatty acids account for 7–13% of the total fatty acids of all strains of the genus *Meiothermus* examined (Tenreiro et al., 1995a; Nobre et al., 1996a; Chung et al., 1997; M. Y. Chen et al., 2002). The strains of *M. ruber* and *M. cerbereus* also contain appreciable amounts of branched-chain 3-hydroxy fatty acids (Table 4), but these are practically absent in *M. chliarophilus* and *M. silvanus* (Nobre et al., 1996a; Chung et al., 1997).

With the exception of the strains of *T. thermophilus*, none of the organisms of the genera *Thermus* and *Meiothermus* grow in media containing over 1% NaCl. The majority of the strains of *T. thermophilus* are halotolerant and grow in yeast extract-containing media with 3–6% NaCl. Trehalose is the primary compatible solute, but lower amounts of mannosylglycerate also contribute to the osmotic balance during salt stress (Nunes et al., 1995; Empadinhas et al., 2003; Silva et al., 2003). Three structurally linked genes for the synthesis of trehalose, namely *otsA* (encoding trehalose-phosphate synthase), *otsB* (encoding trehalose-6-phosphate phosphatase), and *treS* (encoding the maltose-converting trehalose synthase), lead to the synthesis of trehalose, and structurally linked *mpgs* (encoding mannosylphosphoglycerate synthase) and *mpgp* (encoding mannosylphosphoglycerate synthase), lead to the synthesis of mannosylglycerate in strains RQ-1, Fiji3 A1, PRQ-14, B and T-2. These organisms grow in a defined medium (without yeast extract, as a source of trehalose) containing up to 6% NaCl. Strains HB8, GK24 and AT-62 that do not have a complete *otsA* gene and strain HB27 that does not possess the *otsA-otsB-treS* gene cluster do not synthesize trehalose and do not grow in media containing more than 2% NaCl. Strain CC-16, which lacks *mpgs-mpgp* but possesses *otsA*, *otsB* and *treS*, only grows in media containing up to 1% NaCl. Apparently, mannosylglycerate is important during low level osmotic adjustment, while trehalose is a compatible solute with a role in osmotic adjustment in media containing higher concentrations of salt (Silva et al., 2003; S. Alarico et al., unpublished results). The species of the genera *Marinithermus*, *Vulcanithermus* and *Oceanithermus*, in contrast to the species of *Thermus* and *Meiothermus*, inhabit marine hydrothermal vents and are all slightly halophilic.

The Genera *Marinithermus*, *Vulcanithermus* and *Oceanithermus*

Species belonging to the genera *Marinithermus*, *Vulcanithermus* and *Oceanithermus* have been recently described (Miroshnichenko et al., 2003a; Miroshnichenko et al., 2003b; Mori et al., 2004; Sako et al., 2003). Surprisingly, independent manuscripts describing two of these clearly distinct genera with the same generic epithet were submitted almost simultaneously to the *International Journal of Systematic and Evolutionary Microbiology*, leading to a change in one name while the manuscripts were being reviewed. These organisms originate from abyssal hydrothermal areas on the East Pacific Rise, the Suiyu Seamount off Japan, and the Mid-Atlantic Ridge and are thermophilic, with an optimum growth temperature range of 60–70°C. All species require at least 0.5% NaCl for growth and have an optimum NaCl concentration for growth of about 3%. The colonies are nonpigmented or cream-colored. The species

Table 5. Characteristics that distinguish the species of the genera *Marinithermus*, *Vulcanithermus* and *Oceanithermus*.

Characteristic	*Marinithermus hydrothermalis* T1^T	*Vulcanithermus mediatlanticus* TR^T	*Oceanithermus profundus* 506^T	*Oceanithermus desulfurans* St55B^T
Habitat	Deep sea hydrothermal chimney in the Izu-Bonine Arc, Japan	Deep sea hydrothermal chimney in Mid-Atlantic Ridge	A hydrothermal vent site in the Eastern Pacific	A hydrothermal vent site in the Western Pacific
Morphology	Rods or filamentous (rotund bodies not present)	Rods (rotund bodies not present)	Rods (rotund bodies present)	Rods (rotund bodies present)
Pigmentation	Whitish colonies	Cream colonies	Colorless colonies	Colorless colonies
Gram stain	–	–	–	–
Sensitivity to O_2	Aerobic	Microaerophilic	Microaerophilic and anaerophilic	Microaerophilic and anaerophilic
G+C content (mol%)	68.6	68.4	68.6	71.1
Major respiratory quinone	MK-8	MK-8	MK-8	MK-8
Cellular fatty acids (FAs)				
Major FAs	*iso*-C15:0 and *iso*-C17:0	*iso*-C15:0 and *iso*-C17:0	*iso*-C15:0 and *iso*-C16:1	*iso*-C15:0 and *anteiso*-C15:0
Unsaturated FAs (%)	nd	27	37	18
Presence of C11:0 3-OH	+	–	–	–
Optimum growth conditions				
Temperature (°C)	67.5	70.0	60.0	60.0
pH	7.0	6.7	7.5	6.5
NaCl concentration (%)	3	3	3	3
Electron acceptors				
O_2	nd	+	+	+
NO_3^-	nd	+	+	+
NO_2^-	nd	+		+
S°	nd	+	+	+
Oxidation of H_2	–	+	+	+
Carbon and energy source				
Yeast extract	+	+	–	+
Tryptone	+	+	+	+
Fructose	–	+	+	–
Galactose	–	+	+	–
Glucose	–	+	+	–
Sucrose	+	+	+	–
Xylose	–	+	+	–
Acetate	+	+	+	–
Pyruvate	+	+	+	+

Symbols: +, present; and –, absent.

Abbreviations: MK-8, 2-methyl-3-prenyl-1,4-naphthoquinone, a lipophilic nonprotein component of the electron transport chain in bacteria, with a prenyl side chain containing eight isoprene units; *iso*-$C_{15:0,}$; *iso*-$C_{17:0,}$; *iso*-$C_{16:1,}$; *anteiso*-$C_{15:0,}$; 3-OH $C_{11:0,}$; and nd, not determined.

From Miroshnichenko et al. (2003a, 2003b), Sako et al. (2003), and Mori et al. (2004).

Marinithermus hydrothermalis is strictly aerobic and heterotrophic (Sako et al., 2003), but *Oceanithermus profundus* (Miroshnichenko et al., 2003a), *O. desulfurans* (Mori et al., 2004) and *Vulcanithermus mediatlanticus* (Miroshnichenko et al., 2003b) are microaerophilic, are facultatively anaerobic, and, with the exception of *O. desulfurans*, can utilize molecular hydrogen as a source of electrons in the presence of an organic carbon source (Table 5). These organisms have optimum O_2 concentrations for growth of 2–6%; electron acceptors for anaerobic respiration depend on the organism but include nitrate, nitrite or elemental sulfur. Fermentation has not been detected.

Interestingly, the cells of these species resemble those of *Thermus* and *Meiothermus*. Some species, namely the two species of *Oceanithermus*, form "rotund bodies" similar to those seen in *Thermus* strains. The fatty acid composition of these novel organisms, composed primarily of saturated *iso-* and *anteiso*-branched chains, closely resembles that of *Thermus* spp. and *Meiothermus* spp. However, *V. mediatlanticus*, *O. profundus* and *O. desulfurans* have very high levels of unsaturated fatty acids that can reach over 30% of the total fatty acids. Moreover, branched chain cyclopropane fatty acids derived from the unsaturated fatty acids were also detected in small amounts in *V. mediatlanticus*. The polar lipids of *V. mediatlanticus* are composed of phospholipids and glycolipids that appear to be very similar if not identical to those of *Thermus* spp., but *O. profundus* has only phospholipids, which is a novel characteristic within the family Thermaceae, whose members also possess glycolipids.

Applications

The exploitation of thermostable enzymes is a major goal of biotechnology; however, the main thrust is directed to the utilization of enzymes from organisms that grow at or near the boiling point of water, leaving out organisms that grow in the temperature range of of the *Thermus*-like organisms. Despite all the interest in the biotechnological exploitation of hyperthermophilic organisms, *T. aquaticus* YT-1 produces one of the most valuable enzymes in scientific and economic terms. This enzyme is, of course, the Taq polymerase. This enzyme made the polymerase chain reaction (PCR) and thereby recombinant DNA technology possible, and even other polymerases from hyperthermophiles have not replaced the Taq polymerase.

Compatible solutes such as glycerol and trehalose have been extensively used to protect proteins, enzymes, and cells from thermal denaturation, from desiccation, and during long-term storage. Other compatible solutes, like ectoine, have also been found to be useful in cosmetics, and additional functions may be forthcoming as applied research finds new solutions for modern needs. Mannosylglycerate has been found to be more efficient than other solutes in protecting model enzymes from heat denaturation and freeze-drying and may, in the future, replace other compatible solutes for these purposes (Ramos et al, 1997; Borges et al., 2002). *Thermus thermophilus* appears to be the organism of choice for the production of this compatible solute since all other thermophilic or hyperthermophilic organisms that produce this compatible solute grow very poorly or are too fragile for industrial production. Moreover, recombinant production of the compound remains elusive in many industrial strains.

Literature Cited

Alfredsson, G. A., and J. K Kristjánsson. 1995. Ecology, distribution, and isolation of Thermus. *In:* R. Sharp and R. A. D. Williams (Eds.) Thermus Species. Plenum Press. New York, NY. 43–66.

Balkwill, D. L., T. L. Kieft, T. Tsukuda, H. M. Kostandarithes, T. C. Onstott, S. Macnaughton, J. Bownas, and J. K. Fredrickson. 2004. Identification of iron-reducing Thermus strains as Thermus scotoductus. Extremophiles 8:37–44.

Bateson, M. M., K. J. Thibault, and D. M. Ward. 1990. Comparative analysis of 16S ribosomal RNA sequences of Thermus species. Syst. Appl. Microbiol. 13:8–13.

Battista, J. R., and F. A. Rainey. 2001. The genus Deinococcus. *In:* D. R. Boone and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:396–403.

Becker, R. J., and M. J. Starzyk. 1984. Morphology and rotund body formation in Thermus aquaticus Microbios, 41:115–129.

Beffa, T., M. Blanc, P.-F. Lyon, G. Vogt, M. Marchiani, J. L. Fischer, and M. Aragno. 1996. Isolation of Thermus strains from hot composts (60 to 80°C). Appl. Environ. Microbiol. 62:1723–1727.

Borges, N., A. Ramos, N. D. Raven, R. J. Sharp, and H. Santos. 2002. Comparative study of the thermostabilizing properties of mannosylglycerate and other compatible solutes on model enzymes. Extremophiles 6:209–216.

Brock, T. D., and H. Freeze. 1969. Thermus aquaticus gen. n. and sp. n., a nonsporulating extreme thermophile. J. Bacteriol. 98:289–297.

Brock, T. D., and M. R. Edwards. 1970. Fine structure of Thermus aquaticus, an extreme thermophile. J. Bacteriol. 104:509–517.

Brock, T. D., and K. L. Boylen. 1973. Presence of thermophilic bacteria in laundry and domestic hot-water heater. Appl. Microbiol. 25:72–76.

Brock, T. D. 1984. Genus Thermus. *In:* N. R. Krieg and J. G. Holt (Eds) Bergey's Manual of Systematic Bacteriology, 1st ed. Williams & Wilkins. Baltimore, MD. 1:333–337.

Carreto, L., R. Wait, M. F. Nobre, and M. S. da Costa. 1996. Determination of the structure of a novel glycolipid from Thermus aquaticus 15004 and demonstration that hydroxy fatty acids are amide linked to glycolipids in Thermus spp. J. Bacteriol. 178:6479–6486.

Castenholz, R. W. 1969. Thermophilic blue-green algae and the thermal environment. Bacteriol. Rev. 33:476–504.

Castón, J. R., J. L. Carrascosa, M. A. de Pedro, and J. Berenguer. 1988. Identification of a crystalline surface layer on the cell envelope of the thermophilic eubacterium Thermus thermophilus. FEMS Microbiol. Lett. 51:225–230.

Chen, C., L. Lin, Q. Peng, K. Ben, and Z. Zhou. 2002b. Meiothermus rosaceus sp nov isolated from Tengchong hot spring in Yunnan, China. FEMS Microbiol. Lett. 216:263–268.

Chen, M. Y., G. H. Lin, Y. T. Lin, and S. S. Tsay. 2002a. Meiothermus taiwanensis sp. nov., a novel filamentous, thermophilic species isolated in Taiwan. Int. J Syst. Evol. Microbiol. 52:1647–1654.

Chung, A. P., F. Rainey, M. F. Nobre, J. Burghardt, and M. S. da Costa. 1997. Meiothermus cerbereus sp. nov., a new slightly thermophilic species with high levels of 3-hydroxy fatty acids. Int. J. Syst. Bacteriol. 47:1225–1230.

Chung, A. P., F. A. Rainey, M. Valente, M. F. Nobre, and M. S. da Costa. 2000. Thermus igniterrae sp. nov. and Thermus antranikianii sp. nov., two new species from Iceland. Int. J. Syst. Evol. Microbiol. 50:209–217.

Collins, M. D., and D. Jones. 1981. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. Microbiol. Rev. 45:316–354.

da Costa, M. S., and F. A. Rainey. 2001a. Thermaceae fam. nov. *In:* D. R. Boone and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:403.

da Costa, M. S., M. F. Nobre, and F. A. Rainey. 2001b. The genus Thermus. *In:* D. R. Boone and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:404–414.

Das, S. K., A. K. Mishra, B. J. Tindall, F. A. Rainey, and E. Stackebrandt. 1996. Oxidation of thiosulfate by a new bacterium, Bosea thiooxidans (strain BI-42) gen. nov., sp. nov.: Analysis of phylogeny based on chemotaxonomy and 16S ribosomal DNA sequencing. Int. J. Syst. Bacteriol. 46:981–987.

Degryse, E., N. Glansdorff, and A. Piérard. 1978. A comparative analysis of extreme thermophilic bacteria belonging to the genus Thermus. Arch. Microbiol. 177: 189–196.

Denman, S., K. Hampson, and B. K. Patel. 1991. Isolation of strains of Thermus aquaticus from the Australian artesian basin and a simple and rapid procedure for the preparation of their plasmids. FEMS Microbiol. Lett. 66:73–78.

Donato, M. M., E. A. Seleiro, and M. S. da Costa. 1990. Polar lipid and fatty acid composition of strains of the genus Thermus. Syst. Appl. Microbiol. 13:234–239.

Donato, M. M., E. A. Seleiro, and M. S. da Costa. 1991. Polar lipid and fatty acid composition of strains of Thermu ruber. Syst. Appl. Microbiol. 14:235–239.

Embley, T. M., A.G. O'Donnel, R. Wait, and J. Rostron. 1987. Lipid and cell wall amino acid composition in the classification of members of the genus Deinococcus. Syst. Appl. Microbiol. 10:20–27.

Embley, T. M., R. H. Thomas, and R. A. D. Williams. 1993. Reduced thermophilic bias in the 16S rDNA sequence from Thermus ruber provides further support for a relationship between Thermus and Deinococcus. Syst. Appl. Microbiol. 16:25–29.

Empadinhas, N., L. Albuquerque, A. Henne, H. Santos, and M. S. da Costa. 2003. The bacterium Thermus thermophilus, like hyperthermophilic archaea, uses a two-step pathway for the synthesis of mannosylglycerate. Appl. Environ. Microbiol. 69:3272–3279.

Faraldo, M. M., M. A de Pedro, and J. Berenguer. 1992. Sequence of the S-layer gene of Thermus thermophilus HB8 and functionality of its promoter in Escherichia coli J. Bacteriol. 174:7458–7462.

Fernández-Herrero, L. A., G. Olabarría, and J. Berenguer. 1997. Surface proteins and a novel transcription factor regulate the expression of the S-layer gene in Thermus thermophilus HB8 Mol. Microbiol. 24:61–72.

Ferraz, A. S., L. Carreto, S. Tenreiro, M. F. Nobre, and M. S. da Costa. 1994. Polar lipids and fatty acid composition of Thermus strains from New Zealand. Ant. v. Leeuwenhoek 66:357–363.

Ferreira, A. M., R. Wait, M. F. Nobre, and M. S. da Costa. 1999. Caracterization of glycolipids from Meiothermus spp. Microbiology 145:1191–1199.

Georganta, G., K. E. Smith, and R. A. D. Williams. 1993. DNA/DNA homology and cellular components of Thermus filiformis and other strains of Thermus from New Zealand hot springs. FEMS Microbiol. Lett. 107:145–150.

Henne, A., H. Bruggemann, C. Raasch, A. Wiezer, T. Hartsch, H. Liesegang, A. Johann, T. Lienard, O. Gohl, R. Martinez-Arias, C. Jacobi, V. Starkuviene, S. Schlenczeck, S. Dencker, R. Huber, H. P. Klenk, W. Kramer, R. Merkl, G. Gottschalk, and H. J. Fritz. 2004. The genome sequence of the extreme thermophile Thermus thermophilus. Nature Biotechnol. 22:547–553.

Hensel, R., W. Demharter, O. Kandler, R. M. Kroppenstedt, and E. Stackebrandt. 1986. Chemotaxonomic and molecular-genetic studies of the genus Thermus: Evidence for a phylogenetic relationship of Thermus aquaticus and Thermus ruber to the genus Deinococcus. Int. J. Syst. Bacteriol. 36:444–453.

Hoshino, T., Y. Yoshino, E. D. Guevarra, S. Ishida, T. Hiruta, R. Fujii, and T. Nakahara. 1994. Isolation and partial characterization of carotenoid underproducing and overproducing mutants from an extremely thermophilic Thermus thermophilus HB27. J. Ferm. Bioengin. 7:131–136.

Hudson, J. A., H. W. Morgan, and R. M. Daniel. 1986. A numerical classification of some Thermus isolates. J. Gen. Microbiol. 132:531–540.

Hudson, J. A., H. W. Morgan, and R. M. Daniel. 1987a. Numerical classification of some Thermus isolates from Icelandic hot springs. Syst. Appl. Microbiol. 9: 218–223.

Hudson, J. A., H. W. Morgan, and R. M. Daniel. 1987b. Thermus filiformis sp. nov., a filamentous caldoactive bacterium. Int. J. Syst. Bacteriol. 37:431–436.

Hudson, J. A., H. W. Morgan, and R. M. Daniel. 1989. Numerical classification of Thermus isolates from globally distributed hot springs. Syst. Appl. Microbiol. 11:250–256.

Kieft, T. L., J. K. Fredrickson, T. C. Onstott, Y. A. Gorby, H. M. Kostandarithes, T. J. Bailey, D. W. Kennedy, S. W. Li, A. E. Plymale, C. M. Spadoni, and M. S. Gray. 1999. Dissimilatory reduction of Fe(III) and other electron acceptors by a Thermus isolate. Appl. Environ. Microbiol. 65:1214–1221.

Kristjánsson, J. K., and G. A. Alfredsson. 1983. Distribution of Thermus spp. in Icelandic hot springs and a thermal gradient. Appl. Environ. Microbiol. 45:1785–1798.

Kristjánsson, J. K., G. O. Hreggvidsson, and G. A. Alfredsson. 1986. Isolation of halotolerant Thermus scotoductus spp. from submarine hot springs in Iceland. Appl. Environ. Microbiol. 52:1313–1316.

Kristjánsson, J. K., S. Hjörleifsdóttir, V. T. Marteinsson, and G. A. Alfredsson. 1994. Thermus scotoductus, sp. nov., a pigment-producing thermophilic bacterium from hot tap water in Iceland and including Thermus sp. X-1. Syst. Appl. Microbiol. 17:44–50.

Loginova, L. G., L. A. Egorova, R. S. Golovacheva, and L. M. Seregina. 1984. Thermus ruber sp. nov., nom. rev. Int. J. Syst. Bacteriol. 34:498–499.

Manaia, C. M., and M. S. da Costa. 1991. Characterization of halotolerant Thermus isolates from shallow marine hot springs on S. Miguel, Azores. J. Gen. Microbiol. 137: 2643–2648.

Manaia, C. M., B. Hoste, M. C. Gutierrez, M. Gillis, A. Ventosa, K. Kersters, and M. S. da Costa. 1994. Halotolerant Thermus strains from marine and terrestrial hot springs belong to Thermus thermophilus (ex Oshima and Imahori, 1974) nom. rev. emend. Syst. Appl. Microbiol. 17:526–532.

Marteinsson, V. T., J.-L. Birrien, J. K. Kristjánsson, and D. Prieur. 1995. First isolation of thermophilic aerobic nonsporulating heterotrophic bacteria from deep-sea hydrothermal vents. FEMS Microbiol. Ecol. 18:163–174.

Marteinsson, V. T., J.-L. Birrien, G. Raguénès, M. S. da Costa, and D. Prieur. 1999. Isolation and characterization of Thermus thermophilus Gy1211 from a deep-sea hydrothermal vent. Extremophiles 3:247–251.

Merkel, G. J., S. S. Stapleton, and J. J. Perry. 1978. Isolation and peptidoglycan of Gram-negative hydrocarbon-utilizing thermophilic bacteria. J. Gen. Microbiol. 109: 141–148.

Miroshnichenko, M. L., S. L'Haridon, C. Jeanthon, A. N. Antipov, N. A .Kostrikina, B. J. Tindall, P. Schumann, S. Spring, E. Stackebrandt, and E. A. Bonch-Osmolovskaya. 2003a. Oceanithermus profundus gen. nov., sp. nov., a thermophilic, microaerophilic, facultatively chemolithoheterotrophic bacterium from a deep-sea hydrothermal vent. Int. J. Syst. Evol. Microbiol. 53:747–752.

Miroshnichenko, M. L., S. L'Haridon, O. Nercessian, A. N.Antipov, N. A., Kostrikina, B. J Tindall, P. Schumann, S. Spring, E. Stackebrandt, E. A. Bonch-Osmolovskaya, and C. Jeanthon. 2003b. Vulcanithermus mediatlanticus gen. nov., sp. nov., a novel member of the family Thermaceae from a deep-sea hot vent. Int. J. Syst. Evol. Microbiol. 53:1143–1148.

Mori, K., T. Kakegawa, Y. Higashi, K. Nakamura, A. Maruyama, and S. Hanada. 2004. Oceanithermus desulfurans sp. nov., a novel thermophilic, sulfur-reducing bacterium isolated from a sulfide chimney in Suiyo Seamount. Int. J. Syst. Evol. Microbiol. 54:1561–1566.

Munster, M. J., A. P. Munster, J. R. Woodrow, and R. J. Sharp. 1986. Isolation and preliminary taxonomic studies of Thermus strains isolated from Yellowstone National Park, USA. J. Gen. Microbiol. 132:1677–1683.

Nobre, M. F., L. Carreto, R. Wait, S. Tenreiro, O. Fernandes, R. J. Sharp, and M. S. da Costa. 1996a. Fatty acid composition of the species of the genera Thermus and Meiothermus. Syst. Appl. Microbiol. 19:303–311.

Nobre, M. F., H. G. Trüper, and M. S. da Costa. 1996b. Transfer of Thermus ruber (Loginova et al. 1984), Thermus silvanus (Tenreiro et al. 1995), and Thermus chliarophilus (Tenreiro et al. 1995) to Meiothermus gen. nov. as Meiothermus ruber comb. nov., Meiothermus silvanus comb. nov., and Meiothermus chliarophilus comb. nov., respectively, and emendation of the genus Thermus. Int. J. Syst. Bacteriol. 46:604–606.

Nobre, M. F., and M. S. da Costa. 2001. The genus Meiothermus. *In:* D. R. Boone and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:414–420.

Nold, S. C., and D. M. Ward. 1995. Diverse Thermus species inhabit a single hot spring microbial mat. Syst. Appl. Microbiol. 18:274–278.

Nunes, O. C., C. M. Manaia, M. S. da Costa, and H. Santos. 1995. Compatible solutes in the thermophylic bacteria Rhodothermus marinus and "Thermus thermophilus." Appl. Environ. Microbiol. 61:2351–2357.

Olabarría, G., L. A. Fernández-Herrero, J. L. Carrascosa, and J. Berenguer. 1996. slpM, a gene coding for an "S-layer-like array" overexpressed in S-layer mutants of Thermus thermophilus HB8. J. Bacteriol. 178:357–365.

Oshima, M., and T. Yamakawa. 1974. Chemical structure of a novel glycolipid from an extreme thermophile, Flavobacterium thermophilum. Biochemistry 13:1140–1146.

Oshima, T., and K. Imahori. 1974. Description of Thermus thermophilus (Yoshida and Oshima) comb. nov., a nonsporulating thermophilic bacterium from a Japanese thermal spa. Int. J. Syst. Bacteriol. 24:102–112.

Pask-Hughes, R., and R. A. D. Williams. 1975. Extremely thermophilic Gram-negative bacteria from hot tap water. J. Gen. Microbiol. 88:321–328.

Pask-Hughes, R. A., and R. A. D. Williams. 1977. Yellow-pigmented strains of Thermus spp. from Icelandic hot springs. J. Gen. Microbiol. 102:375–383.

Pask-Hughes, R. A., and R. A. D. Williams. 1978. Cell envelope components of strains belonging to the genus Thermus. J. Gen. Microbiol. 107:65–72.

Pond, J. P., T. A. Langworthy, and G. Holzer. 1986. Long-chain diols: A new class of membrane lipids from a thermophilic bacterium. Science 231:1134–1136.

Pond, J. P., and T. A. Langworthy. 1987. Effect of growth temperature on the long-chain diols and fatty acids of Thermomicrobium roseum. J. Bacteriol. 169:1328–1330.

Prado, A., M. S. da Costa, and V. M. C. Madeira. 1988. Effect of the growth temperature on the lipid composition of two strains of Thermus sp. J. Gen. Microbiol. 134:1653–1660.

Rainey, F. A., M. F. Nobre, P. Schumann, E. Stackebrandt, and M. S. da Costa. 1997. Phylogenetic diversity of the deinococci as determined by 16S ribosomal DNA sequence comparison. Int. J. Syst. Bacteriol. 47:510–514.

Rainey, F. A., and M. S. da Costa. 2001. Thermales ord. nov. *In:* D. R. Boone and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:403.

Ramaley, R. F., and J. Hixson. 1970. Isolation of a nonpigmented, thermophilic bacterium similar to Thermus aquaticus. J. Bacteriol. 103:527–528.

Ramos, A., N. D. H. Raven, R. J. Sharp, S. Bartolucci, M. Rossi, R. Cannio, J. Lebbink, J. van der Oost, W. M. De Vos, and H. Santos. 1997. Stabilization of enzymes against thermal stress and freeze-drying by mannosylglycerate. Appl. Environ. Microbiol. 63:4020–4025.

Saiki, T., R. Kimura, and K. Arima. 1972. Isolation and characterization of extremely thermophilic bacteria from hot springs. Agric. Biol. Chem. 36:2357–2366.

Sako, Y, S. Nakagawa, K. Takai, and K. Horikoshi. 2003. Marinithermus hydrothermalis gen. nov., sp. nov., a strictly aerobic, thermophilic bacterium from a deep-sea hydrothermal vent chimney. Int. J. Syst. Evol. Microbiol. 53:59–65.

Santos, M. A., R. A. D. Williams, and M. S. da Costa. 1989. Numerical taxonomy of Thermus isolates from hot springs in Portugal. Syst. Appl. Microbiol. 12:310–315.

Schleifer, K.-H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. 36:407–477.

Sharp, R. J., and R. A. D. Williams. 1988. Properties of Thermus ruber strains isolated from Icelandic hot springs and DNA:DNA homology of Thermus ruber and Thermus aquaticus. Appl. Environ. Microbiol. 54:2049–2053.

Sharp, R., D. Cossar, and R. Williams. 1995. Physiology and metabolism of Thermus. *In:* R. Sharp, and R. A. D. Williams (Eds.) Thermus Species. Plenum Press. New York, NY. 67–91.

Silva, Z., S. Alarico, A. Nobre, R. Horlacher, J. Marugg, W. Boos, A. I. Mingote, and M. S. da Costa. 2003. Osmotic adaptation of Thermus thermophilus RQ-1: Lesson from a mutant deficient in synthesis of trehalose. J. Bacteriol. 185:5943–5952.

Skirnisdottir, S., G. O. Hreggvidsson, O. Holst, and J. K. Kristjansson. 2001. Isolation and characterization of a mixotrophic sulfur-oxidizing Thermus scotoductus. Extremophiles 5:45–51.

Stramer, S. L., and M. J. Starzyk. 1981. The occurrence and survival of Thermus aquaticus. Microbios. 32:99–110.

Tabata, K., S. Ishida, T. Nakahara, and T. Hoshino. 1994. A carotenogenic gene cluster exists on a large plasmid in Thermus thermophilus. FEBS Lett. 341:251–255.

Taguchi, H., M. Yamashita, H. Matsuzawa, and T. Ohta. 1982. Heat stable and fructose 1,6-biphosphate-activated L-lactate dehydrogenase from an extremely thermophilic bacterium. J. Biochem. 91:1343–1348.

Tenreiro, S., M. F. Nobre, and M. S. da Costa. 1995a. Thermus silvanus sp. nov. and Thermus chliarophilus sp. nov., two new species related to Thermus ruber but with lower growth temperatures. Int. J. Syst. Bacteriol. 45:633–639.

Tenreiro, S., M. F. Nobre, B. Hoste, M. Gillis, J. K. Kristjánsson, and M. S. da Costa. 1995b. DNA:DNA hybridization and chemotaxonomic studies of Thermus scotoductus. Res. Microbiol. 146:315–324.

Tenreiro, S., M. F., Nobre, F. A. Rainey, C. Miguel, and M. S. da Costa. 1997. Thermomena rossianum sp. nov., a new thermophilic and slightly halophilic species from saline hot springs in Naples, Italy. Int. J. Syst. Bacteriol. 47:122–126.

Van de Peer, Y., J.-M. Neefs, P. De Rijk, P. De Vos, and R. De Wachter. 1994. About the order of divergence of the major bacterial taxa during evolution. Syst. Appl. Microbiol. 17:32–38.

Wait, R., L. Carreto, M. F. Nobre, A. M. Ferreira, and M. S. da Costa. 1997. Characterization of novel long-chain 1,2-diols in Thermus species and demonstration that Thermus strains contain both glycerol-linked and diol-linked glycolipids. J. Bacteriol. 179:6154–6162.

Waring, G. A. 1965. Thermal Springs of the United States and Other Countries of the World: A Summary. US Government Printing Office. Washington, DC.

Weisburg, W. G., S. J. Giovannoni, and C. R. Woese. 1989. The Deinococcus-Thermus phylum and the effect of rRNA composition on phylogenetic tree construction. Syst. Appl. Microbiol. 11:128–134.

Williams, R. A. D., and M. S. da Costa. 1992. The genus Thermus and related microorganisms. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 3745-3753.

Williams, R. A. D., K. E. Smith, S. G. Welch, J. Micallef, and R. J. Sharp. 1995. DNA relatedness of Thermus strains, description of Thermus brockianus sp. nov., and proposal to reestablish Thermus thermophilus (Oshima and Imahori). Int. J. Syst. Bacteriol. 45:495–499.

Williams, R. A. D., K. E. Smith, S. G. Welch, and J. Micallef. 1996. Thermus oshimai sp. nov., isolated from hot springs in Portugal, Iceland, and the Azores, and comment on the concept of a limited geographical distribution of Thermus species. Int. J. Syst. Bacteriol. 46:403–408.

Yoshida, M., and T. Oshima. 1971. The thermostable allosteric nature of fructose 1,6-diphosphatase from an extreme thermophile. Biochem. Biophys. Res. Comm. 45:495–500.

PART 10

Chloroflexaceae and Related Bacteria

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CHAPTER 10.1

The Family Chloroflexaceae

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Introduction

The first multicellular filamentous anoxygenic phototrophic bacterium was discovered in thermal environments (Pierson and Castenholz, 1971) and was soon described as a new genus and species with the name of "*Chloroflexus aurantiacus*" (Pierson and Castenholz, 1974a). The organism showed gliding motility and contained pigment-bearing vesicles called "chlorosomes." Chlorosomes are generally observed in green sulfur bacteria that are strictly anaerobic phototrophs sensitive to oxygen. The pigment-bearing vesicles in *Chloroflexus aurantiacus* were very similar, both structurally and functionally, to those in green sulfur bacteria (Schmidt, 1980a). Aside from this pigment resemblance, however, significant differences were found between the filamentous organism and green sulfur bacteria. In addition, this new phototroph apparently differed in many phenotypic characteristics from purple bacteria, which is another type of anoxygenic photosynthetic bacteria. Therefore, the new family Chloroflexaceae was created for this filamentous phototroph containing prominent features (Trüper, 1976).

Following the description of *Chloroflexus aurantiacus*, related mesophilic filamentous phototrophs were found in freshwater habitats: Two genera, *Chloronema* (Dubinina and Gorlenko, 1975) and *Oscillochloris* (Gorlenko and Pivovarova, 1977; Keppen et al., 1994), were individually proposed for the isolates. Like *Chloroflexus aurantiacus*, these mesophiles are morphologically filamentous, show gliding motility, and contain chlorosomes as light-harvesting components. On the other hand, a chlorosome-less filamentous phototroph, *Heliothrix oregonensis*, was also found in a hot spring (Pierson et al., 1984a). The *Chloroflexus*-like bacterium is an interesting organism in that it does not contain bacteriochlorophyll (Bchl) *c* but does contain as its sole chlorophyll species Bchl *a*. Recently, another chlorosome-less filamentous phototroph was newly isolated from a hot spring and proposed as *Roseiflexus castenholzii* (Hanada et al., 2002b). Although *Roseiflexus castenholzii*, like *Heliothrix oregonensis*, lacks Bchl *c* and chlorosomes, genotypic and phenotypic comparisons showed that they were phylogenetically distant from each other.

Filamentous morphology and gliding motility are typical features of these organisms, whereas these properties are seldom observed in any other anoxygenic photosynthetic bacteria, i.e., purple bacteria, green sulfur bacteria, and the heliobacteria. The phylogenetic analysis based on 16S rRNA sequence also supported the solitary taxonomic position of this group: Oyaizu et al. revealed that *Chloroflexus aurantiacus* belonged to a deep branching lineage (phylum) of the domain Bacteria together with the genera *Herpetosiphon* and *Thermomicrobium*, which are comprised of nonphototrophic bacteria, and that *Chloroflexus aurantiacus* was phylogenetically distant from any other photosynthetic group (Oyaizu et al., 1987).

The phototrophic group (as well as the phylogenetic phylum) was at first called "green nonsulfur bacteria," because the first discovery possessed chlorosomes, a trait typical of green sulfur bacteria. However, this appears to be a misnomer for the following reasons: 1) the group is clearly distant from green sulfur bacteria and 2) two of the total of five phototrophic genera have no chlorosomes and indeed are not green but red to orange. Therefore, two new general terms representing the photosynthetic group have been proposed: "multicellular filamentous green bacteria" (Pfennig, 1989) and "filamentous anoxygenic phototrophs" (Pierson and Castenholz, 1995). Considering the common features of the members (and desiring to avoid calling them "green") in this chapter, we use the term "filamentous anoxygenic phototrophs" (FAPs) to refer to this phototrophic group composed of the family Chloroflexaceae.

FAP organisms have been observed in marine and hypersaline environments as well as in hot springs and freshwater habitats (Stolz, 1983; D'Amelio et al., 1987; D'Amelio et al., 1989; Mack and Pierson, 1988; Venetskaya and Gerasimenko, 1988; Garcia-Pichel et al., 1994; Pierson et al., 1994). These halophiles are morphologi-

cally diverse: They vary in cell diameter, and some hypersaline filaments have complex internal membranes seldom observed in *Chloroflexus aurantiacus*. Most marine and hypersaline FAPs contain chlorosomes as well as Bchl *a* and *c* as photosynthetic pigments (and sometimes Bchl *d*). The one exception to this is the marine purple filaments described by D'Amelio et al. (1987). J. A. Klappenbach and B. K. Pierson (unpublished observation) found that the FAPs previously described in enrichment cultures (Pierson et al., 1994) were phylogenetically related to other chlorosome-containing FAPs. Nübel et al. (2001) investigated, by rRNA-based comparison, the phylogeny of hypersaline *Chloroflexus*-like organisms that formed microbial mats in evaporation ponds in a saltern. They revealed that these hypersaline filaments were phylogenetically related to FAPs. As mentioned above, FAPs are distributed throughout various environments and have significant physiological, morphological and taxonomic diversity.

FAPs are important to the study of the evolution of photosynthesis. It is clear that FAPs are phylogenetically distant from purple bacteria and are phenotypically very distant from them as well. However, these two groups resemble each other structurally and functionally in their photochemical reaction centers (the reaction center is an essential protein that contains bacteriochlorophylls for the primary action in photosynthesis). On the other hand, several FAPs obviously possess chlorosomes as a light-harvesting system like that of green sulfur bacteria, in spite of the phylogenetic distance between these two photosynthetic groups. Xiong et al. (2000) reported that the gene sequences of encoded enzymes for the biosynthesis of bacteriochlorophylls were closely related between the green sulfur bacteria and the FAPs. These findings indicate that FAPs are so-called "chimeric organisms" endowed with the purple bacterial photochemical system and the green bacterial light-harvesting system. This presents a puzzle in understanding the evolution of photosynthesis, since these three photosynthetic groups are phylogenetically diverse from one another. To explain the discrepancy between the taxonomic distance and photosynthetic similarity, the possibility of horizontal transfers of photosynthetic genes between these groups was suggested (Blankenship, 1992).

A similar discrepancy between the phototrophic groups is also found in the pathways of carbon dioxide fixation. Recently, Keppen et al. (2000) separated a mesophilic species *Oscillochloris trichoides* from the family Chloroflexaceae and proposed a new family name Oscillochloridaceae for the mesophilic species. One of the notable features of this organism is the presence of a reductive pentose phosphate cycle, i.e., the Calvin-Bassham-Benson cycle (Ivanovsky et al., 1999). *Chloroflexus aurantiacus* does not use this type of pathway to fix carbon dioxide, but uses the 3-hydroxypropionate cycle (Holo, 1989; Strauss and Fuchs, 1993) or the reductive dicarbonate cycle (Ivanovsky et al., 1993) as do the early branched-off bacteria and chemoautotrophs of Archaea. It is quite interesting that *Oscillochloris trichoides* includes the reductive pentose phosphate cycle, because this carbon dioxide fixing pathway is found generally in autotrophs that belong to the Proteobacteria or Cyanobacteria.

As mentioned above, there are interesting evolutionary peculiarities in the photosynthetic group that includes the family Chloroflexaceae. Therefore, it is possible that FAPs may be key organisms in resolving the process of evolution in carbon dioxide fixation as well as in photosynthetic systems.

Phylogeny

Until the early 1990s, only one 16S rRNA sequence, that of *Chloroflexus aurantiacus*, was available for phylogenetic analysis in the family Chloroflexaceae. Over the past decade, however, sequence data have been increasing for the filamentous anoxygenic phototrophs (FAPs). Molecular techniques containing enzymatic amplification of nucleotides and cloning have made possible the determination of rRNA (or its gene sequence) of members in mixed cultures or natural microbial mats. Weller et al. (1992) determined a partial 16S rRNA sequence of *Heliothrix oregonensis* in coculture with *Isosphaera pallida* and succeeded in obtaining several *Chloroflexus*-related clone sequences from microbial mats in a hot spring. Similarly, Nübel et al. (2001) analyzed the phylogeny of filamentous anoxygenic phototrophs in hypersaline environments and gave us phylogenetic information about halophilic species. Through culture-independent rRNA sequence analysis, Boomer et al. (2002) determined the taxonomic position of filamentous phototrophs that formed the distinct red bacterial mats in hot springs. Gich et al. (2001) were successful in sequencing the 16S rRNA gene of a possible *Chloronema giganteum* in a mixed culture through molecular techniques. *Chloronema giganteum* is thought to be a freshwater species, though it remains to be isolated. Phylogenetic analyses, not only on axenic cultures but also on enriched cocultures and natural microbial communities, are key to understanding the phylogenetic diversity of FAPs and the relationships between them.

Figure 1 shows the FAP phylogenetic tree based on 16S rRNA sequences of its members,

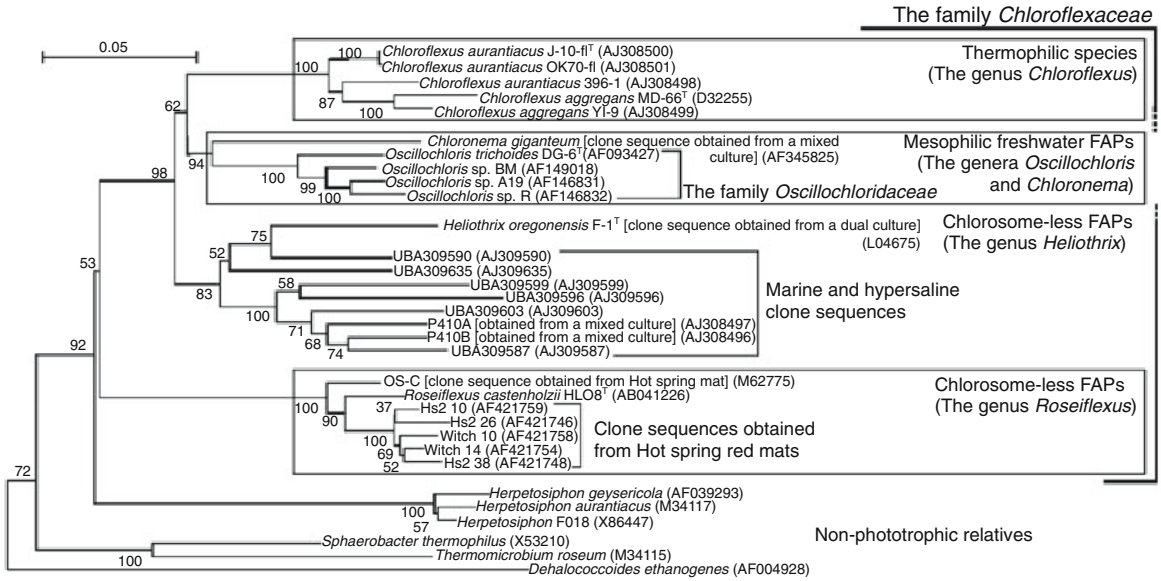

Fig. 1. A phylogenetic tree showing the relationship among the filamentous anoxygenic phototorophs (FAPs) and related non-phototrophic species based on 16S rRNA gene sequence. Bootstrap confidence values from 100 bootstrap replicates are indicated at the branching points. Bar = 5 nucleotide substitutions in 100 nucleotides in 16S rRNA sequences. The accession number of each reference species is shown in parentheses.

with clone sequence data obtained from mixed cultures and natural microbial mats. The tree indicates that each of the four phenotypes (thermophilic, mesophilic, halophilic or chlorosome-less filaments) forms an individual phylogenetic cluster or clusters and does not intermingle with the others. Two mesophilic freshwater species, *Oscillochloris trichoides* and *Chloronema giganteum* (the clone sequence was obtained from a mixed culture), are related to each other, and these are placed beside a coherent cluster composed of thermophilic *Chloroflexus aurantiacus* and *Chloroflexus aggregans*. Chlorosome-less filaments are divided into two lineages, one represented by *Heliothrix oregonensis* and the other by *Roseiflexus castenholzii*, with a great phylogenetic distance between them. The *Roseiflexus* lineage contains clone sequences obtained from microbial mats in Yellowstone National Park (United States): five clone sequence series, e.g., Hs2 and Witch, from the red-colored bacterial mats that comprised chlorosome-less filamentous phototrophs (Boomer et al., 2002) and a clone sequence, named "OS-C," from microbial mats in Octopus Spring in the Lower Geyser Basin, Yellowstone National Park (Weller et al., 1992). *Heliothrix oregonensis* is related to clone sequences retrieved from hypersaline environments by Nübel et al. (2001). This cluster is a widely diverse assemblage composed mainly of a lot of clone sequences obtained from microbial mats in evaporation ponds with various salinities (5.5–16%). It also includes two different 16S rRNA gene sequences (P4-1-0 clone A and P4-1-0 clone B) recovered from an enrichment of halophilic FAPs. Nübel et al. (2001) reported that the enriched halophilic filaments in medium containing 9% sea salt had in vivo absorption maxima at 850 and 755 nm (which were due to Bchl *a* and *c*, respectively), showed gliding motility, and had filaments resembling those of the "marine *Chloroflexus*-like organisms" (MCLOs) described by Pierson et al. (1994). The sequence similarities between *Heliothrix oregonensis* and the hypersaline clone sequences are approximately 84–86%. Although these values are very low, a high bootstrap confidence (83%) at the branching point strongly suggests that *Heliothrix oregonensis* and halophiles belong to the same assemblage, in spite of their clear phenotypic differences. One must be cautious in this interpretation, however, because the 16S rRNA data from *Heliothrix* include only a partial sequence. Because of this, it was omitted from all phylogenetic trees published in *Bergey's Manual of Systematic Bacteriology* (Pierson, 2001).

The divergence within the 16S rRNA sequence of the FAPs that comprise the family Chloroflexaceae and its relatives is very large (more than 22%). Although this group contains only five genera, phylogenetically it is as diverse as larger taxa, such as Proteobacteria and Gram-positive bacteria. Recently, and chiefly by taking account of the rRNA sequence difference, a mesophilic freshwater species, *Oscillochloris trichoides*, was separated from the family Chlo-

roflexaceae. A new family name, Oscillochloridaceae, was proposed for it. From a phylogenetic viewpoint, still more families might be separately created for a few of the five genera, e.g., *Heliothrix* and *Roseiflexus*, and also marine or hypersaline relatives, as species become isolated.

Within the Bacteria, the phylum that includes the FAPs as shown in Fig. 1 also includes filamentous chemotrophs but not the more distantly related unicellular chemotrophs included in Fig. 1. The phototrophs form a cohesive group. Species of *Herpetosiphon* are the closest chemotrophic relatives. Together these phototrophs and filamentous chemotrophs have similar morphological and gliding properties, and the species that have been analyzed (the genera *Chloroflexus* and *Herpetosiphon*) have similar wall structure.

Within the phototrophs, the "green" genera which contain chlorosomes and accessory light-harvesting Bchl *c* or *d* appear to form a branch distinct from the "red" genera which lack chlorosomes and have only Bchl *a*. The genera *Chloroflexus*, *Oscillochloris*, *Chloronema*, the hypersaline MCLO (marine and hypersaline *Chloroflexus*-like organism) and other hypersaline strains are all closely grouped. Each genus has no more than 85% similarity to the others, suggesting that they might represent four different clades at the level of family. The genus *Roseiflexus* and the red mat sequences reported by Boomer et al. (2002) appear to group together (distinct from the green line) but also do not show tremendous closeness. The one exception to this generalization is the genus *Heliothrix*, which lacks chlorosomes and falls within the "green" group in Fig. 1. Cautious interpretation is required, however, since the *Heliothrix* sequence is limited in length. The lack of sequence data for the large red filaments studied by Boomer et al. (2000) is unfortunate, and it will be interesting to see whether they are eventually placed in the "red line." The red line appears to be distant from either the chemoheterotrophic genus *Herpetosiphon* or the green line.

This chapter emphasizes the 16S rRNA phylogeny but other relevant phylogenies that include *Chloroflexus aurantiacus* and examine its relationships to other phototrophs have been constructed using Hsp60 and Hsp70 signature sequences (Gupta et al., 1999), group 1 and 2 σ factors (Gruber and Bryant, 1997; Gruber and Bryant, 1998a), and RecA sequences (Gruber et al., 1998b). The position of *Chloroflexus* in a deeper branch of the 16S rRNA tree than other phototrophs does not correlate with the suggested pattern of evolution of photosynthetic genes obtained from sequence analysis of pigment synthesis genes (Xiong et al., 2000). Since much of the photosynthetic apparatus may have been acquired in different lines of descent by lateral gene transfer, phylogeny of the organisms does not necessarily correlate with evolution of photosynthetic ability.

Taxonomy

Five genera have been described as filamentous anoxygenic phototrophs (FAPs): *Chloroflexus*, *Chloronema*, *Oscillochloris*, *Roseiflexus* and *Heliothrix*. The genus "*Chlorocrinis*" (Ward et al., 1998) does not exist. This was a tentative name originally proposed for the organism subsequently named "*Chloroflexus aggregans*." Two additional organisms have been well characterized and will certainly constitute new genera, the marine and hypersaline *Chloroflexus*-like organism (MCLO; Pierson et al., 1994) and the red hot spring mat filaments (RCR; Boomer et al., 2000). Although most of the genera are readily distinguished from each other by 16S rRNA (Fig. 1), distinguishing phenotypic features are not always very obvious and some of the differences are subtle (see Table 1). These features are discussed in more detail in the section "IDENTIFICATION." This section highlights the taxonomic difficulties posed by the relative lack of and overlap of phenotypic characters.

The genera *Chloroflexus*, *Oscillochloris*, *Chloronema* and the MCLO are readily distinguished from *Heliothrix*, *Roseiflexus* and the RCR because the first four organisms all contain Bchl *c* or *d* and chlorosomes in addition to Bchl *a*. However, distinguishing among these four green organisms by phenotypic characters is not easy. *Oscillochloris* species appear to be sulfide-dependent and to grow primarily photolithoautotrophically or photoheterotrophically. They do not grow well aerobically, although some strains may grow well under microaerobic conditions. *Chloroflexus* species, however, grow best photoheterotrophically and also grow well under aerobic conditions. Gas vesicles are frequently observed in the genus *Oscillochloris* and have not been reported in the genus *Chloroflexus*. *Oscillochoris chrysea* has incomplete septa lined with chlorosomes. The genus *Chloroflexus* has its chlorosomes confined to the cell periphery. *Oscillochloris* species have been observed in muds and in algo-bacterial mats up to 45°C. *Chloroflexus* species are found in hot spring mats in the presence or absence of sulfide at temperatures around 45°C and at much higher temperatures. However, organisms appearing identical to the genus *Chloroflexus* have been observed in mesophilic freshwater (Gorlenko, 1976) and marine habitats (Mack and Pierson, 1988). The genus *Chloronema* also has gas vesicles and is larger in diameter than *Chloroflexus* species and

Table 1. Differential characteristics of the validly described filamentous anoxygenic phototrophs.

Physiological subgroup	*Chloroflexus* species			Mesophilic freshwater forms				Chlorosome-less FAPs	
Genus	*Chloroflexus*			*Oscillochloris* (the family *Oscillochlortdaceae*)		*Chlaronema*		*Heliothrix*	*Roselflexus*
Species	*Cfl. auranitacus*	*Cfl. auranitacus* var. *mesophilus*	*Cfl. aggregans*	*Osc. irtehoides*	*Osc. chrysea*	*Cln. giganteum*	*Cln. spirodtum*	*Hir. oregonensis*	*Rfl. castenholzu*
Type strain	J-10-fl (ATCC 29366, DSM 635)	Not type culture is available	MD-66 (DSM 9485)	DG-6	No type culture is available	No type culture is available	No type culture is available	ISF-1 (co-culture with *Isophaera pallida*)	HLOS (DSM 13941, JCM 11240)
Morphology	Straight filaments	Straight filaments	Straight filaments	Straight filaments	Straight filaments	Straight or spiral filaments	Spiral filaments	Straight filaments	Straight filaments
Cell diameter (μm)	0.7–1.2	0.5–0.7	1.0–1.5	1.0–1.5	4.5–5.5	2.0–2.5	1.5–2.0	1.5	0.8–1.0
Sheath	± (occ. thinly sheathed)	+	–	±	–	+	+	–	–
Gas vesicles	–	–	–	+	+	+	+	–	–
Gram staining	Negative	ND	Negative	Variable	Positive	ND	ND	ND	Negative
Optimal growth temperature (°C) [temperature range for the growth]	55	20–25	55	28–30	[10–20]	[4–15]	mesophilic	40–55	50
Metabolism:									
Photoheterotroph	+	+	+	+ (photolithoheterotroph)	ND	ND	ND	+	+
Photoautotroph	± (in some strains)	–	–	+	+?	+	ND	ND	–
O_2 respiration	+	+	+	–	+?	+	ND	ND	+
Chlorosomes	+	+	+	+	+	+	+	–	–
Bacteriochlorophyll(s)	a, c	c*	a, c	a, c	a, c	d (and c)*	d (and c)*	a	a
Remarkable peaks (nm) in *in vivo* absorption spectra of IR region	740, 808, 868	755	740, 808, 868	748, 852	ND	720	ND	795, 865	801, 878
Major carotenoids	γ-carotene, β-carotene, OH-γ-caroteneglucoside ester	ND	γ-carotene, β-carotene, OH-γ-caroteneglucoside ester	γ-carotene, β-carotene	ND	ND	ND	keto-OH-γ-carotene, keto-myxocoxanthin, myxobactene	methoxy-keto-myxocoxanthin, keto-myxocoxanthin glycoside ester
Major cellular fatty acids	C18:0, C16:0, C18:1	ND	ND	C18:1, C16:0, C16:1	ND	ND	ND	ND	C16:0, C14:0, C15:0
Major quinone	MK-10	ND	MK-10 (and MK-4)	MK-10	ND	ND	ND	ND	MK-11
DNA G+C content (mol %)	53.1–54.9 (Bd), 56.9–57.1 (HPLC)	ND	56.7–57.0 (HPLC)	59.2	ND	ND	ND	ND	62.0
References	Pierson and Castenholz, 1974; Halfen et al., 1972	Gorlenko, 1995	Hanada et al., 1995	Keppen et al., 1993, 1994, 2000, 2001; Gorlenko and Pivovarova, 1977	Gorlenko and Pivovarova, 1977; Keppen et al., 2001	Dubinina and Gorlenko, 1975; Gorlenko and Pierson, 2001	Dubinina and Gorlenko, 1975; Gorlenko and Pierson, 2001	Pierson et al., 1984, 1985	Hanada et al., 2002; Takaichi et al., 2001

Symbols; +, positive; –, negatives; ND, not determined. *Each organism would also contain another bacteriochlorophyll, possibly Bchl *a*, that is essential to its photosynthesis.

Oscillochloris trichoides, but is narrower than *Oscillochloris chrysea*. *Chloronema* species are planktonic, unlike all the other filamentous anoxygenic phototrophs. The MCLO is readily distinguished from the other three green genera on the basis of habitat (found in hypersaline microbial mats), dependence on high sulfide, and its distinctive ultrastructure with infoldings of the cell membrane forming pockets lined with chlorosomes on the cytoplasmic side (Pierson et al., 1994).

The three organisms lacking chlorosomes are more readily distinguished from each other. All contain Bchl *a* and are found in hot spring microbial mats. *Roseiflexus castenholzii* has narrow filaments (1.0 μm or less in diameter) and lacks internal membranes (Hanada et al., 2002). *Heliothrix oregonensis* is larger (1.5 μm in diameter) and also lacks internal membranes (Pierson et al., 1984a; Pierson et al., 1985b). The RCR filaments are also 1.5 μm in diameter, but the cells contain abundant internal membranes (Boomer et al., 2000).

The genus *Chloroflexus* has two species, *Chloroflexus aurantiacus* and *aggregans*. The species are readily distinguished by 16S rRNA but phenotypically are very similar except for the aggregating ability of *Chloroflexus aggregans* (Hanada et al., 1995a).

The genus *Oscillochoris* has two species, *Oscillochoris trichoides* and *chrysea*, which can be distinguished on the basis of size and ultrastructure. *Oscillochoris chrysea* is very large (4–5 μm in diameter) and has partial septa bearing chlorosomes.

The genus *Chloronema* has the species *Chloronema giganteum* and the incompletely described species *Chloronema spiroideum*.

A higher level taxonomy has recently been described in the *Bergey's Manual of Systematic Bacteriology* (Castenholz, 2001; Garrity and Holt, 2001). The phylum formerly described as the green nonsulfur bacteria has been named the "phylum Chloroflexi" after the genus *Chloroflexus*, the most thoroughly studied genus in this group. The phylum contains one class, the "Chloroflexi." All members are Gram-negative filamentous gliding bacteria lacking a lipopolysaccharide-containing outer membrane and containing L-ornithine instead of the diaminopimelic acid in the peptidoglycan. These organisms are phylogenetically related by 16S rRNA analysis and have been divided into two orders: the "Chloroflexales," which contains all the phototrophic organisms (or at least the organisms containing Bchl), and the "Herpetosiphonales," which are not photosynthetic and contain no Bchl. The "Chloroflexales" contains only one family, the "Chloroflexaceae," which includes all the filamentous anoxygenic phototrophic bacteria. The phylogenetic distances between the genera in the 16S rRNA tree, however, are great enough to warrant taxonomic distinction at the level of family. Consequently, one proposal already exists to create a new family, "Oscillochloridaceae," for the genus *Oscillochloris*. The current descriptions of these two families will need refining before any new families are created, partly because of the paucity of distinguishing phenotypic characters. It may be that new families will be appropriate for all of the genera described here. The phylogenetic position of *Heliothrix oregonensis* is more uncertain because only a partial sequence of its 16S rRNA has been determined. The RCR has yet to be sequenced. Nevertheless, on purely phenotypic characters, one could suggest the creation of two suborders, one including the "green line" of *Chloroflexus*, *Oscillochloris*, *Chloronema*, and the MCLOs, all of which contain Bchl *c* or *d* and chlorosomes. The other suborder would include the "red line" of the genera *Heliothrix*, *Roseiflexus*, and the RCR, which lack Bchl *c* and *d* and chlorosomes. The uncertain taxonomy of this group is a reflection of the limited number of species that were available for study until very recently. Our current suggestions are tentative as we gather more information about newly emerging genera, and as cultivation techniques and sequence analyses improve, for strains previously described on the basis of field observations only.

Habitat

The filamentous anoxygenic phototrophs (FAPs) belonging to the family Chloroflexaceae or the related family Oscillochloridaceae are widely distributed throughout various environments such as hot and cold springs, freshwater lakes, river water and sediments, and both marine and hypersaline environments.

The Genus *Chloroflexus*

Chloroflexus species, which are unbranched filamentous anoxygenic phototrophic bacteria with a diameter of about 1 μm, are generally found in neutral to alkaline hot springs. Both authentic species in this genus, *Chloroflexus aurantiacus* (Pierson and Castenholz, 1974a) and *Chloroflexus aggregans* (Hanada et al., 1995a), have been isolated from hot springs. Almost all strains in the genus *Chloroflexus* are thermophiles grown at around 55°C, and these organisms are distributed worldwide. In natural hot springs, the organisms formed yellow-orange-greenish mats with cyanobacteria. The mats vary in morphology and color, depending on temperature, pH,

Fig. 2. The microbial mat consists of a dark green layer (cyanobacteria) and yellow streamers (*Chloroflexus* sp.). The mat was collected from Okukinu Meotobuchi Hot Spring in Japan (57°C, pH 7.0). The length of yellow streamers is about 10–15 mm.

and sulfide concentration (Castenholz, 1984; Jørgensen and Nelson, 1988; Castenholz and Pierson, 1995). The *Chloroflexus* mats appear in hot spring effluents as thin orange films or yellow-orange streamers formed on the surface of dark green cyanobacterial mats (Fig. 2).

In North America, *Chloroflexus* populations with cyanobacteria are found up to 70–72°C, and the lower temperature limits are 30–40°C, with a pH between 5.5 and 10 (Pierson and Castenholz, 1995). These conditions are nearly the same even in hot springs in other regions, such as Japan, Italy and Iceland. In the extensive survey in Japanese hot springs, *Chloroflexus* strains were isolated from hot spring water at temperatures of 50–70°C with pH of 6.4–8.2 (Hanada et al., 1995b). Pentecost investigated a microbial community in Italian hot springs with travertine deposits (Pentecost, 1995). *Chloroflexus* spp. were predominantly observed in these hot springs at 40–60°C and were isolated from 39.5–63.4°C (most abundantly from 55–60°C). Jørgensen and Nelson studied microbial mats in Icelandic sulfide-rich hot springs with pH of 7.5 and 8.5 (Jørgensen and Nelson, 1988; Keppen et al., 1994). They reported that *Chloroflexus* species were well developed at 55–66°C. Almost all researchers also mentioned observing these *Chloroflexus*/cyanobacterial mats in hot springs with sulfide concentrations under 100 μm. In natural hot springs, *Chloroflexus* spp. appear to grow photoheterotrophically or chemoheterotrophically by using substrates derived from cyanobacteria, since all isolated strains belonging to the genus *Chloroflexus* show good growth both in photoheterotrophic conditions (anaerobically in the light) and chemoheterotrophic conditions (aerobically in the dark).

However, several researchers have reported a *Chloroflexus* strain growing independently without cyanobacteria. A dark green mat of nearly pure *Chloroflexus* strains was found in Yellowstone National Park (Castenholz, 1988b). The hot water at that site contains a large amount of sulfide (up to 1000 μm), and this high concentration prevents cyanobacteria from growing. This *Chloroflexus* strain, designated the "GCF strain," would grow photoautotrophically using sulfide as an electron donor in the hot springs. A physiological study of the GCF strain mat in the laboratory revealed that the strain showed sulfide-dependent photoautotrophic growth but no respiratory growth in the presence of oxygen. A similar case was reported in Nakabusa Hot Spring, Nagano Prefecture, Japan (Sugiura et al., 2001), in which the authors found pure *Chloroflexus* mats devoid of cyanobacteria. This type of mat was well developed at the high temperature zone (71–77°C; pH 8.5) preventing the growth of cyanobacteria (in Japan, no cyanobacteria are able to grow at a temperature of more than 70°C, although cyanobacteria commonly can be seen at up to 73–74°C in western North America). The growth of the *Chloroflexus* mats is considered to be sulfide-dependent and photoautotrophic, since the hot spring effluent includes sulfide. The high photoautotrophic activity, susceptibility to oxygen, and thermotolerance of these cyanobacteria-independent *Chloroflexus* mats make them clearly different from any known strains of *Chloroflexus aurantiacus* or *Chloroflexus aggregans*. Therefore, these autotrophic strains may be classified as a new species in the genus *Chloroflexus*, although they have yet to be axenically isolated or phylogenetically analyzed.

There is no evidence to support that *Chloroflexus* spp. grow in acidic (less than pH 6) or brackish-saline hot springs. In a survey of various hot springs over a wide area of Japan, no *Chloroflexus* mat was observed in such hot springs (Hanada et al., 1995b). In Japan, the organism also appeared to be absent in springs with high iron contents. However, Pierson and Parenteau (2000) found a *Chloroflexus* strain in an iron-depositing hot spring (Chocolate Pots, Yellowstone National Park). The hot water contained a large amount of ferrous iron (the concentration was more than 100 μm). The *Chloroflexus* strain formed a microbial mat with a cyanobacterium, *Synechococcus* sp., in the hot water stream at 49–54°C. Since the *Chloroflexus* mats contain abundant iron deposits, it is quite possible that the *Chloroflexus* strain oxidizes ferrous iron as an electron donor for photosynthesis, which is known to occur in some species of purple bacteria and green sulfur bacteria (Ehrenreich and Widdel, 1994; Heising et al., 1999).

In addition to the thermophiles, a mesophilic variety of *Chloroflexus aurantiacus*, called *Chloroflexus aurantiacus* var. "*mesophilus*," was also reported (Gorlenko, 1976; Pivovarova and Gorlenko, 1977). The variety grows in the temperature range of 10–40°C with an optimum at 20–25°C. The mesophile was isolated from mat communities in the bottom mud of freshwater lakes. Gorlenko (1976) mentioned that mesophilic *Chloroflexus* spp. are commonly observed in the anoxic zones of freshwater lakes.

Mesophilic Freshwater Forms (the Genera *Oscillochloris* and *Chloronema*)

Mesophilic freshwater species of the filamentous anoxygenic phototrophs, the genera *Oscillochloris* and *Chloronema*, have been observed mainly in anaerobic environments containing significant amounts of sulfide, such as algobacterial mats formed on mud surfaces or the anoxic zones of freshwater lakes. In their habitats, they are often associated with purple sulfur bacteria and green sulfur bacteria, *Beggiatoa* and *Oscillatoria*.

Almost no freshwater mesophiles have been isolated as pure cultures, and *Oscillochloris trichoides* is the sole species proposed and described on the basis of axenic cultures (the family Oscillochloridaceae was newly created for the organism; Keppen et al., 1994; Keppen et al., 2000). Although strains of *Oscillochloris trichoides* are basically freshwater forms, they have been observed in various fresh or brackish water environments, e.g., microbial mats in a sulfide-containing warm spring, shallow river water, a littoral estuary, and a brackish alkaline soda lake. The organism is a mesophile with optimal growth temperatures of 28–30°C, but it may occur in natural warm springs with temperatures up to 45°C. Another species, *Oscillochloris chrysea*, with wide filamentous morphology (the cell diameters are 4.5–5.5 μm) has been found on the surface of mud in freshwater streams with high organic content from domestic sewage effluents (Gorlenko and Pivovarova, 1977; Gorlenko, 1988).

Chloronema spp. have been observed below the chemoclines of freshwater lakes. One species in the genus, *Chloronema giganteum*, was found in the metalimnion and upper hypolimnion of shallow stratified freshwater lakes (Dubinina and Gorlenko, 1975; Gorlenko and Lokk, 1979b). The lake waters contained ferrous iron with high concentrations and no or low concentrations of hydrogen sulfide. Borrego et al. (1998) studied natural populations of anoxygenic phototrophs in the anoxic hypolimnion of 17 holomictic lakes in the United States and Spain and indicated that *Chloronema* spp. were predominant in 13 of those lakes during summer stratification.

Chlorosome-less Filaments (the Genera *Heliothrix* and *Roseiflexus*)

Possession of chlorosomes is one of the conspicuous features of most FAPs. However, the group also contains two photosynthetic species lacking chlorosomes, *Heliothrix oregonensis* and *Roseiflexus castenholzii*. Both species are thermophiles and have been isolated from hot springs. *Heliothrix oregonensis* is found in some hot springs in western North America at temperatures up to 55°C, at approximately pH 8.5, without hydrogen sulfide (Pierson et al., 1984a; Pierson et al., 1985b). In the natural hot springs, the organism forms bright orange masses on the layer of cyanobacteria. Puffs and tufts often form on the surface of its mat where it is exposed to oxygen (Castenholz, 1988b). *Heliothrix oregonensis* is impressively dominant in small alkaline pools at the Warm Springs Indian Reservation (Oregon), and an apparently identical organism is also common in several alkaline hot springs in Yellowstone National Park.

Another chlorosome-less filament is an organism described as *Roseiflexus castenholzii* (Hanada et al., 2002b). Phylogenetic analyses and phenotypic comparisons revealed that the organism was not closely related to *Heliothrix oregonensis*, although both lack chlorosomes and contain Bchl *a* as the sole photosynthetic pigment. *Roseiflexus castenholzii* was first found in Japan's Nakabusa Hot Spring. The organism forms a distinct, dense red layer underneath the mats of cyanobacteria and *Chloroflexus* spp. The red layer is dominated by *Roseiflexus* filaments. In Nakabusa hot spring, the *Roseiflexus* mat is observed at temperatures of 45.5–68.5°C at pH 7.8–8.2 (Sugiura et al., 2001). The red mat is also observed in other Japanese hot springs, such as Meotobuchi (Tochigi Prefecture; 57°C, pH 6.8) and Atagawa (Shidzuoka Prefecture; 44°C, pH 8.0). The morphological and spectroscopic properties of the filaments in these hot springs are completely identical. Boomer et al. (Boomer et al., 2000; Boomer et al., 2002) reported on red mats in alkaline hot springs of Yellowstone National Park. The temperature and pH ranges of hot springs containing the mats were 30–50°C and 7.5–8.7, respectively. The phylogenetic analysis based on 16S rRNA sequence revealed that the Yellowstone filaments were closely related to *Roseiflexus castenholzii*, but there were clear differences between these filaments and those of *Roseiflexus castenholzii* in morphological and spectroscopic characteristics. These differences suggest that the Yellowstone filaments belong

not to *Roseiflexus castenholzii* but to a novel species related to it.

In addition, it was reported that a FAP containing only Bchl *a* was observed in hypersaline mats (the salinity of brine is about 8%). The organism was found in close association with a cyanobacterium, *Microcoleus chthonoplastes* (D'Amelio et al., 1987). Little is known about this hypersaline organism except for its morphological properties.

FAPs in Marine and Hypersaline Environments

Chlorosome-bearing filamentous bacteria have been frequently observed in marine or hypersaline environments (Pierson et al., 1994), although no pure culture has been isolated to date. In the marine intertidal environment, these organisms were most notably observed in association with species of *Thiocapsa*, *Chloroherpeton*, *Oscillatoria* and *Beggiatoa* (Mack and Pierson, 1988). It was demonstrated that these FAPs showed diel migration in the intertidal microbial mats. During the day, these filaments were found below the layer of a purple sulfur bacterium, *Thiocapsa* sp.; at night, they migrated to the surface. The migration could be induced artificially by changing the intensity of the illumination (Mack and Pierson, 1988).

A lot of FAPs have been found globally in hypersaline environments, such as evaporation ponds of salterns. Stolz has identified the organisms involved in hypersaline microbial mats in Laguna Figueroa, Baja California, Mexico (Stolz, 1983; Stolz, 1984; Stolz, 1990) by transmission electron microscopy. Using similar methods, D'Amelio et al. (1989) confirmed their occurrence in Guerrero Negro in Baja California and in Solar Lake, on the Sinai Peninsula of Egypt. Venetskaya and Gerasimenko (1988) also detected them in the hypersaline lagoons near the Sea of Azov, Ukraine.

The FAPs formed olive-green layers within the hypersaline microbial mats. The mats were composed of cyanobacteria, *Beggiatoa*, and some purple sulfur bacteria. The mats occurred in evaporation ponds at 18–21°C and pH 7.8–8.4. Temperatures in the mats themselves often exceeded 30°C, and they reached 40°C in summer. The salinities ranged from about 7 to 14% (Pierson et al., 1994).

These filaments were identified only morphologically, but the pigment analysis on the mats in Guerrero Negro revealed the presence of γ-carotene in particular layers (Palmisano et al., 1989); this carotenoid is typical of FAPs. The molecular analysis of hypersaline mats in Guerrero Negro by Nübel et al. (2001) suggested that these filaments were obviously related to the FAPs. In addition, uptake experiments using ^{14}C-labeled bicarbonate indicated that marine filaments clearly showed the light-dependent assimilation of carbon (Pierson et al., 1994).

Isolation

Of the filamentous anoxigenic phototrophs (FAPs), the following species have been isolated as axenic monocultures or as dual-cultures with a nonphototrophic bacterium: *Chloroflexus aurantiacus* and *Chloroflexus aggregans* isolated from hot springs; *Oscillochloris trichoides*, a mesophile from freshwater environments; and *Heliothrix oregonensis* and *Roseiflexus castenholzii*, chlorosome-less thermophiles from hot springs. In this section, we show mainly the procedures of enrichment, isolation, cultivation, and maintenance of these isolated species (especially the genus *Chloroflexus*, the first discovery among the FAPs). Also, several researchers have succeeded in enriching other FAPs in fresh and saline habitats, although no axenic culture has been obtained. We therefore provide helpful information for enriching these other FAPs in freshwater, marine and hypersaline environments.

The Genus *Chloroflexus*

Facultative Thermophilic Species: Enrichment and Isolation Almost all strains that have been isolated axenically are able to grow photoheterotrophically and chemoheterotrophically and inhabit microbial mats in hot springs with cyanobacteria. Microbial mats formed in hot springs (at approximately 30–70°C) should be collected in vials along with the hot water and brought to the laboratory at normal temperature (12–25°C) in darkness. The collected *Chloroflexus* strains retain their viability under these conditions. Cooler or warmer temperatures, or light irradiation, will usually shorten the survival time. Freezing as well as extensive exposure to oxygen should be avoided. Enrichment and isolation of these *Chloroflexus* strains are generally done according to the following procedures.

Co-enrichment with cyanobacteria is a simple and usually successful method. A sample of a microbial mat is inoculated and incubated in a liquid medium for cyanobacteria, e.g., D medium. The incubation is done in a cotton-plugged flask under low-intensity light (1000–3000 lux; fluorescent or incandescent bulbs) at an appropriate temperature (e.g., 45–55°C). The one or more species of cyanobacteria grown in the medium will excrete organic compounds, which will support the heterotrophic growth of *Chloroflexus* strains. While these cultures also contain other non-phototrophic bacteria, the

coexistence of *Chloroflexus* strains with cyanobacteria is very stable.

A coculture can also be established on 1.5% agar-solidified D medium. Cyanobacteria grown on agar plates often glide out from the initial inoculum spot, sometimes forming fascicles or circling aggregates. These cyanobacteria may be transferred to fresh plates or to a similar liquid medium. The transferred cyanobacterial filaments invariably include *Chloroflexus* filaments. Occasionally, a bi-culture composed of a *Chloroflexus* strain and a cyanobacterium without other non-phototrophic contaminants may be obtained.

Using an agar medium like the one described above, the photoheterotrophic *Chloroflexus* strains may be simply isolated from these cocultures with cyanobacteria or directly from microbial mats even in hot springs. A small piece (4–16 mm^2) of the cocultured cyanobacteria or a collected mat sample is placed on 1.5% agar DG medium (D medium with 0.8 g/liter of glycylglycine as buffer; the pH is adjusted to 8.2 before autoclaving) and incubated at 45–50°C under illumination (fluorescent or incandescent bulbs; 10–100 W/m^2). After one to a few days, some of the cyanobacteria will have spread by gliding motility. *Chloroflexus* strains may be carried along with the cyanobacterial trichomes, since these trichomes generally glide more slowly than do cyanobacteria. However, wisps of gliding trichomes of *Chloroflexus* species will form frequently beyond the edge of the cyanobacteria (Fig. 3). These wisps of *Chloroflexus* strains may then be picked up with a watchmaker's forceps and streaked on a new DG plate that contains yeast extract (0.02–0.2 g/liter). An axenic culture can be obtained by repeatedly picking fine trichomes, if possible a single filament, from the tips of speading wisps and streaking them (Fig. 4).

For direct isolation of *Chloroflexus* strains, another method, using PE medium, was reported (Hanada et al., 1995b). PE medium is a newly designed medium containing acetate, glutamate, succinate, casamino acids, and yeast extract as carbon sources as well as some vitamins. As a buffering agent, phosphate is substituted for the glycylglycine used in previous methods for *Chloroflexus*, and the pH of the medium is adjusted to 7.5. Instead of the nitrate present in medium D, ammonium sulfate is added as a nitrogen source. The medium includes thiosulfate for possible utilization. The PE medium can therefore support the heterotrophic growth of *Chloroflexus* strains independent of cyanobacteria.

The collected bacterial mats may be dispersed and directly streaked on 1.5% agar plates of PE medium and then incubated at 55°C under incandescent light (30 W/m^2). Green or orange gliding trichomes will appear on the plate in a few days. The new medium, however, also will enrich aerobic or fermentative non-phototrophic bacteria. To selectively enrich *Chloroflexus* strains, agar plates should be incubated alternately under aerobic and anaerobic conditions after every transfer. *Chloroflexus* strains can sustain their growth under both conditions because they are generally facultative bacteria able to grow photoheterotrophically under anaerobic conditions and chemotrophically under aerobic conditions by respiration. An easy and convenient way to achieve anaerobic conditions is to use an oxygen absorber called "the Ageless S-100" (Mitsubishi Gas Chemical Co., Tokyo, Japan) with a sealed nylon bag. By the repetition of alternate aerobic and anaerobic culturing, bacteria other than

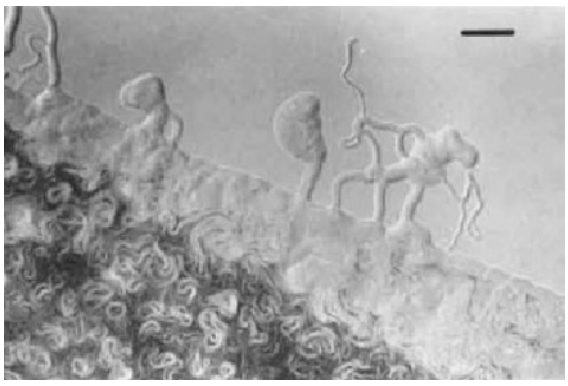

Fig. 3. Dual culture of *Chloroflexus aurantiacus* (NZ-Mar. 62-fl) and the cyanobacterium *Synechococcus lividus* (OH-53-s) on an agar surface (DG medium, 45°C). The dark area at the lower left is the population of *Synechococcus*. The lighter swirls and wisps of filaments advancing toward the upper right are *Chloroflexus*. Bar = 0.2 mm.

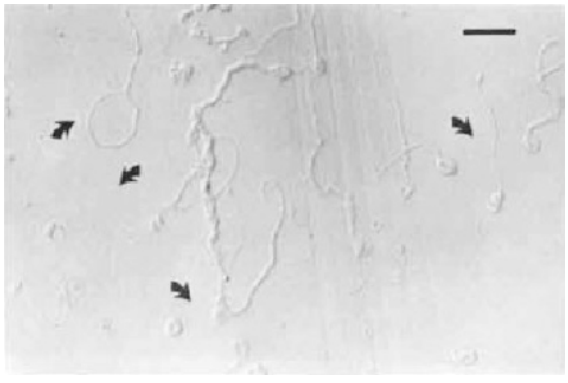

Fig. 4. Culture of *Chloroflexus aurantiacus* (OK-70-fl) on an agar surface at 45°C, 10 days after streak dilution. Single and double filaments are indicated by arrows. Bar = 0.2 mm.

Chloroflexus species will be efficiently removed. This is an efficient and reproducible method for isolating facultative *Chloroflexus* strains capable of growing both photoheterotrophically and chemoheterotrophically.

Facultative Thermophilic Species: Cultivation and Maintenance For the cultivation of *Chloroflexus* strains, variations of D medium are commonly used. D medium is prepared as a 20-fold concentrated stock (pH 3) and can be stored at 4°C without autoclaving. The culture medium (DG medium) is prepared by diluting the stock and adding glycylglycine (0.8 g/liter) as a buffer. Yeast extract (2.0 g/liter) and NH_4Cl (0.2 g/liter) are usually supplemented for liquid axenic cultures in screw-cap tubes or flasks. The pH should be adjusted to 8.0–8.2 with NaOH before autoclaving. To exclude nitrate, the base of D medium has also been modified (Castenholz, 1988a), since *Chloroflexus* strains require reduced forms of nitrogen (Brock, 1978) and the ND medium serves as an adequate substitute for D-based medium. In the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, the German Collection of Microorganisms and Cell Cultures), *Chloroflexus* strains are usually grown photoheterotrophically in D-based medium with 0.5 g of glycylglycine, 0.5 g of yeast extract, and 0.4–0.5 g of $Na_2S \cdot 9H_2O$ per liter. To eliminate oxygen, sodium sulfide is added from a separate sterile solution after autoclaving and cooling. In all cases, the *Chloroflexus* medium is adjusted to pH 8.2 with NaOH prior to autoclaving. For photoheterotrophic growth, screw-cap tubes or flasks should be filled to the top with preheated medium before incubation. All inoculated media are incubated and maintained at about 52–60°C under incandescent light (10–30 W/m^2). Cultures at 55°C should be transferred every 7–14 days. Agar-shake cultures (containing 1.0% agar) and agar plates (1.5% agar) are also used for maintaining these *Chloroflexus* species.

Facultative *Chloroflexus* strains are tolerant of oxygen and may also be grown as chemoheterotrophs by respiration. The same basic medium with yeast extract and casamino acids may be used with vigorous shaking or with an aeration system, using sterile-filtered line air. Aerobic growth has been sustained in DG medium plus 0.2 g of NH_4Cl, 0.5 g of yeast extract, 1.0 g of casamino acids, and 2.0 g of sodium acetate per liter (Sirevåg and Castenholz, 1979).

PE medium is another appropriate culture medium for all facultative *Chloroflexus* strains (Hanada et al., 1995b). The medium can be used for both photoheterotrophic and chemoheterotrophic culturing. *Chloroflexus* strains grow with a good rate and yield in the medium whether in the anaerobic condition in light or in the aerobic condition in the dark.

PE medium is also suitable for the maintenance of *Chloroflexus* cultures. The viability of most *Chloroflexus* strains can be extended to a few months by using PE medium as a maintenance medium in liquid anaerobic culture under low light intensity (less than 5 W/m^2) at 55°C. Under these conditions, *C. aurantiacus* J-10-fl survived in a screw-cap tube for 3 months with little degradation of its pigments.

Cultivation of Strictly Photoautotrophic Species For species that are strictly photoautotrophic, e.g., the GCF strain from sulfidic hot springs (Castenholz, 1973; Giovannoni et al., 1987), a sulfide-containing inorganic medium, e.g., DGN medium with $Na_2S \cdot 9H_2O$ (0.5 g/liter or about 2 mM final concentration) and $NaCO_3$ or $NaHCO_3$ (2.0 g/liter) as an inorganic carbon source (Madigan and Brock, 1977a; Brock, 1978) can be used. Since this physiological type of the genus *Chloroflexus* is very sensitive to oxygen, anaerobic procedures are necessary both for preparing media (e.g., purging with an anaerobic gas mixture) and for handling samples. Hungate tubes or other sealed anaerobic vessels with butyl rubber stoppers may be used for cultivation. Giovannoni et al. (1987) reported that the best liquid medium for photoheterotrophic growth was a DGN medium modified by adding 0.2–0.5 g of yeast extract and 1.0 g of D-glucose per liter. All strictly photoautotrophic cultures should be maintained at moderately thermophilic temperatures (45–55°C) in dim light (5–10 W/m^2).

Cultivation of a Mesophilic Variety Gorlenko (1976) succeeded in the enrichment of a freshwater, mesophilic variety of *Chloroflexus aurantiacus* found in lake-bottom water and sediment columns supplemented with Na_2SO_4 (0.6 g/liter), $Na_2S \cdot 9H_2O$ (0.1 g/liter), yeast extract (0.025 g/liter), and casein hydrolysate (0.025 g/liter) at a temperature of 25–30°C under illumination (<30 W/m^2). To isolate mesophilic strains, a technique similar to that used for thermophilic strains can be used, although Gorlenko (1976) did not explain how he isolated the mesophile from an initial enrichment. A mesophilic *Chloroflexus* strain was cultured (Pivovarova and Gorlenko, 1977) within soft agar medium (0.4%) with mineral salts and supplemented with 0.4 g of Na_2SO_4, 0.5 g of tryptone, and 0.5 g of yeast extract per liter (pH 7.0). The culture grew in the temperature range of 10–40°C. Pivovarova and Gorlenko (1977) maintained the mesophilic strain in soft-agar (0.4%) shake cultures in cotton-plugged tubes that were kept illuminated at approximately 30 W/m^2 and warmed to 28°C.

Other Information Other information useful for enrichment, isolation, and maintenance is as follows. First, the addition of 3,4-dichlorophenyl-1,1-dimethylurea (DCMU) to 5–10 μm is a good way to selectively enrich *Chloroflexus* strains without cyanobacteria or to remove cyanobacteria from mixed cultures, because the chemical compound prevents the cyanobacterial oxygenic photosynthesis. Second, manually picking an individual filament makes it possible to obtain a single clone of a strain. Finally, a dual culture with a cyanobacterium may offer the advantages of safe and stable maintenance of *Chloroflexus* strains.

The Genus *Oscillochloris* (the family Oscillochloridaceae)

Strain SR-1, the original type strain of *Oscillochloris trichoides*, was isolated from algobacterial mats by serial dilutions in test tubes (Gorlenko and Korotkov, 1979a). The isolation medium was a slightly modified version of a medium commonly used for green sulfur bacteria. The modified medium contained the following compounds: additional anhydrous Na_2SO_4, 330 mg/liter; yeast extract, 25 mg/liter; tryptone, 25 mg/liter; vitamin B_{12}, 20 μg/liter; $Na_2S \cdot 9H_2O$, 300 mg/liter; agar, 0.2–0.4%; and some trace elements (pH 7.5). The incubation was done at 25–35°C under a light intensity of 2000 lux. By serial dilutions (3–4 times), associated phototrophic bacteria, mainly purple nonsulfur bacteria, were removed and mono-phototrophic cultures were established. The cultures still contained some non-phototrophic contaminants, including facultative anaerobic fermenting bacteria and sulfur- and sulfate-reducers.

Whereas the enrichment of strain SR-1 was subsequently lost, another strain, DG-6, was isolated in pure culture and was described as the neotype strain of *Oscillochloris trichoides* (Keppen et al., 1993; Keppen et al., 1994). Following this description, further related strains have been isolated from fresh- and brackish-water environments (Keppen et al., 2000). A slightly modified DGN medium (see above) was used for the isolation of these strains: Nitrates were omitted, and KH_2PO_4 (1 g/liter), 0.2–0.25% $NaHCO_3$, 0.02–0.1% $Na_2S \cdot 9H_2O$ and a vitamin mixture (Madigan et al., 1974) were added (occasionally 0.25% Na_2CO_3 was also added). The pH was adjusted to 8.2–9.0. Cultures were inoculated with bacterial mats from warm springs, river water, or lake water and were incubated under strictly anaerobic conditions at 28°C with illumination of 1000 lux.

All strains of *Oscillochloris trichoides* are maintained in the same medium and routinely cultivated in screw-cap bottles completely filled with medium under the same conditions as those used in the isolation. These strains are basically photoautotrophic growers, but their growth is promoted by the addition of some organic compounds, i.e., acetate, pyruvate, casein hydrolysate, or yeast extract.

Another species in the genus, *Oscillochloris chrysea*, has not been grown in semisolid agar media, thus no pure culture of this species has been obtained.

The Genus *Chloronema*

Although *Chloronema* spp., which are Bchl *d*-containing mesophilic organisms, generally dominate in the hypolimnion of freshwater lakes, no pure culture has been established and knowledge about cultivation of those filaments is also very limited. Recently, Gich et al. (2001) reported on the phylogenetic analysis of an enrichment of *Chloronema* established by Rémy Guyoneaud. They have also obtained different enrichments from Little Long Lake, Wisconsin, and are studying properties of the *Chloronema* enrichments (F. Gich, personal communication).

The Genus *Heliothrix*

Heliothrix oregonensis was isolated from microbial mats at the Warm Springs Indian Reservation, Oregon (Pierson et al., 1984a; Pierson et al., 1985b). The chlorosome-less filamentous organism is closely associated with a nonphotosynthetic aerobic bacterium, *Isosphaera pallida* (belonging to the family Planctomycetaceae in the phylum Planctomycetes). The organism can, therefore, be brought into coculture with *I. pallida*. The medium used for isolation and enrichment is IMC medium (Pierson et al., 1984a) containing 0.025% glucose and 0.025% casamino acids as energy sources, along with some vitamins. *Heliothrix* filaments can be collected from the orange-red microbial mat by a syringe with a 17- or 18-gauge needle. The collected material should be washed immediately to remove unicellular cyanobacteria and other non-phototrophic cells. Since the filaments are large enough to be seen with a dissecting microscope at high resolving power, they can be picked up manually. A single, axenic filament picked up is then inoculated into a pure culture of *I. pallida* (strain IS-1B) on an agar plate and incubated aerobically at 45°C in the light.

The coculture can be maintained on slants or plates of agar-solidified IMC medium (pH 8.1) at 45°C under a moderate light intensity (3000 lux) in an atmosphere of 95% air and 5% CO_2. The culture can be maintained by monthly transfers.

The Genus *Roseiflexus*

Another chlorosome-less filamentous anoxygenic phototroph, *Roseiflexus castenholzii*, is a facultative bacterium able to grow photoheterotrophically in the light and chemoheterotrophically in the dark; it forms a distinct red layer within the microbial mats (Hanada et al., 2002b). The red layer is composed predominantly of the organism, whereas other phototrophs, i.e., cyanobacteria and *Chloroflexus* spp., are rarely found in this layer. A collected red layer is cut into a small piece (about 100 mm^3) and gently dispersed with a sterile homogenizer in buffer. The moderately dispersed filaments are then washed with sterile buffer and harvested by low-speed centrifugation. Most non-phototrophic unicellular contaminants may be removed by repeating this rinse process a few times. The rinsed *Roseiflexus* fragments are inoculated on PE medium (pH 8.0) solidified with 1.5% agar (see "The Genus *Chloroflexus*" in this section) and anaerobically incubated at 50°C in incandescent light (30 W/m^2). Red to reddish-brown colonies may be formed on the plate after a few weeks of incubation. The colonies may still contain non-phototrophic contaminants, e.g., thermophilic *Bacillus* spp. and *Thermus* spp. These contaminants can be removed by alternating incubation under aerobic and anaerobic conditions after every transfer (see "The Genus *Chloroflexus*" in this section).

Roseiflexus castenholzii is cultivated and maintained in the same PE medium in screw-cap bottles under incandescent light (30 W/m^2) or in flasks shaken vigorously (200 rpm) in the dark at 50°C. The pH of the medium should be adjusted to 7.5. Also, 02YE medium (pH 7.5) has been used to culture the strain. This medium contains 0.2% yeast extract, 0.38 g of KH_2PO_4, 0.39 g of K_2HPO_4, 0.5 g of $(NH_4)_2SO_4$, 1 ml of a vitamin mixture, and 5 ml of a basal salt solution per liter. The vitamin mixture and basal salt solution are the same as those used in PE medium. The aerobic and anaerobic cultures should be transferred every week and two weeks, respectively.

Enrichments of Marine and Hypersaline FAPs

No axenic culture of marine and hypersaline filamentous anoxygenic phototrophs (FAPs) has yet been obtained. Likewise no general, standard methods to enrich them have been established. However, several mixed cultures containing these organisms have been enriched and maintained for several years (Pierson et al., 1994). A marine FAP from an intertidal habitat on Mellum Island, Germany, was maintained in a medium of normal seawater salinity. The medium is modified from the standard medium for marine purple and green sulfur bacteria (Pfennig and Trüper, 1992). The modification includes the addition of acetate and yeast extract and the omission of sulfate. Aluminum phosphate is also added to the medium as an artificial sediment for the adhesion of gliding bacteria (Widdel and Bak, 1992). Hypersaline FAPs from evaporation ponds in salterns with various salinities have also been enriched successfully. The compositions of media used in successful enrichments vary, since they should be fitted to the chemical characteristics of their habitats. Most media, however, are based on the medium designed for the marine FAP mentioned above. The hypersaline FAP cultures obtained from microbial mats in Baja California have been enriched in a similar medium with additional NaCl (the final concentrations are approximately 13–16%). Some hypersaline FAPs can also grow photoautotrophically in the presence of sulfide. For such strains, the range of sulfide concentrations is 0.25–10 mM, and growth is optimized in 2.5 mM sulfide. The optimal temperature for growth is 35–40°C, and no growth has been observed above 42°C. The cultures grow best under dim incandescent light (10 W/m^2). More details on the cultivation of marine and hypersaline FAPs are given in Pierson et al. (1994).

Nübel et al. (2001) reported the successful enrichment of hypersaline FAPs from evaporation ponds in Guerrero Negro, Baja California, using a different medium. In that case, they used the inorganic medium for cyanobacteria (Garcia-Pichel et al., 1998) containing 9% commercial sea salts. The medium was also modified by adding NH_4Cl (200 mg/liter), yeast extract (250 mg/liter), $Na_2S_2O_4$ (70 mg/liter), $Na_2S \cdot 9H_2O$ (150 mg/liter), DCMU (1.2 mg/liter), and resazurin (0.0001%) as a redox indicator. The incubation was done anaerobically at room temperature (20–25°C) under natural daylight. They observed that Bchl *c*-containing gliding filaments with a diameter of about 1 μm were grown in the enrichments. However, no growth was seen after subsequent transfers to fresh medium, and no cultures have been maintained.

Identification

All filamentous anoxygenic phototrophs (FAPs) have the following features: 1) unbranched multicellular filamentous morphology; 2) gliding motility; and 3) anoxygenic photosynthetic activity. Currently, an organism with this combination of features would be classified as a FAP, possibly in the family Chloroflexaceae or the family Oscillochloridaceae. The differential characteris-

tics of valid genera and species belonging to the FAPs are summarized in Table 1. The five FAP genera can be differentiated from each other by their pigmentation, morphology, and growth temperatures. Of these FAP genera, the presence of chlorosomes is a notable trait in the three genera *Chloroflexus*, *Oscillochloris* and *Chloronema*. These chlorosome-containing FAPs also include a typical carotenoid, β-carotene, as well as Bchl *c* (or *d* in the genus *Chloronema*) as photopigments, while no β-carotene is detected in the chlorosome-less FAPs (the genera *Heliothrix* and *Roseiflexus*). The chlorosome-containing FAPs can be classified further into two groups, depending on their growth temperatures: the genus *Chloroflexus* on the one hand and, on the other, the mesophilic freshwater forms. *Chloroflexus* consists of thermophiles that grow well at 55°C, with the sole exception of *Chloroflexus aurantiacus* var. "*mesophilus*" (Dubinina and Gorlenko, 1975). In spite of the difference in growth temperature, the mesophilic variety of *Chloroflexus* is more similar morphologically and physiologically to the thermophilic *Chloroflexus* species than to the mesophilic freshwater genera, i.e., both mesophilic and thermophilic *Chloroflexus* species have slender filaments, respond similarly to oxygen, and lack gas vesicles. On the other hand, the mesophilic freshwater forms, which include the genera *Oscillochloris* and *Chloronema* species, have relatively thick morphologies and intracellular gas vesicles and in general are not highly tolerant to oxygen. Further information on identification and classification within these subgroups and genera is provided below.

The Genus *Chloroflexus*

The genus *Chloroflexus* contains two species, *Chloroflexus aurantiacus* and *Chloroflexus aggregans*. *Chloroflexus aurantiacus* is the type species of this genus, and almost all strains in the species are thermophilic filamentous bacteria with a diameter of about 1 μm. They are generally found in neutral to alkaline hot springs, where they form microbial mats with or without cyanobacteria. *Chloroflexus aurantiacus* contains Bchl *a* and *c* as photosynthetic pigments, and the latter is present in intracellular vesicles (i.e., chlorosomes). The organism has a conspicuous peak at 740 nm in the in vivo absorption spectrum of the near infrared (IR) region that is attributable to chlorosomes (Bchl *c*) and also shows absorption peaks at 808 and 868 nm in the near IR region that are due to Bchl *a*. It contains β- and γ-carotene and OH-γ-carotene glucoside esters as carotenoid pigments. *Chloroflexus aurantiacus* can grow photoheterotrophically in the light and chemoheterotrophically in the dark. Some strains, e.g., strain OK-70-fl, are able to grow photoautotrophically by fixing carbon dioxide as the sole carbon source in the presence of sulfide. The respiratory quinone of the species is predominantly menaquinone (MK)-10, and the major cellular fatty acids are palmitic ($C_{16:0}$), stearic ($C_{18:0}$), and oleic acids ($C_{18:1}$). The mol% G+C of the DNA is 53.1–54.9% (by buoyant density [Bd]) or 56.9–57.1% (by high pressure liquid chromatography [HPLC]).

Another species, *Chloroflexus aggregans*, is phenotypically very similar to the type species. However, there are clear differences between the two species: 1) the cells of *Chloroflexus aggregans* are wider than those of *Chloroflexus aurantiacus*; 2) *Chloroflexus aggregans* contains mainly γ-carotene and its derivatives, with only trace amounts of β-carotene, whereas β-carotene is a major component in *Chloroflexus aurantiacus*; 3) *Chloroflexus aggregans* includes a small but significant amount of MK-4 in addition to the dominant homologue MK-10. Besides these phenotypic differences, the sequence similarity of 16S rRNAs between the two species is very low (92.8%), warranting classification as different species.

The Genera *Oscillochloris* and *Chloronema*

The mesophilic freshwater forms, the genera *Oscillochloris* and *Chloronema*, are classified mainly according to their morphological and pigmentation properties. Like *Chloroflexus* species, the *Oscillochloris* species are straight flexible filaments containing Bchl *c* as the major light-harvesting pigment. The *Oscillochloris* filaments have no sheath or a thin sheath. Another genus, *Chloronema*, consists of members containing Bchl *d* as the major light-harvesting pigment. The *Chloronema* filaments are often spirally twisted and thickly sheathed.

The Genus *Oscillochloris*

Of these mesophilic freshwater forms, *Oscillochloris trichoides* is the sole species whose description is based on axenic cultures. *Oscillochloris trichoides* strains have been isolated from fresh- and brackish-water environments. The filaments are 0.8–1.4 μm wide. The filaments may or may not have a thin sheath, or they may be surrounded by a thin layer of slime. Intracellular gas vesicles are formed along the cell septa (cross-walls). The organism is a mesophile grown optimally at 28–30°C, at pH 6.8–9.0. The type strain is strictly anaerobic and obligately phototrophic. Its growth under photolithoautotrophic conditions can be detected in the presence of sulfide and/or molecular hydrogen as the electron donors. The optimal concentration of sulfide ($Na_2S \cdot 9H_2O$) is 0.5–0.7 g/liter. The in vivo absorption spectra of cell suspen-

sions have maxima at 748 and 852 nm in the IR region. The photosynthetic pigment is Bchl *c*, with a small amount of Bchl *a*. *Oscillochloris trichoides* also contains β-carotene and γ-carotene. The predominant quinone is MK-10. The mol% G+C of the DNA is 59.2% (by the melting temperature method [Tm]).

A different species, *Oscillochloris chrysea*, was also proposed within the genus. The organism has a large cell morphology (4.5–5.5 μm wide). The cell wall has no outer membrane but a thick peptidoglycan layer, and the cells are stained Gram positive. Phototrophic and probable chemotrophic growth occurs. The organism develops at 10–20°C, at pH 7.5–8.5. It contains Bchl *a* and *c*, as well as some carotenoids. Since no pure culture is available, little is known about other physiological and chemotaxonomic properties of this species.

The Genus *Chloronema* *Chloronema* species are gas-vesicle-containing straight or spiral-shaped filaments found in hypolimnion-metalimnion of freshwater stratified lakes. The notable feature of these filaments is the presence of Bchl *d*, which is not found in any other FAPs. An in vivo absorption peak of chlorosomes in this group is obviously blue shifted (720 nm). This genus contains two species, *Chloronema giganteum* and *Chloronema spiroideum*. Since neither species has been isolated or cultivated successfully, the classification is based solely on morphology. *Chloronema giganteum* has a filamentous morphology with a diameter of 2.0–2.5 μm, while the diameter of *Chloronema spiroideum* filaments is slightly smaller (1.5–2.0 μm).

The Genera *Heliothrix* and *Roseiflexus*

The chlorosome-less FAPs consist of two genera (*Heliothrix* and *Roseiflexus*) that are phylogenetically distant from each other (the sequence similarity of 16S rRNA is 78.6%). Despite this distance, the pigment profiles of these two genera resemble each other. Both contain Bchl *a* as the sole photosynthetic pigment, and both lack any other bacteriochlorophylls. They have mainly γ-carotene derivatives (precisely, keto-3,4-didehydro-OH-γ-carotene; myxocoxanthin) as major carotenoids and, unlike the chlorosome-containing filaments, contain no β-carotene. Each of these chlorosome-less filaments inhabits hot springs and forms distinct red or red-orange microbial mats in thermal environments. Other characteristics of these organisms appear below.

The Genus *Heliothrix* The organism *Heliothrix oregonensis* was isolated as a stable coculture with a non-phototrophic aerobic bacterium, *Isosphaera pallida*. The coculture could grow at 40–55°C. The growth was supported by mainly photoheterotrophic metabolism. The diameter of *Heliothrix* filaments is 1.5 μm, and the septa (cross-walls) can be seen with a phase-contrast microscope. The cells frequently contain large amounts of polyhydroxybutyrate granules. The in vivo absorption spectrum has conspicuous peaks at 795 and 865 nm (due to Bchl *a*) in the IR region.

The Genus *Roseiflexus* *Roseiflexus castenholzii* is a filament (diameter of 0.8–1.0 μm) and a thermophilic facultative phototroph that grows at 45–55°C and pH 7–9. The organism grows photoheterotrophically under anaerobic and light conditions, and aerobic growth in the dark occurs at full atmospheric oxygen tension. Neither photoautotrophic nor fermentative growth is observed. No invagination of intracytoplasmic membrane is present. The cell suspension shows absorption maxima at 801 and 878 nm in the IR region. Pigmentation occurs under both aerobic and anaerobic conditions. The cellular fatty acids consist of $C_{16:0}$, $C_{14:0}$, and $C_{15:0}$, and no unsaturated fatty acids are detected. The respiratory quinone system is occupied predominantly by MK-11. The mol% G+C of the DNA is 62.0% (HPLC).

Preservation

All strains deposited in the German culture collection (DSMZ) and belonging to *Chloroflexus aurantiacus* or *Chloroflexus aggregans* can be preserved in liquid nitrogen (–196°C) under aerobic conditions when 5% dimethylsulfoxide, 5% methanol, or 10% glycerol is used as a cryoprotectant (Malik, 1998). The cultures are also successfully freeze-dried by avoiding exposure to oxygen during the process. Cultures thus preserved show good survival rates after drying and in long-term storage.

Little information is available about the long-term preservation of other FAPs, but *Roseiflexus castenholzii* can be safely stored in 15% glycerol at –80°C for at least a year.

Physiology

Among the filamentous anoxygenic phototrophs (FAPs), physiological information centers mostly on *Chloroflexus aurantiacus*, since the organism was identified early and the first to be isolated as a pure culture. In this section, we present a number of physiological findings of the extensive range of studies on *Chloroflexus aurantiacus*. We also mention important physiological properties that have been investigated in

several species, i.e., *Chloroflexus aggregans*, *Heliothrix oregonensis*, *Oscillochloris trichoides* and *Roseiflexus castenholzii*.

Autotrophy

While *Chloroflexus aurantiacus* generally grows well under photoheterotrophic conditions, some strains in this species can grow photoautotrophically. Madigan and Brock (1975) revealed that *Chloroflexus aurantiacus* strain OK-70-fl could oxidize sulfide photoheterotrophically, with fixing of carbon dioxide. Photoautotrophic growth is also achieved with hydrogen as an electron donor instead of sulfide (Holo and Sirevåg, 1986). In the *Chloroflexus aurantiacus* strain OK-70-fl, the unique 3-hydroxypropionate cycle is operated for fixing carbon dioxide (Sirevåg and Castenholz, 1979; Holo, 1989; Strauss et al., 1992; Eisenreich et al., 1993; Strauss and Fuchs, 1993). Glyoxylate is formed from acetyl-CoA after the fixation of two molecules of CO_2 by two respective enzymes, acetyl-CoA and propionyl-CoA carboxylases, while acetyl-CoA is regenerated in this cycle (Menendez et al., 1999). Another fixing cycle, the reductive dicarboxylic acid cycle, is also proposed in a different *Chloroflexus* strain (Ivanovsky et al., 1993). Similar CO_2 fixing pathways are widely found in autotrophic *Crenarchaeota*, e.g., *Sulfolobus*, *Metallosphaera* and *Acidianus* (Menendez et al., 1999).

Although photoautotrophic growth of the *Chloroflexus* strain is a very slow process, this type of metabolism apparently occurs in some hot springs (Castenholz, 1973; Giovannoni et al., 1987). The GCF strain, a strain that grows without cyanobacteria in natural hot springs, shows a high level of sulfide-dependent phototrophic activity even in the laboratory. The GCF strain is unable to grow by respiration. The sulfide- or hydrogen-dependent photoautotrophy seen not only in GCF strain but also in *Chloroflexus aurantiacus* strain OK-70-fl is not observed in any other strains, e.g., strain J-10-fl, the type strain of *Chloroflexus aurantiacus*, or in any strains of the closely related species, *Chloroflexus aggregans*. The inability of the GCF strain to respire may be due to its adaptation to anaerobic environments without oxygenic cyanobacteria. Alternatively, the other *Chloroflexus* strains may have acquired a respiratory ability and high tolerance to oxygen after living in environments containing cyanobacteria.

The freshwater mesophile *Oscillochloris trichoides* can grow photolithoautotrophically using sulfide or hydrogen as an electron donor and carbon dioxide as a carbon source (the preferable mode is a photolithoheterotrophic growth in the presence of some organic acids). Because the type strain of this species (strain DG-6) clearly shows activities of ribulosebisphosphate carboxylase and phosphoribulokinase, it appears to fix carbon dioxide by the Calvin-Bassham-Benson cycle (Ivanovsky et al., 1999). The fixing cycle is generally found in autotrophs belonging to the Proteobacteria and cyanobacteria.

Roseiflexus castenholzii, one of the chlorosome-less FAPs, is a "true" heterotroph and requires yeast extract for good phototrophic growth. No photoautotrophic growth has been observed in any cultural conditions (Hanada et al., 2002b).

In marine and hypersaline FAPs, light-dependent uptake of bicarbonate was reproducibly demonstrated in situ and in the laboratory (Pierson et al., 1994).

Heterotrophy

Almost all strains in the genus *Chloroflexus* can grow heterotrophically using various organic compounds (a number of sugars, organic acids, and amino acids) under either aerobic (in the dark) or anaerobic (in the light) conditions in the presence of folic acid and thiamine as growth factors and in the presence of inorganic carbon (Madigan et al., 1974; Pierson and Castenholz, 1974a; Pierson and Castenholz, 1974b; Hanada et al., 1995a). The complex organic substrates (yeast extract and casamino acids) can substantially support the growth of both under either condition.

In *Chloroflexus aurantiacus*, the tricarboxylic acid (TCA) cycle appears to operate under either aerobic or anaerobic conditions, either to supply reductant for respiration or to support the reductive assimilation of substrates, respectively (Sirevåg and Castenholz, 1979). Krasil'nikova et al. (1986) found all enzymatic activities for the complete TCA cycle, whereas the activity was low for only a single enzyme, α-keto-glutarate dehydrogenase. The organism also contains the two key enzymes of the glyoxylate shunt, isocitrate lyase and malate synthetase, and both activities are increased when cells grow on acetate (Løken and Sirevåg, 1982; Krasil'nikova et al., 1986).

Chloroflexus aurantiacus has the Embden-Meyerhof pathway for glycolysis (Krasil'nikova et al., 1986; Krasil'nikova and Kondrat'eva, 1987b). The cells grown on glucose showed higher activities of phosphofructokinase and fructose-diphosphate aldolase than those grown on acetate. Two enzymes in the pentose monophosphate pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, were also detected (Krasil'nikova et al., 1986). Some strains can grow by fermentation of sugar or pyruvate (Krasil'nikova and Kondrat'eva, 1987b). End products of the fermenta-

tion on glucose are acetate, pyruvate, lactate, malate, ethanol, and occasionally formate (Krasil'nikova and Kondrat'eva, 1987b).

In the presence of sulfide and bicarbonate, *Oscillochloris trichoides* can grow photolithoheterotrophically on acetate, pruvate, casein hydrolysate, or yeast extract (Keppen et al., 2000). However, it can use only a limited number of organic compounds because it lacks a complete TCA cycle and a glyoxylate shunt. The organism lacks activities of 2-oxoglutarate hydrogenase and isocitrate lyase, which are key enzymes in the cycle and shunt. *Oscillochloris trichoides* is also incapable of chemoheterotrophic growth under aerobic or microaerobic conditions in the dark.

Roseiflexus castenholzii can grow photoheterotrophically and chemoheterotrophically. Yeast extract is a good substrate for growth under either aerobic or anaerobic conditions. Citrate, glucose, and casamino acids are each also used as a sole carbon and energy source, though only under anaerobic light conditions, but the cell yields are clearly less than that obtained with yeast extract.

Nitrogen Metabolism

Chloroflexus aurantiacus grows well when ammonia is the sole nitrogen source, but nitrate does not support its growth (Heda and Madigan, 1986). Heda and Madigan (1986) also reported that several amino acids (alanine, aspartate, glutamate, glutamine, glycine and serine) supported good growth in all *Chloroflexus* strains tested. Cysteine supported good growth for most strains. Tyrosine and valine supported growth for all strains but with lower growth yields than those supported by the amino acids listed above. Proline, adenine, and urea did not serve as nitrogen sources for any of the strains tested. Neither nitrogen fixation nor nitrogenase activity was detected in any strains of *Chloroflexus aurantiacus* (Heda and Madigan, 1986).

The pathways of nitrogen metabolism in this organism are partially understood. Ammonia assimilation apparently proceeds differently in *Chloroflexus aurantiacus* than in other phototrophic green or purple bacteria. The only enzyme present for ammonia assimilation is a glutamine synthetase, which is not repressed when cells are grown in high-ammonia culture media (Kaulen and Klemme, 1983). Although the glutamate synthase (GOGAT) generally collaborated with glutamine synthetase in assimilating ammonia, this enzyme was not detected in the same preparation. This may be due to problems of preparation (Klemme, 1989). Details of this aspect of nitrogen assimilation remain to be clarified.

In *Chloroflexus aurantiacus*, glutamate, alanine, and isoleucine are the main constituents of the intracellular amino acid pool in cells grown photosynthetically in minimal medium (Klemme et al., 1988). Two different enzymes with L-threonine (L-serine) dehydratase activity were found in *Chloroflexus aurantiacus* (Laakmann-Ditges and Klemme, 1986). One was sensitive to inhibition by isoleucine and could be biosynthetic for isoleucine, since the other key enzymes for its biosynthesis (asparto-kinase, homoserine dehydrogenase, and acetohydroxy acid synthase) were also present in cells grown photoautotrophically (Laakmann-Ditges and Klemme, 1988).

Although nitrogen metabolism has been little investigated in other FAPs, nitrogenase activity has been detected in *Oscillochloris trichoides* by an acetylene reduction assay (Keppen et al., 1989). This finding suggests that the freshwater mesophile fixes molecular nitrogen for its growth. The organism can also use ammonium salts, urea, glycine, glutamate, glutamine, asparagine, and casein hydrolysate as nitrogen sources.

Sulfur Metabolism

Chloroflexus aurantiacus can use sulfate as a good source of sulfur for biosynthesis, and thiosulfate can also be used during photoheterotrophic growth (Krasil'nikova, 1987a; Kondrat'eva and Krasil'nikova, 1988). Under photoheterotrophic conditions, cysteine, glutathione, methionine, or sulfide can serve as a sulfur source (Krasil'nikova, 1987a). With sulfate as a sulfur source, high levels of ATP sulfurylase activity were found, with optimal activity at 60–70°C (Krasil'nikova, 1987a).

The utilization of sulfur compounds as electron donors in photosynthetic growth has been reported by Madigan and Brock (1975) and Giovannoni et al. (1987). In some strains of *Chloroflexus aurantiacus*, sulfide was oxidized to elemental sulfur both photoautotrophically and photoheterotrophically. The produced sulfur was accumulated outside the cells. Sulfur granules affixed to the cells were observed frequently with a microscope.

Oscillochloris trichoides can also use sulfide as an electron donor for photosynthesis (Keppen et al., 1993). The oxidation product of sulfide is elemental sulfur, and further oxidation of sulfur does not occur. The organism utilizes sulfide, cystine, and cysteine as sources of sulfur. Sulfate, however, does not support its growth (Keppen et al., 2000).

Sulfide also appears to support photoautotrophy even in marine and hypersaline FAPs (Pierson et al., 1994).

Motility

Gliding motility, a feature common to all FAPs, is a way of moving on a solid or semisolid surface without flagella-like propulsive organs (Castenholz, 1982). It is a smooth movement observed typically among other multicellular filamentous bacteria (e.g., cyanobacteria and flexibacteria) or, sometimes, unicellular organisms (e.g., cytophaga and myxobacteria). The gliding rates vary among the FAPs: *Chloroflexus aurantiacus* glides at a rate of 0.01–0.04 μm/sec on a 1.5% agar surface (Pierson and Castenholz, 1974a); *Oscillochloris trichoides* glides at a rate of 0.2 μm/sec (Keppen et al., 1993); *Chloronema* species are fast gliders, achieving speeds of about 10 μm/sec (Gorlenko and Pierson, 2001); *Heliothrix oregonensis* exhibits a gliding rate of 0.1–0.4 μm/sec (Pierson et al., 1985b). Gliding motility enables the organisms to form microbial mats together with cyanobacteria and other bacteria in natural environments.

Chloroflexus aggregans isolated from bacterial mats in hot springs is able to form bacterial mat-like dense cell aggregates rapidly in an axenic culture (Hanada et al., 1995a; Hanada et al., 2002a). Aggregate formation, which was observed among growing cells in a liquid medium in a bottle, occurred every time within 20–30 min after the cells were dispersed by shaking. This has not been seen in any strains of the related species, *Chloroflexus aurantiacus*. However, *Heliothrix oregonensis* shows a similar rapid aggregation in cell suspensions (Pierson et al., 1984a). The bacterium has a fast gliding motility (1–3 μm/s) that is 100 times faster than that of *Chloroflexus aurantiacus* strains. The distinct rapid cell aggregation of this organism appears to be due to its fast gliding. The cell aggregation rate is accelerated by increased cyclic AMP concentration in the cells (Hanada et al., 2002a). The similar acceleration of gliding and cell aggregation by cAMP has been reported among some gliding cyanobacteria (Ohmori et al., 1992).

Gliding motility is also observed in the marine and hypersaline FAPs. These filaments show diel migration within microbial mats by their gliding movement (Mack and Pierson, 1988).

Cellular Lipids and Cell Envelope

The biochemical composition of the cell envelope and the composition of cellular lipids in *Chloroflexus aurantiacus* exhibit some unique properties. The polar lipids consist mainly of phosphatidylglycerol, phosphatidylinositol, monogalactosyldiglyceride, and diglycosyldiglyceride (Kenyon and Gray, 1974; Knudsen et al., 1982). The predominance of phosphatidylinositol in *Chloroflexus aurantiacus* is rarely found in other phototrophic bacteria.

The cellular fatty acids of *Chloroflexus aurantiacus* are predominantly straight-chain saturated and monounsaturated with $C_{16–20}$ chain lengths and with no detectable hydroxylated or cyclopropane-substituted chains (Knudsen et al., 1982). The major components of these acids are $C_{18:0}$, $C_{16:0}$, and $C_{18:1}$. The fatty acid profile of *Oscillochloris trichoides* is somewhat similar. The mesophile lacks longer-chain fatty acids ($C_{19–20}$) and contains a significant amount of unsaturated fatty acids. The ratio of unsaturated to saturated fatty acids was 3.3–3.8 in *Oscillochloris trichoides* and 0.63 in *Chloroflexus aurantiacus*, respectively (Keppen et al., 2000). The presence of highly saturated and somewhat longer-chain fatty acids is consistent with the thermophilic nature of *Chloroflexus aurantiacus*. The fatty acid profile was also determined in *Roseiflexus castenholzii*. The organism contained predominantly saturated fatty acids ($C_{14–16}$) and no unsaturated fatty acids (Hanada et al., 2002b). The thermophile grows at slightly lower temperatures than *Chloroflexus aurantiacus*. This difference may cause *Roseiflexus castenholzii* to include shorter-chain fatty acids than those found in *Chloroflexus aurantiacus*.

The lipid extract of *Chloroflexus aurantiacus* contains a large variety of wax esters (C_{28}–C_{38}; Knudsen et al., 1982). Similar wax esters (C_{37}–C_{40}) are found in *Roseiflexus castenholzii* (M. T. J. van der Meer, submitted). The presence of the wax esters is observed infrequently among other bacteria, with a few exceptions. Although not clear, the function or role of the wax esters in these bacteria might be related to thermophily.

The cell envelope of *Chloroflexus aurantiacus* apparently lacks the lipopolysaccharide (LPS) typical of Gram-negative bacteria. No hydroxylated fatty acids and no saccharides usually contained in outer-membrane LPS were detected in this organism (Knudsen et al., 1982). These features in the cell envelope of *Chloroflexus aurantiacus* are very unlike the structural organization found in typical Gram-negative bacterial envelopes and more strongly resemble the features found in Gram-positive bacterial walls (Jürgens et al., 1987). The abundant complex polysaccharide bound to the rigid peptidoglycan layer contains sugar O-methyl ethers of hexoses and contains rhamnose, mannose, glucose, galactose, xylose and arabinose (Jürgens et al., 1987). Although the peptidoglycan of *Chloroflexus* contains *N*-acetyl-glucosamine, *N*-acetyl-muramic acid (about 15% of which is phosphorylated), L-alanine, D-alanine, and D-glutamic acid, the presence of L-ornithine instead of diaminopimelic acid in the peptidoglycan of *Chloroflexus* also distinguishes it from the other

Gram-negative bacteria and substantiates the similarity in wall composition with Gram-positive bacteria (Jürgens et al., 1987). The unusual biochemical composition of the envelope of *Chloroflexus aurantiacus* is particularly interesting, since the cells stain Gram negative and ultrastructurally the envelope appears to be multilayered. Absent, however, is a well-defined outer membrane structure typical of Gram-negative cell envelopes (Pierson and Castenholz, 1974a; Staehelin et al., 1978).

Interestingly, cells of *Oscillochloris chrysea* stain Gram-positive and have a very thick peptidoglycan layer with no outer membrane (Gorlenko, 1988; Gorlenko, 1989a). Although the cells of *Oscillochloris trichoides* have the Gram-negative type cell wall, they are variably stained in Gram staining. The hypersaline organisms also lack a well-defined outer membrane and are Gram-variable when stained (Pierson et al., 1994). No well-defined outer membrane structure has been recognized in the cells of *Roseiflexus castenholzii*, which are apparently negatively stained.

Pigment Synthesis and Regulation

BACTERIOCHLOROPHYLLS *Chloroflexus* species contain bacteriochlorophylls *a* and *c* as photosynthetic pigments. These bacteriochlorophylls are generally esterified with some long chain alcohol: Bchl *a* is esterified to phytol. Takaichi et al. (1995) reported that Bchl *c* in *Chloroflexus aurantiacus* existed as mixtures of stearyl, phytyl, geranylgeranyl, and cetyl/oleyl esters. The mixing ratio of esters depended on light intensity. Phytyl and stearyl esters were dominant in low-light growth conditions, while phytyl and geranylgeranyl esters dominated at high light intensity (Takaichi et al., 1995).

These bacteriochlorophylls (both Bchl *a* and *c*) in *Chloroflexus aurantiacus* are synthesized via the glutamate-C_5 pathway as in the cyanobacteria and the green and purple sulfur bacteria, rather than via the pathway involving δ-aminolevulinic acid, as in some species of nonsulfur purple bacteria (Avissar et al., 1989; Kern and Klemme, 1989).

Little is known, however, about the regulation of pigment synthesis at the molecular level in *Chloroflexus aurantiacus*. However, the synthesis of bacteriochlorophylls is apparently controlled by light intensity and oxygen concentration. Bacteriochlorophyll content decreases in the presence of high light intensity or oxygen (Pierson and Castenholz, 1974b; Oelze, 1992a), and both light and oxygen affect the structural development of the photosynthetic apparatus containing these pigments (Schmidt et al., 1980b; Sprague et al., 1981a; Sprague et al., 1981b; Feick et al., 1982). The change in the ratio of Bchl *c* to *a* in cells grown at different light intensities and adapted to reductions in oxygen levels suggested independent regulation of the two pigments (Pierson and Castenholz, 1974b; Sprague et al., 1981b). Light intensity may influence pigment synthesis via an indirect effect on growth rate. By growing *Chloroflexus aurantiacus* in a chemostat and controlling the growth rate by regulating the dilution factor while keeping the light intensity constant, Oelze and Fuller (1987) showed that the specific contents of Bchl *a* and *c* increased at lower growth rates. Since light intensity has a direct effect on growth rate, it is difficult to distinguish the relationships among these parameters without a chemostat. Cell protein levels, as well as specific Bchl *a* content, increased linearly with a decreasing growth rate, while the specific Bchl *c* content increased exponentially with a decreasing growth rate. Oelze and Söntgerath (1992b) also reported that pigment synthesis was affected by amino acids supplemented in the growth culture. The addition of serine, glutamate, or alanine to the growth medium stimulated the synthesis of bacteriochlorophylls.

In a mesophilic FAP, *Oscillochloris trichoides*, a similar effect on the synthesis of bacteriochlorophylls by light and oxygen was also observed (Gorlenko and Pierson, 2001).

CAROTENOIDS *Chloroflexus aurantiacus* contains large amounts of carotenoids such as β- and γ-carotene as well as γ-carotene derivatives. Takaichi et al. (1995) revealed that the γ-carotene derivatives were OH-γ-carotene glucoside ester and its intermediates. Halfen et al. (1972) detected no OH-γ-carotene glucoside ester but rather OH-γ-carotene glucoside in *Chloroflexus* cells. However, the absence of carotenoid ester was probably due to saponification (which degrades carotenoid glucoside esters into carotenoid glucosides) in their preparation (Takaichi et al., 1995). Some of the β- and γ-carotenes were in the cytoplasmic membrane, but most were in the chlorosomes, while the OH-γ-carotene glucoside esters were the predominant carotenoid in the membrane (Schmidt et al., 1980b). This OH-γ-carotene derivative increased relative to the other carotenoids in cells grown at higher light intensities (Schmidt et al., 1980b) and may have a role in protecting the membrane-bound photochemical systems from the damaging effects of high light intensities.

Like the *Chloroflexus* species, *Oscillochloris trichoides* also contains primarily β- and γ-carotene. However, *Heliothrix oregonensis* and *Roseiflexus castenholzii* lack β-carotene and contain predominantly unique γ-carotene derivatives, i.e., myxocozanthin derivatives (Pierson et al., 1984a; Takaichi et al., 2001). Takaichi et al.

(2001) suggested that the absence of β-carotene resulted from the absence of chlorosomes in these organisms because the pigment is located mainly in chlorosomes in *Chloroflexus* species.

PHOTOCHEMICAL REACTION CENTER AND LIGHT-HARVESTING APPARATUS The photochemical reaction center and primary light-harvesting apparatus of *Chloroflexus aurantiacus* have very interesting similarities to components within two diverse groups of phototrophs, purple bacteria and green sulfur bacteria, respectively.

The photochemical reaction center of *Chloroflexus aurantiacus* is very similar to the reaction centers of the purple bacteria, despite some significant differences. The *Chloroflexus* reaction center is the smallest one known, being composed of two essential polypeptides, designated "L" and "M" subunits, and an additional membrane-bound cytochrome *c* complex. This reaction center apparently lacks a polypeptide, the H subunit, which is usually found in all purple bacteria (Pierson and Thornber, 1983; Shiozawa et al., 1987). The pigment composition also differs from that of purple bacterial reaction centers. *Chloroflexus* has a photoreactive special pair or dimer of Bchl *a* molecules as do the purple bacteria, but it has only one additional Bchl *a* molecule and three bacteriopheophytin *a* molecules, rather than the two additional Bchl *a* and two bacteriopheophytin molecules found in the purple bacteria (Pierson and Thornber, 1983; Blankenship et al., 1984). Furthermore, the *Chloroflexus* reaction center lacks carotenoid pigments (Pierson and Thornber, 1983), and menaquinone is the electron acceptor for photochemistry, rather than ubiquinone (Vasmel and Amesz, 1983). Functionally, the reaction center of *Chloroflexus aurantiacus* is very similar to that of purple bacteria, and the intermediate electron acceptor in both is bacteriopheophytin *a* (Blankenship et al., 1983; Blankenship et al., 1984; Kirmaier et al., 1983). The arrangement of the chromophores appears to be similar to that in purple bacteria, on the basis of spectroscopic measurements (Vasmel et al., 1986). The two subunits of the *Chloroflexus* reaction center are probably structurally similar to those of the purple bacteria, since the L subunits exhibit a 40% deduced amino acid sequence homology and the M subunits 42% (Ovchinnikov et al., 1988a; Ovchinnikov et al., 1988b; Shiozawa et al., 1989). Within the pigment-binding regions of the two subunits, the sequence homology is higher (59–75%; Shiozawa et al., 1989). Thus, although *Chloroflexus aurantiacus* is phylogenetically different from purple bacteria, their reaction centers have strong structural and functional similarities. The lack of the H subunit in the *Chloroflexus* reaction center, slight differences in pigment composition, and intrinsic thermal stability have little to do with the apparently highly conserved structural and functional characteristics of the sites directly involved in the primary photochemical act.

The molecular organization of the primary light-harvesting system is particularly interesting, since it has a complex antenna involving accessory Bchl *c* housed in chlorosomes adjacent to the cell membrane. Although the size, composition, and ultrastructure of the chlorosomes of *Chloroflexus aurantiacus* differ somewhat from those of green sulfur bacteria, both contain three low-molecular-weight chlorophyll-binding proteins, and the sequence homology between *Chloroflexus aurantiacus* and green sulfur bacteria is about 30% (Wechsler et al., 1985b; Wagner-Huber et al., 1988). The chlorosomes of *Chloroflexus* also appear to contain a small component of Bchl *a*, called a "baseplate" (Schmidt, 1980a; Sprague et al., 1981a; Feick et al., 1982), that appears to be involved in transferring excitation energy to additional antenna components in the cell membrane (Van Dorssen and Amesz, 1988). The baseplate was also found in green sulfur bacterial chlorosomes, but these bacteria transfer excitation energy to the reaction center via a unique water soluble Bchl *a* protein not found in *Chloroflexus*.

The green sulfur bacteria, however, do not have an intracytoplasmic membrane light-harvesting system similar to those found in *Chloroflexus aurantiacus* and purple bacteria. The Bchl *a* antenna in the cell membrane of *Chloroflexus aurantiacus* is very similar to that in the intracytoplasmic membranes of purple bacteria. In addition to harvesting light energy directly, however, this antenna also funnels excitation energy from chlorosomes to the reaction center (Brune et al., 1987; Van Dorssen and Amesz, 1988; Wittmershaus et al., 1988). The absorption properties of this light-harvesting complex, with maxima at 805 and 865 nm, are similar to those of many purple bacteria, and the two polypeptides that bind the Bchl *a* molecules show significant sequence homology (27–40%) to the comparable polypeptides in purple bacteria (Wechsler et al., 1985a; Wechsler et al., 1987). Watanabe et al. (1995), however, revealed that the transcriptional organizations differed between *Chloroflexus aurantiacus* and purple bacteria. In purple bacteria, genes of the Bchl *a*, containing light-harvesting polypeptides and reaction center polypeptides, form the same operon, but in *Chloroflexus aurantiacus*, the similar operon is divided into two. These two operons (genes for reaction center polypeptides on the one hand and, on the other, genes for hight-harvesting polypeptides and tetraheme cytochrome *c*554) are at least 3 kb distant from each other.

Among all other FAPs, very few attempts have been made at structural, functional, and genetic studies of photochemical reaction centers or light-harvesting apparatuses. However, recent structural and genetic studies of the reaction center in *Roseiflexus castenholzii* have been undertaken, and so far it has been made clear that the *Roseiflexus* reaction center resembles that of *Chloroflexus aurantiacus* in structural and spectroscopic respects (Takaichi et al., 2001). Further research on this organism would give useful information about the enigmatic photosynthetic nature of FAPs.

Electron Transport System

The electron transport chains of both phototrophically and chemotrophically grown cells of *Chloroflexus aurantiacus* were reviewed by Zannoni (1995). Phototrophic *Chloroflexus* cells contain multiple *b* and *c* type cytochromes, including the electron donor to the reaction center, tetraheme cytochrome c_{554} (Zannoni and Ingledew, 1985; Wynn et al., 1987). There is strong evidence for the presence of a putative cytochrome bc_1 complex in the membranes of phototrophically grown cells (Zannoni and Ingledew, 1985), as well as for the presence of cytochrome *c*-CO binding activity (Pierson, 1985a; Zannoni and Ingledew, 1985; Wynn et al., 1987). A membrane-diffusible quinone (menaquinone-10) is also abundantly involved in the cells.

The cells include neither a soluble cytochrome c_2 nor a high-potential iron protein (HiPIP; Zannoni, 1987), one and/or the other of which are essential electron transporters for either photosynthesis or respiration in purple bacteria. The *Chloroflexus* cells contain two types of the novel blue copper protein, designated "auracyanin," instead of these electron transporters (McManus et al., 1992). An analogous protein, plastocyanin, is present in cyanobacteria and chloroplasts. However, the crystal structure of an auracyanin (auracyanin B) recently reported by Bond et al. (2001) indicated that the blue copper protein was related more closely to azurin (a blue copper protein found in some proteobacteria; Baker, 1988) than to plastocyanin.

The electron cycle process in the photosynthesis of *Chloroflexus aurantiacus* would be as follows: Owing to excitation by photons, an electron in a "special pair" of Bchl *a* molecules is transferred to a bacteriopheophytin molecule (Becker et al., 1991) and then passes to a quinone within a reaction center core complex; a reduced quinone then diffuses from the reaction center into the membrane (Vasmel and Amesz, 1983; Mulkidjanian et al., 1998) and gives an electron to a putative membrane-bound cytochrome bc_1 complex; an electron is returned by a blue copper protein to the reaction center via a cytochrome *c*554 that binds directly to the reaction center.

Chemotrophically grown cells lack a photochemical reaction center and the tetraheme cytochrome *c*554 that closely binds to this core complex (Wynn et al., 1987). Also, a blue copper protein, auracyanin, is not present under the aerobic conditions (Trost et al., 1988). Two types of terminal oxidase for aerobic respiration have been reported, cytochrome aa_3 (Pierson, 1985a; Zannoni, 1986) and cytochrome *a* (Wynn et al., 1987; Knaff et al., 1988). Cytochrome aa_3 generally operates in cells grown at less than saturated oxygen level, and cytochrome *a* is present at higher oxygen levels (Zannoni, 1995).

As a membrane-diffusible electron transporter, menaquinone-10 is usually used in *Chloroflexus* species and *Oscillochloris trichoides*. However, *Roseiflexus castenholzii* uses mainly menaquinone-11 under both phototrophic and respiratory conditions (Hanada et al., 2002b).

Thermophily

The thermophilic aspects of the physiology of *Chloroflexus aurantiacus* have been studied in several systems. The photochemical reaction center of *Chloroflexus aurantiacus* also exhibits thermal stability (Pierson and Thornber, 1983; Nozawa and Madigan, 1991). The reconstituted membranes containing the isolated reaction centers can develop a proton motive force in the presence of light under anaerobic conditions (Speelmans et al., 1993).

Activities of some soluble enzymes, such as dehydrogenases of amino or organic acids and ATP sulfurylase, determined as a function of temperature, were found to be maximal between 45 and 70°C (Krasil'nikova et al., 1986; Krasil'nikova, 1987a; Laakmann-Ditges and Klemme, 1988). The functioning of membrane-bound proteins at elevated temperatures, however, depends on the properties of the membrane as well as on the temperature characteristics of the proteins themselves. Oelze and Fuller (1983) determined the temperature characteristics of growth and the membrane-bound enzyme activities of NADH oxidase, succinate 2,6-dichlorophenol-indophenol reductase, ATPase, and light-induced proton extrusion. Enzymatic activities were maximal at 65–70°C, and a major lipid phase transition occurred near 40°C when cells were grown at 50°C. Membrane phenomena were significantly altered at temperatures above 60°C, which may reflect the denaturation of bulk membrane proteins (Oelze and Fuller, 1983). Although individual enzyme activities were high in this

temperature range, proton extrusion was not thermostable above 60°C and growth rates did not increase in this range.

Tvermyr et al. (1998) succeeded in cloning of a DNA polymerase I gene in *Chloroflexus aurantiacus* and in expressing the recombinant protein in *Escherichia coli*. The recombinant *Chloroflexus* protein showed an efficient, heat-stable proofreading and polymerase activity at 70°C. The proofreading, i.e., 3-5 exonuclease activity, found in *Chloroflexus* polymerase is lost in some thermophilic DNA polymerases such as *Taq* polymerase (from *Thermus aquaticus*), *Bst* polymerase (from *Bacillus stearothermophilus*), and *Streptococcus pneumoniae* DNA polymerase I.

Genetics

While knowledge about the genetics of *Chloroflexus* species and other FAPs is limited, some gene sequences and their organization for various proteins, mainly relating to photosynthesis, have been investigated in *Chloroflexus aurantiacus* (see the section "Physiology" in this Chapter). Fundamental information about the genetic analysis of *Chloroflexus aurantiacus* was reviewed by Shiozawa (1995).

In *Chloroflexus aurantiacus*, chemical or UV mutagenesis appears to be one of several useful methods for obtaining mutants deficient in the production of bacteriochlorophylls or lacking carotenoids (Pierson et al., 1984b). Mutagenesis is helpful for the genetic analysis of pigment synthesis. Furthermore, using a similar technique, it would be possible to obtain mutants deficient in or lacking activities of enzymes other than those for the synthesis of photopigments. Susceptibility to antibiotics is also important for the screening of recombinants. The type strain of *Chloroflexus aurantiacus* is sensitive to penicillin and ampicillin but is resistent to carbenicillin up to 100 μg/ml (Pierson et al., 1984b).

The genome project on *Chloroflexus aurantiacus* is now progressing at the Joint Genome Institute operated by the University of California for the United States Department of Energy. Up to the present (May 2002), 3 million nucleotides have been determined, and more than 3000 candidate protein-encoding genes have been found in the determined sequence. The draft sequence data can be obtained at the web page (http://www.jgi.doe.gov/JGI_microbial/html/). In the near future, whole genome data will enable comparisons between *Chloroflexus aurantiacus* and other photosynthetic bacteria, and the findings obtained from this comparison will provide a number of keys to resolving the phylogenetically enigmatic nature of this organism as well as the complex evolutionary puzzle of photosynthesis.

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Medium Lists

Medium D, DG, DGN, and ND

Double-distilled water	1 liter
NTA (sodium nitrilotriacetate)	0.1 g
Micronutrient solution (see below)	0.5 ml
$FeCl_3$ solution (0.29 g/liter)	1.0 ml
$CaSO_4 \cdot 2H_2O$	0.06 g
$MgSO_4 \cdot 7H_2O$	0.10 g
NaCl	0.008 g
KNO_3	0.10 g
$NaNO_3$	0.70 g
Na_2HPO_4	0.11 g

Micronutrient Solution

Double-distilled water	1 liter
H_2SO_4 (concentrated)	0.5 ml
$MnSO_4 \cdot H_2O$	2.28 g
$ZnSO_4 \cdot 7H_2O$	0.50 g
H_3BO_3	0.50 g
$CuSO_4 \cdot 5H_2O$	0.025 g
$Na_2MoO_4 \cdot 2H_2O$	0.025 g
$CoCl_2 \cdot 6H_2O$	0.045 g

Medium DG is medium D to which 0.8 g of glycylglycine per liter has been added as buffer. The pH is adjusted to 8.2 before autoclaving. Medium DGN is medium D to which 0.8 g of glycylglycine and 0.2 g of NH_4Cl have been added per liter. Medium ND (lacks combined N except as NTA) is medium D without KNO_3 and $NaNO_3$, but with 0.07 g of Na_2HPO_4 and 0.036 g of KH_2PO_4 per liter.

Medium PE

Basal salts solution (see below)	5 ml
Phosphate solution	5 ml
Vitamin solution	1 ml
$(NH_4)_2SO_4$	0.5 g
Sodium glutamate	0.5 g
Sodium succinate	0.5 g
Sodium acetate	0.5 g
Yeast extract	0.5 g
Casamino acids	0.5 g
Sodium thiosulfate	0.5 g

Dilute with distilled water up to 1 liter and adjust pH to 7.2–7.5.

Basal Salts Solution

Na_3EDTA	4.12 g
$FeSO_4 \cdot 7H_2O$	1.11 g
$MgSO_4 \cdot 7H_2O$	24.65 g
$CaCl_2 \cdot 2H_2O$	2.94 g
NaCl	23.4 g
Trace elements solution	10 ml

Dilute with distilled water up to 1 liter.

Phosphate Solution

KH_2PO_4	75 g
K_2HPO_4	78 g

Dilute with distilled water up to 1 liter.

Vitamin Solution

Nicotinic acid	0.1 g
Thiamine	0.1 g
Biotin	0.005 g
p-Amino-benzoic acid	0.05 g
Vitamin B_{12}	0.001 g
Calcium panthothenate	0.05 g
Pyridoxine HCl	0.05 g
Folic acid	0.05 g
Na_3EDTA	0.02 g

Dilute with distilled water up to 100 ml.

Trace Elements Solution

$MnSO_4 \cdot 4H_2O$	5.58 g
$ZnSO_4 \cdot 7H_2O$	1.44 g
$Co(NO_3)_2 \cdot 6H_2O$	1.46 g
$CuSO_4 \cdot 5H_2O$	1.26 g
$Na_2MoO_4 \cdot 2H_2O$	1.21 g
H_3BO_3	1.55 g
Na_3EDTA	20.6 g

Dilute with distilled water to 500 ml.

Medium IM Solution A

$CaCl_2 \cdot 2H_2O$	0.32 g
$MgSO_4 \cdot 7H_2O$	0.40 g
KCl	0.5 g
NaCl	1.0 g
$(NH_4)_2SO_4$	0.5 g
KH_2PO_4	0.3 g
$FeCl_3$	0.292 mg
Micronutrient solution SL7	10 ml
Vitamin B_{12}	0.005 mg

Dilute with distilled water up to 1 liter and adjust pH to 7.6. Solution A and B are separately prepared and stored. Solution B contains 0.05 M $NaHCO_3$ (in water). It is sterilized by autoclaving, followed by bubbling vigorously with CO_2 for 1 h. A total of 250 ml of solution A is added to 650 ml of distilled water. After autoclaving and cooling, 100 ml of solution B is added.

Medium IMC is medium IM to which 0.25 g of glucose, 0.25 g of vitamin-free casamino acids and 0.5 ml of vitamin solution (see below) have been added per liter.

Vitamin Solution

Nicotinic acid	2 mg
Thiamine hydrochloride	1 mg
p-Amino-benzoic acid	0.2 mg
Biotin	0.02 mg

Dilute with distilled water up to 1 ml.

Literature Cited

Avissar, Y. J., J. G. Ormerod, and S. I. Beale. 1989. Distribution of δ-aminolevulinic acid biosynthetic pathways among phototrophic bacterial groups. Arch. Microbiol. 151:513–519.

Baker, E. N. 1988. Structure of azurin from Alcaligenes denitrificans: Refinement at 1.8 Å resolution and comparison of the two crystallographically independent molecules. J. Molec. Biol. 203:1071–1095.

Becker, M., V. Nagarajan, D. Middendorf, W. W. Parson, J. E. Martin, and R. E. Blankenship. 1991. Temperature-dependence of the initial electoron-transfer kinetics in photosynthetic reaction centers of Chloroflexus aurantiacus. Biochem. Biophys. Acta. 1057:299–312.

Blankenship, R. E., R. Feick, B. C. Bruce, C. Kirmaier, D. Holten, and R. C. Fuller. 1983. Primary photochemistry in the facultative green photosynthetic bacterium Chloroflexus aurantiacus. J. Cell. Biol. 22:251–261.

Blankenship, R. E., L. J. Mancino, R. Feick, R. C. Fuller, J. Machnicki, H. A. Frank, C. Kirmaier, and D. Holten. 1984. Primary photochemistry and pigment composition of reaction centers isolated from the green photosynthetic bacterium Chloroflexus aurantiacus. *In:* C. Sybesma (Ed.) Advances in Photosynthesis Research. M. Nijhoff/Dr. W. Junk. The Hague, The Netherlands. 203–206.

Blankenship, R. E. 1992. Origin and early evolution of photosynthesis. Photosynth. Res. 33:91–111.

Bond, C. S., R. E. Blankenship, H. C. Freeman, J. M. Guss, M. J. Maher, F. M. Selvaraj, M. C. Wilce, and K. M. Willingham. 2001. Crystal structure of auracyanin, a "blue" copper protein from the green thermophilic photosynthetic bacterium Chloroflexus aurantiacus. J. Molec. Biol. 306:47–67.

Boomer, S. M., B. K. Pierson, R. Austinhirst, and R. W. Castenholz. 2000. Characterization of novel bacteriochlorophyll-a-containing red filaments from alkaline hot springs in Yellowstone National Park. Arch. Microbiol. 174:152–161.

Boomer, S. M., D. P. Lodge, B. E. Dutton, and B. K. Pierson. 2002. Molecular characterization of novel red green nonsulfur bacteria from five distinct hot spring communities in Yellowstone National Park. Appl. Environ. Microbiol. 68:346–355.

Borrego, C. M., J. Garcia-Gil, X. P. Cristina, X. Vila, and C. A. Abella. 1998. Occurrence of new bacteriochlorophyll d forms in natural populations of green photosynthetic sulfur bacteria. FEMS Microbiol. Ecol. 26:257–267.

Brock, T. D. 1978. Thermophilic Microorganisms and Life at High Temperatures. Springer-Verlag. New York, NY.

Brune, D. C., G. H. King, A. Infosino, T. Steiner, M. L. W. Thewalt, and R. E. Blankenship. 1987. Antenna organization in green photosynthetic bacteria. 2: Excitation transfer in detached and membrane-bound chlorosomes from Chloroflexus aurantiacus. Biochemistry 26:8652–8658.

Castenholz, R. W. 1973. The possible photosynthetic use of sulfide by the filamentous phototrophic bacteria of hot springs. Limnol. Oceanogr. 18:863–876.

Castenholz, R. W. 1982. Motility and taxes. *In:* N. G. Carr and B. A. Whitton (Eds.) The Biology of Cyanobacteria. University of California Press. Los Angeles, CA. 413–439.

Castenholz, R. W. 1984. Composition of hot spring microbial mats: A summary. *In:* Y. Cohen, R. W. Castenholz, and H. O. Halvorson (Eds.) Microbial Mats: Stromatolites. Alan R. Liss. New York, NY. 101–119.

Castenholz, R. W. 1988a. Culturing methods (cyanobacteria). *In:* L. Packer and A. N. Glazer (Eds.) Methods in Enzymology: Cyanobacteria. Academic Press. San Diego, CA. 68–93.

Castenholz, R. W. 1988b. The green sulfur and nonsulfur bacteria of hot springs. *In:* J. M. Olson, J. G. Ormerod, J. Amesz, E. Stackebrandt, and H. G. Trüper (Eds.) Green Photosynthetic Bacteria. Plenum Press. New York, NY. 243–255.

Castenholz, R. W., and B. K. Pierson. 1995. Ecology of thermophilic anoxygenic phototrophs. *In:* R. E. Blanken-

ship, M. T. Madigan, and C. E. Bauer (Eds.) Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers. Dordrecht, The Netherlands. 87–103.

Castenholz, R. W. 2001. Class I: "Chloroflexi". *In:* D. R. Boone, R. W. Castenholz, and G. M. Garrity (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:427.

D'Amelio, E. D., Y. Cohen, and D. J. Des Marais. 1987. Association of a new type of gliding, filamentous, purple phototrophic bacterium inside bundles of Microcoleus chthonoplastes in hypersaline cyanobacterial mats. Arch. Microbiol. 147:213–220.

D'Amelio, E. D., Y. Cohen, and D. J. Des Marais. 1989. Comparative functional ultrastructure of two hypersaline submerged cyanobacterial mats: Gueerrero Negro, Baja California Sur, Mexico, and Solar Lake, Sinai, Egypt. *In:* Y. Cohen and E. Rosenberg (Eds.) Microbial Mats: Physiological Ecology of Benthic Microbial Communities. American Society for Microbiology. Washington, DC. 97–113.

Dubinina, G. A., and V. M. Gorlenko. 1975. New filamentous photosynthetic green bacteria containing gas vacuoles. Microbiology 44:452–458.

Ehrenreich, A., and F. Widdel. 1994. Anaerobic oxidation of ferrous iron by purple bacteria, a new-type of phototrophic metabolism. Appl. Environ. Microbiol. 60:4517–4526.

Eisenreich, W., G. Strauss, U. Werz, G. Fuchs, and A. Bacher. 1993. Retrobiosynthetic analysis of carbon fixation in the phototrophic eubacterium Chloroflexus aurantiacus. FEBS Eur. J. Biochem. 215:619–632.

Feick, R. G., M. Fitzpatrick, and R. C. Fuller. 1982. Isolation and characterization of cytoplasmic membranes and chlorosomes from the green bacterium Chloroflexus aurantiacus. J. Bacteriol. 150:905–915.

Garcia-Pichel, F., M. Mechling, and R. W. Castenholz. 1994. Diel migrations of microorganisms within a benthic, hypersaline mat community. Appl. Environ. Microbiol. 60:1500–1511.

Garcia-Pichel, F., U. Nübel, and G. Muyzer. 1998. The phylogeny of unicellular, extremely halotolerant cyanobacteria. Arch. Microbiol. 169:469–482.

Garrity, G. M., and J. G. Holt. 2001. Phylum BVI: Chloroflexi phy. nov. *In:* D. R. Boone, R. W. Castenholz, and G. M. Garrity (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:427.

Gich, F., J. Garcia-Gil, and J. Overmann. 2001. Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. Arch. Mikrobiol. 177:1–10.

Giovannoni, S. J., N. P. Revsbech, D. M. Ward, and R. W. Castenholz. 1987. Obligately phototrophic Chloroflexus: primary production in anaerobic hot spring microbial mats. Arch. Microbiol. 147:80–87.

Gorlenko, V. M. 1976. Characteristics of filamentous phototrophic bacteria from freshwater lakes. Microbiology 44:682–684.

Gorlenko, V. M., and T. A. Pivovarova. 1977. On the belonging of bluegreen alga Oscillatoria coerulescens Gicklhorn, 1921 to a new genus of Chlorobacteria Oscillochloris nov. gen. Izv. Akad. Nauk SSSR, Ser. Biol. 3:396–409.

Gorlenko, V. M., and S. A. Korotkov. 1979a. Morphological and physiological features of the new filamentous gliding green bacterium Oscillochloris trichoides nov. comb. Izv. Acad. Nauk. Uzb. SSSR Ser. Biol. 5:848–857.

Gorlenko, V. M., and S. I. Lokk. 1979b. Vertical distribution and characteristics of the species composition of microorganisms from some stratified Estonian lakes. Mikrobiologiya 48:351–359.

Gorlenko, V. M. 1988. Ecological niches of green sulfur and gliding bacteria. *In:* J. M. Olson, J. G. Ormerod, J. Amesz, E. Stackebrandt, and H. G. Trüper (Eds.) Green Photosynthetic Bacteria. Plenum Press. New York, NY. 257–267.

Gorlenko, V. M. 1989a. Genus "Oscillochloris." *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 1703–1706.

Gorlenko, V. M., and B. K. Pierson. 2001. Genus II: Chloronema. *In:* D. R. Boone, R. W. Castenholz, and G. M. Garrity (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:437–438.

Gruber, T. M. and D. A. Bryant. 1997. Molecular systematic studies of Eubacteria, using 70-type sigma factors of group 1 and group 2. J. Bacteriol. 179:1734–1747.

Gruber, T. M., and D. A. Bryant. 1998a. Characterization of the group 1 and group 2 sigma factors of the green sulfur bacterium Chlorobium tepidum and the green nonsulfur bacterium Chloroflexus aurantiacus. Arch. Microbiol. 170:285–296.

Gruber, T. M., J. A. Eisen, K. Gish, and D. A. Bryant. 1998b. Phylogenetic relationships of Chlorobium tepidum and Chloroflexus aurantiacus based upon their RecA sequences. FEMS Microbiol. Lett. 162:53–60.

Gupta, R. S., T. Mukhtar, and B. Singh. 1999. Evolutionary relationships among photosynthetic prokarytoes (Heliobacterium chlorum, Chloroflexus aurantiacus, cyanobacteria, Chlorobium tepidum, and proteobacteria): Implications regarding the origin of photosynthesis. Molec. Microbiol. 32:893–906.

Halfen, L. N., B. K. Pierson, and G. W. Francis. 1972. Carotenoids of a gliding organism containing bacteriochlorophylls. Arch. Mikrobiol. 82:240–246.

Hanada, S., A. Hiraishi, K. Shimada, and K. Matsuura. 1995a. Chloroflexus aggregans sp. nov., a filamentous phototrophic bacterium which forms dense cell agregates by active gliding movement. Int. J. Syst. Bacteriol. 45:676–681.

Hanada, S., A. Hiraishi, K. Shimada, and K. Matsuura. 1995b. Isolation of Chloroflexus sp. and related thermophilic photosynthetic bacteria from hot springs using an improved isolation procedure. J. Gen. Appl. Microbiol. 41:119–130.

Hanada, S., K. Shimada, and K. Matsuura. 2002b. Active and energy-dependent rapid formation of cell aggregates in the thermophilic photosynthetic bacterium Chloroflexus aggregans. FEMS Microbiol. Lett. 208:275–279.

Hanada, S., S. Takaichi, M. K., and N. K. 2002b. Roseiflexus castenholzii gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium which lacks chlorosomes. Int. J. Syst. Evol. Microbiol. 52:187–193.

Heda, G. D., and M. T. Madigan. 1986. Utilization of amino acids and lack of diazotrophy in the thermophilic anoxygenic phototroph Chloroflexus aurantiacus. J. Gen. Microbiol. 132:2469–2473.

Heising, S., L. Richter, W. Ludwig, and B. Schink. 1999. Chlorobium ferrooxidans sp nov., a phototrophic green sulfur bacterium that oxidizes ferrous iron in coculture with a "Geospirillum" sp strain. Arch. Microbiol. 172: 116–124.

Holo, H., and R. Sirevåg. 1986. Autotrophic growth and CO_2 fixation of Chloroflexus aurantiacus. Arch. Microbiol. 145:173–180.

Holo, H. 1989. Chloroflexus aurantiacus secretes 3-hydroxypropionate, a possible intermediate in the assimilation of CO_2 and acetate. Arch. Microbiol. 151:252–256.

Ivanovsky, R. N., E. N. Krasil'nikova, and Y. G. Fal. 1993. A pathway of the autotrophic CO_2 fixation in Chloroflexus aurantiacus. Arch. Microbiol. 159:257–264.

Ivanovsky, R. N., Y. I. Fal, I. A. Berg, N. V. Ugolkova, E. N. Krasilnikova, O. I. Keppen, L. M. Zakharchuc, and A. M. Zyakun. 1999. Evidence for the presence of the reductive pentose phosphate cycle in a filamentous anoxygenic photosynthetic bacterium, Oscillochloris trichoides strain DG-6. Microbiology 145:1743–1748.

Jürgens, U. J., J. Meiszner, U. Fischer, W. A. König, and J. Weckesser. 1987. Ornithine as a constituent of the peptidoglycan of Chloroflexus aurantiacus, diaminopimelic acid in that of Chlorobium vibrioforme f. thiosulfatophilum. Arch. Microbiol. 148:72–76.

Jørgensen, B. B., and D. C. Nelson. 1988. Bacterial zonation, photosynthesis, and spectral light distribution in hot spring microbial mats of Iceland. Microb. Ecol. 16:133–147.

Kaulen, H., and J.-H. Klemme. 1983. No evidence of covalent modification of glutamine synthetase in the thermophilic phototrophic bacterium Chloroflexus aurantiacus. FEMS Microbiol. Lett. 20:75–79.

Kenyon, C. N., and A. M. Gray. 1974. Preliminary analysis of lipids and fatty acids of green bacteria and Chloroflexus aurantiacus. J. Bacteriol. 120:131–138.

Keppen, O. I., N. V. Lebedeva, O. Y. Troshina, and Y. V. Rodionov. 1989. The nitrogenase activity of a filamentous phototrophic green bacterium. Mikrobiologiya 58:520–521.

Keppen, O. I., O. I. Baulina, A. M. Lysenko, and E. N. Kondrat'eva. 1993. New green bacterium belonging to family Chloroflexaceae. Mikrobiologiya 62:179–185.

Keppen, O. I., O. I. Baulina, and E. N. Kondratieva. 1994. Oscillochloris trichoides neotype strain DG-6. Photosynth. Res. 41:29–33.

Keppen, O. I., T. P. Tourova, B. B. Kuznetsov, R. N. Ivanovsky, and V. M. Gorlenko. 2000. Proposal of Oscillochloridaceae fam. nov. on the basis of a phylogenetic analysis of the filamentous anoxygenic phototrophic bacteria, and emended description of Oscillochloris and Oscillochloris trichoides in comparison with further new isolates. Int. J. Syst. Evol. Microbiol. 50:1529–1537.

Kern, M., and J.-H. Klemme. 1989. Inhibition of bacteriochlorophyll biosynthesis by gabaculin (3-amino,2,3-dihydrobenzoic acid) and presence of an enzyme of the C5-pathway of d-aminolevulinate synthesis in Chloroflexus aurantiacus. Z. Naturforsch. 44c:77–80.

Kirmaier, C., D. Holten, R. Feick, and R. E. Blankenship. 1983. Picosecond measurements of the primary photochemical events in reaction centers isolated from the facultative green photosynthetic bacterium Chloroflexus aurantiacus: Comparison with the purple bacterium Rhodopseudomonas sphaeroides. FEBS Lett. 158:73–78.

Klemme, J.-H., G. Laakmann-Ditges, and J. Mertschuweit. 1988. Ammonia assimulation and amino acid metabolism in Chloroflexus aurantiacus. *In:* J. M. Olson, J. G. Ormerod, J. Amesz, E. Stackebrandt, and H. G. Trüper (Eds.) Green Photosynthetic Bacteria. Plenum Press. New York, NY. 173–174.

Klemme, J.-H. 1989. Organic nitrogen metabolism of phototrophic bacteria. Ant. v. Leeuwenhoek 55:197–219.

Knaff, D. B., R. M. Wynn, T. E. Redlinger, R. E. Blankenship, J. M. Foster, R. W. Shaw, and R. C. Fuller. 1988. Electron transport chains of phototrophically and chemotrophically grown Chloroflexus aurantiacus. *In:* J. M. Olson, J. G. Ormerod, J. Amesz, E. Stackebrandt, and H. G. Trüper (Eds.) Green Photosynthetic Bacteria. Plenum Press. New York, NY. 145–147.

Knudsen, E., E. Jantzen, K. Bryn, J. G. Ormerod, and R. Sirevåg. 1982. Quantitative and structural characteristics of lipids in Chlorobium and Chloroflexus. Arch. Microbiol. 132:149–154.

Kondrat'eva, E. N., and E. N. Krasil'nikova. 1988. Utilization of thiosulfate by Chloroflexus aurantiacus. Microbiology 57:291–294.

Krasil'nikova, E. N., O. I. Keppen, V. M. Gorlenko, and E. N. Kondrat'eva. 1986. Growth of Chloroflexus aurantiacus on media with different organic compounds and pathways of their metabolism. Microbiology 55:325–329.

Krasil'nikova, E. N. 1987a. ATP Sulfurylase activity in Chloroflexus aurantiacus and other photosynthesizing bacteria as a function of temperature. Microbiology 55:418–421.

Krasil'nikova, E. N., and E. N. Kondrat'eva. 1987b. Growth of Chloroflexus aurantiacus under anaerobic conditions in the dark and the metabolism of organic substrates. Microbiology 56:281–285.

Laakmann-Ditges, G., and J.-H. Klemme. 1986. Occurrence of two L-threonine (L-serine) dehydratases in the thermophile Chloroflexus aurantiacus. Arch. Microbiol. 144:219–221.

Laakmann-Ditges, G., and J.-H. Klemme. 1988. Amino acid metabolism in the thermophilic phototroph, Chloroflexus aurantiacus: properties and metabolic role of two L-threonine (L-serine) dehydratases. Arch. Microbiol. 149:249–254.

Løken, Ø., and R. Sirevåg. 1982. Evidence for the presence of the glyoxylate cycle in Chloroflexus. Arch. Microbiol. 132:276–279.

Mack, E. E., and B. K. Pierson. 1988. Preliminary characterization of a temperate marine member of the Chloroflexaceae. *In:* J. M. Olson, J. G. Ormerod, J. Amesz, E. Stackebrandt, and H. G. Trüper (Eds.) Green Photosynthetic Bacteria. Plenum Publishing. New York, NY. 237–241.

Madigan, M. T., S. R. Petersen, and T. D. Brock. 1974. Nutritional studies on Chloroflexus, a filamentous photosynthetic, gliding bacterium. Arch. Microbiol. 100:97–103.

Madigan, M. T., and T. D. Brock. 1975. Photosynthetic sulfide oxidation by Chloroflexus aurantiacus, a filamentous, photosynthetic gliding bacterium. J. Bacteriol. 122:782–784.

Madigan, M. T., and T. D. Brock. 1977a. CO_2 fixation in photosynthetically-grown Chloroflexus aurantiacus. FEMS Microbiol. Lett. 1:301–304.

Malik, K. A. 1998. Preservation of Chloroflexus by deep-freezing and liquid-drying methods. J. Microbiol. Meth. 32:73–77.

McManus, J. D., D. C. Brune, J. Han, J. Sanders-Loehr, T. E. Meyer, M. A. Cusanovich, G. Tollin, and R. E. Blankenship. 1992. Isolation, characterization, and amino acid sequences of auracyanins, blue copper proteins from the green photosynthetic bacterium Chloroflexus aurantiacus. J. Biol. Chem. 267:6531–6540.

Menendez, C., Z. Bauer, H. Huber, N. Gad'on, K. O. Stetter, and G. Fuchs. 1999. Presence of acetyl coenzyme A (CoA) carboxylase and propionyl-CoA carboxylase in autotrophic Crenarchaeota and indication for operation of a 3-hydroxypropionate cycle in autotrophic carbon fixation. J. Bacteriol. 181:1088–1098.

Mulkidjanian, A., G. Venturoli, A. Hochkoeppler, D. Zannoni, B. A. Melandri, and L. Drachev. 1998. Photosynthetic electrogenic events in native membranes of chloroflexus-aurantiacus—flash-induced charge displacements within the reaction-center cytochrome c(554) complex. Photosynth. Res. 41:35–143.

Nozawa, T., and M. T. Madigan. 1991. Temperature and solvent effects on reaction centers from Chloroflexus aurantiacus and Chromatium tepidum. J. Bacteriol. 110:588–594.

Nübel, U., M. M. Bateson, M. T. Madigan, M. Kuhl, and D. M. Ward. 2001. Diversity and distribution in hypersaline microbial mats of bacteria related to Chloroflexus spp. Appl. Environ. Microbiol. 67:4365–4371.

Oelze, J., and R. C. Fuller. 1983. Temperature dependence of growth and membrane-bound activities of Chloroflexus aurantiacus energy metabolism. J. Bacteriol. 155:90–96.

Oelze, J., and R. C. Fuller. 1987. Growth rate and control of development of the photosynthetic apparatus in Chloroflexus aurantiacus. Arch. Microbiol. 148:132–136.

Oelze, J. 1992a. Light and oxygen regulation of the synthesis of bacteriochlorophylls a and c in Chloroflexus aurantiacus. J. Bacteriol. 174:5021–5026.

Oelze, J., and B. Söntgerath. 1992b. Differentiation of the photosynthetic apparatus of Chloroflexus aurantiacus depending on growth with different amino acids. Arch. Microbiol. 157:141–147.

Ohmori, K., M. Hirose, and M. Ohmori. 1992. Function of cAMP as a mat-forming factor in the cyanobacterium Spirulina platensis. Plant Cell Physiol. 33:21–25.

Ovchinnikov, Y. A., N. G. Abdulaev, B. E. Shmukler, A. A. Zargarov, M. A. Kutuzov, I. N. Telezhinskaya, N. B. Levina, and A. S. Zolotarev. 1988a. Photosynthetic reaction centre of Chloroflexus aurantiacus. II. Primary structure of M-subunit. FEBS Lett. 232:364–368.

Ovchinnikov, Y. A., N. G. Abdulaev, A. S. Zolotarev, B. E. Shmukler, A. A. Zargarov, M. A. Kutuzov, I. N. Telezhinskaya, and N. B. Levina. 1988b. Photosynthetic reaction centre of Chloroflexus aurantiacus. I: Primary structure of L-subunit. FEBS Lett. 231:237–242.

Oyaizu, H., B. Devrunner-Vossbrinck, L. Mandelko, J. A. Studier, and C. R. Woese. 1987. The green non-sulfur bacteria: a deep branching in the eubacterial line of descent. System. Appl. Microbiol. 9:47–53.

Palmisano, A. C., S. E. Cronin, E. D. D'Amelio, E. Munoz, and D. J. Des Marais. 1989. Distribution and survival of lipophilic pigments in a laminated microbial mat community near Guerrero Negro, Mexico. *In:* Y. Cohen and E. Rosenberg (Eds.) Microbial Mats: Physiological Ecology of Benthic Microbial Communities. American Society for Microbiology. Washington, DC. 138–152.

Pentecost, A. 1995. The micribial ecology of some italian hot-spring travertines. Microbios 81:45–58.

Pfennig, N. 1989. Multicellular filamentous green bacteria. *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams and Wilkins. Baltimore, MD. 3:1697–1707.

Pfennig, N., and H. G. Trüper. 1992. The family Chlomatiaceae. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 3200–3221.

Pierson, B. K., and R. W. Castenholz. 1971. Bacteriochlorophylls in gliding filamentous prokaryotes from hot springs. Nature 223:25–27.

Pierson, B. K., and R. W. Castenholz. 1974a. A phototrophic gliding filamentous bacterium of hot springs, Chloroflexus aurantiacus, gen. and sp. nov. Arch. Microbiol. 100:5–24.

Pierson, B. K., and R. W. Castenholz. 1974b. Studies of pigments and growth in Chloroflexus aurantiacus, a phototrophic filamentous bacterium. Arch. Microbiol. 100:283–305.

Pierson, B. K., and J. P. Thornber. 1983. Isolation and spectral characterization of photochemical reaction centers from the thermophilic green bacterium Chloroflexus aurantiacus strain J-10-fl. Proc. Natl. Acad. Sci. USA 80:80–84.

Pierson, B. K., S. J. Giovannoni, and R. W. Castenholz. 1984a. Physiological ecology of a gliding bacterium containing bacteriochlorophyll a. Appl. Environ. Microbiol. 47:576–584.

Pierson, B. K., L. M. Keith, and J. G. Leovy. 1984b. Isolation of pigmentation mutants of the green filamentous photosynthetic bacterium Chloroflexus aurantiacus. J. Bacteriol. 159:576–584.

Pierson, B. K. 1985a. Cytochromes in Chloroflexus aurantiacus grown with and without oxygen. Arch. Microbiol. 143:260–265.

Pierson, B. K., S. J. Giovannoni, D. A. Stahl, and R. W. Castenholz. 1985b. Heliothrix oregonensis, gen. nov., sp. nov., a phototrophic filamentous gliding bacterium containing bacteriochlorophyll a. Arch. Microbiol. 142:164–167.

Pierson, B. K., D. Valdez, M. Larsen, E. Morgan, and E. E. Mack. 1994. Chloroflexus-like organisms from marine and hypersaline environments: Distribution and diversity. Photo. Res. 41:35–52.

Pierson, B. K., and R. W. Castenholz. 1995. Taxonomy and physiology of filamentous anoxygenic phototrophs. *In:* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (Eds.) Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers. Dordrecht, The Netherlands. 31–47.

Pierson, B. K., and M. T. Parenteau. 2000. Phototrophs in high iron microbial mats: microstructure of mats in iron-depositing hot springs. FEMS Microbiol. Lett. 32:181–196.

Pierson, B. K. 2001. Family I: "Chloroflexaceae," filamentous anoxygenic phototrophic bacteria. *In:* D. R. Boone, R. W. Castenholz, and G. M. Garrity (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:427–444.

Pivovarova, T. A., and V. M. Gorlenko. 1977. Fine structure of Chloroflexus aurantiacus var. mesophilus (Nom. prof.) grown in light under aerobic and anaerobic conditions. Microbiology 46:276–282.

Schmidt, K. 1980a. A comparative study on the composition of chlorosomes (chlorobium vesicles) and cytoplasimic membranes from Chloroflexus aurantiacus strain OK-70-fl and Chlorobium limicola f. thiosulfatophilum strain 6230. Arch. Microbiol. 124:21–31.

Schmidt, K., M. Maarzahl, and F. Mayer. 1980b. Development and pigmentation of chlorosomes in Chloroflexus aurantiacus strain Ok-70-fl. Arch. Microbiol. 127:87–97.

Shiozawa, J. A., F. Lottspeich, and R. Feick. 1987. The photochemical reaction center of Chloroflexus aurantiacus

is composed of two structurally similar polypeptides. Eur. J. Biochem. 167:595–600.

Shiozawa, J. A., F. Lottspeich, D. Oesterhelt, and R. Feick. 1989. The primary structure of the Chloroflexus aurantiacus reaction-center polypeptides. Eur. J. Biochem. 180:75–84.

Shiozawa, J. A. 1995. A foundation for the genetic analysis of green sulfur, green filamentous and heliobacteria. *In:* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (Eds.) Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers. Dordrecht, The Netherlands. 1159–1173.

Sugiura, M., M. Takano, S. Kawakami, T. K., and S. Hanada. 2001. Application of a portable spectrophotometer to microbial mat studies: Temperature dependence of the distribution of cyanobacteria and photosynthetic bacteria in hot spring water. Microb. Environ. 16:255–261.

Sirevåg, R., and R. W. Castenholz. 1979. Aspects of carbon metabolism in Chloroflexus. Arch. Microbiol. 120:151–153.

Speelmans, G., D. Hillenga, B. Poolman, and W. N. Konings. 1993. Application of thermostable reaction centers from Chloroflexus aurantiacus as a proton motive force generating system. Biochim. Biophys. Acta 1142:269–276.

Sprague, S. G., L. A. Staehelin, M. J. DiBartolomeis, and R. C. Fuller. 1981a. Isolation and development of chlorosomes in the green bacterium Chloroflexus aurantiacus. J. Bacteriol. 147:1021–1031.

Sprague, S. G., L. A. Staehelin, and R. C. Fuller. 1981b. Semiaerobic induction of bacteriochlorophyll synthesis in the green bacterium Chloroflexus aurantiacus. J. Bacteriol. 147:1032–1039.

Staehelin, L. A., J. R. Golecki, R. C. Fuller, and G. Drews. 1978. Visualization of the supramolecular architecture of chlorosomes (Chlorobium type Vesicles) in freeze-fractured cells of Chloroflexus aurantiacus. Arch. Microbiol. 119:269–277.

Stolz, J. F. 1983. Fine structure of the stratified microbiol community at Laguna Figueroa, Baja California, Mexico. I: Methods of in situ study of the laminated sediments. Precambrian Res. 20:479–492.

Stolz, J. F. 1984. Fine structure of the stratified microbiol community at Laguna Figueroa, Baja California, Mexico. II: Transmission electron microscopy as a diagnostic tool in studying microbial community in situ. *In:* Y. Cohen, R. W. Castenholz, and H. O. Halvorson (Eds.) Microbial Mat: Stromatolites. Alan R. Liss. New York, NY. 23–38.

Stolz, J. F. 1990. Distribution of phototrophic microbes in the flat laminated microbial mat at Laguna Figureoa, Baja, California, Mexico. Biosystems 23:345–357.

Strauss, G., W. Eisenreich, A. Bacher, and G. Fuchs. 1992. 13C-NMR study of autotrophic CO_2 fixation pathways in the sulfur-reducing Archaebacterium Thermoproteus neutrophilus and in the phototrophic Eubacterium Chloroflexus aurantiacus. Eur. J. Biochem. 205:853–866.

Strauss, G., and G. Fuchs. 1993. Enzymes of a novel autotrophic CO_2 fixation pathway in the phototrophic bacterium Chloroflexus aurantiacus, the 3-hydroxypropionate cycle. FEBS Eur. J. Biochem. 215:633–643.

Takaichi, S., K. Tsuji, K. Matsuura, and K. Shimada. 1995. A monocyclic carotenoid glucoside ester is a major carotenoid in the green filamentous bacterium Chloroflexus aurantiacus. Plant Cell Physiol. 36:773–778.

Takaichi, S., T. Maoka, M. Yamada, K. Matsuura, Y. Haikawa, and S. Hanada. 2001. Absence of carotenes and presence of a tertiary methoxy group in a carotenoid from a thermophilic filamentous photosynthetic bacterium Roseiflexus castenholzii. Plant Cell Physiol. 42:1355–1362.

Trost, J. T., J. D. McManus, J. C. Freeman, B. L. Ramakrishna, and R. E. Blankenship. 1988. Auracyanin, a blue copper protein from the green photosynthetic bacterium chloroflexus-aurantiacus. Biochemistry 7858–7863.

Trüper, H. G. 1976. Higher taxa of the phototrophic bacteria: Chloroflexaceae fam. nov., a family for the gliding, filamentous, phototrophic "green" bacteria. Int. J. Syst. Bacteriol. 26:74–75.

Tvermyr, M., B. E. Kristiansen, and T. Kristensen. 1998. Cloning, sequence analysis and expression in E-coli of the DNA polymerase I gene from Chloroflexus aurantiacus, a green nonsulfur eubacterium. Genet. Anal. Tech. Appl. 14:75–83.

Van Dorssen, R. J., and J. Amesz. 1988. Pigment organization and energy transfer in the green photosynthetic bacterium Chloroflexus aurantiacus. III: Energy transfer in whole cells. Photosynth. Res. 15:177–189.

Vasmel, H., and J. Amesz. 1983. Photoreduction of menaquinone in the reaction center of the green photosynthetic bacterium Chloroflexus aurantiacus. Biochim. Biophys. Acta 724:118–122.

Vasmel, H., J. Amesz, and A. J. Hoff. 1986. Analysis by exciton theory of the optical properties of the reaction center of Chloroflexus aurantiacus. Biochim. Biophys. Acta 852:159–168.

Venetskaya, S. L., and L. M. Gerasimenko. 1988. Electron-microscopic study of microorganisms in a halophilic cyanobacterial community. Microbiology 57:377–383.

Wagner-Huber, R., R. Brunisholz, G. Frank, and H. Zuber. 1988. The BChlc/e-binding polypeptides from chlorosomes of green photosynthetic bacteria. FEBS Lett. 239:8–12.

Ward, D. M., M. J. Ferris, S. C. Nold, and M. M. Bateson. 1998. Natural view of microbial biodiversity within hot spring cyanobacterial mat communities. Microbiol. Molec. Biol. Rev. 62:1353–1370.

Watanabe, Y., R. G. Feick, and J. A. Shiozawa. 1995. Cloning and sequencing of the genes encoding the light-harvesting B806-866 polypeptides and initial studies on the transcriptional organization of puf2B, puf2A and puf2C in Chloroflexus aurantiacus. Arch. Microbiol. 163:124–130.

Wechsler, T., R. Brunisholz, F. Suter, R. C. Fuller, and H. Zuber. 1985a. The complete amino acid sequence of a bacteriochlorophyll a binding polypeptide isolated from the cytoplasmic membrane of the green photosynthetic bacterium Chloroflexus aurantiacus. FEBS Lett. 191:34–38.

Wechsler, T., F. Suter, R. C. Fuller, and H. Zuber. 1985b. The complete amino acid sequence of the bacteriochlorophyll c binding polypeptide from chlorosomes of the green photosynthetic bacterium Chloroflexus aurantiacus. FEBS Lett. 181:173–178.

Wechsler, T. D., R. A. Brunisholz, G. Frank, F. Suter, and H. Zuber. 1987. The complete amino acid sequence of the antenna polypeptide B806-866- from the cytoplasmic membrane of the green bacterium Chloroflexus aurantiacus. FEBS Lett. 210:189–194.

Weller, R., M. M. Bateson, B. K. Heimbuch, Kopczynski, and D. M. Ward. 1992. Uncultivated cyanobacteria, Chloroflexus-like inhabitants, and spirochete-like inhabitants of a hot spring microbial mat. Appl. Environ. Microbiol. 58:3964–3969.

Widdel, F., and F. Bak. 1992. Gram-negative mesophilic sulfate-reducing bacteria. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (Eds.) The Prokaryotes, 2nd ed. Springer-Verlag. New York, NY. 3352–3378.

Wittmershaus, B. P., D. C. Brune, and R. E. Blankenship. 1988. Energy transfer in Chloroflexus aurantiacus: Effects of temperature and anaerobic conditions. *In:* H. Scheer and S. Schneider (Eds.) Photosynthetic Light-harvesting Systems. Walter de Gruyter Press. Berlin, Germany. 543–554.

Wynn, R. M., T. E. Redlinger, J. M. Foster, R. E. Blankenship, R. C. Fuller, R. W. Shaw, and D. B. Knaff. 1987. Electron-transport chains of phototrophically and chemotrophically grown Chloroflexus aurantiacus. Biochim. Biophys. Acta 891:216–226.

Xiong, J., W. M. Fischer, K. Inoue, M. Nakahara, and C. E. Bauer. 2000. Molecular evidence for the early evolution of photosynthesis. Science 289:724–1730.

Zannoni, D., and W. J. Ingledew. 1985. A thermodynamic analysis of the plasma membrane electron transport components in photoheterotrophically grown cells of Chloroflexus aurantiacus. FEBS Lett. 193:93–98.

Zannoni, D. 1986. The branched respiratory chain of heterotrophically dark-grown Chloroflexus aurantiacus. FEBS Lett. 198:119–124.

Zannoni, D. 1987. The interplay between photosynthesis and respiration in facultative anoxygenic phototrophic bacteria. *In:* S. Papa, B. Chance, and L. Ernster (Eds.) Cytochrome Systems. Plenum Press. New York, NY. 575–583.

Zannoni, D. 1995. Aerobic and anaerobic electron transport chains in anoxygenic phototrophic bacteria. *In:* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (Eds.) Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers. Dordrecht, The Netherlands. 949–971.

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CHAPTER 10.2

The Genus *Thermoleophilum*

JEROME J. PERRY

Introduction

Thermoleophilum, a genus of eubacteria obligate for aerobiosis and thermophily, is distinguished by the ability to grow solely on *n*-alkane substrates ranging in length from C_{13} to C_{20} (Merkel et al., 1978a; Merkel et al., 1978b; Zarilla and Perry, 1984; Zarilla and Perry, 1986). Mud samples obtained from hot springs and from environmental niches generally considered nonthermal have both yielded isolates of *Thermoleophilum*. Thermal elution profiles of DNA duplexes delineate two species within the genus, *T. album* and *T. minutum* (Zarilla and Perry, 1984; Zarilla and Perry, 1986). The sequence of 16S rRNA from *T. album* strain NM indicated that these bacteria are unique phylogenetically (C. Woese, personal communication). Further study (Brown and Haas, 1997) placed this genus in a grouping with the green nonsulfur bacteria (Fig. 1). Then the gene sequence that encodes RNase P RNA was cloned and RNA transcribed from this clone was catalytically active in vitro when reconstituted with a specific oligonucleotide that regenerated the secondary structure. A phylogenetic tree based on the sequencing of the cloned RNase P RNA is consistent with the tree based on small subunit rRNA. The RNase P in *T. album* is of type A, the common ancestral form. Organisms in the green nonsulfur group are progeny of a deep divergence in the eubacterial line of descent. Isolation of strains from environments separated by considerable geographic distance indicates that *Thermoleophilum* strains may be of fairly common occurrence. Efforts to isolate a mesophilic counterpart of *Thermoleophilum* that would also be restricted to growth on *n*-alkane substrates have been unsuccessful.

Thermoleophilum species are Gram-negative, slow growing, small, nonmotile bacteria. They cannot utilize sugars, amino acids, fatty acids, or any substrate tested (except the *n*-alkanes) as source of carbon or energy. During active growth on *n*-heptadecane, the organism can incorporate a limited amount of acetate into cell material (less than 10% of total cell carbon). Members of this genus have a unique tetrahydrogenated menaquinone that has not been observed in the respiratory pigments of bacteria of any other species. The catalase from *Thermoleophilum* is a manganese-containing enzyme relatively resistant to cyanide inhibition.

Habitat

Thermoleophilum strains have been isolated from mud samples obtained from both thermal and nonthermal environments. The thermal environments included the outlet stream of hot springs in the states of Wyoming, Arkansas, and New Mexico (Table 1). The nonthermal environments were mud samples from Roanoke Rapids, NC, and Beaufort, NC. All mud samples were taken from aquatic environments and the temperature of sampling sites in Yellowstone, WY, and Hot Springs, AR, were near the optimum for growth of the strains isolated (YS-3, YS-4, and HS-5) (Merkel et al., 1978b; Zarilla and Perry, 1984). The nonthermal environmental samples were obtained from dark mud that was regularly exposed to the sun (strains PTA-1 and RR-D). Solar heating can raise the temperature of such dark surfaces to 60°C or higher (Brock, 1970). Growth of these obligate thermophiles in nonthermal environments would probably occur sporadically when the transient temperatures rose to a suitable level.

Attempts to isolate a mesophilic organism restricted to growth solely on *n*-alkane substrates have been unsuccessful. The presence of fast-growing, *n*-alkane-utilizing organisms in most environments of moderate temperature might well preclude the isolation of organisms with growth characteristics equivalent to *Thermoleophilum* species.

Isolation and Enrichment

The primary enrichment medium for the isolation of *Thermoleophilum* species was prepared by adding *n*-heptadecane at 0.1% (v/v) to a mineral (L [liquid]-salts) basal medium modified

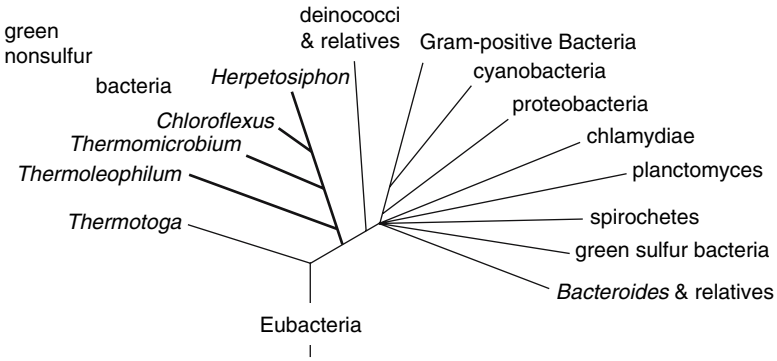


Fig. 1. The phylogenetic position of the genus *Thermoleophilum* based on 16S rRNA sequence analysis. Note the genus resides in the "green-nonsulfur-bacteria" branch of the Eubacteria (indicated by boldface). The tree was constructed based on maximum likelihood analysis.

Table 1. Locations where *Thermophilum* strains have been isolated.

Strain	Source of inoculum	Temperature
T. album		
HS-5	Hot Spring, AR	61°C
NM	Faywood Hot Springs, NM	>50°C[a]
YS-3	Yellowstone National Park, WY	60°C
RR-D	Roanoke Rapids, NC	Ambient
T. minutum		
YS-4	Yellowstone National Park, WY	63°C
PTA-1	Beaufort, NC	Ambient

[a]Close to the water source and above 50°C.
Adapted from Zarilla and Perry, 1984.

from that of Leadbetter and Foster (1958). The recipe for this medium is given below. Nitrogen was provided in the modified medium by adding 1 g/liter of each of NH_4Cl and $NaNO_3$ (Zarilla and Perry, 1984). Mud samples employed as inocula were obtained from both thermal and nonthermal environments (Table 1). Incubation was at 60°C in stationary culture until turbidity was apparent. After repeated transfer in the liquid medium, a pure culture was obtained by streaking on plates of 3.0% agar containing the modified L-salts medium. The *n*-heptadecane substrate was introduced by inverting the inoculated plate and placing 0.2-ml *n*-heptadecane in the cover (Perry, 1985). The plates were placed in plastic bags (to prevent desiccation) and incubated at 60°C for days. The colonies were small white, dry and flat. Axenic cultures were difficult to obtain as the organisms grow poorly on agar surfaces.

Mineral-Salts Medium (Modified L-Salts) for *Thermoleophilum* Species

NH_4Cl	11.0 g
$NaNO_3$	1.0 g
$MgSO_4 \cdot 7H_2O$	200 mg
Na_2HPO_4	210 mg
NaH_2PO_4	90 mg
$CaCl_2$	15 mg
KCl	140 mg
$FeSO_4 \cdot 7H_2O$	1 mg
$CuSO_4 \cdot 5H_2O$	5 mg
H_3BO_3	10 mg
$MnSO_4 \cdot 5H_2O$	10 mg
$ZnSO_4 \cdot 7H_2O$	70 mg
MoO_3	10 mg

Distilled water 1 liter

Cultivation

Growth of the isolates occurred between 45 and 70°C (optimally at 58–62°C) and at a pH between 6.5 and 7.5 (Table 2). The generation time was difficult to determine accurately but exceeded 6 h in every case. Strains are all strict aerobes but do not respond favorably to increased oxygen availability. Under optimal conditions of temperature (60°C), pH (7.0), and substrate (*n*-heptadecane), the cell yield was 0.1–0.3 g/liter (Zarilla and Perry, 1984; Zarilla and Perry, 1986).

Substrate Utilization Patterns

None of the *Thermoleophilum* strains grew in the presence of any substrate tested other than *n*-alkanes (C_{13}–C_{20}). No growth occurred on shorter or longer chains of *n*-alkanes, nor on 1-alkenes or alcohols of a chain length C_{13}–C_{20}. Homologous ketones and branched alkanes (C_{13}–C_{17}) were not utilized. The following substrates did not support growth: arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, melibiose, rhamnose, ribose, sorbose, sucrose, trehalose, xylose, glycerol, mannitol, sorbitol, acetate, propionate, butyrate, citrate, pyruvate, succinate, acetone, nutrient broth, peptone, yeast extract, or tryptone plus yeast extract. All of the strains utilized ammonium chloride as a nitrogen source. Strains HS-5, NM and RR-D also could utilize sodium nitrate as sole nitrogen source. Two strains,

Table 2. Properties of *T. album* and *T. minutum*.

Strain	Cell length (μm)	Generation time (h)	n-Alkane substrate range	GC content (mol%)
T. album				
HS-5	0.9	6.5	C_{13}–C_{20}	70.4
NM	1.0	9	C_{14}–C_{20}	68.8
YS-3	0.7	6	C_{13}–C_{20}	69
RR-D	1.0	7.5	C_{14}–C_{20}	70
T. minutum				
YS-4	1.5	6	C_{13}–C_{20}	70
PTA-1	1.1	7.5	C_{14}–C_{19}	68.8

HS-5 and NM, could derive their nitrogen from glycine but growth was poor, and none of the carbon of the glycine was incorporated. Neither alanine nor glutamate could serve as source of nitrogen. Addition of growth factors, e.g., B-vitamins or amino acids, to the growth medium did not alter the growth rate or total growth. Mineral salts medium similar in composition to the hot springs water of the original sampling site in Yellowstone did not affect growth rate or cell yield.

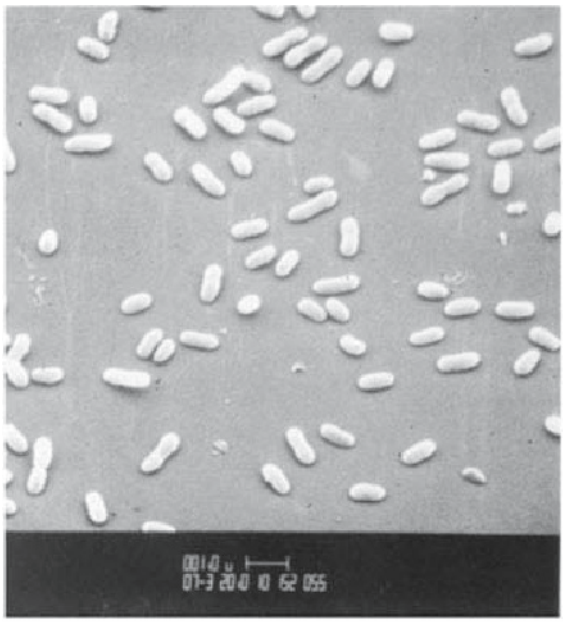
Identification

Thermoleophilum species are very small, Gram-negative rods (Table 1 and Fig. 2). The organisms are nonmotile and have no pigmentation. They have been isolated only on *n*-alkane enrichment substrates (C_{13}–C_{20}) and are readily identified by their inability to grow on any other substrate. *Thermoleophilum* strains have a G+C content of 68.8 to 70.4 mol%. The major diamino acid in the cell wall peptidoglycan of the genus *Thermoleophilum* is diaminopimelic acid. All strains grew to confluence on agar surfaces with few individual colonies formed. Colonies were small, round, and white and generally dry and flaky. They were often closely associated with pink-pigmented thermophilic strains on original isolation, and separating the slow-growing *Thermoleophilum* species was a tedious process.

The DNA hybridization studies indicated that *T. album* and *T. minutum* are of the same genus but different species (Zarilla and Perry, 1986; Table 3). There was no detectable homology with *Thermomicrobium roseum*, *Bacillus thermoleovorans* (Zarilla and Perry, 1987) or *Thermus aquaticus*. Data presented in Fig. 3 confirm that there are two species within this genus.

Physiological Characteristics

Menaquinones are widely distributed in the plasma membranes of prokaryotes and are generally distinguished by the length of their isopre-



RRD

Fig. 2. Electron micrograph of *Thermoleophilum* album strain RR-D.

Table 3. Relative binding in reassociation reactions with [α-^{32}P]deoxycytidine triphosphate-labeled DNA from *T. album* (HS-5) and *T. minutum* (YS-4).

	Relative binding (%) with the following source of radiolabeled DNA[a]			
	T. album HS-5		*T. minutum* YS-4	
Strain	62°C	76°C	62°C	76°C
T. album				
HS-5	100[b]	100	44	27
YS-3	81	76	42	27
NM	101	94	42	28
RR-D	86	79	39	28
T. minutum				
YS-4	43	25	100	100
PTA-1	44	25	99	99

[a]Average of duplicate determinations.
[b]Temperature at which reassociation occurred. 62°C is the optimal temperature; 70°C is the stringent temperature. Adapted from Zarilla and Perry, 1986.

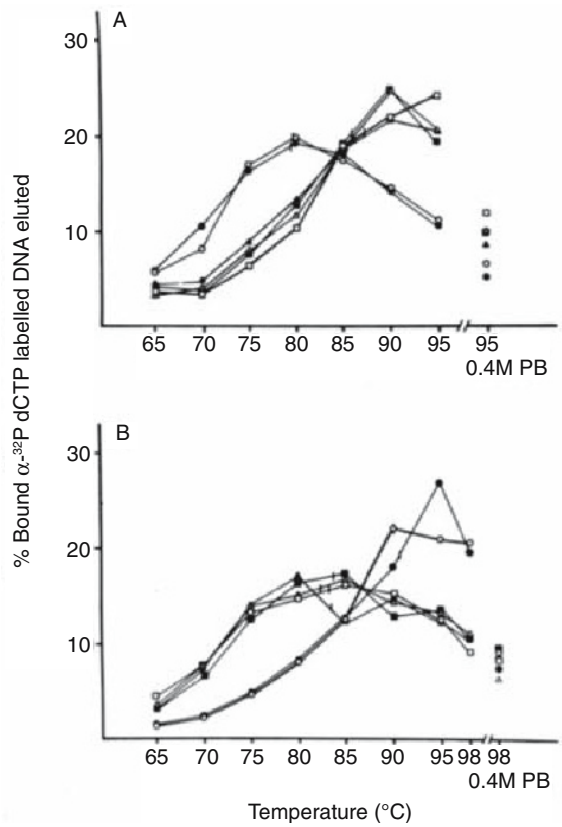


Fig. 3. Thermal elution profiles of *Thermoleophilum* DNA duplexes reassociated at 62°C. One strand of each DNA was made up of [α-^{32}P]deoxycytidine triphosphate (dCTP)-labeled DNA from either *Thermoleophilum album* strain HS-5T (A) or *Thermoleophilum minutum* strain YS–4T (B). The other strand was made up of DNA from strain HS-5T (□), YS-3 (▲), YS-4T (○), NM (▲), RR-D (■), or PTA-1 (●). The homologous duplex is indicated by a thick line. The thermal stability values are indicated by a vertical line on each curve and represent the temperature at which 50% of the double-stranded DNA was eluted from the hydroxyapatite. The DNA was eluted with 0.14 M phosphate buffer (PB) containing 0.1% sodium dodecyl sulfate until the maximum temperature was attained, at which point any remaining-bound DNA was eluted by using 0.4 M phosphate buffer. Adapted from Zarilla and Perry (1986).

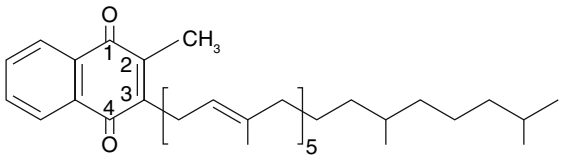


Fig. 4.

nyl side chain. The number of isoprenoid units varies from 1 to 15, and species also differ in the number of these isoprenoids that are saturated/unsaturated (Collins and Jones, 1981). Generally Gram-negative eubacterial menaquinones are composed of unsaturated isoprenoid units but a few Gram-negative bacterial species have dihydrogenated menaquinones. Examination of the respiratory quinones of *T. album* strain NM led to the isolation of a novel tetrahydrogenated menaquinone, 2 methyl-3-VI,VII-tetrahydrohepta-prenyl-1, 4-naphthoquinone, as depicted in Fig. 4 (Collins et al., 1986). The presence of this naphthoquinone in *T. album* readily distinguishes the genus from other Gram-negative, aerobic, obligately thermophilic groups. The genus *Thermus* and *Thermomicrobium roseum* contain saturated menaquinone as their major respiratory quinone.

Superoxide dismutase (SOD) is induced in aerobic bacteria by high oxygen stress. This enzyme, together with catalase and peroxidase, removes toxic oxygen intermediates generated as byproducts in the univalent reduction of molecular oxygen. These highly reactive intermediates of oxygen reduction are predominantly superoxide anion, hydrogen peroxide, and the hydroxyl radical. Growth of aerobic organisms at higher oxygen tensions or in the presence of methyl viologen (a superoxide generator) will effectively induce SOD (DiGuiseppi and Fridovich, 1984). Studies suggest that thermophilic bacteria generally react unfavorably to increased oxygen tension (Allgood and Perry, 1985b). As a consequence of this observation, an examination was made of the effect of oxygen stress on the level of SOD, catalase and peroxidase in *T. album* NM. Growth of strain NM in the presence of methyl viologen did not affect the level of cellular SOD but caused a marked increase in catalase activity. Peroxidase activity increased a small amount. Increasing the oxygen level also led to higher levels of catalase with a decrease in total growth (Allgood and Perry, 1985a; Allgood and Perry, 1985b; Allgood and Perry, 1986b). The level of oxygen defense enzymes is comparable with that in other aerobic bacteria. The products of O_2 reduction do not seem to be responsible for the negative response to increased aeration.

The catalase from *T. album* NM has been isolated, purified (>96%), and characterized (Allgood and Perry, 1986a). The major properties of the enzyme are presented in Table 4. For comparison the properties of the equivalent enzyme from *Escherichia coli*, *Rhodopseudomonas sphaeroides* and *Lactobacillus plantarum* are included. The catalase from *T. album* is thermoactive and thermostable. It has a low Mr, and the metal present is manganese. This is the only report (Allgood and Perry, 1986a) of a manganese-containing catalase in an aerobic

Table 4. Comparison of the properties of the catalase from *T. album* with that from other bacterial species.

Organism	Metal present	M_r	Number of subunits
Thermoleophilum album	Mn	141,000	4
Escherichia coli	Fe	337,000	4
Rhodopseudomonas sphaeroides	Fe	232,000	4
Lactobacillus plantarum	Mn	172,000	6

organism. The significance of such a catalase in this ancient thermophile is not understood at this time.

The electrophoretic mobilities of several enzymes in crude cell-free extracts from *T. album* strains were markedly different from the equivalent enzyme from other thermophiles (Zarilla and Perry, 1986). Among these enzymes of differing mobility were malate dehydrogenase, catalase, esterase and SOD. Electrophoresis of purified catalase and malate dehydrogenase from *T. album* NM resulted in an equivalent pattern.

Two enzymes from *Thermoleophilum* sp. were purified and characterized. These were the NADP-dependent isocitrate dehydrogenase (IDH) from *T. minutum* YS-4 (Novotny and Perry, 1991) and the malate dehydrogenase (MDH) from *T. album* NM (Novotny and Perry, 1990). Purification was by affinity chromatography and electroelution from a nondenaturing polyacrylamide gel. Both enzymes were thermostable and under nondenaturing conditions had distinctly different electrophoretic mobilities than the equivalent enzyme from other genera tested, including other thermophiles. The IDH had an Mr of 60,000 and was composed of two identical subunits. The optimum pH for activity was 7.2, at a temperature of 75°C. The enzyme required Mn^{+2} or Mg^{+2} for activity and NAD could not substitute for NADP. The amino acid composition of the IDH had an Arg/Lys ratio of 4.1 and very high levels of glycine. The purified MDH had an Mr of 61,000 and consisted of two subunits, each with an Mr of 32,500. Optimal activity occurred at 60°C and a pH of 7.5. The *T. album* MDH differed in amounts of arginine, lysine, glycine, proline, and histidine from the MDHs of other thermophilic and mesophilic microorganisms.

Other enzymes have been partially purified from *T. album* including a novel NADH-ferricyanide oxidoreductase (Vrana et al., 1999) and an alcohol dehydrogenase (Cacek et al., 1995). The NADH reductase catalyzes the transfer of electrons to mammalian cytochrome *c*.

The *T. minutum* YS-4 extracts contained all of the enzymes involved with the tricarboxylic acid cycle except α-ketoglutarate dehydrogenase (J. G. McCarthy and J. J. Perry, unpublished observation). Exhaustive attempts to demonstrate this enzyme in any of the *Thermoleophilum* strains have been unsuccessful. An active isocitrate lyase was present in extracts of *Thermoleophilum*, suggesting that an active glyoxylate cycle is present. All strains had NADH and NADPH oxidase activity. The *T. album* NM extracts had the large-sized citrate synthase typical of Gram-negative bacteria (Weaver et al., 1987). Addition of ^{14}C-acetate to nonproliferating cells of *T. album* NM resulted in little incorporation of radiolabel. Addition of radiolabeled acetate to a culture during active growth on *n*-heptadecane did result in some incorporation of acetate, but at a level of less than 10% of the total cell carbon. Increasing the level of acetate from 0.1 mM to 50 mM did not lead to any increase in incorporation (Weaver et al., 1987). The inability of the organism to incorporate more added acetate and the absence of α-ketoglutarate dehydrogenase are reminiscent of these activities in the obligate autotrophs. Possibly, the genus *Thermoleophilum* cannot obtain energy from substrates other than the *n*-alkanes normally utilized.

Polyamines are a constituent of the hyperthermophilic and the moderate thermophilic bacteria and the thermophilic Archaea (Hamana et al., 1998). The cellular polyamines present in *Thermoleophilum* sp. have been characterized (Hamana et al., 1990; Allgood and Perry, 1992; Allgood and Perry, 1998). Two strains of *T. album* (HS-5 and YS-3) and two of *T. minutum* (YS-4 and PTA-1) were examined and all four strains had an equivalent pattern of polyamines. The major polyamine in this genus was N^4-bis (aminopropyl) spermidine. The distribution pattern of polyamines in *Thermoleophilum* was unique when compared with other thermophiles. The role of these compounds in thermophilic microorganisms is not known at the present time.

Literature Cited

Allgood, G. S., and J. J. Perry. 1985a. Paraquat toxicity and effect of hydrogen peroxide on thermophilic bacteria. J. Free Rad. Biol. Med. 1:233–237.

Allgood, G. S., and J. J. Perry. 1985b. Oxygen defense systems in obligately thermophilic bacteria. Can. J. Microbiol. 31:1006–1010.

Allgood, G. S., and J. J. Perry. 1986a. Characterization of a manganese-containing catalase from the ob1igate ther-

mophile Thermoleophilum album. J. Bacteriol. 168:563–567.

Allgood, G. S., and J. J. Perry. 1986b. Effect of methyl viologen and oxygen concentration on thermophilic bacteria. J. Basic Microbiol. 26:379–382.

Brock, T. D. 1970. High temperature systems. *In:* R. F. Johnston, P. W. Frank, and C. D. Michner (Eds.) Annual Review of Ecology and Systematics. 1:Annual Reviews. Palo Alto, CA. 191–220.

Brown, J. W., and E. S. Haas. 1997. RNase P RNAs of Green Non-sulfur Bacteria. Oxford University Press. London, Nucleic Acids Symposium Series No. 36:73–75.

Cacek, R. A., and M. T. Werth. 1995. Isolation of a novel alcohol dehydrogenase from Thermoleophilum album. Abstracts of the 210th American Chemical Society Meeting *In:* Washington DC, CHED-136:

Collins, M. D., and D. Jones. 1981. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. Microbiol. Rev. 45:316–354.

Collins, M. D., O. W. Howarth, and J. J. Perry. 1986. A new respiratory quinone, 2-methyl-3-VI, VII-tetrahydroheptaprenyl-1, 4-naphthoquinone isolated from Thermoleophilum album. FEMS Microbiol. Lett. 34:167–171.

DiGuiseppi, J., and I. Fridovich. 1984. The toxicity of molecular oxygen. CRC Critical Reviews in Toxicology 12: 315–342.

Hamana, K., S. Matsuzaki, M. Niitsu, and K. Samejima. 1990. Two tertiary branched tetraamimines, N4-aminopropylnorspermidine and N4-aminopropylspermidine are the major polyamines in thermophilic eubacteria, Thermoleophilum. FEMS Microbiol. Lett. 66:35–38.

Hamana, K., M. Niitsu, S. Matsuzaki, K. Samejima, Y. Igarashi, and T. Kodama. 1992. Novel linear and branched polyamines in the extremely thermophilic eubacteria Thermoleophilum, Bacillus, and Hydrogenobacter. Biochem. J. 284:741–747.

Hamana, K., M. Niitsu, K. Samejima, T. Itoh, H. Hamana, and T. Shinozawa. 1998. Polyamines of the thermophilic eubacteria belonging to the genera Thermotoga, Thermodesulfovibrio, Thermoleophilum, Thermus, Rhodothermus, and Meiothermus and the thermophilic archaebacteria belonging to the genera Aeropyrum, Picrophilus, Methanobacterium, and Methanococcus. Microbios. 94:7–21.

Leadbetter, E. R., and J. W. Foster. 1958. Studies on some methane-utilizing bacteria. Arch. Microbiol. 30:91–118.

Merkel, G. J., S. S. Stapleton, and J. J. Perry. 1978a. Isolation and peptidoglycan of Gram-negative hydrocarbon-utilizing thermophilic bacteria. J. Gen. Microbiol. 109: 141–148.

Merkel, G. J., W. H. Underwood, and J. J. Perry. 1978b. Isolation of thermophilic bacteria capable of growth solely on long-chain hydrocarbons. FEMS Microbiol. Lett. 3:81–83.

Novotny, J. F., and J. J. Perry. 1990. Characterization of the malate dehydrogenase from Thermoleophilum album NM. Arch. Microbiol. 154:304–307.

Novotny, J. F., and J. J. Perry. 1991. Characterization of a heat-stable NADP-dependent isocitrate dehydrogenase from the obligate thermophile Thermoleophilum minutum YS-4. Appl. Microbiol. Biotechnol. 35:461–465.

Perry, J. J. 1985. Isolation and characterization of thermophilic hydrocarbon-utilizing bacteria. *In:* H. W. Jannasch and P. J. L. Williams (Eds.) Advances in Aquatic Microbiology. Academic Press. London, 3:109–139.

Vrana, A. M., J. L. Linder, and M. T. Werth. 1999. Partial purification and characterization of a novel NADH-reductase from Thermoleophilum album. *In:* Abstracts of the 217th American Chemical Society Meeting. Anaheim, CA. CHED-276.

Weaver, K. L., K. C. Terlesky, and J. J. Perry. 1987. Metabolism in the obligately hydrocarbonoclastic genus Thermoleophilum. *In:* Abstracts of the 87th General Meeting of the American Society for Microbiology. Washington DC, Abstr. I-136:195.

Zarilla, K. A., and J. J. Perry. 1984. Thermoleophilum album gen. nov. and sp. nov., a bacterium obligate for thermophily and n-alkane substrates. Arch. Microbiol. 137:286–290.

Zarilla, K. A., and J. J. Perry. 1986. Deoxyribonucleic acid homology and other comparisons among obligately thermophi1ic hydrocarbonoclastic bacteria, with a proposal for Thermoleophilum minutum sp. nov. Int. J. Syst. Bacteriol. 36:13–16.

Zarilla, K. A., and J. J. Perry. 1987. Bacillus thermoleovorans, sp. nov., a species of obligately thermophilic hydrocarbon utilizing endosporeforming bacteria. System. Appl. Microbiol. 9:258–264.

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CHAPTER 10.3

The Genus *Thermomicrobium*

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Introduction

Thermomicrobium roseum (ATCC 27502), the sole representative of a phylogenetically distinct branch of the eubacteria, is an obligate thermophile originally isolated from the effluent of a hot spring in Yellowstone National Park, WY (Jackson et al., 1973). The grouping of *T. roseum* with the green nonsulfur (GNS) bacteria is based on ribosomal RNA sequence comparisons (Oyaizu et al., 1987). The sequence of 5S rRNA from *T. roseum* (Van den Eynde et al., 1990) affirms that it should be clustered with *Chloroflexus*. *Herpetosiphon* and *Chloroflexus*, the other representatives in this branch, are markedly different phenotypically from *T. roseum* (for a review see Kristjansson and Alfredsson, 1992). Although the GNS bacteria share some common ribosomal characters, they are the progeny of a deep phylogenetic divergence (Gibson et al., 1985); *Herpetosiphon* is a mesophile and more rapidly evolving than either *Chloroflexus* or *Thermomicrobium*. The deepest branching in eubacterial evolution is predominantly represented by thermophiles (see Fig. 1, *Thermoleophilum* chapter) and these organisms evolved more slowly than the mesophiles. They have consequently retained more of their ancestral character (Achenbach-Richter et al., 1987). Recent results (Haas and Brown, 1998) indicate that most of the unusual structural elements of type B RNase P RNAs found in low G+C Gram-positive bacteria evolved independently (apparently by convergent molecular means) in *T. roseum*. The gene for heat shock protein (HSP70) present in *T. roseum* was cloned and the protein generated from this was sequenced. The results of sequencing affirm the relatedness of *T. roseum* to other GNS bacteria (Gribaldo et al., 1999; Gupta et al., 1997).

Isolation and Enrichment

The primary enrichment medium for *T. roseum* was composed of 0.1% tryptone and 0.1% yeast extract (TYE) in Allen's salts (see recipe below; Allen, 1959). Enrichment media were inoculated with samples of bacterial mat or water from various springs in Yellowstone National Park and were incubated at 70, 75, 80 and 85°C. After a suitable time, the enrichments were streaked on plates of TYE agar (3.0%) containing Castenholz's salts solution (Jackson et al., 1973; Castenholz, 1969). The enrichment culture of bacterial mat taken from Toadstool Spring in Yellowstone and incubated at 70°C was the only one that yielded unusual colonies. These compact pink colonies were distinctly different from colonies of other isolates, which appeared to be mostly of the genus *Thermus*. Transfer by streaking on the TYE agar medium at pH 8.5 with as much as 0.5% tryptone and yeast extract led to a pure culture of *T. roseum* (Jackson et al., 1973).

Allen's Salts Solution (mg/liter)

$(NH_4)_2SO_4$	1,300
KH_2PO_4	280
$MgSO_4 \cdot 7H_2O$	247
$CaCl_2 \cdot 2H_2O$	74
$FeCl_3 \cdot 6H_2O$	19
$MnC1_2 \cdot 4H_2O$	1.8
$Na_2B_4O_7 \cdot 10H_2O$	4.4
$ZnSO_4 \cdot 7H_2O$	0.22
$CuCl_2 \cdot H_2O$	0.05
$Na_2MoO_4 \cdot 2H_2O$	0.03
VCl_2	0.03

Dissolve in deionized distilled water and adjust the pH to 7.8.

Castenholz's Salts Solution (mg/liter)

Nitrilotriacetic acid	100
$CaSO_4 \cdot 2H_2O$	60
$MgSO_4 \cdot 7H_2O$	100
NaCl	9
KNO_3	103
$NaNO_3$	689
Na_2HPO_4	111
$FeCl_3$	0.28
$MnSO_4 \cdot H_2O$	2.2
$ZnSO_4 \cdot 7H_2O$	0.5
H_3BO_3	0.5
$CuSO_4$	0.016
$Na_2MoO_4 \cdot 2H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.045

Dissolve in deionized distilled water.

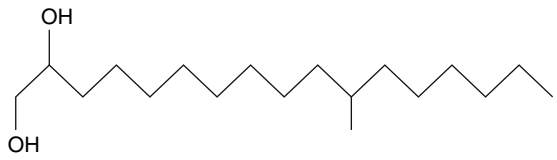

Fig. 1. Typical structure of a long chain 1,2 diol.

Habitat

Toadstool Spring (no longer active) was located in the Lower Geyser Basin of Yellowstone National Park. *Thermomicrobium roseum* was isolated from a sample taken near the source of the spring, where the water temperature was 74°C and pH was 8–9. The inoculum was from an orange/red bacterial mat attached to a silica substrate. The mat had accumulated under a piece of wax paper debris that may have offered some protection from the high light intensity and rapidly moving water. Although Jackson et al. (1973) suggested that glass slides immersed in the water near the source of the spring had exhibited microbial growth that appeared similar to *T. roseum*, microbes have not yet been cultivated from these sources. Biologically active layers of a cyanobacterial mat have been analyzed for various apolar lipid, glycolipid and phospholipid content (Zeng et al., 1992). At 1–2 and 2–4 mm intervals, long-chain diols were detected which were apparently derived from glycolipids of microorganisms such as *T. roseum.*

A thermophilic organism was isolated from soil by enrichment with *n*-alkanes as substrate and was tentatively assigned to the genus *Thermomicrobium* (Phillips and Perry, 1976). Subsequent DNA–DNA hybridization studies indicated that this isolate should be placed in an entirely different genus (Zarilla and Perry, 1986; Zarilla and Perry, 1987).

Substrate Utilization Patterns

Complex media are the preferred substrate for *T. roseum*. A maximum cell yield was attained with tryptone and yeast extract each added at 0.5%, with less (0.3%) supporting the most rapid growth. Little growth occurred with the substrates added at 1.0%. Levels higher than 1.0% completely inhibited growth. Growth on peptone, casein hydrolysate, brain heart infusion, nutrient broth, trypticase soy broth, and on tryptone or yeast extract (substrate at 0.2%) required inclusion of NH_4^+ (in Castenholz's salts solution) as nitrogen source. Of these, only nutrient broth and yeast extract yielded growth with NO_3^- (in Allen's salts solution) as the source of nitrogen. Growth on defined medium with selected substrates occurred only when 0.2% glutamate was added with nitrogen source NO_3^-. Glycerol and sucrose were adequate carbon sources under these conditions. Sparse growth occurred with glutamate as sole substrate (in Allen's salts solution). Fructose, succinate, mannitol, acetate and citrate were inadequate as growth substrates (Jackson et al., 1973). The organism grew consistently better with Allen's than with Castenholz's salts solution. Apparently, some ammonia was lost from the latter at this pH and temperature.

Cultivation

Growth was optimum at 70–75°C at pH 8.2–8.5 and was possible at temperatures as low as 45–48°C, at pH 7–8.7. Under optimum conditions with tryptone and yeast extract (0.3%) as substrates, the organism had a generation time of 5 to 5.5 h.

Identification

T. roseum cells are generally pleomorphic (with some that are dumbbell shaped) and occur as short, irregularly shaped (single or paired) rods. The organism is 1.3 to 1.8 μm in diameter and 3 to 6 μm in length. The G+C content of the DNA is 64.3 mol%. The colonies on agar are compact and pink-pigmented. Although some slight motility was observed by light microscopy, no flagella were seen by electron microscopy, and the organism is considered to be nonmotile (Jackson et al., 1973). Endospores are not visible in *T. roseum* by light, phase contrast or electron microscopy. The organism is Gram-negative, and thin sections viewed longitudinally have a layered cell wall consistent with this staining character. Electron-dense hexagonal structures were visible in thin sections that may have been bacteriophages, but no lytic activity was observed (Jackson et al., 1973).

DNA hybridization reactions were employed to determine whether *T. roseum* DNA reassociated with DNA from thermophilic *Bacillus* strains, *Thermus aquaticus* and *Thermoleophilum album*. The results confirmed that *T. roseum* is not related to any of these other organisms (Zarilla and Perry, 1986; Zarilla and Perry, 1987). Antibiotic sensitivity tests indicate that *T. roseum* is sensitive to kanamycin, neomycin and penicillin, but less sensitive to erythromycin and chloromycetin (Jackson et al., 1973).

The pigments extracted from *T. roseum* with acetone were analyzed spectrophotometrically. The absorbance maxima at 470, 494 and 530 nm indicate that *T. roseum* carotenoid pigments are

similar to torulene and 3,4-dehydrolycopene. Extracted pigment from the bacterial mats that served as the enrichment inocula had equivalent absorptive properties (Jackson et al., 1973).

Physiological Properties

Membrane Lipid Composition

Virtually all eubacterial and eukaryotic cells have membrane lipids composed of fatty acids esterified to glycerophosphate. The membranes of archaebacteria differ markedly in that the lipids of these organisms are composed of isoprenoid chains joined to glycerol by an ether linkage. The membrane lipids of *T. roseum* have neither ester nor ether linkages, but are composed of a series of straight-chain and internally branched l,2-diols. A typical 1,2-diol is shown in Fig. 1. The carbon length of the diols ranges from C_{18} to C_{23} (Pond et al., 1986). Similar long-chain 1,2 diols have been characterized from strains of the genus *Thermus* (Wait et al., 1997). These thermophiles also contained both glycerol-linked and diol-linked glycolipids.

The cellular lipids in *T. roseum* account for about 3% of the dry weight of the cell, and, of this, 22.6% is nonpolar neutral lipid and 77.1% is polar glycolipids or phospholipids. Polar lipids were hydrolyzed to remove the polar head groups, and the apolar residues were analyzed (Pond et al., 1986). The distribution of the l,2-diols present in the lipid of *T. roseum* are listed in Table 1. These structures are analogous to the glycero-lipids in that the terminal hydroxyl can link to the polar-head group, and long-chain fatty acids can be esterified to the secondary hydroxyl. This process provides the hydrophobic chains necessary for a lipid bilayer (Pond et al., 1986).

Analysis of the fatty acid constituents of these unusual lipids indicated that they too had normal and internally methylated hydrocarbon chains (Pond and Langworthy, 1987). The distribution of fatty acids in *T. roseum* grown at 70°C is presented in Table 2. Methyl-branched-chain fatty acids are also common constituents of eubacterial lipids but generally they are *iso-* or *anteiso-*methylated fatty acids.

The effect of temperature on the relative amounts of normal to branched-chain fatty acids and diols was determined (Pond and Langworthy, 1987) and the results are presented in Table 3. As the growth temperature was increased, the relative percentage of unbranched fatty acids and diols increased, while the relative amount of the methyl-branched constituents decreased (Pond and Langworthy, 1987). The longer chain, normal fatty acids have a higher melting point than the branched homologs, so increased branching as the temperature is lowered may be a mechanism whereby membrane fluidity is retained at lower temperatures.

Polyamines have been detected as constituents of *T. roseum* (Hamana et al., 1990). These unusual polyamines are penta- and hexamines and their function is not yet known.

Cell Wall Structure

Electron microscopy of negatively stained outer cell wall preparations from *T. roseum* indicated

Table 1. Distribution of 1,2-diols in the lipids of *Thermomicrobum roseum* after growth at 75°C.

Compound	Percent of total
11-Methyl-1,2-heptadecanediol	<0.5
1,2-Nonadecanediol	10.6
13-Methyl-1,2-nonadecanediol	21.1
1,2-Cosanediol	6.1
13-Methyl-1,2-cosanediol	2.5
15-Methyl-1,2-cosanediol	0.9
1,2-Eicosanediol	48.5
15-Methyl-1,2-eicosanediol	7.8
1,2-Docosanediol	1.0
1,2-Tricosanediol	1.1
1,2-Tetracosanediol	<0.5

Adapted from Pond *etal.* (1986).

Table 2. Distribution of fatty acids in *Thermomicrobium roseum* after growth at 70°C.

Compound	Percent of total
n-Hexadecanoic acid	1.2
10-Methyl-pentadecanoic acid	1.5
n-Heptadecanoic acid	0.5
10-Methyl-hexadecanoic acid	0.7
n-Octadecanoic acid	24.2
12-Methyl-heptadecanoic acid	57.3
n-Nonadecanoic acid	2.6
12-Methyl-octadecanoic acid	1.9
n-Cosanoic acid	8.4
14-Methyl-nonadecanoic acid	1.2

Adapted from Pond and Langworthy (1987).

Table 3. Effect of temperature on the percentage of normal and branched-chain fatty acids and diols in *Thermomicrobium roseum*.

Growth temperature (°C)	Percent normal	Percent branched
Fatty Acids		
60	24.1	75.6
75	46.3	52.4
Diols		
60	62.2	37.1
75	87.3	12.5

Adapted from Pond and Langworthy (1987).

Table 4. Amino acid composition of the two fractions of the cell wall of *Thermomicrobium roseum*.

Component	Fraction A (%)	Fraction B (%)
Threonine	8.1	3.6
Serine	6.2	4.0
Proline	8.1	14.0
Muramic acid	3.1	0
Glutamic acid	12.8	11.9
Glycine	19.3	33.8
Alanine	16.8	11.5
Valine	4.0	1.8
Diaminopimelic acid	1.2	0
Leucine	6.2	3.2
Isoleucine	0	1.8
Tyrosine	4.7	2.2
Galactosamine	3.1	0
Histidine	1.2	3.2
Arginine	3.7	Trace
Lysine	0	Trace
Ornithine	0	7.2
Phenylalanine	0	1.8

[a]Expressed as % of the total amino acid/sugar present. From Merkel et al., 1978.

that there were regular, repeating structures present in the cell envelope. These structures appear in a consistent mosaic pattern (Ramaley et al., 1978). Analysis of purified cell walls from *T. roseum* suggested that the organism, although sensitive to penicillin (Jackson et al., 1973), has low levels of peptidoglycan (Merkel et al., 1978; Merkel et al., 1980). Cell walls were removed from *T. roseum* and purified. This purified cell wall material was readily separated into two fractions (A and B), which were analyzed for amino acid composition (Table 4). Fraction A was composed of an array of amino acids with alanine, glutamic acid and glycine predominating; lesser amounts of diaminopimelic acid, galactosamine and muramic acid were detected.

Fraction B was a cell wall protein (M_r of 75,000) that accounted for 60% of the total cell wall amino-reactive material. The amino acid composition of this protein is presented in Table 4. This protein is probably the principal structural component of the cell wall revealed by electron microscopy (Ramaley et al., 1978; Merkel et al., 1980). How this protein might contribute to the thermostability of *T. roseum* is unknown.

Metabolism

Although *T. roseum* is a strict aerobe, it does not respond to increased oxygen levels by more rapid growth or higher cell yields. This is a common attribute of aerobic thermophiles and particularly of those that grow on hydrocarbon substrates (Allgood and Perry, 1985a; Allgood and Perry, 1985b; Allgood and Perry, 1986). Growth of *T. roseum* under higher oxygen (O_2) tension did result in increased cellular levels of superoxide dismutase (SOD) and catalase. Addition of methyl viologen to increase superoxide anion generation in cells had little effect on the level of SOD but resulted in increased catalase activity (Allgood and Perry, 1986). That higher O_2 levels do not lead to more rapid growth or cell yield is probably due to factors other than the accumulation of toxic products of O_2 such as superoxide or hydrogen peroxide. Most of the respiratory activity in *T. roseum* is cyanide insensitive. The respiratory pathways in *T. roseum* have not been elucidated further (Allgood and Perry, 1985a; Allgood and Perry, 1985b).

Literature Cited

Achenbach-Richter, A., R. Gupta, K. O. Stetter, and C. R. Woese. 1987. Were the original eubacteria thermophiles? System. Appl. Microbiol. 9:34–39.

Allen, M. B. 1959. Studies with Cyanidium caldarium, an anomously pigmented chlorophyte. Arch. Microbiol. 32:270–277.

Allgood, G. S., and J. J. Perry. 1985a. Paraquat toxicity and effect of hydrogen peroxide on thermophilic bacteria. J. Free Rad. Biol. Med. 1:233–237.

Allgood, G. S., and J. J. Perry. 1985b. Oxygen defense systems in obligately thermophilic bacteria. Can. J. Microbiol. 31:1006–l010.

Allgood, G. S., and J. J. Perry. 1986. Effect of methyl viologen and oxygen concentration on thermophilic bacteria. J. Basic Microbiol. 26:379–382.

Castenholz, R. W. 1969. Thermophilic blue-green algae and the thermal environment. Bacteriol. Rev. 33:476–504.

Gibson. J., W. Ludwig, E. Stackebrandt, and C. R. Woese. 1985. The phylogeny of the green photosynthetic bacteria: Absence of a close relationship between Chlorobium and Chloroflexus. System. Appl. Microbiol. 6:152–156.

Gribaldo, S., V. Lumia, R. Creti, E.C. deMacario, A. Sanangelantoni, and P. Cammarano. 1999. Discontinuous occurrence of the hsp70 (dnaK) gene among Archaea and sequence features of HSP70 to suggest a novel outlook on phylogenies inferred from this protein. J. Bacteriol. 181:434–443.

Gupta, R. S., K. Bustard, M. Falah, and D. Singh. 1997. Sequencing of heat shock protein 70 (DnaK) homologs from Deinococcus proteolyticus and Thermomicrobium roseum and their integration in a protein-based phylogeny of prokaryotes. J. Bacteriol. 179:345–357.

Haas, E. S., and J. W. Brown. 1998. Evolutionary variation in bacterial RNase P RNAs. Nucleic Acids Res. 26:4093–4099.

Hamana, K., S. Matsuzaki, M. Niitsu, and K. Samejima. 1990. Pentamines and hexaamine are present in a thermophilic eubacterium, Thermomicrobium roseum. FEMS Microbiol. Lett. 68:31–34.

Jackson, T. J., R. F. Ramaley, and W. G. Meinschein. 1973. Thermomicrobium, a new genus of extremely thermophilic bacteria. Int. J. Syst. Bacteriol. 23:28–36.

Kristjansson, J. K., and G. A. Alfredsson. 1992. The heterotrophic, thermophilic genera Thermomicrobium, Rhodothermus, Saccharococcus, Acidothermus, and

Scotothermus. *In:* J. K. Kristjansson (Ed.) Thermophilic Bacteria. CRC Press. Boca Raton, FL. 63–76.

Merkel, G. J., S.S. Stapleton, and J. J. Perry. 1978. Isolation and peptidoglycan of Gram-negative hydrocarbon-utilizing thermophilic bacteria. J. Gen. Microbiol. 109: 141–148.

Merkel. G. J., D. R. Durham, and J. J. Perry. 1980. The atypical celI wall composition of Thermomicrobium roseum. Can. J. Microbiol. 26:556–559.

Oyaizu, H., D. Debrunner-Vossbrinck, L. Mandelco. J. A. Studier, and C. R. Woese. 1987. The green non-sulfur bacteria: A deep branching in the eubacterial line of descent. System. Appl. Microbiol. 9:47–53.

Phillips, W. E., and J. J. Perry. 1976. Thermomicrobium fosteri sp. nov., a hydrocarbon-utilizing obligate thermophile. Int. J. System. Bacteriol. 26:220–225.

Pond, J. L.,T. A. Langworthy, and G. Holzer. 1986. Long-chain diols: a new class of membrane lipids from a thermophilic bacterium. Science 231:1134–1136.

Pond, J. L., and T. A. Langworthy. 1987. Effect of growth temperature on the long-chain diols and fatty acids of Thermomicrobium roseum. J. Bacteriol. 169:1328–1330.

Ramaley, R. F., F. R. Turner, L. E. Malick, and R. B. Wilson. 1978. The morphology and surface structure of some extremely thermophilic bacteria found in slightly alkaline hot springs. *In:* S. M. Friedman (Ed.) Biochemistry of Thermophily. Academic Press. New York, NY. 89–102.

Van den Eynde, H., Y. Van de Peer, J. Perry, and R. De Wachter. 1990. 5S rRNA sequences of representatives of the genera Chlorobium, Prosthecochloris, Thermomicrobium, Cytophaga, Flavobacterium, Flexibacter, and Saprospira, and a discussion of the evolution of eubacteria in general. J. Gen. Microbiol. 136:11–18.

Wait, R., L. Carreto, M. F. Nobre, A. M. Ferreira, and M. S. Da Costa. 1997. Characterization of long-chain 1,2-diols in Thermus species and demonstration that Thermus strains contain both glycerol-linked and diol-linked glycolipids. J. Bacteriol. 179:6154–6162.

Zeng, Y. B., D. M. Ward, S. C. Brassell, and G. Eglinton. 1992. Biogeochemistry of hot spring environments 3: Apolar and polar lipids in the biologically active layers of a cyanobacterial mat. Chem. Geol. 95:347–360.

Zarilla, K. A., and J. J. Perry. 1986. Deoxyribonucleic acid homology and other comparisons among obligately thermophilic hydrocarbonoclastic bacteria, with a proposal for Thermoleophilum minutum sp. nov. Int. J. Syst. Bacteriol. 36:13–16.

Zarilla, K. A., and J. J. Perry. 1987. Bacillus thermoleovorans, sp. nov., a species of obligately thermophilic hydrocarbon utilizing endospore forming bacteria. System. Appl. Microbiol. 9:258–264.

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CHAPTER 10.4

The Genus *Herpetosiphon*

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The *Herpetosiphon* species are aerobic, chemoorganotrophic, filamentous bacteria that are Gram negative but do not have a typical Gram negative cell wall. The filaments are very long, unbranched, and multicellular, between 0.5 µm and 1.5 µm wide and usually 100 µm to more than 1200 µm long (Fig. 1). Short transparent sections ("sleeves"; Fig. 2) are seen at the ends of many filaments.

Sleeves are also found in related organisms, like *Chloroflexus*. Depending on the strain, the medium, and the age of the culture, the filaments may fragment into much shorter pieces and ultimately into single cells. The cells in the filaments are tightly attached to one another, and in living filaments, their boundaries can only be seen with a microscope of high resolution at a high magnification, by phase or interference contrast (Fig. 2). They become clearly recognizable if the filament has been fixed to the slide and stained. The cells have the same width as the filaments and measure 1.5–5 µm in length, rarely more. The filaments may perform slow gliding movements; thus, on suitable substrates, the colonies develop into large spreading swarms (Fig. 3). The surface of the swarm is usually felt-like. Long flame-like projections or protruding strands of curled filaments are seen at the edge (Fig. 3). Normally the colonies are colored in shades of yellow, orange, or brick red, but unpigmented strains, especially among *Herpetosiphon*-like strains, do occur (Lee, 2004). A film showing the movements of the filaments and the development of the colonies is available (Reichenbach et al., 1980).

Apparently, *Herpetosiphon* was first discussed in the scientific literature under the name "*Flexibacter giganteus*" (Soriano, 1945; Soriano, 1947). Soriano's isolation techniques, choice of habitats, and description of the filaments, the swarm colonies, and their color correspond to those of the cultures now called "*Herpetosiphon*." However, the first valid description of the genus *Herpetosiphon*, with one species, *H. aurantiacus* (ATCC 23779^T), was provided by Holt and Lewin (1968). The definition was based on three isolates of E. E. Jeffers obtained from the slime coat of a green alga, *Chara* sp., from Birch Lake in Minnesota. Later, a dispute arose about the presence of a sheath in *Herpetosiphon*, and the genus definition was slightly modified to exclude a sheath (Reichenbach and Golecki, 1975). Also, Reichenbach and Golecki (1975) proposed that the species name *aurantiacus* be abandoned in favor of the name *giganteus*, which antedates it. This change could have been done without danger of confusion since Soriano's strain is no longer available. However, since more than one *Herpetosiphon* species may exist, one could argue that Soriano's "*H. giganteus*" was different from *aurantiacus*. The possible existence of different species of *Herpetosiphon* was indicated by another study already during this period when a specimen was isolated from the walls of a sluice of the Neckar River near Heidelberg (Germany). This specimen showed a strong resemblance with *Herpetosiphon aurantiacus* but was not identified as such because of some minor differences in phenotypic characteristics (Brauss et al., 1969). Thus, it was decided to give up the name *giganteus* until more had been discovered about the taxonomy of the genus. In 1970, four new species, one from the vicinity of a hot spring (*H. geysericola* ATCC 23076^T) and three from marine habitats (*Herpetosiphon cohaerens* ATCC 23123^T, *Herpetosiphon nigricans* ATCC 23147^T and *Herpetosiphon persicus* ATCC 23167^T) were added to the genus (Lewin, 1970). However, all three marine species have recently been transferred to a new genus, *Lewinella*, in the phylum *Flexibacter-Bacteroides-Cytophaga* (Sly et al., 1998). Since 1998, over 30 new *Herpetosiphon* and *Herpetosiphon*-like isolates have been described from different environmental habitats, such as freshwater lake systems (Lake Constance, Germany; the strains were previously described as a new *Vitreoscilla* species, *V. proteolytica*; Gräf and Perschmann, 1970; Lee et al. (manuscript in preparation)), sewage plants (Trick and Lingens, 1984; Senghas and Lingens, 1985; Bradford et al., 1996; Kohno et al, 2002; Lee et al. (manuscript in preparation)), and different soil systems (Lee et al. (manuscript in

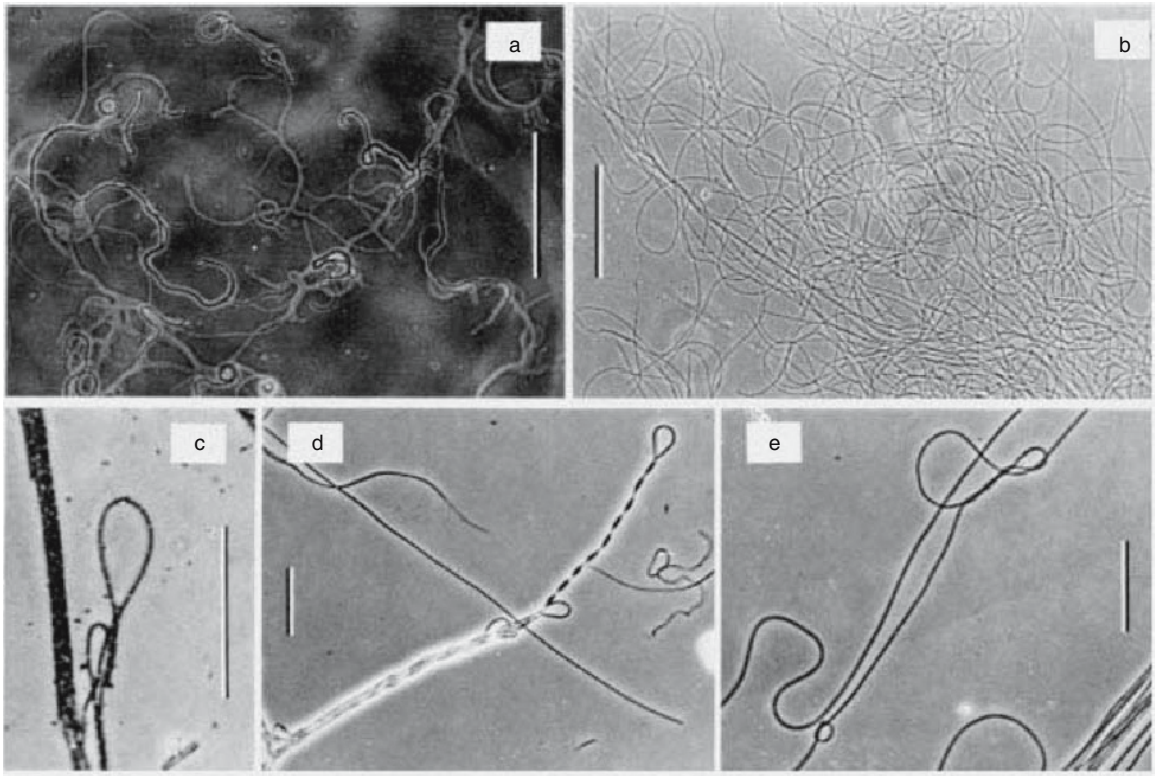

Fig. 1. Filaments of *Herpetosiphon aurantiacus* at low magnifications. a) Filaments with slime tracks in chamber culture. b) Filaments from a liquid culture, slide mount. c) Filaments stained with Loeffler's methylene blue; the individual cells of the filament are clearly visible. d) and e) Twisted filaments from a liquid culture. Zeiss Standard Microscope and Axiomat (d and e), in phase contrast. Bar = 100 μm (for a and b). Bar = 25 μm (for c, d and e).

preparation)). The G+C content of the DNA of about half of these isolates is 48–53 mol%, and the 16S rRNA gene similarity among these isolates is 96.4–99.5% (Sly et al., 1998; Lee et al. (manuscript in preparation)). However, the G+C content of the rest of the *Herpetosiphon*-like isolates, e.g., the strains of Senghas and Lingens (1985) and the strains Hp g6 (DSM 15710), Hp g16 (DSM 15736) and Hp g207 (DSM 15737), are higher (59–75%), which suggests that these isolates may represent novel genera among chloroflexi (Lee et al. (manuscript in preparation)).

A comparison of all present *Herpetosiphon* 16S rRNA gene sequences with all other available 16S rRNA gene sequences from culturable and as yet not cultivatable organisms clearly shows a phylogenetic relationship of *Herpetosiphon* to the third of the four suggested subphyla of the phylum *Chloroflexi*, previously non-green sulfur bacteria (Gibson et al., 1985; Woese et al., 1985; Hugenholtz et al., 1998a; Garrity and Holt, 2001; Lee et al. (manuscript in preparation)). The chloroflexi are well separated from all other bacteria and represent one of the main branches (a phylum) in the bacterial tree of descent. The similarity to other filamentous, gliding bacteria, such as *Vitreoscilla*, *Beggiatoa* and *Leucothrix*, which belong to the Betaproteobacteria and Gammaproteobacteria or the filamentous cyanobacteria, is significantly low (Reichenbach, 1981a; Reichenbach et al., 1986; Lee et al. (manuscript in preparation)). Interestingly, the relationship of *Herpetosiphon* to some chloroflexi members, e.g., *Chloroflexus*, is also reflected in other genes such as the elongation factor Tu or the ATP-synthase beta-subunit genes (Ludwig et al., 1993), as well as in the very unusual structure and composition of the cell walls of the two organisms. Both organisms contain a peptidoglycan in which *meso*-diaminopimelic acid is replaced by L-ornithine and to which a polysaccharide is covalently bound. In addition, both organisms lack a lipopolysaccharide (Jürgens et al., 1987; Jürgens et al., 1989).

Habitats

Typical *Herpetosiphon* strains are regularly found in freshwater, soil, and decaying organic matter (such as rotting wood, dung of herbivorous animals, and compost). *Herpetosiphon* appears to be rather common everywhere in aerobic environments, in the neutral pH range and

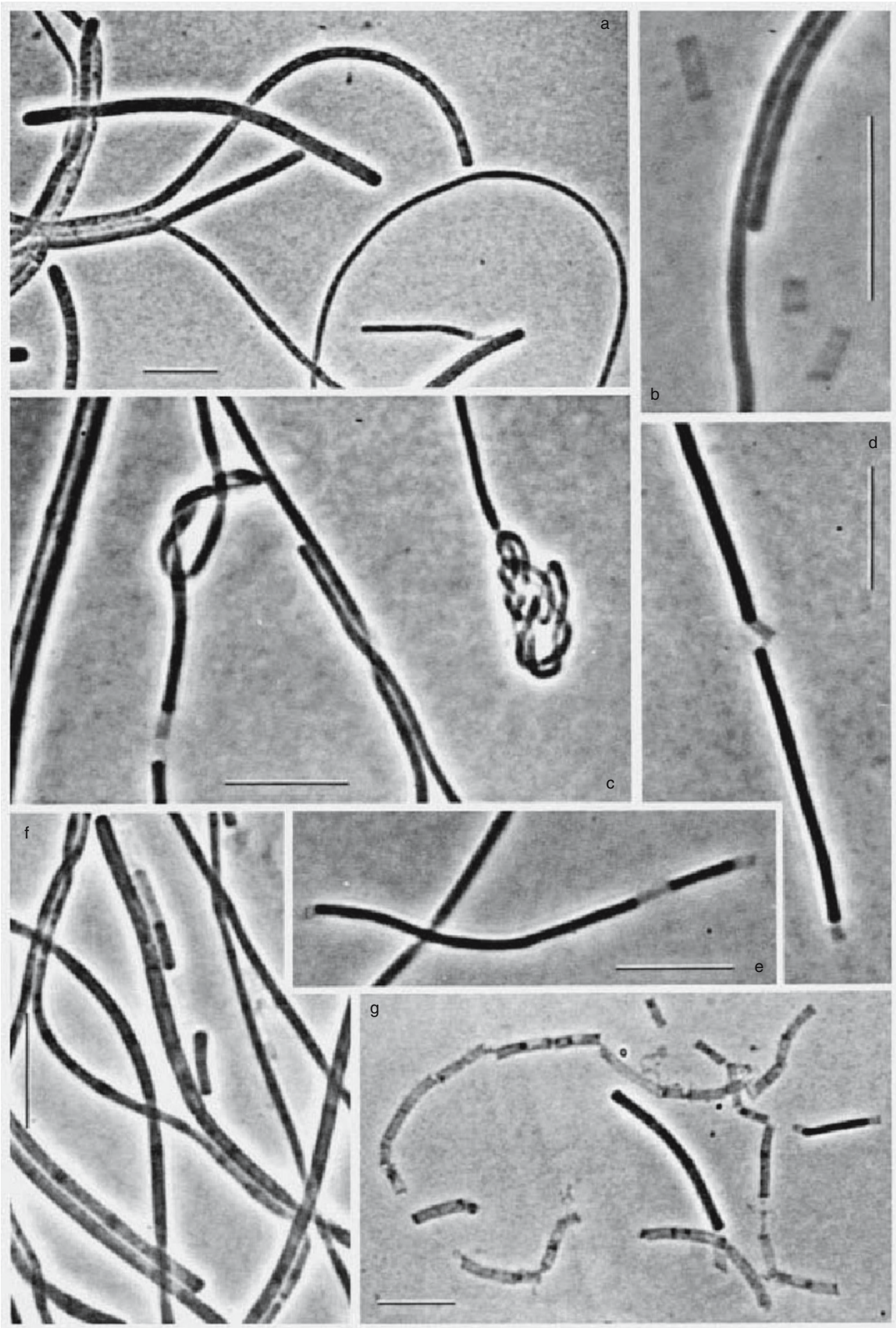
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Fig. 2. Filaments of the type strain of *Herpetosiphon aurantiacus* at high magnifications. a) Filaments from CY agar. Note the enormous variability of the filaments' diameter, which is especially pronounced on peptone media. Slight constrictions at the filaments' surface and dark bands in the interior indicate cell boundaries, which can be particularly clearly recognized in the thick filament in the upper left sector; dark dots seen in many cells may be mesosome-like membrane bodies. b) The translucent segments seen next to the filaments are empty cell-wall cylinders and correspond to the sleeves often found at the ends of the filaments; here, as in other figures of this table, parallel running filaments approach one another very closely, which seems to exclude the presence of a sheath of any significance. c) The knots in the filaments testify to their incredible flexibility; the filament on the left contains a necridium (a dead cell); from VY/2 agar. d) The filament has a short sleeve at the end and a necridium which is almost ready to snap. e) A short filament with sleeves at both ends and a necridium. f) In several filaments, cross-walls are recognizable; there is a one- and a two-celled segment, the latter with a living and a dead cell; from CY agar. g) Decaying filament fragmenting into cell-wall cylinders, each corresponding to one cell. Because the cylinders separate cleanly, there can be no sheath. All micrographs were taken with Zeiss Axiomat, in phase contrast. Bars = 10 µm.

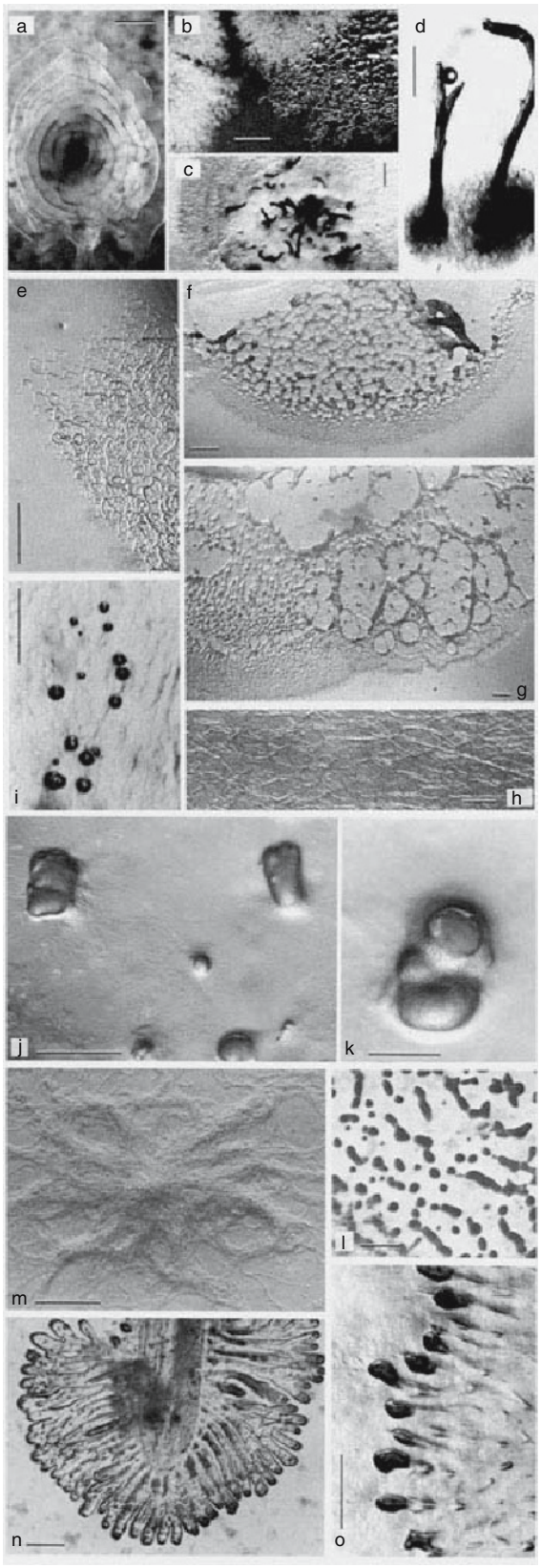

Fig. 3. Various swarm colony types of *Herpetosiphon aurantiacus*. The colonies of *Herpetosiphon* are very variable and occasionally show striking patterns. a) Swarm with a rhythmic growth pattern, on VY/2 agar. Bar = 1 mm. b) Swarm showing surface growth (right), as well as penetration into the agar (light areas). Bar = 1 mm. c) and d) Colonies with proboscis-like protuberances; c) survey picture; d) protuberances at higher magnification. Bar = 1 mm in both c) and d). e) Swarm growing from a streak of autoclaved *Escherichia coli* on water agar; the curly pattern is characteristic for this kind of culture; the cloudy areas in the lower part indicate penetration of the organism into the agar. Bar = 1 mm. f) and g) Large swarm on VY/2 agar; the swarm sheet tends to contract and peel off the agar surface, producing large holes surrounded by ridges; this growth pattern is very characteristic for *Herpetosiphon*. Bar = 1 mm in both f) and g). h) Delicately plicated surface of a swarm sheet on VY/2 agar; this is a relatively unusual pattern. Bar = 100 µm. i) to l) Knobs; they are sometimes produced in large numbers and arranged in patterns (i, l) that make their resemblance to myxobacterial fruiting bodies even more striking; also, their size is in the range of fruiting bodies. Bar = 1 mm in i) and l), 500 µm in j), and 200 µm in k). m) Microcolony showing its composition of long, coiling and interwoven filaments on CY agar. Bar = 100 µm. n) Swarm on a streak of autoclaved *E. coli* (center) on water agar spreading in an uncommon pattern of broad, tape-like tongues. Bar = 1 mm. o) Edge of a swarm growing on a streak of living *E. coli* on water agar; in contrast to the usual pattern on this medium, as shown in b) and e), here the swarm ends in a series of compact knobs with delicate, flame-like extensions (swarm center is to the right). Bar = 1 mm. Pictures made with Leitz Aristophot (a to g); Zeiss Axiomat (h, j, k and m); and Olympus SZH Stereo Microscope (i, l, n and o).

under mesophilic conditions. A study on the distribution of *Herpetosiphon* in the surface layers of Lake Constance (a large, deep, fairly oligotrophic lake in southwestern Germany) demonstrated the organism only in contaminated areas close to cities or at the mouths of rivers, while it was absent in clean stretches of water in the middle of the lake or along uninhabited and undisturbed shores (Gräf and Perschmann, 1970). This suggests that the *Herpetosiphon* population at the lake surface has its origin in the soil of the surrounding area and in contaminated waters running into the lake. The examples mentioned in the introduction show however that *Herpetosiphon* also thrives in the benthos of freshwater bodies. Therefore, its absence in the surface layers may be best explained by a fast sedimentation of the flocs of entangled filaments, which can stay in suspension only in vigorously agitated liquids.

Herpetosiphon and *Herpetosiphon*-like bacteria have also been observed and isolated from activated sludge of sewage plants on several occasions in different parts of the world (Salcher et al., 1982; Trick and Lingens, 1984; Senghas and Lingens, 1985; Bradford et al., 1996; Kohno et al., 2002; Björnsson et al., 2002; Lee et al. (manuscript in preparation)). *Herpetosiphon* may, however, have been observed on many more occasions in sewage material, but it has not been recognized as such. For instance, the organism described from a Dutch sewage plant as belonging to group III (Van Veen, 1973) may have been *Herpetosiphon* (strain Rz in Van Veen's Fig. 12, which shows the typical empty sleeves at the ends of the filaments). Interestingly, those bacteria were practically always present in activated sludge. The role of *Herpetosiphon* in sewage plants is unknown. However, if the filaments observed by Van Veen (1973) were indeed *Herpetosiphon*, it may represent one of the many biological agents for the "bulking" problems in sewage plants (for a review, see Eikelboom [1983] or Jenkins et al. [2003]). On the other hand, recently Björnsson et al. (2002) and later Lee et al. (manuscript in preparation) speculated that filaments belonging to the chloroflexi and, thus among others, *Herpetosiphon*, may be one of the crucial agents for the important backbone structure of activated sludge flocs, thus suggesting a positive role for at least some chloroflexi filaments in sewage plants.

One species, *H. geysericola* (ATCC 23076^T), has been described from the surroundings of a hot spring (Lewin, 1969a; Lewin, 1970). It is represented by just one strain, which in addition appears to be thermotolerant rather than thermophilic (the literature is completely mute about that important point; but the American Type Culture Collection [ATCC] catalog lists a growth temperature of 30°C for the strain). The environment from which *H. geysericola* was isolated is of interest insofar as this is the typical habitat of the related thermophilic phototroph *Chloroflexus*. In a study in a Yellowstone hot spring (Yellowstone National Park, United States), it was shown that the bacterial mat contained two other types of filamentous bacteria besides *Chloroflexus* (Tayne et al., 1987). Unfortunately, the antiserum used to identify *Chloroflexus* was not tested for crossreactions to *Herpetosiphon*. It is thus still unknown whether *Herpetosiphon* may be more common in such environments than has so far been assumed. Even though several molecular surveys on the microbial population in different hot springs and microbial mats have not retrieved any *Herpetosiphon*-related 16S rRNA gene sequences (e.g., Pierson et al., 1994; Hugenholtz et al., 1998b; Boomer et al., 2002; Nübel et al., 2002), this is still not a final proof for their nonexistence in these systems in particular since many of these studies used molecular markers specific only for phototrophic members of chloroflexi (Pierson et al., 1994; Boomer et al., 2002; Nübel et al., 2002). Lee et al. (manuscript in preparation) showed that although it was possible to isolate *Herpetosiphon* from activated sludge as well as observe it *in situ* by microscopy using *Herpetosiphon*-specific oligonucleotide probes, no *Herpetosiphon* affiliated clones could be retrieved from a 16S rRNA clone library based on general *Bacteria* polymerase chain reaction (PCR) primers, although 10% of the obtained clones were indeed affiliated to other as yet not cultivatable members among the chloroflexi. This suggests that *Herpetosiphon* may not be so easily retrieved from the environment in 16S rRNA clone library surveys on the basis of general PCR primers.

While *Herpetosiphon* obviously may be a common inhabitant of freshwater, it is equally at home in nonaquatic environments. One of us has isolated (as a byproduct of myxobacteria isolation) in the course of 20 years more than 400 *Herpetosiphon*-like strains from soil samples, dung pellets, rotting wood, and similar materials collected all over the world in various climate zones, including decidedly hostile, dry, and hot environments, like semi-deserts in Tunisia and Arizona, steppe habitats in Mediterranean countries, the Canary Islands, and gypsum hills on Cyprus (H. Reichenbach, unpublished observations). Twenty-eight of these isolates have been further examined. Ten of these isolates were related to *H. aurantiacus* (16S rRNA gene similarity >97%), whereas the rest most likely represent novel genera among chloroflexi (Lee et al. (manuscript in preparation)). It seems that the diversity of *Herpetosiphon* is larger in soil than in aquatic environments, since all isolates obtained so far from freshwater environments or sewage plants (with the exception of some iso-

lates of Senghas and Lingens [1985], which are unfortunately no longer available for further comparison) are closely related to *H. aurantiacus*, whereas some of the soil isolates represent most likely novel genera (Lee et al. (manuscript in preparation)).

Isolation

Herpetosiphon may be isolated from any of the sources mentioned in the section Habitats in this Chapter. The terrestrial strains usually survive desiccation so that dry samples can also be used. One of us was able to obtain *Herpetosiphon* from rotting wood after eight years storage, from filter-paper preserves of contaminated myxobacteria after six years, and from soil after two years of storage in the dry state at room temperature (H. Reichenbach, unpublished observations). It has been noted that strains from sewage plants may also be extremely resistant to desiccation (Trick and Lingens, 1984).

No specific enrichment techniques are known. The strains on which the definition of the genus is based were isolated by making a single streak from the slimy coat of *Chara* on a plate with 0.3% peptonized milk (Difco) and 1.5% agar. The culture was incubated at room temperature. Evidently, this relatively rich medium can be used only if the sample contains few other microorganisms (Holt and Lewin, 1968). Therefore, for a more successful isolation, use is made of the ability of the organism to glide away from the inoculum and to produce a typical swarm pattern. To elicit gliding and to suppress excessive growth of contaminants, media very low in organic constituents are recommended. The simplest medium is plain water agar, to which cycloheximide may be added to prevent the development of fungi:

WAT Agar

$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

Dissolve the ingredients in distilled water and adjust the pH to 7.2 with KOH. The pH adjustment is much easier if HEPES (20 mM) is added as a buffer. For crude cultures, use WCX agar, which is WAT agar plus a filter-sterilized stock solution of cycloheximide (25 µg/ml of medium).

Several small samples of soil or other sources of inoculum (size of a lentil) are placed on the surface of the dry plate, a few cm from each other, and the culture is incubated at 30°C. After 2–20 days, the typical swarm colonies with long flares and coils of entangled filaments at the edge may be observed (Fig. 3). The very delicate pattern can only be seen if an oblique light beam produced by a tiltable mirror is applied from below. We have had success with WAT agar plates with three thin, parallel streaks of living *Escherichia coli*, each of which is inoculated at one end. *Herpetosiphon* grows in a spreading fashion within the streaks, usually without lysing the *E. coli* cells, and often only appears along the edges of the streaks in the form of long flame-like structures or a loose pattern of separate, coiling strands of filaments. Sometimes, large and more or less dense swarm sheets are produced that spread over much of the plate. An especially high yield of *Herpetosiphon* can sometimes be obtained if such cultures are incubated at 38°C.

For the isolation of planktonic *Herpetosiphon* in freshwater, between 0.5 and 1 liter of lake or river water is passed through a sterile membrane filter (pore size, 0.4 µm). The filter is then cut into pieces, and the segments are placed on a low-nutrient agar medium. In the original study, rabbit dung agar was used but any other lean medium would certainly also do.

Rabbit Dung Agar (Gräf and Perschmann, 1970)

Boil dry dung pellets from wild rabbits (20 g) in 1 liter of distilled water for 20 min. Add 15 g of agar to 1 liter of filtrate. Adjust the pH to 7.2.

The cultures are incubated at 33°C. After about 6 days, swarm colonies begin to spread from the edges of the filter.

Different procedures have been applied for the isolation of *Herpetosiphon* from activated sludge. One procedure consists of streaking a drop of the sludge sample on agar plates or embedding it in agar itself (Trick and Lingens, 1984; Senghas and Lingens, 1985). Another procedure is based on micromanipulation of filaments in the activated sludge (Bradford et al., 1996). Different media have been used, ranging from complex but rather nutrient-poor media, such as BG-11 agar (originally designed for the isolation of cyanobacteria), I agar (Trick and Lingens, 1984; Senghas and Lingens, 1985) or R2A (Bradford et al., 1996) to sludge-based media (Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)). As sewage organisms are notoriously fastidious and adapted to very low nutrient levels, we have listed below all the media used; whether they are really required or could be replaced by simpler recipes has not been determined. The cultures are incubated at 25°C and examined with a phase contrast microscope over several weeks (Salcher et al., 1982; Bradford et al., 1996). Pure cultures can be obtained by transferring filaments from the advancing swarm edge to the same media again, or, as soon as the culture is sufficiently pure, to richer media (see the section Cultivation in this Chapter). We often have good results with transfers to streaks of autoclaved *E. coli* on water agar, although some *Herpetosiphon* strains do

not grow on autoclaved bacteria (see the section Cultivation in this Chapter). As the organism has to be carefully removed from the contaminated plate, transfers are made best by cutting out a piece of the swarm edge on a small agar block using a drawn-out glass rod or, more conveniently, a fine steel injection needle (e.g., on a 1-ml disposable syringe). The procedure is repeated until the strain is pure. Two properties of *Herpetosiphon* may substantially speed up purification: We have found that all *Herpetosiphon* strains tested so far grew at 38°C and are resistant to 250 mg of kanamycin sulfate per liter.

BG-11 Agar (Stanier et al., 1971)

$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA(Na_2-Mg salt)	0.001 g
Na_2CO_3	0.02 g
Trace element solution A5	1 ml
Agar	15 g
Distilled water	1 liter

The trace element stock solution A5 contains per liter: H_3BO_3, 2.8 g; $MnCl_2 \cdot 4H_2O$, 1.8 g; $ZnSO_4 \cdot 7H_2O$, 0.2 g; $Na_2MoO_4 \cdot 2H_2O$, 0.4 g; $CuSO_4 \cdot 5H_2O$, 0.08 g; and $Co(NO_3)_2 \cdot 6H_2O$, 0.05 g.

I Agar (modified from Van Veen, 1973; Salcher et al., 1982)

Glucose	0.15 g
$(NH_4)_2SO_4$	0.5 g
$Ca(NO_3)_2$	0.01 g
K_2HPO_4	0.05 g
$MgSO_4 \cdot 7H_2O$	0.05 g
KCl	0.05 g
$CaCO_3$	0.1 g
Agar	15 g
Distilled water	1 liter

After autoclaving, add 10 ml of a filter-sterilized vitamin solution. The vitamin solution contains per liter: Ca pantothenate, 10 mg; nicotinic acid, 10 mg; biotin, 0.5 mg; cyanocobalamin, 0.5 mg; folic acid, 0.5 mg; pyridoxine·HCl, 10 mg; *p*-aminobenzoic acid, 10 mg; thiamine pyrophosphate, 10 mg; thiamine, 10 mg; inositol, 10 mg; and riboflavin, 10 mg.

R2A Agar (Reasoner and Geldreich, 1985)

Enzymatic digest of casein	0.25 g
Enzymatic digest of animal tissue	0.25 g
Acid hydrolysate of casein	0.5 g
Yeast extract	0.5 g
Glucose	0.5 g
Soluble starch	0.5 g
$KH_2 PO_4$	0.3 g
$MgSO_4 \cdot 7H_2O$	0.1 g
Sodium pyruvate	0.3 g
Agar	15 g
Distilled water	1 liter

Adjust pH to 7.2.

Sludge-based Media (Fuhs and Chen, 1975)

CH_3COOH-Na	5 g
$(NH_4)_2SO_4$	2 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KH_2PO_4	0.25 g
$CaCl_2 \cdot 2H_2O$	0.2 g
Agar	15 g
Mixed liquor (sewage/sludge)	200 ml
Distilled water	800 ml

Adjust pH to 7.0.

VY/2 Agar

Bakers' yeast (fresh weight)	0.5%
$CaCl_2 \cdot 2H_2O$	0.1%
Cyanocobalamin	0.5 mg/liter
Agar	1.5%

Dissolve ingredients in distilled water and adjust to pH 7.2.

CY Agar

Casitone (Difco)	0.3%
Yeast extract	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
Agar	1.5%

Dissolve ingredients in distilled water and adjust to pH 7.2.

CYCAS Agar

Casitone (Difco)	0.3%
Yeast extract	0.3%
$CaCl_2 \cdot 2H_2O$	0.1%
Cellulose powder	0.1%
Soluble starch	0.1%
Acetate	0.1%
Agar	1.5%

Dissolve ingredients in distilled water and adjust to pH 7.2.

ATCC Medium 810 (Myxo 810 Medium)

Skim milk powder	0.5%
Yeast extract	0.05%
Agar	1.5%

Dissolve ingredients in distilled water. Do not adjust the pH.

EC Medium (Enriched Cytophaga Medium; Pate and Chang, 1979)

Tryptone (Difco)	0.2%
Yeast extract (Difco)	0.05%
Na acetate	0.02%
Agar	1.5%

Dissolve ingredients in distilled water and adjust to pH 7.5.

Escherichia coli Overlay Agar

Suspend the growth of four culture plates of *E. coli* (on any suitable medium) in 100 ml of water agar (WAT agar; see the section Isolation in this Chapter). Autoclave the suspension and pour as a thin layer on top of water agar plates.

Medium 2 (Lewin and Lounsbery, 1969b)

Casamino acids	0.1%
Na glycerophosphate	0.01%
Tris buffer	0.1%

KNO3	0.01%
$CaCl_2 \cdot 2H_2O$	0.01%
$MgSO_4 \cdot 7H_2O$	0.01%
Thiamine	1 mg/liter
Cyanocobalamin	1 mg/liter
Agar	1%
Glucose	0.1%

Dissolve all ingredients except for glucose in distilled water and adjust to pH 7.5. Add separately autoclaved glucose after autoclaving the solution of the other ingredients.

Most of the cultivable strains also grow in liquid media, in shake flasks, and in fermentors. A good medium for many strains is HP74 liquid medium. Other strains grow equally well or better in peptone-containing media, e.g., in CAS liquid medium, R2A liquid medium, or MDl liquid medium.

HP74 Liquid Medium

Na glutamate	1%
Yeast extract (Difco)	0.2%
$MgSO_4 \cdot 7H_2O$	0.2%
Glucose	1%

Autoclave the glucose separately as a 20% stock solution. Add 20 ml per liter of separately autoclaved phosphate buffer (50 mM, pH 6.5) to give 1 mM.

CAS Liquid Medium

Casitone (Difco)	1%
$MgSO_4 \cdot 7H_2O$	0.1%

The pH should be 6.8; do not adjust.

MDl Liquid Medium

Casitone (Difco)	0.3%
$CaCl_2 \cdot 2H_2O$	0.07%
$MgSO_4 \cdot 7H_2O$	0.2%
Cyanocobalamin	0.5 mg/liter

Add trace element solution (see below) after autoclaving the MD1 medium. The pH should be 6.8; do not adjust. If Casitone (Difco) is replaced by other enzymatically digested casein peptones, adjust pH to about 7.

Trace Element Solution

$MnCl_2 \cdot 4H_2O$	100 mg
$CoCl_2$	20 mg
$CuSO_4$	10 mg
$Na_2MoO_4 \cdot 2H_2O$	10 mg
$ZnCl_2$	20 mg
LiCl	5 mg
$SnCl_2 \cdot 2H_2O$	5 mg
H_3BO_3	10 mg
KBr	20 mg
KI	20 mg
EDTA, Na-Fe_3^+ salt (trihydrate)	8 g
Water	1 liter

Sterilize by filtration and add 1 ml per liter of medium. The filter-sterilized solution is stable for months at room temperature because of its high EDTA content.

Cultivation

In general, *Herpetosiphon* prefers low nutrient levels. Thus, one of our strains, Hp g175, produced good growth over six transfers on plain water agar, obviously living off minute quantities of contaminating material in the agar but without visibly attacking the agar itself. Rich media, like nutrient agar or nutrient broth, are often not suitable at all. About one out of three strains isolated from soil on streaks of living *E. coli* can be cultivated without difficulty on complex media, such as VY/2 agar, CY agar, CYCAS agar, milk-based agar media (e.g., ATCC medium 810) or R2A agar (see the section Isolation in this Chapter). Isolates obtained from freshwater or sewage systems may also be cultivated on these media and in addition on EC and I agar media (see section Isolation in this Chapter). *Herpetosiphon geysericola* (ATCC 230767^T) was cultivated on medium 2 (Lewin, 1970). This medium was used as a standard medium for the cultivation of many different organisms; thus, not every component may be required by *H. geysericola* (e.g., thiamine). *Herpetosiphon geysericola* however also grows on all of the media mentioned above.

Many strains produce heavy growth and often spread within a few days over the entire plate. Some strains tend to penetrate the agar (Fig. 3). The yeast in VY/2 agar may or may not be lysed. However, a significant number of the soil isolates obtained from water agar media with streaks of living *E. coli* cannot be further cultivated on other media. Only a few of them respond favorably to a reduction of the nutrient concentration and can be grown on media like VY/5 agar (such as VY/2 agar but with yeast concentration reduced to 0.2%). Others grow more or less poorly on *E. coli* overlay agar, but most of them can barely be kept alive on streaks of autoclaved *E. coli* on water agar. Some of these strains survive only on living bacteria, presumably because they require some labile growth factor. The alternative explanation (i.e., *E. coli* removes some inhibiting component) seems less likely because the filaments often creep far away from the *E. coli* streak. All our efforts to identify growth factors have been unsuccessful, although many possibilities have been tested (different vitamins, sugars, and amino acids; plant oil; chitin, catalase, and cell fractions of *E. coli* and of cultivable *Herpetosiphon* strains; anaerobic and microaerophilic conditions; different agar concentrations, temperatures, and pH values; illumination). Recently, we found that four of these fastidious strains, Hp g122 (DSM 15874); Hp g124 (DSM 15875); Hp g150 (DSM 15876), and Hp g174 (DSM 15877) were only distantly related to the type strain *Herpetosiphon aurantiacus* ATCC

23779^T (ca. 80% 16S rRNA gene sequence similarity), which strongly indicates that they represent a new genus among chloroflexi (Lee et al. (manuscript in preparation)).

In agitated cultures the organisms may grow as homogeneous suspensions, but often the filaments stick together to form flakes or tight spherules. One of our strains, Hp a2 (DSM 589), a member of the *H. aurantiacus* species group, forms flakes when inoculated into HP74 liquid medium; the cultures become completely uniform after a few days, but the organism forms flakes again upon transfer into fresh medium. Growth is not particularly fast: The doubling time is in the range of 15–20 h, and the cultures do not enter the stationary phase before 4–6 days of cultivation (30°C, HP74 liquid medium, shake flasks). Wet-weight yields are in the order of 1–3 g/liter in shake flasks and 2–4.5 g/liter in fermentors (HP74 liquid medium). The dry weight amounts to roughly 20% of the wet cell mass. Trick and Lingens (1984) and Senghas and Lingens (1985) obtained similar values (doubling time 20–25 h; cell yield after 5–6 days, 2–3.5 g wet weight per liter and 0.5–1 g dry weight per liter) with their sewage isolates when cultivating them on EC medium at 25°C, their temperature optimum.

All known strains are strictly aerobic. The temperature range differs with different strains. In general, the freshwater strains seem to prefer lower temperatures, around 25°C, while the soil and some freshwater strains grow very well at 30°C and above. All *Herpetosiphon* and *Herpetosiphon*-like soil strains tested by us grew at 38°C, and some *Herpetosiphon*-like strains still grew at 45°C (e.g., Hp g207, DSM 15737; Lee et al. (manuscript in preparation)). The sewage strains showed a temperature minimum at 15°C, a maximum at 37°C, and an optimum at 25°C (Trick and Senghas, 1984; Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)). *Herpetosiphon geysericola* comes from the vicinity of a hot spring but can be cultivated at 30°C (see the section Habitats in this Chapter). The optimal pH range is 7–7.5, although pH values of 8–9 seem occasionally to be tolerated.

Preservation

Most *Herpetosiphon* strains survive on agar media at 30°C or room temperature for up to 3 months. Slant cultures stored at 4°C have been successfully subcultured after 14 months. However, some *Herpetosiphon*-like strains, such as Hp g6 (DSM 15710), Hp g16 (DSM 15736) and Hp g207 (DSM 15737), which represent more likely novel genera of chloroflexi rather than *Herpetosiphon*, are more sensitive to long-term storage at room temperature or at 4°C. These, as well as stock cultures, should be transferred every 3–4 weeks (Lee et al. (manuscript in preparation)).

Herpetosiphon strains may be preserved by any of the standard procedures. A convenient and reliable method is storage at –80°C. The bacteria are suspended in 1 ml of CAS or HP74 liquid medium and simply put into the deep freeze. Thawing should be fast, best accomplished by immersing the tube in cold water, and transfers to a suitable growth medium have to be made immediately after thawing. We have been able to reactivate such preserved cultures with no problems after 8 years of storage, the longest period tested. Alternatively, the bacteria can be frozen in liquid nitrogen. In one study, the filaments were suspended in growth medium (medium 2 as above, without agar), supplemented with 10% of either glycerol or dimethyl sulfoxide (Sanfilippo and Lewin, 1970). All strains survived freezing and thawing with both additives. Only the glycerol preserves were tested again after 1 year, and all strains were still viable. When stored in growth medium without additives at –22°C, three out of four strains survived for 21 weeks (the longest period tested) and one for 6 weeks. Strains dried in skim milk could always be reactivated after storage for up to 12 years (unpublished data). We do not freeze-dry the organism itself but only the skim milk: A few drops of a thick suspension of *Herpetosiphon* filaments, taken from a young plate culture, are added to a plug of freeze-dried skim milk in an ampoule. The plug absorbs the liquid without liquefying itself. The ampoule is then dried at room temperature in a desiccator on a vacuum pump for several hours. After filling the desiccator with nitrogen gas, the ampoules are sealed.

Herpetosiphon strains are currently available from six different cultures collections: ATCC (the American Type Culture Collection, Rockville, USA), ACM (the Microbial Culture Collection of the University of Queensland, Brisbane, Australia), CCUG (Culture Collection University of Göteborg, Sweden), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), NCIMB (the National Collection of Industrial Food and Marine Bacteria, University of Aberdeen, Scotland), and TISTR (the Culture Collection of Bangkok, MIRCEN, Thailand). The largest collection is deposited at DSMZ, encompassing the "Hans Reichenbach Collection" of 460 *Herpetosiphon* and *Herpetosiphon*-like strains; however, only around 30 of these strains have been further characterized (Lee et al. (manuscript in preparation)).

Morphological Characteristics

The filamentous morphology and the "sleeves" of *Herpetosiphon* are rather distinct so that the bacterium can readily be recognized under the microscope. However, some of the other gliding filamentous genera among chloroflexi, e.g., *Chloroflexus*, *Heliothrix* and *Roseiflexus*, share certain morphological features with *Herpetosiphon* (Garrity and Holt, 2001; Lee et al. (manuscript in preparation)). Fortunately, since most of these latter mentioned filaments are mainly thermophilic and facultative phototrophic, a certain distinction may still be possible by knowing the habitat, the physiology or the pigmentation of the organism. Nevertheless, for a final identification, a molecular method, such as 16S rRNA gene analysis or fluorescence in situ hybridization (FISH) using oligonucleotide probes is strongly recommended (for a review of oligonucleotide probes used for detecting different members of *chloroflexi*, see Björnsson et al. [2002] and Lee et al. (manuscript in preparation)).

General Morphology

The morphology of the two acknowledged species (*aurantiacus* and *geysericola*) of *Herpetosiphon* is rather similar. *Herpetosiphon* forms extremely long, unbranched, multicellular filaments of uniform diameter (Fig. 1). The filaments often measure 100 μm to more than 1200 μm in length. The cells in a filament cohere tenaciously so that the filament does not break easily. Unconstrained filaments in a liquid medium appear straight and stiff, but in fact they are extremely flexible and can sharply bend back and twist around themselves in a rope-like fashion or intertwine into plait-like masses (Fig. 1). The width of the filaments varies with different strains (0.5–1.7 μm), mostly between 0.7 μm and 1.2 μm. There is also considerable variation within the same culture (Fig. 2). For instance, we have measured filament diameters between 0.7 μm and 1.6 μm in an 11-day-old culture of the type strain of *H. aurantiacus* on CY agar. In older cultures or under unfavorable conditions, the filaments often swell substantially and may become completely irregular in outline. Furthermore, the filaments are often locally inflated and form large, spherical structures, or "bulbs," with a diameter of 2.5–3.5 μm (Reichenbach and Golecki, 1975; Trick and Lingens, 1984; Fig. 4). Under the electron microscope a membrane body at the end of a septum-like stalk can always

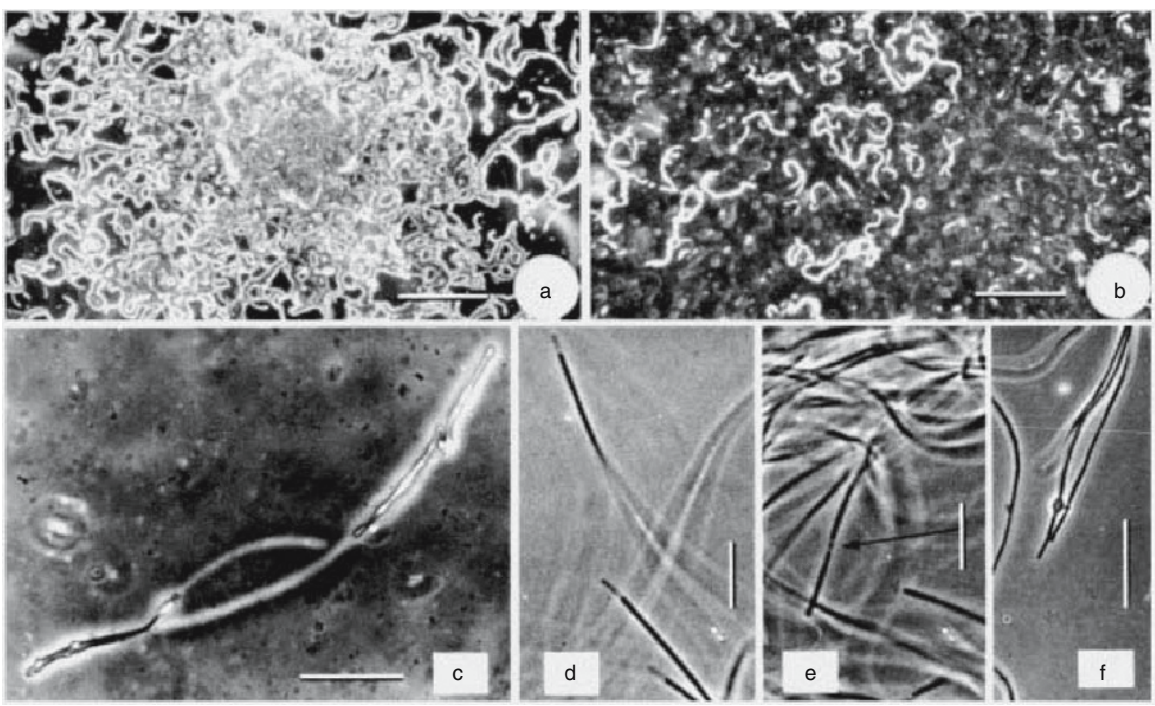

Fig. 4. Special structures of *Herpetosiphon aurantiacus*. (a to c) Desiccation-resistant filaments in a chamber culture. a) and b) Survey pictures; dark field; Bars = 200 μm: a) 9 days old, with the filaments still essentially intact; b) 32 days old, with only remnants of the filaments left. Bar = 200 μm. c) At high magnification, the remaining segments of the old filaments appear irregular in outline and optically refractile; there are conspicuous slime tracks. Bar = 30 μm. d) The empty sleeves, which are visible here at the ends of many filaments, are easy to recognize, even at a relatively low magnification, and thus help to quickly identify an isolate as a *Herpetosiphon*. Bar = 10 μm. e) A necridium (arrow). Bar = 10 μm. f) Bulbs. Bar = 20 μm. Zeiss Standard Microscope (a to c). Zeiss Axiomat (d to f); (c to f) in phase contrast.

be seen within the bulb. Those bulbs seem to be a degeneration phenomenon, and outgrowth or further development has never been observed from them. In old cultures, the filaments also tend to become shorter, especially on peptone-containing media, simply because more and more cells die and the filaments snap at such sites. The fragments can be as short as one cell. In old cultures, particularly on agar, short, optically refractile, irregular filaments are often seen (Fig. 4). As those are the only structures left in old, drying, but still viable cultures, they appear to be desiccation-resistant resting forms. However, their germination has not been directly observed so far.

The filaments consist of cylindrical cells that have the same diameter as the filament, and they are so closely attached to one another that only exceptionally shallow notches can be seen at the surface. Under a microscope with a high resolution using phase or interference contrast, the cells can sometimes be seen in living filaments (Fig. 2). The septa become more conspicuous in older cultures, particularly when the organism is grown on a peptone-rich medium, like CY agar. In such filaments the intracellular membrane bodies (see below) can often also be recognized (Fig. 2). The cells become clearly distinguishable if the filaments are first dried to the slide and then stained, e.g., with Loeffler's methylene blue, crystal violet, or alcoholic fuchsin (rosaniline). The lengths of the cells vary even within one filament, which suggests that the cells do not divide synchronously. The cell length may also vary with the culture medium, the culture age, and the strain. With many strains, cell lengths between 1.5 μm and 4.5 μm have been measured. The type strain of the genus appears to have especially long cells, measuring between 3 μm and 7 μm and sometimes 10 μm.

In wet mounts, *Herpetosiphon* filaments often can be seen to bend slowly and sometimes to slowly oscillate back and forth. In contact with a suitable interface, i.e., one to which the organism can stick, like a glass or an agar surface, the filaments glide slowly and, during this movement, deposit characteristic slime tracks (Fig. 1). Gliding filaments often bend laterally to form hairpin-like loops (Fig. 1). In liquid media, the filaments apparently move along themselves. They may coil around themselves or one another and produce all kinds of loops and knots (Figs. 1 and 2).

Sleeves or Sheathes

A most characteristic feature of *Herpetosiphon* is the presence at the ends of many filaments of transparent sections, which we call sleeves (Fig. 2). The sleeves have the same diameter as the filaments (1.4–5.5 μm) and are usually 1.5–3.5 μm long (the measurements are those for strain Hp a2, DSM 589, member of the *H. aurantiacus* group). The role of those sleeves is still controversial. Some investigators have regarded them as an indication of a sheath (Holt and Lewin, 1968; Skerman et al., 1977) or at least of a microsheath (Trick and Lingens, 1984). We prefer a different explanation (Reichenbach and Golecki, 1975): The filaments appear to multiply by breaking at the sites of necridia, i.e., dead cells along the filament. Such necridia can occasionally be observed in young cultures, and their number increases substantially with the age of the culture (Fig. 2). Their length (1.5–3.7 μm) is in the size range of ordinary cells. Trichome division by breakage at necridial cells is well known from other bacteria and has been described, e.g., for *Oscillatoria* (Lamont, 1969) and *Beggiatoa* (Strohl and Larkin, 1978). When a filament snaps, the empty cell wall cylinder of the necridium remains attached to the end of one daughter filament and gives rise to a sleeve. In strain Hp a2 (DSM 589), the outer wall seems to rupture at one of the ends of the necridium (near the cross-wall rather than across its middle), for the shortest and the longest sleeves correspond exactly to the lengths of the shortest and the longest cells. This would also explain why only some of the filaments' ends bear a sleeve, although it seems that the sleeves may also be shed; often empty sleeves can be seen lying in between the filaments. With the type strain of *H. aurantiacus*, on the other hand, the sleeves are exceptionally short, with a size distribution corresponding approximately to one-half that of the cell length, so that here the necridia seem to break preferentially in their center.

There are at least six more arguments against the presence of a sheath: 1) The filament glides as a whole, with its empty end-pieces in position, which would seem unlikely if the cells were enclosed in a sheath. 2) There is no movement of cells within the filament, e.g., at the site of a necridium, nor a release of cells at the ends of the filament. This was observed by Holt and Lewin (1968) and also by the present authors. But the literature is not in complete agreement on these points. Movement of a filament within its sheath has been described and verified by time-lapse photography (Skerman et al., 1977); however, it is our impression that what was described as a sheath may really have been a slime trail. Also, it was stated that single, gliding cells were released from the filaments (Brauss et al., 1969), but in this case what was seen may simply have been short fragments that broke off the ends of the filament. 3) If the sleeve were a piece of a sheath, it would be difficult to understand why, as a rule, it does not exceed the length

of a cell. 4) If a sheath were present, one could expect an occasional false branching of the filaments. 5) When a whole filament dies and decays, it falls apart into cell-sized pieces (Fig. 2). Obviously, there is no sheath to hold them together. 6) We have never been able to demonstrate anything resembling a real sheath under the electron microscope, with specimens prepared by freeze-etching, negative contrast, metal shadowing, or thin-sectioning after several different fixation protocols (Reichenbach and Golecki, 1975; see also Gräf and Perschmann, 1970). This is also true for the type strain (Fig. 5). The thin, tube-like structure composed of fibrils, which has been described by several investigators and has been interpreted as a microsheath (Skerman et al., 1977; Senghas and Lingens, 1985; Holt, 1989), probably has a different explanation.

How can these differences of opinion about the existence of a sheath in *Herpetosiphon* be reconciled? Differences among strains can probably be ruled out, for we included the type strain in our studies and found no significant divergence from strain Hp a2 (DSM 589). A sheath is a morphologically distinct structure, a hollow cylinder with a discrete outer boundary, excreted by the cells and not covalently bound to their surface. Under the light microscope, a sheath should be clearly recognizable, e.g., after negative staining with nigrosin or India ink. Under the electron microscope, a sheath may be difficult to visualize in thin sections because of poor contrast with the usual techniques, but with negative contrast or metal shadowing, it should be seen. Like all gliding bacteria, *Herpetosiphon* excretes slime, and slime fibrils can always be observed in the neighborhood of the trichomes, but this material is more or less diffuse. Under certain conditions this slime may condense, perhaps as a consequence of dehydration during fixation, and then it may appear as a dense tube. This is probably what has been taken as evidence for a sheath by various investigators (Skerman et al., 1977; Senghas and Lingens, 1985; Holt, 1989). Typically in all those cases cited, a wide gap opens between the filament and the thin, dense tube. Senghas and Lingens (1985) made efforts to demonstrate a sheath in their *Herpetosiphon*-like strains by first lysing the cells in the filament with lysozyme and then staining the remains with crystal violet. In that case, indeed, a long thin tube-like structure could be produced. However, this is still not an unequivocal proof of a sheath, for the technique requires as a prerequisite for reliable results that the organism has a normal bacterial cell wall.

Electron microscopy of thin sections of Hp a2 (DSM 589) reveals, however, that *Herpetosiphon* has an unusual cell wall structure (Reichenbach and Golecki, 1975). Outside the cytoplasmic membrane there is a well-defined peptidoglycan layer, 4–6 nm thick, but no outer membrane can be resolved. In fact, that membrane seems to be absent. Rather there is a thin additional layer, 21–25 nm thick, granular in thin sections, fibrillar in freeze-etch preparations, on top of the peptidoglycan. This layer follows the peptidoglycan into the shallow notches between adjoining cells but not into the cross-septa where only the peptidoglycan layer can be distinguished. We have never found any other defined layer outside the fibrillar layer, and in particular, have found no layer running smoothly across the cell junctions, as would be expected for a sheath. This peculiar wall structure is corroborated by chemical analyses of the cell envelope (see below) and may be directly connected with the sheath problem. Apparently, the additional layer is considerably more resistant to decomposition than the usual lipopolysaccharide-containing outer membrane, and it may give the peptidoglycan, to which it seems to be covalently bound, a higher stability. Within the cells there are extensive intracellular membrane systems, some of which originate at the cross-septa, others from the cytoplasmic membrane along the longitudinal walls. They become especially conspicuous when the negative-contrast technique is applied. As mentioned above, under certain conditions they can even be seen in the light microscope. Recent studies on the cell envelopes of Hp a2 (DSM 589) have revealed further interesting features, showing that ion-permeable channels, formed by a 45-k-Da protein, were present in the cell wall. This suggests that this strain has a permeability barrier on its surface similar to the outer membrane of Gram-negative bacteria, a feature which it shares with certain other Gram positive organisms, such as *Mycobacterium chelonae*, *Corynebacterium glutamicum*, *Streptomyces griseus* and *Micromonospora purpurea* (Harwardt et al., 2004).

Colony Morphology

The colonies, or swarms, usually spread quickly over agar surfaces and may completely cover a culture plate within a few days. *Herpetosiphon*-like strains, such as Hp g16 (DSM 15736) and Hp g207 (DSM 15737) grow more slowly, and may need up to 2–3 weeks to produce a significant spreading growth, although the plate is rarely completely covered (Lee et al. (manuscript in preparation)). On poor media, the migrating filaments remain more or less separate and appear in the form of characteristic curls and whirls (Fig. 3). Such colonies may become extremely delicate and are barely recognizable even if oblique illumination is applied. On media that allow good growth, dense and tough swarm sheets arise, with

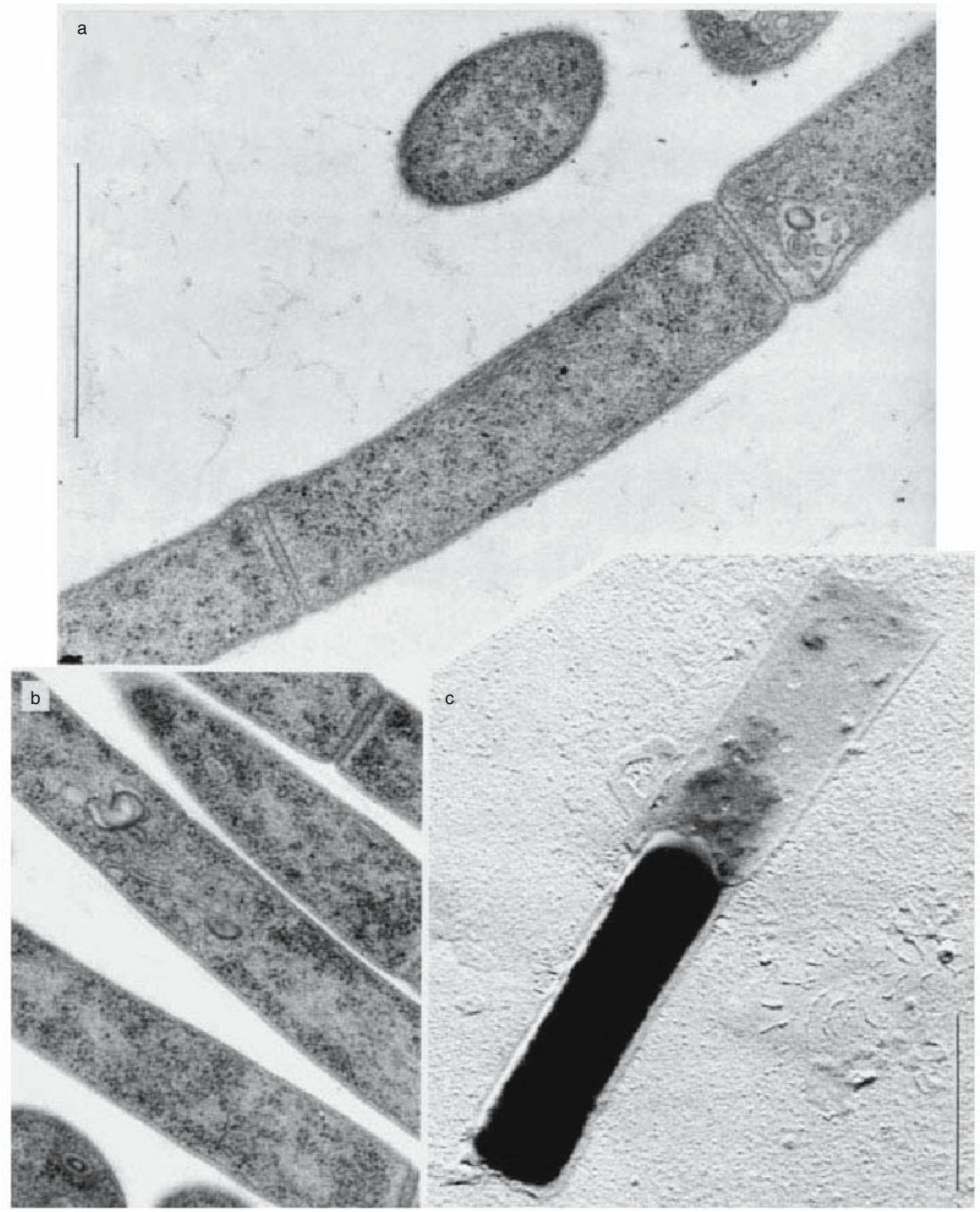

Fig. 5. Electron micrographs of the type strain of *Herpetosiphon aurantiacus*. a) and b) Ultrathin sections; the peptidoglycan layer can be clearly distinguished, but while there is plenty of extracellular slime material, no structure resembling a sheath can be located on the surface; also, the close juxtaposition of the filaments in b) speaks against a sheath. Within the cells, membrane bodies can be seen. c) Shadowed preparation; an end piece with an empty cell-wall cylinder, or sleeve, is visible; again there is no sign of a sheath. Zeiss EM 10B electron microscope. Bars = 1 μm; the magnification is identical for a) and b). (Courtesy of H. J. Hirsch.)

a rough, dry, felt-like surface. Sometimes the swarm surface is completely uniform, with an even, curly, or felt texture. But often elegant networks, massive concentric ridges, a pattern of large and small holes, or large, massive knobs are produced (Fig. 3). These last-mentioned knobs are particularly impressive and have been observed with many different isolates (Holt and Lewin, 1968; Brauss et al., 1969; Reichenbach and Golecki, 1975; Trick and Lingens, 1984; Lee et al. (manuscript in preparation)). Their diameter varies between 200 μm and 2000 μm, and as was noticed repeatedly, they strikingly resemble myxobacterial fruiting bodies, especially since they often are bright red or orange. But the filaments inside those knobs seem not to differ from those in other parts of the swarm, either morphologically or physiologically. Also, the ridges and knobs are not permanent structures but shift continuously from place to place and may even dissolve completely again. This can be seen, e.g., in the movie mentioned earlier (Reichenbach et al., 1980). All those structures seem to originate from chance interactions of the long, migrating filaments that locally pull together, tangle, and pile up. The dynamics of the excreted, drying, and contracting slime may also be involved. Even more spectacular are long, sometimes branched, finger-like protuberances rising up to 5 mm above the swarm surface (Fig. 3). They consist of interwoven filaments and seem to be produced only by certain strains.

Phenotypical and Molecular Characterization

Cell Wall Composition Chemically, the cell wall of *Herpetosiphon aurantiacus* (represented by the type strain ATCC 23779^T and the strain Hp a2 DSM 589) consists of a peptidoglycan that contains L-ornithine in place of diaminopimelic acid, a rather unusual character for a Gram-negative bacterium (Jürgens et al., 1989). A heteropolysaccharide composed of heptose, hexoses, pentoses and *O*-methyl sugars appears to be covalently bound to the peptidoglycan via muramic acid-6-phosphate. This heteropolysaccharide is probably the material seen in electron micrographs as the granular-fibrillar layer on the surface of the longitudinal walls. The peptidoglycan-polysaccharide complex amounts to 20% of the cell dry weight, with a polysaccharide portion of 70%. No evidence for a lipopolysaccharide was found, which would explain the absence of an outer membrane in thin sections; nor was there evidence for the presence of additional sheath material. Unfortunately, the cell wall composition has only been determined for two representants of the *aurantiacus* species group. Nevertheless, the cell wall of *H. aurantiacus* closely resembles that of *Chloroflexus* in both its electron microscopic appearance (Pierson and Castenholz, 1974) and its chemical composition (Jürgens et al., 1987; Meissner et al., 1988). Incidently, the filaments of *Chloroflexus* also often end with a sleeve, which for this organism also has been taken to suggest the existence of a sheath (Pierson and Castenholz, 1974). Hydrolysates of extracellular slime yielded arabinose and glucosamine as the main constituents (for sewage strains; Trick and Lingens, 1984).

Respiratory Quinones The respiratory quinones have only been determined for two strains from the *H. aurantiacus* species group (the type strain *H. aurantiacus* ATCC 23779^T and Hp a2 DSM 589), and for a *Herpetosiphon*-like activated sludge isolate, Wie2, whose genetic relatedness to *Herpetosiphon* (see the sec-tion Taxonomy in this Chapter) is unknown. Mainly menaquinones were detected in these three strains (Kleinig and Reichenbach, 1977; Reichenbach et al., 1978; Senghas and Lingens, 1985). The main menaquinone in *H. aurantiacus* and Hp a2 (DSM 589) is MK-6, and 10% of the respiratory quinone content is MK-7 (Kleinig and Reichenbach, 1977). The respiratory quinones have only been investigated in a few of the culturable representants among chloroflexi. The main common factor seems to be that they contain menaquinones. *Roseiflexus* contains mainly menaquinone-11 (Hanada et al., 2002), *Caldilinea* contains mainly menaquinone-10 under aerobic conditions, whereas no quinones were detected in *Anaerolinea* (Sekiguchi et al., 2003).

Fatty Acid Composition The two type strains of *aurantiacus* and *geysericola* contain the same fatty acids (C16:1, C16:0 and C18:1, cyclopropane C17:0, C17:0 and C18:0), whereas *Herpetosiphon*-like strains show some significant differences to the two type strains (Table 1). Branched-chain fatty acids, which are so typical for many other gliding bacteria, are essentially absent. Hydroxy fatty acids are completely lacking. The novel sulfonolipids, or capnoids, discovered in gliding bacteria of the *Cytophaga* group, were not found in *Herpetosiphon* (Godchaux and Leadbetter, 1983). Only a few fatty acid analyses have been performed on other members of chloroflexi, such as *Chloroflexus*, *Roseiflexus*, *Anaerolinea* and *Caldilinea* (see review in Garrity and Holt [2001] and see Hanada et al. [2002] and Sekiguchi et al. [2003]). However, the results obtained so far clearly indicate that C16:0 is the only common fatty acid present in significant amounts in all these strains and that each strain shows significant differences to the other strains in terms of the composition of other fatty acids.

Table 1. Fatty acid composition in the different species groups of *Herpetosiphon*.

Species	No. of strains	Fatty acid composition
H. aurantiacus[a,b]ATCC 23779[T]	12	C16:1,[a,b] C16:0,[a,b] cyclopropane C17:0,[b] C17:0,[b]C18:1,[a,b] C18:0,[a,b]
H. geysericola[b]ATCC 23076[T]	1	Same composition as the *H. aurantiacus* group
Herpetosiphon-like strains		
Sludge strain Wie2[a]	3	C16:0, C16:1, C18:1, C18:0, C19:0, 8-methyl C17:0
"Soil strains"[b]	10	C14:0, C16:1, C16:0, cyclopropane C17:0, C17:0, C18:1, C18:0, C18:2, C19:1, C19:0, *anteiso* C16

Abbreviation: [T], type strain; C14:0, tetradecanoic acid; C16:0, hexadeconic acid; C17:0, heptadecanoic acid; C18:0, octadecaonic acid; C19:0, nonadecanoic acid.
[a]Senghas and Lingens, 1985.
[b]N. Lee et al., manuscript in preparation.

Pigment Patterns The harvested cell mass and dense colonies on agar plates of *Herpetosiphon* may be brightly colored in shades of yellow-orange to brick-red, but unpigmented strains, especially among *Herpetosiphon*-like strains, do occur (Lee et al. (manuscript in preparation)). However, the pigmentation often depends on the growth medium and is usually more intense on peptone-containing substrates. The pigments are cell bound. Chemically, they are carotenoids (Kleinig and Reichenbach, 1977; Reichenbach et al., 1978). The pigment pattern has so far only been determined for seven strains of the aurantiacus group, including the type strain and two different *Herpetosiphon*-like sludge strains. One of these sewage strains is most likely (unfortunately, these strains are no longer available), on the basis of several phenotypical traits and G+C content, related to the *H. aurantiacus* species group (Trick and Lingens, 1984), whereas the affiliation of the other sewage strain is unknown and is thus placed in an undefined species group (Senghas and Lingens, 1985; see the section Taxonomy in this Chapter). The pigment patterns obtained so far seem however to be uniform since only quantitative (but no qualitative) variation has been observed. The dominant compound has a 4-oxo-beta-psi-chromophore, with a hydroxyl in C-1' to which a disaccharide consisting of glucose is connected via a glycoside bond. One of the sugars bears an ester-bonded fatty acid, in strain Hp a2 (DSM 589) mainly C16:0 and C18:1 (Fig. 6). Composite carotenoids of this type are well known from myxobacteria, but in these latter organisms, only monosaccharides are found as sugar constituents. Carotenoids with a disaccharide component are unique so far among prokaryotic pigments.

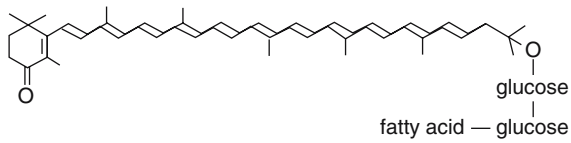

Fig. 6. Chemical structure of the main pigment of *Herpetosiphon aurantiacus*. (From Kleinig and Reichenbach, 1977.)

Metabolic Characteristics Although all known *Herpetosiphon* strains are aerobic, they seem to prefer microaerophilic conditions. All strains, except for the *Herpetosiphon*-like sludge strain of Senghas and Lingens (1985), are catalase- and oxidase positive (Lee et al. (manuscript in preparation)). The nutritional requirements, physiology, and biochemical capabilities of *Herpetosiphon* are not yet fully understood. *Herpetosiphon* is a strict organotroph, but because it is often able to grow on media very low in organic constituents (even on pure water agar, WAT agar; see the section Isolation in this Chapter), the results of substrate-utilization experiments must be interpreted with great care. However, some of the *Herpetosiphon* strains, as well as the *Herpetosiphon*-like sludge strains reported by Senghas and Lingens (1985), do seem to be able to utilize, but not acidify, various hexoses, pentoses and sugar alcohols (Lee et al. (manuscript in preparation)). Organic acids, with the exception of β-hydroxybutyrate, do not support growth.

Apparently, many strains are able to grow on inorganic nitrogen sources (NH_4^+ or NO_3^-), but unequivocal data are often not available. Nitrate reduction to nitrite has only been detected in strains of the *H. aurantiacus* group and some of the *Herpetosiphon*-like sludge organisms (Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)), but only under anaerobic conditions (i.e., in the absence of molecular oxygen), which did not allow growth. Nitrogen fixation has been ruled out in all cases that have been investigated.

Herpetosiphon may contain granules of poly-β-hydroxybutyrate and under certain conditions also of polyphosphate (Trick and Lingens, 1984; Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)), but analytical details have not been given for a final chemical verification of this observation.

Many *Herpetosiphon* strains show impressive hydrolytic capabilities (e.g., of casein, cellulose, chitin, esculin, gelatin, pectin, starch, tributyrin and xylan; Lee et al. (manuscript in preparation)), while some *Herpetosiphon*-like sludge organisms (Trick et al., 1984; Senghas and Lingens, 1985) and sluice strains (Brauss et al., 1969) are less active in this respect. It should be understood that the results of those digestion experiments sometimes critically depend on the medium and the material used. In the case of pectin, a wide variety of preparations are available that give quite different results. With pectin from apple (38% methyl ester), we found pectin degradation by some of the *Herpetosiphon* strains we tested. Cellulose digestion by different *Herpetosiphon* strains is often seen when using the quick agar test based on CEL1 agar (see below), on which large lysis zones are produced within a few days, reaching far beyond the swarm colony. This suggests that cellulose decomposition by *Herpetosiphon* is achieved by diffusible exoenzymes (Lee et al. (manuscript in preparation)). However, only *H. geysericola* (ATCC 23076^T), Hp a1 (DSM number in preparation) and strain So97 (DSM 14854) decompose filter paper, but decomposition is usually slow (recognizable only after 20–30 days at 30°C on ST6 Agar; Lewin, 1970; Lee et al. (manuscript in preparation)).

CEL1 Agar

Cellulose powder	0.5%
$(NH_4)_2SO_4$	0.1%
Agar	1%

Dissolve ingredients in distilled water, adjust to pH 7.2. After autoclaving, pour the medium as a thin layer on top of ST6 agar plates. A suitable cellulose powder, MN 300, is produced by Macherey and Nagel (Germany).

ST6 Agar

Part A

$(NH_4)_2SO_4$	0.1%
$MgSO_4 \cdot 7H_2O$	0.1%
$CaCl_2 \cdot 2H_2O$	0.1%
$MnSO_4 \cdot 2H_2O$	0.01%
$FeCl_3 \cdot 6H_2O$	0.02%
Trace element solution (same as for medium MD1)	1 ml/liter

Ingredients of part A should be dissolved in one-third of the water (*distilled*) volume. Ingredients of part B should be dissolved in two-thirds of the water volume.

Part B

K_2HPO_4	0.1%
Yeast extract	0.002%
Agar	1%

After autoclaving parts A and B separately, combine them and pour the mixture into plates.

With many strains, we have also observed DNA hydrolysis on DNA agar, but in some studies of *Herpetosiphon*-like sludge organisms, tests for DNase were negative (Senghas and Lingens, 1985). Not all strains show β-hemolysis of sheep and human erythrocytes (Gräf and Perschmann, 1970; Trick and Lingens, 1984; Lee et al. (manuscript in preparation)).

Most investigators report that *Herpetosiphon* organisms lyse dead bacteria but also in a few cases live bacteria. However, not all types of bacteria are equally sensitive. For instance, Quinn and Skermann (1980) have reported that some *E. coli*, as well as some Gram-positive strains (*Bacillus subtilis*, *Lactobacillus casei* and *Streptococcus mutans*), turned out to be particularly recalcitrant (Lee et al. (manuscript in preparation)). Not all *Herpetosiphon* strains show identical lysis patterns. The *Herpetosiphon*-like sludge organisms of Trick and Lingens (1984) and Senghas and Lingens (1985) do not lyse bacteria at all; however, the *Herpetosiphon aurantiacus* type sludge organism FC16a (DSM number in preparation) of N. Lee et al. (manuscript in preparation) does lyse bacteria. The autoclaved yeast cells in VY/2 agar are often but not always destroyed. Other yeast species may also be lysed, such as *Pichia stipitis* or *Candida albicans* (Lee et al. (manuscript in preparation)).

The H_2S, indole and acetoin tests were negative for all strains tested, whereas the phosphatase and the urease tests were positive for some of the strains tested (Trick and Lingens, 1984; Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)). Tyrosine in tyrosine agar is often degraded.

Tolerance Tests

The *Herpetosiphon*-like sludge organisms tolerate up to 2% NaCl, but most other *Herpetosiphon* strains are rather sensitive to elevated salt concentrations; growth is rarely observed above 1.5% NaCl. Optimum pH for all strains is around 7; however, growth is tolerable in the pH range 4–10. Optimum temperature for all *Herpetosiphon* strains is 20–30°C, but growth is generally seen in the range 10–37°C. *Herpetosiphon*-like strains (e.g., Hp g207, DSM 15737) may grow at 45°C (Trick and Lingens, 1984; Senghas and Lingens, 1985; Lee et al. (manuscript in preparation)). All *Herpetosiphon* strains investigated so far are resistant to high levels of kanamycin sulfate (250 mg/liter).

Molecular Characterization

The G+C content of the two *Herpetosiphon* species groups (*aurantiacus* and *geysericola*) is 48–53 mol%. However, as already mentioned in the introduction, the G+C content of some *Herpetosiphon*-like isolates, e.g., the strain of Senghas and Lingens (1985), and the strains Hp g6 DSM

15710, Hp g16 DSM 15736 and Hp g207 DSM 15737 is higher (59–75%), which suggests that at least some of these isolates may represent new genera among chloroflexi (Lee et al. (manuscript in preparation)).

The unique multicopy single-stranded DNA (msDNA) common in gliding myxobacteria and also found in *Flexibacter* could not be demonstrated in *Herpetosiphon* (Dhundale et al., 1985). The base sequence of the 5S rRNA gene has only been determined for one of the *Herpetosiphon*-like sludge organisms (strain Wie 2; Van den Eynde et al., 1987). While this 5S rRNA gene sequence corresponds, in general, quite well with the model of eubacterial 5S rRNA structure, there are several deviations—in two cases in highly conserved sites not modified in any other eubacterium. The base sequence of the 16S rRNA gene has been determined for 15 different *Herpetosiphon* strains (Oyaizu et al., 1987; Bradford et al., 1996; Sly et al., 1998; Kohno et al., 2002; Lee et al. (manuscript in preparation)). Comparative 16S rRNA gene sequence analyses confirmed that they all form a specific *Herpetosiphon* cluster within chloroflexi (see the section Taxonomy in this Chapter).

Table 2. 16S rRNA gene sequence similarity values of *Herpetosiphon* strains and other culturable chloroflexi strains.

Culturable chloroflexi strains	Similarity to 16S rRNA gene[a] (%)
H. aurantiacus strains (14 strains[b])	97.8–99.5
H. geysericola	96.4
Herpetosiphon-like strains (7 strains[c])	79–94
Chloroflexus aurantiacus	81.6
Chloroflexus aggregans	80.3
Chloronema giganteum	81.8
Kouleothrix aurantiacus	82.8
Roseiflexus castenholzii	82.6
Oscillochloris trichoides	82.8
Eikelboom type 1851 Ben52	81.3

[a]The gene of *H. aurantiacus* ATCC 23779T.
[b]Hp g8 (DSM 6205), Hp g10 (DSM 6207), HrsBendi 15 FO18 (Bradford et al., 1996), OSI-B2 (Kohno et al., 2002), FC16a (DSM number in preparation), Hp al (DSM number in preparation), Hp a2 (DSM 589), Hp g5 (DSM number in preparation), Hp g156 (DSM 15901), Hp g158 (DSM 15902), Hp g254 (DSM 15903), Hp g269 (DSM 15712), Hp g277 (DSM 15713), and Hp g278 (15714).
[c]Hp g6 (DSM 15710), Hp g16 (DSM 15736), Hp g207 (DSM 15737), Hp g122 (DSM 15874), Hp g124 (DSM 15875), Hp g150 (DSM 15876), and Hp g174 (DSM 1587).
The data for *H. geysericola* are from Sly et al., 1998, whereas the rest of the data are preliminary and from N. Lee et al. (manuscript in preparation).

Taxonomy

The closest culturable relatives of *Herpetosiphon* are only distantly related to *Herpetosiphon* (e.g., *Chloroflexus aurantiacus* and *Roseiflexus aurantiacus*, showing around 81–83% 16S rRNA gene similarity; Table 2). This has been deduced from different comparative 16S rRNA gene sequence studies, from analyses of oligonucleotide catalogs via binary association coefficients (S_{AB} values), through early oligonucleotide signatures (Gibson et al., 1985; Oyaizu et al., 1987) and from a recent comparison of the nearly full 16S rRNA gene sequence of around 15 different strains of *Herpetosiphon* (Lee et al. (manuscript in preparation)), as well as from comparative sequence analysis of elongation factor Tu and ATP–synthase beta-subunit genes (Ludwig et al., 1993). Together with *Anaerolinea* gen. nov., *Caldilinea* gen. nov., *Chloronema*, *Heliothrix*, *Roseiflexus*, *Oscillochloris*, "the Eikelboom type 1851 sludge strain" (Beer et al., 2002), and the *Nostocoida limicola*-like strains of Schade et al. (2002), they represent the few culturable organisms in chloroflexi (<10% of all submitted chloroflexi-affiliated sequences in general molecular databases, June, 2004). Interestingly, most of these culturable strains consist mainly of thermophilic phototrophs (*Chloroflexus*, *Chloronema*, *Heliothrix*, *Roseiflexus* and *Oscillochloris*) or thermophilic organotrophs (*Anaerolinea* gen. nov. and *Caldilinea* gen. nov.). The only exceptions are *Herpetosiphon*, the sludge strains Eikelboom type 1851, the *Nostocoida limicola*-like strains of Schade et al. (2002), and a few strains in different thermophilic genera that are mesophilic organotrophs. At the present stage, the *Herpetosiphon* strains represent the largest group of culturable, nonthermophilic, nonphotosynthetic organisms among the chloroflexi.

Since the discovery of the first *Herpetosiphon* strain in 1968 by Holt and Lewin, the genus *Herpetosiphon* is today still only represented by two rather similar species, *H. aurantiacus* and *H. geysericola,* and some *Herpetosiphon*-like sludge strains (Trick and Lingens, 1984; Senghas and Lingens, 1985; Bradford et al., 1996), whose genetic relatedness to *Herpetosiphon* can no more be determined since the strains are no longer existent. Thus, *Herpetosiphon* is the only genus in the family Herpetosiphonaceae in the second order of the class *Chloroflexi*, *Herpetosiphonales*. As mentioned earlier (see the section Habitats in this Chapter), the three marine "*Herpetosiphon*" strains, which were discovered by Lewin (1970), have recently been transferred to another phylum, *Flexibacter-Bacteroides-Cytophaga* (Sly et al., 1998). Since the discovery of the second culturable *representative of the Class Chloroflexi, Chloroflexus aurantiacus* (Pierson et al., 1974), a vast amount of as yet not cultivatable strains of *chloroflexi*-affiliated organisms have been detected (see, e.g., the review of Björnsson et al., 2002). Only a few of these studies have reported any *Herpetosiphon*-

Table 3. Fatty acid composition, G+C content, 16S rRNA gene similarity and DNA-DNA similarity in different *Herpetosiphon* and *Herpetosiphon*-like strains.

Species groups of *Herpetosiphon*	No. of strains	FA[a]	G+C mol%	16S rRNA similarity[b] (%)	DNA-DNA similarity[b] (%)
H. aurantiacus[c] ATCC 230779^T	12	I	48.1–52.9	97.8–99.5	65.3–95
H. geysericola[c,d] ATCC 23076^T	1	I	51.8	96.4	67.8
Herpetosiphon-like strains:					
"Sludge strains"[e]	3	II	59	ND	ND
"Sludge strains"[f]	5	ND	48.7–49.0	ND	ND
"Soil strains"[c]	10	III	62–75	81–95	<15

Abbreviations: FA, fatty acid composition category; ^{T}, type strain; and ND, not determined, strains no longer available.
[a]Each group (I to III) represent a unique composition; see Table 1.
[b]To the type strain *H. aurantiacus* (ATCC 23779^T).
[c]N. Lee et al. (manuscript in preparation).
[d]Sly et al. (1998).
[e]Senghas and Lingens (1985).
[f]Trick and Lingens (1984).

like organisms, which suggests that the *Herpetosiphon* group might represent a rather stagnant evolutionary group of organisms with a low diversity among the *chloroflexi*. However, recent studies by Lee et al. (manuscript in preparation)) showed that the genetic diversity among the *Herpetosiphon*-like strains is much larger than previously expected, differing up to 19% in 16S rRNA gene sequence from the type strain of *H. aurantiacus*, which suggests that some of these strains represent novel genera among chloroflexi. Thus, the taxonomy of the phylum *Chloroflexi* and the order *Herpetosiphonales* may have to be re-evaluated in the future. Table 3 gives the distinguishing characteristics of the two recognized *Herpetosiphon* species and the *Herpetosiphon*-like organisms.

Summary of the Species Groups of *Herpetosiphon*

H. AURANTICUS. This species group, represented by the type strain ATCC 23779^T and isolated by Lewin and Holt (1968), contains the largest number of strains (14, listed below). The G+C content is 48.1–52.9 mol%. 16S rRNA gene similarity to the type strain is between 97.8% and 99.5% (Table 2). However, the DNA-DNA similarity to the type strain may vary from 65.3% to 95% (Table 3). The fatty acid composition is similar in all strains (Table 1), but the strains may differ in some single phenotypical characteristics (Lee et al. (manuscript in preparation)). Strains have been isolated from freshwater lakes (Lake Constance, Germany: Hp g8 [DSM 6205], Hp g10 [DSM 6207]; Gräf et al., 1970; Lee et al. (manuscript in preparation)); from activated sludge in different parts of the world: HrsBendi 15 FO18 (Bradford et al., 1996), OSI-B2 (Kohno et al., 2002), FC16a [DSM 15711] (Lee et al. (manuscript in preparation)); and from soil from different parts of the world: Hp a1 (DSM number in preparation), Hp a2 (DSM 589), Hp g5 (DSM number in preparation), Hp g156 (DSM 15901), Hp g158 (DSM 15902), Hp g254 (DSM 15903), Hp g269 (DSM 15712), Hp g277 (DSM 15713), Hp g 278 (DSM 15714) by H. Reichenbach and by Lee et al. (manuscript in preparation).

H. GEYSERICOLA. This species is represented by only one strain, the type strain *H. geysericola*, ATCC 23076^T (described by Lewin, 1970). Originally it was suggested (Lewin, 1970) that *H. geysericola* is identical with *Phormidium geysericola* (Copeland, 1936), but this appears very unlikely, because *Phormidium geysericola* was found at the rim of a geyser and in 60–84°C water from various alkaline hot springs in Yellowstone. It thus is definitely a thermophile and would hardly grow at 30°C, as does *H. geysericola*. The validity of *H. geysericola*´s species status has been discussed, since it shares several common phenotypical characteristics (exceptions: urease reaction, nitrate reduction, and hydrolysis of cellulose filter paper), G+C content, and fatty acid composition with the *H. aurantiacus* group (Reichenbach, 1992; Lee et al. (manuscript in preparation)). Recent studies have shown that the 16S rRNA similarity (96.4%; Sly et al., 1998; Table 2) and the DNA-DNA similarity (67.8%, Lee et al. (manuscript in preparation); Table 3) to *H. aurantiacus* is on the border of the present species definition (Stackebrandt et al., 2002). Since the DNA similarity between some of *H. aurantiacus* strains may also range from 65.3% to 95% (Table 3), it is questionable whether *H. geysericola* should remain a distinct species. The main difference between *H. geysericola* and all 14 *H. aurantiacus* strains is that *H. geysericola* was isolated from the vicinity of a hot spring and thus was defined as "thermophilic," although the proper characterization should be thermotolerant. However, thermotol-

erance is not unusual with *aurantiacus* strains, which may also grow at 38–40°C Lee et al. (manuscript in preparation). Further taxonomical studies on *H. geysericola* (in particular investigation of chemotaxonomical characteristics) and, if possible, on more *geysericola*-like strains retrieved from sites in and around hot springs will be needed to determine whether *H. geysericola* should remain a separate species or be included in the *H. aurantiacus* group. Another theoretical possibility is also that some of the present strains within the *H. aurantiacus* group should be transferred to *geysericola*, although they were not isolated from sites in and around hot springs but from soil. The reason for this is that their DNA-DNA similarity values to the *H. aurantiacus* type strain approximates that of the present species definition (i.e., slightly below 70% similarity). Further DNA-DNA similarity studies with these strains would be needed to clarify this question.

Herpetosiphon-LIKE STRAINS. In the first attempt after isolation, several strains have been identified as *Herpetosiphon*-like, but further studies, in particular, utilizing molecular techniques (16S rRNA gene sequence analysis), have revealed that the diversity of *Herpetosiphon*-like strains is much larger than expected. The 16S rRNA gene similarity between *Herpetosiphon*-like strains and *H. aurantiacus* (ATCC 23779^T) is around 79–94%, and the G+C content is around 62–75% (Lee et al. (manuscript in preparation); Table 3). Thus, these strains represent most likely new genera among the chloroflexi, e.g., the strains Hp g6 (DSM 15710), Hp g16 (DSM 15736), Hp g207 (DSM 15737), Hp g122 (DSM 15874), Hp g124 (DSM 15875), Hp g150 (DSM 15876), and Hp g174 (DSM 1587). All these strains have been isolated from soil Lee et al. (manuscript in preparation). The 16S rRNA genes of the *Herpetosiphon*-like sludge organisms described by Trick and Lingens (1984) and Senghas and Lingens (1985) unfortunately cannot be sequenced, since these strains are no longer available. However, previous studies, based on analysis of oligonucleotide catalogs via binary association coefficients (S_{AB} values), showed that the *Herpetosiphon*-like sludge organisms isolated by Senghas and Lingens (1985) are clearly related but not identical to *Herpetosiphon* (S_{AB} values with *Herpetosiphon*: 0.39, for *Chloroflexus*: 0.40, and *Herpetosiphon-Chloroflexus*: 0.31). This is also indicated by the significantly higher G+C mol% value obtained for these organisms (Table 3).

Microbial Ecology

We know virtually nothing about the true distribution, dynamics and activity of *Herpetosiphon* in the environment, as well as about its interactions with other organisms, such as other bacteria or eukaryotic grazers. No reports on bacteriophages of *Herpetosiphon* have been published. All we know is that *Herpetosiphon* may be found in freshwater and sewage systems, in the vicinity of hot springs, as well as in diverse types of soil systems in different countries and islands in many parts of the world (Africa, America, Australia, Europe, India, Japan and Mexico). Our knowledge about the biogeography of *Herpetosiphon* is mainly based on isolation studies or surveys using different chloroflexi specific probes detected by means of FISH (Björnsson et al., 2002; Lee et al. (manuscript in preparation)). Only a few 16S rRNA clone library or denaturing gradient gel electrophoresis screening studies on the microbial community composition in freshwater lakes or sewage systems have reported *Herpetosiphon*-like 16S rRNA genes (Gich et al., 2001; Kohno et al., 2002), despite the fact that a constantly increasing amount of novel, in most cases, chloroflexi-like sequences (from as yet noncultivable organisms, not related to *Herpetosiphon*) are being found in nearly all kinds of environments all over the world (e.g., Pierson et al., 1985; Giovannoni et al., 1996; Snaidr et al., 1997; Chandler et al., 1998; Dojka et al., 1998; Hugentholtz et al., 1998a; Hugentholtz et al., 1998b; Gordon et al., 2000; Sekiguchi et al., 2001; Alfreider et al., 2002; Bano and Hollibaugh, 2002; Boomer et al., 2002; Dunbar et al., 2002; Furlong et al., 2002; Hentschel et al., 2002; Juretschko et al., 2002; Lee et al. (manuscript in preparation)). However, as pointed out earlier (in the section Habitats in this Chapter), this does still not provide the final evidence for the nonexistence of *Herpetosiphon* in any of these systems. It may be that search for *Herpetosiphon* is negatively biased in general molecular screening studies (Lee et al. (manuscript in preparation)), so that specific molecular markers for *Herpetosiphon* must be used to detect them. In those cases, where a large attempt has been undertaken to isolate bacteria from the environment, none of the media used were optimal for the isolation of *Herpetosiphon*.

Whereas virtually nothing is known about the abundance of *Herpetosiphon* in soil, some observations, albeit contradictory, on the abundance of *Herpetosiphon* and other filamentous chloroflexi have been made in sewage plants using different chloroflexi-specific oligonucleotides in FISH (Fig. 7). Björnsson et al. (2002) reported that the amount of chloroflexi filaments in subphylum III (to which *Herpetosiphon* is affiliated) was considerably higher than the amount of other chloroflexi filaments affiliated to other subphyla. N. Lee et al. (manuscript in preparation) found the contrary. Unfortunately, nothing is

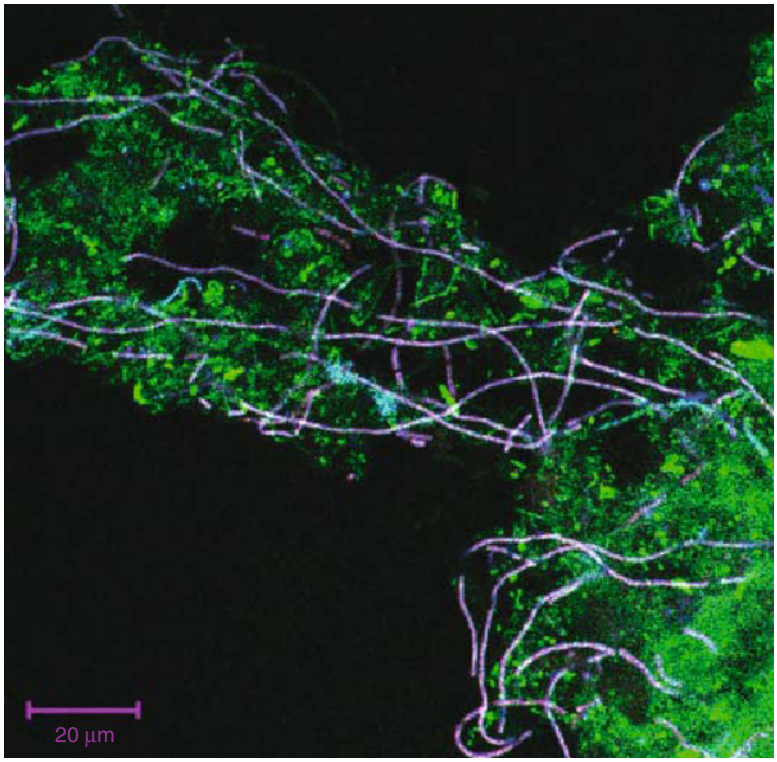

Fig. 7. Image of fluorescence *in situ* hybridization using oligonucleotide probes targeting chloroflexi filaments on the phylum level (probe CFX1223 in Cy3, red) and on the subphylum III level (to which *Herpetosiphon* belongs, CFX784 probe in Cy5, blue) and Bacteria (EUB338, in FLUOS-Prime, green) in activated sludge (photo: N. Lee; for probe references, see Björnsson et al., 2002). The purple-colored filaments are a result of overlapping Cy3 (red) and Cy5 (blue) stained cells. Bar = 20 µm.

known about the *in situ* abundance of *Herpetosiphon* in other sewage systems where sequences of other culturable and as yet not cultivable chloroflexi strains have been obtained (e.g., Bradford et al., 1996; Beer et al., 2002; Juretschko et al., 2002; Kohno et al., 2002; Onuki et al., 2002; Schade et al., 2002). In those cases where isolation attempts were undertaken, no media were used that were optimal for *Herpetosiphon*, and in those cases where FISH was used, no probes targeting specifically *Herpetosiphon* were applied, and so no definitive conclusions about their nonexistence in these systems can be drawn. Thus, the contradiction between the observations made by Björnsson et al. (2002) and Lee et al. (manuscript in preparation) is most likely explained by the effect of unknown geographical factors or operational differences between the different sewage systems. As long as nothing is known about the role of *Herpetosiphon* in sewage systems and about whether significant physiological differences exist among these sewage *Herpetosiphon* strains, any attempt to explain the differences in abundance of *Herpetosiphon* will remain speculative. Considering the hydrolytic capabilities of *Herpetosiphon*, it may however be assumed that the bacterium participates in the degradation of macromolecules and perhaps in the control of microbial populations in nature (Quinn and Skerman, 1980; Lee et al. (manuscript in preparation)).

Molecular Genetics

Nothing is known about the genetics of *Herpetosiphon*. Also, whether *Herpetosiphon* possesses mobile genetic elements or is able to acquire plasmids through lateral gene transfer is unknown. The genome of *Herpetosiphon* has not been sequenced, but the genome of its closest culturable relative, the photosynthetic *Chloroflexus*, is currently being sequenced in a vast program aimed at investigating the evolution and genetics of photosynthesizing prokaryotes (DOE Joint Genome Institute, United States Department of Energy). Future sequencing of the genome of *Herpetosiphon* might however yield interesting insights into the evolution of early life on earth and the divergence of the different members among the present representatives in chloroflexi. A possible case for lateral gene transfer in *Chloroflexus* has already been indicated by a comparative phylogenetic analysis of circadian clock genes in *Archaea* and *Bacteria* showing that these genes might have been laterally transferred from cyanobacteria to different taxa (among them *Chloroflexus*) in *Archaea* and *Bacteria* (Dvornyk et al., 2003).

Given the unique position of the phylum *Chloroflexi*, it is not unlikely that further studies on the molecular genetics and biochemistry of *Herpetosiphon* may reveal interesting facts. This has been shown to be the case in screening studies of

two different enzymes in some *Herpetosiphon* strains from the *H. aurantiacus* species group: the endoribonucleases (Haas and Brown, 1998) and the restriction endonucleases (Mayer and Reichenbach, 1978). The ribonuclease P has been sequenced for several classical phyla of *Bacteria* (encompassing 145 different strains, including three chloroflexi strains, *Chloroflexus*, *Herpetosiphon* and *Thermomicrobium*) for further evolutionary comparisons and revealed interesting insights into the structural evolution of the RNA molecule (Haas and Brown, 1998). A whole family of 17 restriction endonucleases has been more or less completely characterized with respect to their recognition and cleavage specificities (Brown et al., 1980; Kröger et al., 1984; Düsterhöft et al., 1991). All appear to be class II endonucleases, producing cohesive ends, either in the 5′-strand or, in the cases of endonucleases *Hgi* AI and *Hgi* JII, in the 3′-strands, and seem to be located in the periplasm. Most of them interact with hexanucleotide sequences and some with pentanucleotide sequences, but virtually all recognition sequences are degenerate. The availability of such a large family of endonucleases is of considerable theoretical interest because it allows a comparative study of the mechanisms of protein-DNA interaction as well as of molecular evolution. The known *Herpetosiphon* enzymes can be arranged in such a way that their recognition sequences overlap and the whole set thus becomes a continuous system (Kröger et al., 1984). One of the enzymes, *Hgi* EII, has a unique recognition sequence of 2×3 bases separated by 6 unspecified nucleotides. When the recognized bases are read in sequence, they give the recognition sequence of *Hgi* CI, and it has been proposed that enzyme *Hgi* EII originated by an inversion of the two enzyme subunits so that the recognition sites are now on the surface rather than in the center of the enzyme. Isoschizomeres can be found for almost all *Herpetosiphon* recognition sequences, e.g., *Sal* I enzyme is isoschizomere to *Hgi* CIII and *Hgi* DII. Interestingly, no hybridization was found between *Sal* I gene probes and *Herpetosiphon* DNA, whereas two other producers of *Sal* I isoschizomeres, *Rhodococcus rhodochrous* and *Xanthomonas amaranthicola*, showed clear DNA similarity under stringent conditions (Rodicio and Chater, 1988). While this suggests that the latter organisms and *Streptomyces albus* G have at least partially homologous enzymes, the high G+C difference of 20 mol% between *Herpetosiphon* and *Streptomyces* could have obscured, through codon replacement, a structural relationship between their enzymes. A comparison of the base sequences of the genes or of the amino acid sequences of the various enzymes should answer the question.

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Literature Cited

Alfreider, A., C. Vogt, and W. Babel. 2002. Microbial diversity in an in situ reactor system treating monochlorobenzene contaminated groundwater as revealted by 16S ribosomal DNA analysis. System. Appl. Microbiol. 25:232–240.

Bano, N., and J. T. Hollibaugh. 2002. Phylogenetic composition of bacterioplankton assemblages from the Arctic Ocean. Appl. Environ. Microbiol. 68:505–518.

Beer, M., E. M. Seviour, Y. Kong, M. Cunningham, L. L. Blackall, and R. J. Seviour. 2002. Phylogeny of the filamentous bacterium Eikelboom type 1851, and design and application of a 16S rRNA targeted oligonucleotide probe for its fluorescence in situ identification in activated sludge. FEMS Microbiol. Lett. 207:179–183.

Björnsson, L., P. Hugenholtz, G. W. Tyson, and L. L. Blackall. 2002. Filamentous Chloroflexi (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal. Microbiology 148: 2309–2318.

Boomer, S. H., D. P. Lodge, B. E. Dutton, and B. Pierson. 2002. Molecular characterization of novel red green nonsulfur bacteria from five distince hot spring communities in Yellowstone National Park. Appl. Environ. Microbiol. 68:346–355.

Bradford, D., P. Hugenholtz, E. M. Seviour, M. Cunningham, H. Stratton, R. J. Seviour, and L. L. Blackall. 1996. 16S rRNA analysis of isolates obtained from Gram-negative, filamentous bacteria micromanipulated from activated sludge. System. Appl. Microbiol. 19:334–343.

Brauss, F. W., W. Heyne, and I. Heyne-Katzenberger. 1969. Beschreibung eines neuen lytisch-aktiven Bakterienstammes. Arch. Hyg. Bacteriol. 153:457–459.

Brown, N. L., M. McClelland, and P. R. Whitehead. 1980. Hgi AI: A restriction endonuclease from Herpetosiphon giganteus HP 1023. Gene 9:49–68.

Chandler, D. P., F. J. Brockman, and J. K. Fredrickson. 1998. Phylogenetic diversity of Archaea and Bacteria in a deep subsurface paleosol. Microb. Ecol. 36:37–50.

Copeland, J. J. 1936. Yellowstone thermal Myxophyceae. Ann. NY Acad. Sci. 36:1–232.

Dhundale, A. R., T. Furuichi, S. Inouye, and M. Inouye. 1985. Distribution of multicopy single-stranded DNA among myxobacteria and related species. J. Bacteriol. 164:914–917.

Dojka, M. A., P. Hugenholtz, S. K. Haack, and N. R. Pace. 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64: 3869–3877.

Dunbar, J., S. M. Barns, L. O. Ticknor, and C. R. Kuske. 2002. Empirical and theoretical bacterial diversity in four Arizona soils. Appl. Environ. Microbiol. 68:3035–3045.

Düsterhoft, A., D. Erdmann, and M. Kröger. 1991. Stepwise cloning and molecular characterization of the HgiD1 restriction-modification system from Herpetosiphon giganteus Hp a2. Nucleic Acids Res. 19:1049–1056.

Dvornyk, V., O. Vinogradova, and E. Nevo. 2003. Origin and evolution of circadian clock genes in prokaryotes. Proc. Natl. Acad. Sci. USA 100:2495–2500.

Eikelboom, D. H., van Buijsen, H. J. J. 1983; Microscopic sludge investigation manual. TNO Institute of Environmental Sciences, Delft.

Fuhs, G. W., and M. Chen. 1975. Microbiological basis of phosphate removal in the activated sludge process for the treatment of wastewater. Microb. Ecol. 2:119–138.

Furlong, M. A., D. R. Singleton, D. C. Coleman, and W. B. Whitman. 2002. Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm Lumbricus rubellus. Appl. Environ. Microbiol. 68:1265–1279.

Garrity, G., and J. G. Holt. 2001. Chloroflexi phy. nov. *In:* D. R. Boone and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed., Volume 1: The Archaea and the Deeply Branching and Phototrophic Bacteria. Springer-Verlag. New York, NY. 427–446.

Gibson, J., W. Ludwig, E. Stackebrandt, and C. R. Woese. 1985. The phylogeny of the green photosynthetic bacteria: Absence of a close relationship between Chlorobium and Chloroflexus. System. Appl. Microbiol. 6:152–156.

Gich, F., J. Garcia-Gil, and J. Overmann. 2001. Previously unknown and phylogenetically diverse members of the green nonsulfur bactera are indigenous to freshwater lakes. Arch. Microbiol. 177:1–10.

Giovannoni, S. J., M. S. Rappé, K. L. Vergin, and N. L. Adair. 1996. 16S rRNA genes reveal stratified opean ocean bacterioplankton populations related to the green nonsulfur bacteria. Proc. Natl. Acad. Sci. USA 93:7979–7984.

Godchaux, W., and E. R. Leadbetter. 1983. Unusual sulfonolipids are characteristic of the Cytophaga-Flexibacter group. J. Bacteriol. 153:1238–1246.

Gordon, D. A., J. Priscu, and S. Giovannoni. 2000. Origin and phylogeny of microbes living in permanent Antarctic lake ice. Microb. Ecol. 39:197–202.

Gräf, W., and G. Perschmann. 1970. Über eine neue Spezies von Vitreoscilla (Vitreoscilla proteolytica) im Bodensee. Arch. Hyg. Bacteriol. 154:128–137.

Haas, E. S., and J. W. Brown. 1998. Evolutionary variation in bacterial Rnase P RNAs. Nucleic Acids Res. 26:4093–4099.

Hanada, S., S. Takaichi, K. Matsuura, and K. Nakamura. 2002. Roseiflexus castenholzii gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium that lacks chlorosomes. Int. J. Syst. Evol. Microbiol. 52:187–193.

Harwardt, R., E. Maier, H. Reichenbach, R. Weckesser, and R. Benz. 2004. Channel-forming (Porin) activity in Herpetosiphon aurantiacus Hp a2. J. Bacteriol. 186:6667–6670.

Hentschel., U., J. Hopke, M. Horn, A. B. Friedrich, M. Wagner, J. Hacker, and B. S. Moore. 2002. Molecular evidence for a uniform microbial community in sponges from different oceans. Appl. Environ. Microbiol. 68: 4431–4440.

Holt, J. G., and R. A. Lewin. 1968. Herpetosiphon aurantiacus gen. et sp. n., a new filamentous gliding organism. J. Bacteriol. 95:2407–2408.

Holt, J. G. 1989. Genus Herpetosiphon Holt and Lewin, 1965, 2408. *In:* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (Eds.) Bergey's Manual of Systematic Bacteriology. Williams & Wilkins. Baltimore, MD. 3:2136–2138.

Hugenholtz, P., B. M. Goebel., and N. R. Pace. 1998a. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180: 4765–4774.

Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace. 1998b. Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. 180:366–376.

Jenkins, D., M. G. Richard, and G. Daigger. 2003. Manual on the Causes and Control of Activated Sludge Bulking, Bulking Foaming and Other Solids Separation Problems, 3rd ed. Lewis Publishers. Boca Raton, FL, USA.

Juretschko, S., A. Loy, A. Lehner, and M. Wagner. 2002. The microbial community composition of a nitrifiying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. System. Appl. Microbiol. 25:84–99.

Jürgens, U. J., J. Meissner, U. Fischer, W. A. König, and J. Weckesser. 1987. Ornithine as a constituent of the peptidoglycan of Chloroflexus aurantiacus, diaminopimelic acid in that of Chlorobium vibrioforme f. thiosulfatophilum. Arch. Microbiol. 148:72–76.

Jürgens, U. J., J. Meissner, H. Reichenbach, and J. Weckesser. 1989. L-Ornithine containing peptidoglycan-polysaccharide complex from the cell wall of the gliding bacterium Herpetosiphon aurantiacus. FEMS Microbiol. Lett. 60:247–250.

Kleinig, H., and H. Reichenbach. 1977. Carotenoid glucosides and menaquinones from the gliding bacterium Herpetosiphon giganteus Hp a2. Arch. Microbiol. 112:307–310.

Kohno, T., K. Sei, and K. Mori. 2002. Characterization of type 1851 organism isolated from activated sludge. Water Sci. Technol. 46:111–114.

Kröger, M., G. Hobom, H. Schütte, and H. Mayer. 1984. Eight new restriction endonucleases from Herpetosiphon giganteus—divergent evolution in a family of enzymes. Nucleic Acids Res. 12:3127–3141.

Lamont, H. C. 1969. Sacrificial cell death and trichome breakage in an oscillatorian blue-green alga: The role of murein. Arch. Microbiol. 69:239–259.

Lee, N., I. Tjernberg, R. Rossello-Mora, B. Sponzar, L. Larsson, F. Rainey, and H. Reichenbach. 2005. Manuscript in preparation.

Lewin, R. A. 1969a. A classification of flexibacteria. J. Gen Microbiol. 58:189–206.

Lewin, R. A., and D. M. Lounsbery. 1969b. Isolation, cultivation and characterization of flexibacteria. J. Gen. Microbiol. 58:145–170.

Lewin, R. A. 1970. New Herpetosiphon species (Flexibacterales). Can. J. Microbiol. 16:517–520.

Ludwig, W., J. Neumaier, N. Klugbauer, E. Brockmann, C. Roller, S. Jilg, K. Reetz, I. Schachtner, A. Ludvigsen, M. Bachleitner, U. Fischer, and K.-H. Schleifer. 1993. Phylogenetic relationships of Bacteria based on comparative sequence analysis of elongation factor Tu and ATP-synthase β-subunit genes. Ant. v. Leeuwenhoek 64:285–305.

Mayer, H., and H. Reichenbach. 1978. Restriction endonucleases: General survey procedure and survey of gliding bacteria. J. Bacteriol. 136:708–713.

Meissner, J., J. H. Krauss, U. J. Jürgens, and J. Weckesser. 1988. Absence of a characteristic cell wall lipopolysaccharide in the phototrophic bacterium Chloroflexus aurantiacus. J. Bacteriol. 170:3213–3216.

Nübel, U., M. M. Bateson, V. Vandieken, A. Wieland, M. Kühl, and D. M. Ward. 2002. Microscopic examination of distribution and phenotypic properties of phylogenetically diverse Chloroflexaceae-related bacteria in hot spring microbial mats. Appl. Environ. Microbiol. 68:4593–46003.

Onuki, M., H. Satoh, and T. Mino. 2002. Analysis of microbial community that performs enhanced biological phosphorus removal in activated sludge. Water Sci. Technol. 46:145–154.

Oyaizu, H., B. Debrunner-Vossbrink, L. Mandelco, J. A. Studier, and C. A. Woese. 1987. The green non-sulfur bacteria: A deep branching in the eubacterial line of descent. System. Appl. Microbiol. 9:47–53.

Pate, J. L., and L. Y. E. Chang. 1979. Evidence that gliding motility in prokaryotic cells is driven by rotary assemblies in the cell envelopes. Curr. Microbiol. 2: 59–64.

Pierson, B. K., and R. W. Castenholz. 1974. A phototrophic gliding filamentous bacterium of hot springs, Chloroflexus aurantiacus, gen. and sp. nov. Arch. Microbiol. 100:5–24.

Pierson, B. K., S. J. Giovannoni, D. A. Stahl, and R. W. Castenholz. 1985. Heliothrix oregonensis, gen. nov., sp. nov., a phototrophic filamentous gliding bacterium containing bacteriochlorophyll a. Arch. Microbiol. 142:164–167.

Pierson, B. K., D. Valdez, M. Larsen, E. Morgan, and E. E. Mack. 1994. Chloroflexus-like organisms from marine and hypersaline environments. Photosynth. Res. 41:35–52.

Quinn, G. R., and V. B. D. Skerman. 1980. Herpetosiphon—nature's scavenger? Curr. Microbiol. 4:57–62.

Reasoner, D. J., and E. E. Geldreich. 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49:1–7.

Reichenbach, H., and J. R. Golecki. 1975. The fine structure of Herpetosiphon, and a note on the taxonomy of the genus. Arch. Microbiol. 102:281–291.

Reichenbach, H., P. Beyer, and H. Kleinig. 1978. The pigments of the gliding bacterium Herpetosiphon giganteus. FEMS Microbiol. Lett. 3:144–156.

Reichenbach, H., H. K. Galle, and H. H. Heunert. 1980. Herpetosiphon giganteus (Leucotrichales): Wachstum und Bewegung [film]. Institut für den wissenschaftlichen Film. Göttingen, Germany. Encyclopaedia Cinematographica E 2420.

Reichenbach, H. 1981a. Taxonomy of the gliding bacteria. Ann. Rev. Microbiol. 35:339–364.

Reichenbach, H., and M. Dworkin. 1981b. The order Cytophagales (with addenda on the genera Herpetosiphon, Saprospira, and Flexithrix). *In:* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (Eds.) The Prokaryotes, Springer-Verlag. Berlin, Germany. 1:356–379.

Reichenbach, H., W. Ludwig, and E. Stackebrandt. 1986. Lack of relationship between gliding cyanobacteria and filamentous gliding heterotrophic eubacteria: Comparison of 16S rRNA catalogues of Spirulina, Saprospira, Vitreoscilla, Leucothrix, and Herpetosiphon. Arch. Microbiol. 145:391–395.

Reichenbach, H. 1992. The Genus Herpetosiphon. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. Berlin, Germany. 1:3785–3805.

Rodicio, M. R., and K. F. Chater. 1988. Cloning and expression of the Sal I restriction-modification genes of Streptomyces albus G. Molec. Gen. Genet. 213:346–353.

Salcher, O., G. Scheff, E. Senghas, I. Trick, and F. Lingens. 1982. The nutritional pattern of filamentous bacteria isolated from bulking sludge. Zbl. Bakt. Hyg., I. Abt. Orig. C 3:450–456.

Sanfilippo, A., and R. A. Lewin. 1970. Preservation of viable flexibacteria at low temperatures. Can. J. Microbiol. 16:441–444.

Schade, M., C. Beimfohr, and H. Lemmer. 2002. Phylogenetic and physiological characterization of a Nostocoida limicola-like organism isolated from activated sludge. Water Sci. Technol. 46:91–97.

Sekiguchi, Y., H. Takahashi, Y. Kamagata, A. Ohashi, and H. Harada. 2001. In situ detection, isolation, and physiological properties of a thin filamentous microorganism abundant in methanogenic granular sludges: A novel isolate affiliated with a clone cluster, the green nonsulfur bacteria, subdivision I. Appl. Environ. Microbiol. 67:5740–5749.

Sekiguchi, Y., T. Yamada, S. Hanada, A. Ohashi, H. Harada, and Y. Kamagata. 2003. Anaerolinea thermophila gen. nov., sp. nov., and Caldilinea aerophila gen. nov., sp. nov., two novel filamentous thermophiles that represent a previously uncultured lineage of the domain Bacteria at the subphylum level. Int. J. Syst. Evol. Microbiol. 53:1843–1851.

Senghas, R., and F. Lingens. 1985. Characterization of a new Gram-negative filamentous bacterium isolated from bulking sludge. Appl. Microbiol. Biotechnol. 21:118–124.

Skerman, V. B. D., G. R. Quinn, L. I. Sly, and J. V. Hardy. 1977. Sheath formation by strains of Herpetosiphon species. Int. J. Syst. Bacteriol. 27:274–278.

Sly, L. I., M. Taghavi, and M. Fegan. 1998. Phylogenetic heterogeneity within the genus Herpetosiphon: Transfer of the marine species Herpetosiphon coherens, Herpetosiphon nigricans and Herpetosiphon persicus to the genus Lewinella gen. nov. in the Flexibacter-Bacteroides-Cytophaga phylum. Int. J. Syst. Bacteriol. 48:731–737.

Snaidr, J., R. Amann, I. Huber, W. Ludwig, and K.-H. Schleifer. 1997. Phylogenetic analysis and in situ identification of bacteria in activated sludge. Appl. Environ. Microbiol. 63:2884–2896.

Soriano, S. 1945. El nuevo orden Flexibacteriales y la clasificación de los órdenes de las bacterias. Rev. Argent. Agron. (Buenos Aires) 12:120–140.

Soriano, S. 1947. The Flexibacteriales and their systematic position. Ant. v. Leeuwenhoek 12:215–222.

Stackebrandt, E., W. Frederiksen, G. M. Garrity, P. A. D. Grimonat, P. Kämpfer, M. C. J. Maiden, X. Nesme, R. Rosselló-Mora, J. Swings, H. G. Trüper, L. Vauterin, A. C. Ward, and W. B., Whitman. 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst Evol. Microbiol. 52:1043–1047.

Stanier, R. Y., K. Kunisawa, M. Mandel, and G. Cohen-Bazire. 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol. Rev. 35:171–205.

Strohl, W. R., and J. M. Larkin. 1978. Cell division and trichome breakage in Beggiatoa. Curr. Microbiol. 1:151–155.

Tayne, T. A., J. E. Cutler, and D. M. Ward. 1987. Use of Chloroflexus-specific antiserum to evaluate filamentous

bacteria of a hot spring microbial mat. Appl. Environ. Microbiol. 53:1982–1984.

Trick, I., and F. Lingens. 1984. Characterization of Herpetosiphon spec.—a gliding filamentous bacterium from bulking sludge. Appl. Microbiol. Biotechnol. 19:191–198.

Van den Eynde, H., E. Stackebrandt, and R. de Wachter. 1987. The structure of the 5S ribosomal RNA of a member of the phylum of green non-sulfur bacteria and relatives. FEBS Lett. 213:302–303.

Van Veen, W. L. 1973. Bacteriology of activated sludge, in particular the filamentous bacteria. Ant. v. Leeuwenhoek 39:189–205.

Woese, C. R., E. Stackebrandt, R. J. Macke, and G. E. Fox. 1985. A phylogenetic definition of the major eubacterial taxa. System. Appl. Microbiol. 6:143–151.

PART 11

Verrucomicrobium

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CHAPTER 11.1

The Phylum Verrucomicrobia: A Phylogenetically Heterogeneous Bacterial Group

HEINZ SCHLESNER, CHERYL JENKINS AND JAMES T. STALEY

Introduction

General Phylogeny and Taxonomy

The Verrucomicrobia were proposed as a new division within the bacterial domain by Hedlund et al. (1997) and more recently ranked as a phylum (Garrity and Holt, 2001). They represent a distinct lineage within the phylogenetic trees and contain a number of environmental species as well as a small number of cultured species assigned to four genera (Table 1): *Verrucomicrobium* (Fig. 1a, b), *Prosthecobacter* (Fig. 2), *Opitutus* and *Victivallis*.

Verrucomicrobia have shown a moderate degree of relationship to the Planctomycetes and Chlamydiae, but the significance of the common branching is low and the relationships among the three phyla may change as additional species are characterized. The comparative phylogenetic analyses of members of the order Planctomycetales and the type strains of *Verrucomicrobium spinosum* and *Prosthecobacter fusiformis*, as well as *Prosthecobacter* sp. strain FC-2, did not indicate a statistically significant phylogenetic relationship between the Planctomycetes and the Verrucomicrobia and further provided evidence that the Chlamydiae are no more related to both phyla than they are to members of other bacterial lineages.

Knowledge of the phylum Verrucomicrobia is limited owing to the relatively few species that have been obtained in pure culture and characterized. However, through the application of molecular ecology techniques it has become apparent that the Verrucomicrobia are ubiquitous in a wide range of aquatic and terrestrial habitats. The phylogenetic tree based on sequence analyses of the 16S rDNA is dominated by sequences derived from the extraction of total DNA from various habitats. Hugenholtz et al. (1998a) recognized five subdivisions, of which three were characterized by clone sequences only. Since then the phylogenetic tree has been expanded to seven subdivisions, although only three contain validly described taxa (Fig. 3).

The genera of the prosthecate bacteria *Verrucomicrobium* and *Prosthecobacter* are grouped in subdivision 1. Bacteria designated "*Candidatus* Xiphinematobacter" are in subdivision 2. These bacteria are obligate endosymbionts of nematodes in the genus *Xiphinema* (Vandekerckhove et al., 2000). Another group of bacteria living in close connection to eukaryotes are the epixenosomes (Figs. 4 and 5), which are obligate ectosymbionts living on the dorsal surface of marine ciliates of the genus *Euplotidium*. They possess so-called "extrusomes," harpoon-like organelles, and so provide their hosts with a weapon against predatory protozoa (Petroni et al., 2000; Chin et al., 2001). The epixenosomes are members of subdivision 4 together with *Opitutus terrae* (Chin et al., 2001) and three strains of ultramicrobacteria with dwarf cells isolated from anoxic rice paddy soil. The latest described organism, *Victivallis vadensis*, is placed in subdivision 7.

Only a few other strains have been isolated. Janssen et al. (2002) were able to cultivate strain Ellin 428 from Australian pasture soil (which could be grouped in subdivision 2), and Sakai et al. (2003) reported of a fucoidan-degrading marine bacterium (provisional name "*Fucophilus fucoidanolyticus*") from the gut contents of the sea cucumber *Stichopus japonicus*.

Ecology

Verrucomicrobia were first observed in aquatic habitats (Henrici and Johnson, 1935) but are now known to exist in many other habitats, the majority of which are eutrophic or even heavily polluted (Table 2). They were found not only at moderate temperatures but also at cold temperatures in the deep sea and in Antarctica. A member of the Verrucomicrobia was also identified from a hot spring (75–95°C). Several reports are of Verrucomicrobia from extreme environments such as sulfide-rich water and sediments and a soda lake. Interestingly, a member of the Verrucomicrobia was identified in the chitin tubes of the giant vent worm *Riftia pachyptila*, illustrating another connection between this group and thermal environments, such as the hydrothermal vent fauna of the deep sea.

Table 1. Characteristics differentiating the genera of the phylum Verrucomicrobia.

Genus	Habitat	Cell morphology	Color	Motility	Oxygen requirement	G+C (mol%)	References
Verrucomicrobium	Freshwater	Rods with numerous fimbrated prosthecae	Light yellow	–	Aerobic and fermentative	59	Schlesner, 1987
Prosthecobacter	Freshwater; Raw sewage	Fusiform shaped cells with 1 polar prostheca	White or yellow	–	Obligate aerobe	54–60	Staley et al., 1976
Opitutus	Rice paddy soil	Coccoid dwarf cells (0.4–0.6 μm)	Colorless	+	Obligate anaerobe, fermentative	74	Chin et al., 2001
Victivallis	Human fecal sample	Coccoid, 0.5–1.3 μm	Colorless	–	Obligate anaerobe, fermentative	59	Zoetendal et al., 2003
"*Candidatus* Xiphinematobacter"	Ectosymbionts in nematodes	Rod-shaped, 0.7–1.0 $\times$ 2.1–3.2 μm	ND	ND	ND	ND	Vandekerckhove et al., 2000

Symbols: +, present; – absent; and ND, no data.

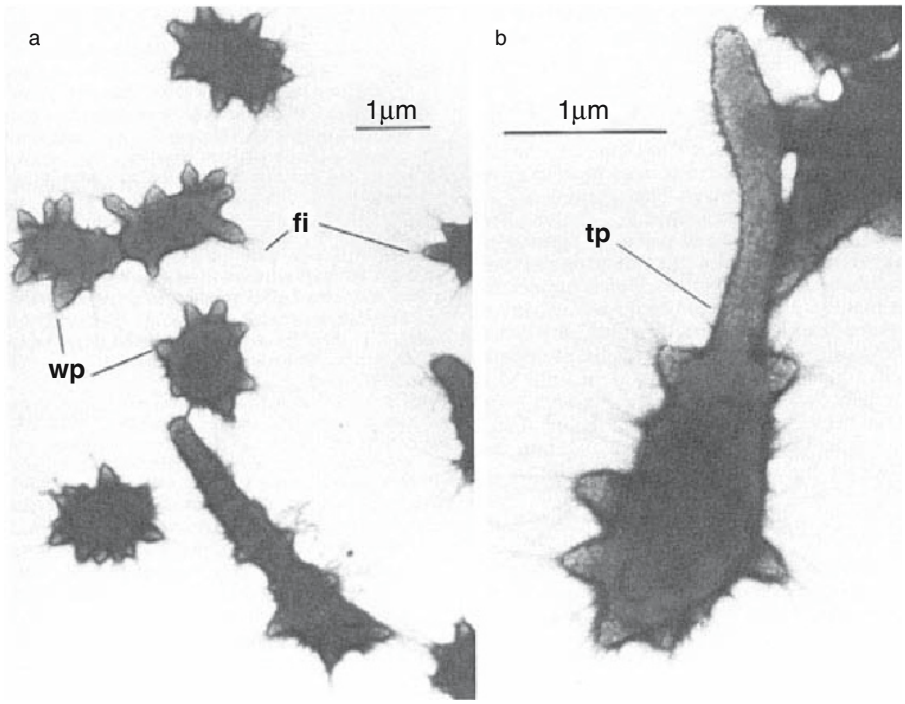

Fig. 1. Morphology of *Verrucomicrobium spinosum*. a) Cells with wart-like prosthecae (wp), from the tips of which fimbriae (fi) extend. b) A cell with a polarly inserted tube-like prostheca (tp).

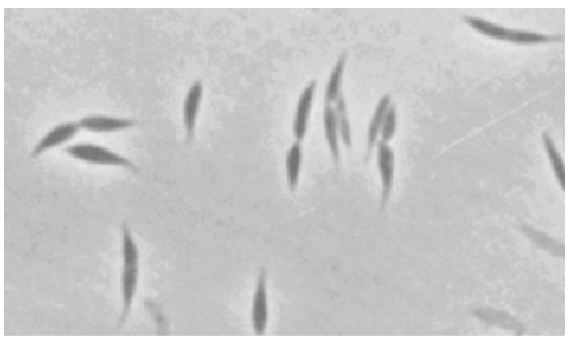

Fig. 2. Phase contrast micrograph of *Prosthecobacter dejongeii*.

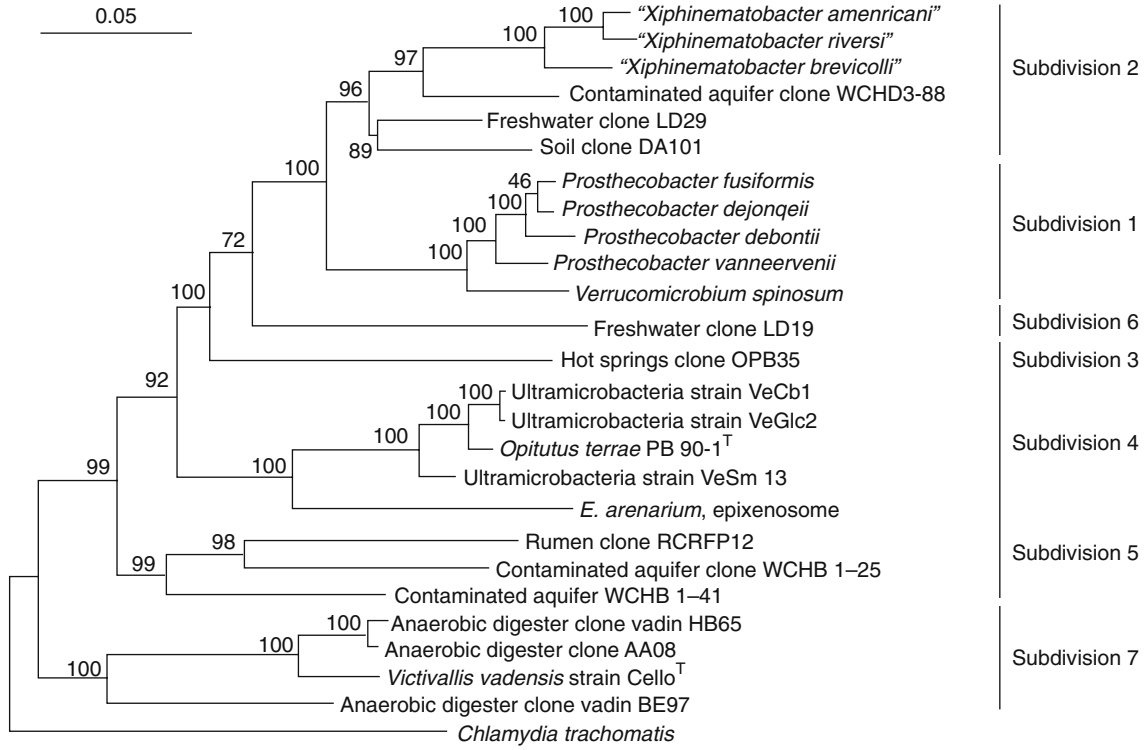

Fig. 3. Phylogenetic tree of the 16S rRNA genes of members of the seven subdivisions of the Verrucomicrobia. The tree was constructed using the DNAdist and Neighbor programs in PHYLIP. The scale bar indicates 0.05 nucleotide substitutions per site.

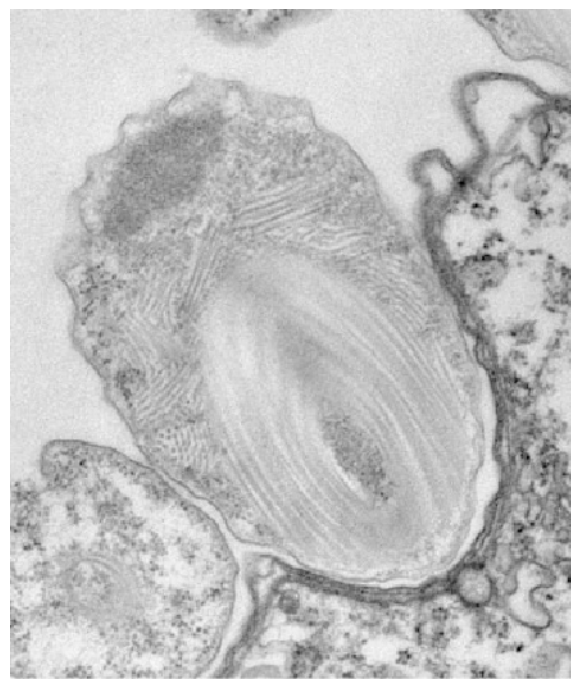

Fig. 4. Transmission electron micrograph of a thin section of a stage II epixenosome displaying the ribbon-like extrusive apparatus and surrounding microtubule-like structures.

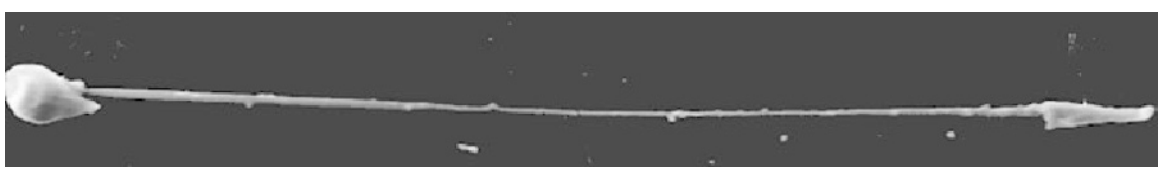

Fig. 5. Scanning electron micrograph of an epixenosome following extrusion of its harpoon-like structure.

Table 2. Selected environ taxa phylogenetically related to the phylum Verrucomicrobia.

Source	Sequence accession nos.	References
Terrestrial		
Pasture soil, Ellinbank, (Victoria, Australia)	AJ132479	O'Farrell and Janssen, 1999
Forest soil (Australia)	X64382	Liesack and Stackebrandt, 1992
Soil of soy bean field (Japan)	D26194	Ueda et al., 1995
Agricultural soil (Washington, United States)	U51864	Lee et al., 1996
Peat sample (Germany)	X97110	Rheims et al., 1996
Mature and forest soil (Amazonia)	U68592	Bomemann and Triplett, 1997
Grassland soil (The Netherlands)	Y07576	Felske and Akkermans, 1998
Soil of grass pasture (Scotland)	AF078263	McCaig et al., 1999
Anoxic rice paddy soil (laboratory experiment)	AJ229212	Hengstmann et al., 1999
Marine		
Sediment from Sagami Bay (Japan)	AB02267	Urakawa et al., 1999
Mesocosms experiment retrieved from Gulf of Lyon (Mediterranean Sea)	AJ240909	Schäfer et al., 2000
Sulfide-rich mud		Tanner et al., 2000
Brackish habitat (East San Francisco Bay)	AF211330	
Hypersaline marsh (South San Francisco Bay)	AF211331	
Arctic Ocean, depth 55m	AY08220	Bano and Hollibaugh, 2002
Arctic Ocean, depth 131m	AY08221	
Anoxic zone of the Cariaco Basin, water depth 500m (Northern coast of Venezuela)	AF224823	Madrid et al., 2001
Anoxic marine sediment, Loch Duich (Scotland)	AY114321	Freitag and Prosser, 2003
Freshwater		
Lake in Adirondack range (New York, United States)	U58170	Hioms et al., 1997
Sediment of Caroline Bay (South Carolina, United States)	U62825	Wise et al., 1997
Hot spring (Obsidian Pool, Yellowstone, United States)	AF027005	Hugenholtz et al., 1998b
Aquifer contaminated with hydrocarbons and chlorinated solvents (Michigan, United States)	AF050557	Dojka et al., 1998
Lake Loosdrecht (The Netherlands)	AF009974	Zwart et al., 1998
Columbia River (United States)	AF141385	Crump et al., 1999
Aquifer contaminated with livestock wastewater (Korea)	AF175648	Cho and Kim, 2000
Moated region of Lake Fryxell (Antarctica)	AJ287649	Brambilla et al., 2001
Humic Lake Fuchskuhle (Germany)	AF009975	Glöckner et al., 2002
Other sources		
Activated sludge from an industrial sewage treatment plant (Germany)	AF234742	Juretschko et al., 2002
Sulfide-rich spring, north of Zodletone Mountain (Oklahoma, United States)	AY327234	Elshahed et al., 2003
Cryoconite hole in Canada Glacier, McMurdo (Antarctica)	AY124349	Christner et al., 2003
Soda lake: Mono Lake (Califomia, United States)		Humayoun et al., 2003
23m (top of chemocline)	AF507902	
35m (monimolimnion)	AF507901	
Organism-associated		
Human gut	AF132232	Suau et al., 1999
Chitin tube of *Riftia pachyptila*	AF449255	Lopez-Garcia, 2002
Mouse gastrointestinal microflora, large intestine	AJ400275	Salzman et al., 2002

Molecular Ecology Studies

Since novel groups of bacteria were first revealed by analysis of genetic material obtained from forest soil (Liesack and Stackebrandt, 1992), a large number of sequences of 16S rDNA have been obtained from a variety of sources (Table 2). Another method that enables the study of bacterial communities in the environment is fluorescence in situ hybridization (FISH), which entails the application of appropriate fluorescence-labeled gene probes. With this method Ravenschlag et al. (1999) identified members of the Verrucomicrobia in permanently cold sediment in the Arctic Ocean off the coast of Spitsbergen, Norway. Since these probes bind to ribosomal RNA, only physiologically active cells are detected. Because the DNA is much more stable even after the death of the cell, it is not easy to prove the activity of cells in a biotope at the time of sampling. Similarly, Gremion et al. (2003) identified in heavy metal-contaminated bulk and rhizosphere soil clones belonging to the phyla Verrucomicrobia, *Firmicutes*, and *Cytophaga-*

Flavobacterium-Bacterioides (but only in rDNA clone libraries, not in rRNA libraries originating from the same samples). On this basis, they concluded that these bacteria might not represent active constituents in their samples.

Morphology and Physiology

The isolated members of the phylum Verrucomicrobia are Gram-negative bacteria with a coccoid or rod-shaped morphology. Cells in the genera *Verrucomicrobium* and *Prosthecobacter* are prosthecate (Figs. 1 and 2; Table 1). Multiplication occurs by binary fission or unequal cell division (e.g., in *Verrucomicrobium*).

The most discriminating physiological character of the genera is the oxygen requirement. Organisms are strict aerobes, facultative anaerobes, or strict anaerobes (Table 1). The carbon and energy sources are mainly carbohydrates as sugars. *Opitutus terrae* strain ACB hydrolyzes cellulose and "Fucophilus fucoidanolyticus" degrades fucoidan, a mixture of sulfated fucose-containing polysaccharides, which are components of many seaweeds.

Genus *Verrucomicrobium*

Introduction

Bacteria with multiple prosthecae are grouped in different phyla: the phototrophic *Ancalochloris* (Gorlenko and Lebedeva, 1971) and *Prosthecochloris* (Gorlenko, 1970) are members of the phylum Chlorobi (Garrity and Holt, 2001), while the heterotrophic bacteria *Ancalomicrobium*, *Prosthecomicrobium* (Staley, 1968) and *Stella* (Vasilyeva, 1985) are *Proteobacteria* (Garrity and Holt, 2001).

New isolates of heterotrophic bacteria with bundles of fimbriae extending from the tips of the prosthecae were described as *Verrucomicrobium spinosum* (Schlesner, 1987; Schlesner, 1992).

Phylogeny

Oligonucleotide cataloguing and reverse transcriptase sequencing of 16S rRNA (Albrecht et al., 1987) as well as sequencing of the 5S rRNA (Bomar and Stackebrandt, 1987) indicated that *V. spinosum* is a representative of a novel phylum. This finding was supported by the 16S rDNA sequence (Ward-Rainey et al., 1995).

Taxonomy

Verrucomicrobium spinosum is the only species of the genus *Verrucomicrobium*.

Habitat

Prosthecate bacteria with fimbriae extending from the tips of prosthecae have rarely been observed. Such bacteria occurred in an enrichment culture of Lake Plußsee water to which vitamin solution no. 6 (see below) was added (P. Hirsch, personal communication). Lake Plußsee is a small eutrophic lake near Plön, Holstein, Germany. The type strain of *V. spinosum* was isolated from Lake Vollstedter See (Holstein, Germany), a shallow (max. depth 2 m) eutrophic lake. Another strain came from the "Schrevenparkteich," a pond in a public park in Kiel, Germany. This pond was very eutrophic because flocks of water fowl lived there and were fed by visitors to the park.

Isolation

Verrucomicrobium spp. can be enriched in Erlenmeyer flasks with 50 ml of enrichment medium over a sediment of $CaCO_3$.

Verrucomicrobium Enrichment Medium

N-Acetylglucosamine	1.0 g
Hutner's basal salts	20 ml
Vitamin solution no. 6	10 ml
Distilled water	1 liter

Combine ingredients, dilute to 1 liter with distilled water, and adjust pH to 9.7. Autoclave, cool to room temperature, then add $NaH_2PO_4 \cdot H_2O$ aseptically to give a concentration of 0.65 mmol/liter.

Hutner's Basal Salts (Cohen-Bazire, 1957)

Nitrilotriacetate (NTA)	10.00 g
$MgSO_4 \cdot 7H_2O$	29.70 g
$CaCl_2 \cdot 2H_2O$	3.34 g
$NaMoO_4 \cdot 2H_2O$	12.67 g
$FeSO_4 \cdot 7H_2O$	99.00 mg
Metal salt solution "44"	50.00 ml
Double distilled water	900.00 ml

Dissolve the NTA first by neutralization with KOH. Add the other salts. Adjust pH to 7.2 with KOH or H_2SO_4. Adjust volume to 1000 ml with double distilled water. Store cold (5°C) and clean. The solution is clear.

Metal Salts Solution "44"

Ethylene diaminotetraacetate (EDTA)	250.0 mg
$ZnSO_4 \cdot 7H_2O$	1095.0 mg
$FeSO_4 \cdot 7H_2O$	500.0 mg
$MnSO_4 \cdot H_2O$	154.0 mg
$CuSO_4 \cdot 5H_2O$	39.2 mg
$CoCl_2 \cdot 6H_2O$	20.3 mg
$Na_2B_4O_7 \cdot 10H_2O$	17.7 mg
Double distilled water	1000.0 ml

Add a few drops of H_2SO_4 to retard precipitation before diluting to volume. Store cold (5°C).

Vitamin Solution No. 6 (Staley, 1968)

Biotin	4.0 mg
Pyridoxine hydrochloride	20.0 mg
Thiamine hydrochloride	10.0 mg
Ca-pantothenate	10.0 mg
p-Aminobenzoic acid	10.0 mg
Folic acid	4.0 mg
Riboflavin	10.0 mg
Nicotinamide or nicotinic acid	10.0 mg
Vitamin B_{12}	0.2 mg
Double distilled water	1000 ml

Stir the mixture to improve dissolution. Sterilize by filtration only. Store dark and cold (5°C).

When subsequent microscopic checks indicate an increase in prosthecate bacteria, streak onto agar-solidified medium M 13 (Schlesner, 1986):

M 13

Peptone	0.25 g
Yeast extract	0.25 g
Glucose	0.25 g
Hutner's basal salts	20 ml
Vitamin solution no. 6	10 ml
Buffer (0.1 M Tris/HCl, pH 7.5)	50 ml
Artificial seawater	250 ml
Distilled water	670 ml

Artificial Sea Water (Lyman and Fleming, 1940)

NaCl	23.477 g
$MgCl_2$	4.981 g
Na_2SO_4	3.917 g
$CaCl_2$	1.102 g
KCl	0.664 g
$NaHCO_3$	0.192 g
KBr	0.096 g
H_3BO_3	0.026 g
$SrCl_2$	0.024 g
NaF	0.003 g

Upcoming colonies have to be examined microscopically for prosthecate cells. This is conveniently done by applying the time-saving toothpick-procedure (Hirsch et al., 1977) which even allows the examination of very small colonies: A sterile wooden toothpick is dipped onto a colony and then on the agar surface of a Petri dish containing the appropriate medium, thus inoculating the agar. To allow inoculation of a single Petri dish with bacteria from a large number of colonies, a grid can be drawn with a marker on the backside of the Petri dish with areas of about 5×5 mm. After inoculation of the agar medium the toothpick generally contains enough bacteria to prepare a smear for microscopic examination. Three specimens can be placed on one slide.

Identification

When studied with a phase contrast microscope, *Verrucomicrobium* spp. resemble *Prosthecomicrobium* spp., i.e., the unicellular organisms have conical prosthecae extending in all directions from the cell surface. These prosthecae are about 0.5 µm long (Fig. 1a). Occasionally, one or two longer prosthecae (up to 2 µm) may occur, and often one of them is polarly inserted (Fig. 1b). Under the electron microscope, bundles of fimbriae of varying number and length protruding from the tips of the prosthecae are detected. The Gram-negative cells are nonmotile. Colonies on medium M 13 are light yellow. Growth of *V. spinosum* is optimal between 26°C and 33°C, and the maximum growth temperature is 34°C. The organism is stenohaline (i.e., able to tolerate 50% artificial seawater or 1% NaCl). The cell wall contains *m*-diaminopimelic acid. The main respiratory quinone is MK10 (a menaquinone with 10 isoprenoid units); MK9, MK10(H_2), and MK11 are minor quinones. Characteristic fatty acids are n14/15:1d11 and n16/17:1d5 (Sittig and Schlesner, 1993). The following phospholipids were identified: phosphatidylglycerol and phosphatidylmethylethanolamine as major components, biphosphatidylglycerol in less amounts, and phosphatidylethanolamine and phosphatidylcholine only in traces. Phosphatidyldimethylethanolamine was not detected (Sittig and Schlesner, 1993). The DNA base composition is 58.6 ± 0.2 mol% G+C (T_m) for strain IFAM 1439^T = DSM 4136^T = ATCC 43997^T. The presence of *dnaK* (HSP70) multigene family was reported (Ward-Rainey et al., 1997).

Cultivation

The strains are growing aerobically with medium M 13 at a temperature of 30°C. Liquid cultures can be shaken.

Preservation

The strains can be kept on agar slants at 4–6°C for several months. For long term preservation, lyophilization or storage in liquid nitrogen is recommended. Deep freezing at –70°C with 50% glycerol is also possible.

Physiology

Verrucomicrobium spinosum is a facultative anaerobe. Various sugars are fermented without gas formation, but nitrate is not reduced under anaerobic conditions.

Only a limited number of substrates can be utilized as sole carbon and energy source, mainly hexoses, di- or trisaccharides or derivatives of glucose. C_1- or C_2-compounds are not utilized,

nor are fatty acids or amino acids (Schlesner, 1987). Carbon sources include: maltose, mannose, melibiose, melizitose, rhamnose, raffinose, trehalose, cellobiose, fructose, galactose, glucose, lactose, xylose, sucrose, *N*-acetylglucosamine, glucuronate, amygdalin, salicin, esculin and glycerol. Carbon sources not utilized are: D-arabinose, L-arabinose, sorbose, inulin, lyxose, fucose, ribose, methanol, ethanol, mannitol, sorbitol, inositol, acetate, adipate, butyrate, caproate, 2-oxoglutarate, pyruvate, succinate, valerate, formate, β-hydroxybutyrate, lactate, malate, propionate, aspartate, aspartic acid, glutamine, glutamic acid, histidine, proline, serine and threonine. The following carbohydrates could be utilized fermentatively (acid, but no gas formation): glucose, lactose, sucrose, maltose, mannose, melibiose, rhamnose, trehalose, cellobiose, fructose, galactose, xylose, and *N*-acetylglucosamine. Ammonia, urea, nitrate, and *N*-acetylglucosamine are suitable nitrogen sources. *Verrucomicrobium spinosum* produces exoenzymes which can be demonstrated by the hydrolysis of gelatin and starch. However, casein or Tween 80 is not hydrolyzed. Catalase, cytochromoxidase, phosphatase, and urease are produced. H_2S from thiosulfate or NH_3 from peptone is not produced.

Genus *Prosthecobacter*

Introduction

The genus *Prosthecobacter* contains nonmotile fusiform-shaped bacteria (Fig. 2). Since the previous edition of *The Prokaryotes* (Staley, 1992) and *Bergey's Manual of Systematic Bacteriology* (Poindexter and Staley, 1989), much has been learned about the phylogenetic diversity of these bacteria and their position as members of the phylum Verrucomicrobia in the Tree of Life (Hedlund et al., 1996).

Prosthecobacter was first reported and photographed by Henrici and Johnson (1935) attached to glass slides that had been submerged and incubated in situ in lakes. However, pure culture isolates were not reported until 1970 (DeBont et al., 1970). Strains were subsequently isolated from ponds, lakes and raw sewage (Staley et al., 1976). Similar organisms have also been observed in sea ice samples from Antarctica (Sullivan and Palmisano, 1984) but, to our knowledge, none yet has been cultivated. Cells undergoing division elongate and differentiate to produce a second prostheca at the dividing pole. Typically at the time of cell division, the two daughter cells are mirror images of one another.

Phylogeny

On the basis of 16S rDNA sequence analyses, the genus *Prosthecobacter* belongs to the same subgroup of the Verrucomicrobia as the genus *Verrucomicrobium* (Fig. 3). These two genera resemble one another in that they are both aerobic, prosthecate bacteria. In contrast to *Verrucomicrobium spinosum*, which has several prosthecae per cell, *Prosthecobacter* has only a single polar prostheca.

Prosthecobacter is the only genus among the Bacteria or Archaea so far known to contain the genes *btuba* and *btubb*, which are homologs for α- and β-tubulin genes, respectively (Jenkins et al., 2002). Reverse transcriptase polymerase chain reaction (RT-PCR) results indicate that the genes are expressed, but the proteins have not as yet been identified in cells. The evolution of these genes is poorly understood and the topic of ongoing investigation. Likewise, their function has not yet been studied.

Taxonomy

Four strains, each representing a separate species, have been cultivated. In terms of their 16S rDNA sequences, the four strains are sufficiently different from one another that they comprise different species (Hedlund et al., 1997). *Prosthecobacter fusiformis* $FC4^T$ is the type strain and species for the genus. The other species are *P. vanneervenii* strain FC2, *P. debontii* FC3 and *P. dejongeii* FC1.

Habitat

Prosthecobacter spp. have been isolated from ponds in Michigan and Washington states as well as a lake and raw sewage (North Carolina in the United States), indicating they are widespread and likely common freshwater organisms. Electron micrographs of water samples from Lake Washington indicate that they are commonly found attached to other organisms including diatoms.

Marine organisms resembling *Prosthecobacter* species have also been reported from sea ice communities (Sullivan and Palmisano, 1984; J. T. Staley, unpublished observation), but to our knowledge, strains have not yet been isolated in pure culture.

Isolation

Dilute peptone enrichment cultures have been used to isolate *Prosthecobacter* strains. These are typically set up in sterilized 150-ml beakers covered with aluminum foil lids. The beakers are inoculated with 100 ml of water from the site of

interest and an autoclaved solution of Bacto-Peptone is added to provide a final concentration of 0.01% (w/v). The enrichment cultures are incubated at room temperature for one to two weeks and observed by phase microscopy periodically to determine if fusiform cell shapes typical of the genus are present. If positive, the material is streaked onto Dilute Peptone Agar (see the chapter The Genera Prosthecomicrobium, Ancalomicrobium, Stella and Blastobacter in Volume 5) plates. Following incubation, wet mounts are made of small white to yellow colonies, which should be examined using the microscope to determine whether they have the characteristic morphology of the organism. They can be readily distinguished from *Caulobacter* cells because they are nonmotile and have a wider prostheca with a distinctly bulbous tip. Furthermore, prior to cell division, a prostheca can been seen to extend from the pole of each cell undergoing division, making the dividing cells symmetrical at this stage, unlike that of dividing *Caulobacter* spp. Appropriate colonies or wet mount preparations are restreaked until a pure culture is achieved.

For routine maintenance, *Prosthecobacter* spp. can be grown on MMB medium (see the chapter The Genera *Prosthecomicrobium*, *Ancalomicrobium*, *Stella*, and *Blastobacter* in the second edition). Slant cultures can be maintained in the refrigerator for about one month, and lyophilization or deep freezing can be used for longer storage.

Identification

The four different species can be differentiated from one another by phenotypic tests (Table 3). In addition, their 16S rDNA sequences are sufficiently different to possibly be of use in distinguishing novel strains.

Morphology

Morphologically the cells are about 0.5–1.0 μm in diameter and 3–10 μm in length. Cells may be straight to curved, fusiform shapes. The cells may aggregate to form small rosettes in pure culture. The tip of the prostheca is bulbous and the prostheca diameter is about 0.2 μm, somewhat wider than those of *Caulobacter* and *Asticacaulis*. Also, the *Prosthecobacter* prostheca has crossbands unlike those of *Caulobacter* and *Asticacaulis*.

Nutrition

A variety of mono- and disaccharides are used as sole carbon sources for growth and are helpful in distinguishing known species (Table 3).

Genus *Opitutus* and the Ultramicrobacteria

The recently named genus, *Opitutus*, has at least three strains (PB90-1^T, PB90-3 and ACB90) of obligately anaerobic bacteria isolated from rice paddy soil (Chin et al., 2001). Also isolated from this habitat are three strains of "ultramicrobacteria" (strains VeCb1, VeGlc2 and VeSm13), anaerobic dwarf cells with cell volumes of 0.03–0.04 μm^3 (Janssen et al., 1997). Unlike members of the genera *Verrucomicrobium* and *Prosthecobacter*, *Opitutus* and the ultramicrobacteria are non-prosthecate.

Phylogeny

Both the genus *Opitutus* and the ultramicrobacteria belong to subdivision 4 of the Verrucomicrobia, as proposed by Hugenholtz et al. (1998a). Of all the Verrucomicrobia strains isolated in pure culture, members of the genus *Opitutus* are most closely related to the ultramicrobacteria. *Opitutus* and ultramicrobacteria strains share 96–97% 16S rDNA sequence similarity, and thus they may be sufficiently similar to be considered members of the same genus. Phylogenetic trees based on partial (~275 bp) 16S rDNA sequences indicate that the ultramicrobacteria are also

Table 3. Differentiation among *Prosthecobacter* species.

Property	*P. fusiformis*	*P. dejongeii*	*P. vanneervenii*	*P. debontii*
Strain number	FC4	FC1	FC2	FC3
Cell shape	Straight, long cells	Straight, wide cells	Straight, long cells	Curved cells
Source	Marl pond, Michigan	Raw sewage, North Carolina	University Lake, North Carolina	Pond, Washington State
Carbon source utilization[a]				
D-Ribose	−	+	+	+
D-Arabinose	+	−	−	+
D-Fructose	−	−	+	+
D-Raffinose	−	−	+	−
Glycogen	−	+	+	−
N-Acetylglucosamine	−	+	+	+

[a]Sole carbon source utilization data from Hedlund et al. (1997).

closely related to the soybean soil clone, pad7 (Janssen et al., 1997).

Also in subdivision 4 of the Verrucomicrobia are ectosymbionts of ciliated protozoa termed "epixenosomes" (Petroni et al., 2000; Chin et al., 2001). The epixenosomes of the ciliate hypotrich, *Euplotidium arenarium*, share 83% sequence similarity with *Opitutus* strains.

Taxonomy

The genus *Opitutus* is currently represented by a single species, *Opitutus terrae*. The three strains of *O. terrae* (PB90-1^T, PB90-3 and ACB90) contain identical 16S rDNA sequences. PB90-1^T is the type strain of *O. terrae*.

Given the level of 16S rDNA sequence similarity between *O. terrae* and the ultramicrobacteria, it is likely that the ultramicrobacteria are also members of the genus *Opitutus* but may represent separate species.

Habitat

All strains of *O. terrae* and ultramicrobacteria in pure culture were isolated from anoxic (flooded) rice paddy soil microcosms where they appear to be relatively abundant. In the environment from which they were isolated, *Opitutus* spp. were found to comprise a significant proportion of the population (5%) of polysaccharolytic or saccharolytic fermenting bacteria (Chin et al., 1999). Furthermore, the discovery of 16S rDNA sequences of related organisms in clone libraries constructed from soybean field soil and peat bog soil (Rheims et al., 1996a) suggests that these organisms may be widespread in anoxic terrestrial environments.

Isolation

Both members of the genus *Opitutus* and the ultramicrobacteria can be isolated using liquid serial dilution culture (most probable number [MPN]) techniques. Rice plants (*Oryzae sativa*, var. Roma, type japonica) are grown in the laboratory using flooded soil obtained from wetland rice fields. Rice plants should be grown for 90 days before the soil is sampled. Soil samples are taken using a coring device to a depth of approximately 15 cm. Only the lower 10-cm portion of the soil core is used as inoculum. MPN tubes containing sulfide-reduced, bicarbonate buffered medium (SM) supplemented with pectin or amorphous cellulose as carbon source are used for the isolation of *Opitutus* strains. Dilute medium can be used for the isolation of ultramicrobacteria.

SM Medium

Mineral Salts Solution	
NaCl	1.0 g
$MgCl_2 \cdot 6H_2O$	0.40 g
NH_4Cl	0.10 g
KH_2PO_4	0.20 g
$CaCl_2 \cdot 2H_2O$	0.10 g
KCl	0.50 g
Distilled water	1 liter

Dissolve ingredients and dilute up to 1 liter with distilled water. Autoclave the mineral salts solution at 121°C for 45 min and cool under a headspace of N_2/CO_2 (80:20 [vol/vol]). Supplement the medium with 30 mL of 1 M sodium bicarbonate solution, which has been autoclaved under a headspace of CO_2, 3 ml of 0.5 M Na_2S (autoclaved under N_2), and 1 ml of each vitamin solution. Adjust the medium to pH 7.2–7.3 and dispense into either sterile screw capped bottles or serum bottles. Add carbon sources (such as pectin or amorphous cellulose). If the medium is to be dispensed into MPN tubes, gas the headspace with N_2/CO_2 and add sodium dithionate to a final concentration of 100 μM.

Vitamin Solution 1

4-Aminobenzoic acid	40 mg
D(+)-Biotin	10 mg
Calcium D(+)-pantothenate	100 mg
Nicotinic acid	50 mg
Pyridoxine hydrochloride	100 mg
Folic acid	30 mg
DL-*a*-Lipoic acid	10 mg
Sodium phosphate buffer (50 mM, pH 7.1)	1 liter

Dissolve ingredients and dilute in sodium phosphate buffer up to a 1 liter. Dissolve the vitamins in sodium phosphate buffer and filter sterilize.

Vitamin Solution 2

Cyanocobalamin	50 mg
Distilled water	1 liter

Dissolve cyanocobalamin and dilute up to 1 liter with distilled water. Filter sterilize this solution.

Vitamin Solution 3

Thiamine chloride dihydrochloride	10 mg

Dissolve vitamin powder in 100 ml of 25 mM sodium phosphate buffer (pH 3.4) and filter sterilize.

Trace Element Solution

HCl (2M)	50 ml
$FeCl_2 \times 4H_2O$	1000 mg
$CoCl_2 \cdot 6H_2O$	130 mg
$MnCl_2 \cdot 2H_2O$	80 mg
$ZnCl_2$	70 mg
H_3BO_3	6 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$CuCl_2 \cdot 2H_2O$	2 mg
Distilled water	1 liter

Dissolve the $FeCl_2 \cdot 4H_2O$ in the HCl, then add the distilled water and the rest of the components up to a total of 1 liter. Autoclave this solution under air.

Dilute Medium

Mineral Salts Solution	
NaCl	0.25 g
$MgCl_2$	0.20 g
KCl	0.10 g
NH_4Cl	0.10 g
KH_2PO_4	0.05 g
$CaCl_2 \cdot 2H_2O$	0.10 g
Distilled water	1 liter

Dissolve components and dilute up to 1 liter with distilled water. Autoclave the mineral salts solution at 121°C for 40 min and cool under a headspace of N_2/CO_2 (80:20 [vol/vol]) with an overpressure of 6 kPa. Once the mineral salts solution is cool, add 30 ml of 1 M sodium bicarbonate and 3 ml of 0.5 M Na_2S, which have been autoclaved in vessels sealed with rubber stoppers under headspaces of CO_2 and N_2, respectively. In addition, add 5 ml of vitamin solution 1 and 1 ml each of vitamin solution 2, trace element solution, and selenite/tungstate solution. Then, adjust pH to 7.2 with sterile HCl or 0.5 M Na_2CO_3. Fill serum bottles part way with medium and seal. Gas the headspace with N_2/CO_2 (80:20 [vol/vol]). Alternatively, dispense the medium into sterile screw capped bottles, leaving only a small gas bubble. Add appropriate carbon sources as desired. Different combinations of carbon sources have been used to isolate ultramicrobacteria, including glucose at [4 mM], cellobiose at [2 mM] and a mixture of seven sugars (1 mM each of glucose, cellobiose, fructose, xylose, arabinose, maltose and galactose).

Vitamin Solution 1

4-Aminobenzoate	40 mg
(+)-Biotin	10 mg
Nicotinic acid	100 mg
Hemicalcium D-(+)-pantothenate	50 mg
Pyridoxamine hydrochloride	150 mg
Thiamine chloride hydrochloride	100 mg
Cyanocobalamin	50 mg
Distilled water	1 liter

Dissolve and dilute ingredients up to 1 liter with distilled water. Filter sterilize this solution.

Vitamin Solution 2

DL-6,8-thioctic acid	10 mg
Riboflavin	10 mg
Folic acid	4 mg
Distilled water	1 liter

Dissolve and dilute ingredients up to 1 liter with distilled water. Filter sterilize this solution.

Trace Element Solution

HCl (25%)	10 mL
$FeCl_2 \cdot 4H_2O$	1.5 g
$CoCl_2$	190 mg
$MnCl_2 \cdot 4H_2O$	100 mg
$ZnCl_2$	70 mg
H_3BO_3	6 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$CuCl_2 \cdot 2H_2O$	2 mg
Distilled water	1 liter

Dissolve the $FeCl_2 \cdot 4H_2O$ in the HCl, then add the distilled water and the rest of the components up to 1 liter. Autoclave this solution under air.

Selenite/Tungstate Solution

NaOH	0.5 g
$Na_2SeO_3 \cdot 5H_2O$	3 mg
$Na_2WO_4 \cdot 2H_2O$	4 mg
Distilled water	1 liter

Dissolve and dilute ingredients up to 1 liter with distilled water. Autoclave this solution under air.

Identification

Morphological and Cytological Characteristics

Opitutus terrae Unlike some other cultured members of the Verrucomicrobia, *O. terrae* strains are non-prosthecate. Cells are coccoid but sometimes have the appearance of short rods under the phase contrast microscope because of the formation of chains of three or four cells. Diplococci are commonly observed, with one cell of the pair possessing a subpolar flagellum. Cells of *O. terrae* are 0.4–0.6 μm in diameter. Cells stain Gram negative and test positive for the aminopeptidase reaction and the KOH test. In agar deeps, *Opitutus* colonies appear granular and are nonpigmented. Spore formation has not been observed. Mol% G+C of the type strain is 73.7 ± 0.3.

Ultramicrobacteria
As with *Opitutus* strains, the ultramicrobacteria are non-prosthecate. Cells are ellipsoid to spherical in shape and often appear as diplococci. Cells are approximately 0.5 μm long and 0.35 μm wide. By definition, ultramicrobacteria have cell volumes of less than 0.1 μm^3; ultramicrobacteria in the phylum Verrucomicrobia typically have cell volumes of between 0.03 and 0.04 μm^3. Mature cells are motile via a single subpolar flagellum that can be up to 8.5 μm in length. Cells stain Gram negative and test positive for the KOH test. Colonies in agar deeps are white and lens-shaped. Spores have not been observed. Mol% G+C ratio ranges from 63.3–63.5.

Metabolic and Biochemical Properties

Opitutus strains possess a fermentative metabolism and are obligate anaerobes. Ultramicrobacteria strains are also fermentative but are oxygen tolerant. Growth of the type strain of *O. terrae* and the ultramicrobacteria is supported by mono-, di- and polysaccharides (Table 4).

Substrates that do not support the growth of any strains listed in Table 4 include ribose, cellulose (although *O. terrae* strain ACB90 hydrolyzes cellulose), pyruvate, lactate, fumarate, malate, citrate, crotonate, tartrate, glycerol (with or without acetate), alanine, glutamate, lysine, aspartate and leucine. Of the compounds tested, ultramicrobacteria strains VeCb1 and VeGlc2 have identical substrate ranges for growth, while VeSm13

Table 4. Carbon sources supporting the growth of *O. terrae* and the ultramicrobacteria.

Substrate	*O. terrae* PB90-1^T	Ultramicrobacteria strains		
		VeCb1	VeGlc2	VeSm13
Glucose (4mM)	+	+	+	+
Fructose (4mM)	+	–	–	–
Galactose (4mM)	+	+	+	+
Mannose (4mM)	+	–	–	–
Galacturonic acid (4mM)	+	ND	ND	ND
Mannitol (4mM)	+	–	–	–
Arabinose (4mM)	+	+	+	+
L-Rhamnose (4mM)	ND	–	–	+
Xylose (4mM)	–	+	+	+
Cellobiose (2mM)	+	+	+	–
Sorbose (2mM)	–	ND	ND	ND
Maltose (2mM)	+	+	+	–
Sucrose (2mM)	+	+	+	–
Lactose (2mM)	+	+	+	+
Melibiose (2mM)	+	+	+	–
Sorbose (2mM)	–	ND	ND	ND
Methyl α-glucopyranoside (2mM)	–	ND	ND	ND
Arabinogalactan (0.1%)	–	ND	ND	ND
Xylan (0.1%)	+	+	+	–
Starch (0.1%)	+	+	+	–
Pectin (0.1%)	+	+	+	+
Chitin (0.1%)	–	ND	ND	ND
D,L-3-hydroxybutyrate (20mM)	ND	–	–	–
Succinate (20mM)	ND	–	–	–
Ethylene glycol (20mM)	ND	–	–	–
1,2-Propanediol (20mM)	ND	–	–	–
Acetoin (20mM)	ND	–	–	–
Glycine (20mM)	ND	–	–	–
Threonine (20mM)	ND	–	–	–
Valine (20mM)	ND	–	–	–
Isoleucine (20mM)	–	ND	ND	ND
Proline(20mM)	–	ND	ND	ND

Symbols: +, present; –, absent; and ND, no data.

has a more limited range. *Opitutus terrae* PB90-1^T is capable of using almost all of the same substrates as VeCb1 and VeGlc2 but is able to use more monosaccharides.

For all three ultramicrobacteria strains, the fermentation end products are acetate, propionate and succinate. *Opitutus terrae* produces propionate and acetate as the major end products of fermentation but also produces smaller amounts (0.2 mol per mol of growth substrate) of succinate, lactate, ethanol and H_2. When grown on pectin, *O. terrae* also produces methanol. *Opitutus terrae*, but not the ultramicrobacteria strains, can use nitrate as an electron acceptor which is reduced solely to nitrite.

Sulfur, sulfate and thiosulfate are not reduced by any strains. The ultramicrobacteria hydrolyze urea and esculin, while *O. terrae* is only capable of esculin hydrolysis. Gelatin is not hydrolyzed by any strains, and catalase activity was not detected for any of the strains.

The type strain of *O. terrae* grows optimally at a pH of 7.5–8.0 but is able to grow within the range of 5.5–9.0. Ultramicrobacteria strain VeGlc2 is able to grow at pH values between 6.6 and 8.0, although other values have not been tested. *Opitutus terrae* PB90-1^T and VeGlc2 are capable of growth at temperatures between 10°C and 37°C but not at 40°C. Growth of *O. terrae* was possible in media supplemented with NaCl (30 g but not 40 g per liter). Ultramicrobacteria strain VeGlc2 tolerated NaCl (15 g but not 20 g per liter).

Strain VeGlc2 is resistant to bacitracin and benzylpenicillin at 1000 $\mu g \cdot ml^{-1}$ but is sensitive to ampicillin and other antibiotics.

Epixenosomes

Epixenosomes are Verrucomicrobia that reside as ectosymbionts on the dorsal surface of marine hypotrich ciliates in the genus *Euplotidium*. As epixenosomes are obligate symbionts, they have not been isolated in pure culture and therefore studies on these organisms have been limited. Studies to date have involved the epixenosomes of *E. itoi* and *E. arenarium*. These epixenosomes possess a complex internal ultrastructure consisting of a ribbon-like extrusive apparatus and bun-

dles of associated microtubule-like structures (Rosati et al., 1993; Fig. 4). In the natural environment the harpoon-like extrusomes of epixenosomes (Fig. 5) are believed to perform a defensive role for the host ciliates against predatory protozoa such as *Litonotus lamella*. Indeed, *in vitro* experiments have demonstrated that *Euplotidium* sp. containing epixenosomes are never ingested by *Litonotus* unless the extrusive apparatus is inhibited. In contrast, ingestion of *Euplotidium* occurs readily when their adhering epixenosomes have been removed (Rosati et al., 1999).

Phylogeny

The complexity of the internal organization of epixenosomes combined with the presence of microtubule-like structures initially led to confusion about whether these organisms were prokaryotic or eukaryotic in nature. More recently, the 16S rRNA gene of the *E. arenarium* epixenosome was sequenced, and phylogenetic trees constructed using these data showed that these organisms are bacteria that cluster in Verrucomicrobia subdivision 4 along with *Opitutus* and ultramicrobacteria strains (Petroni et al., 2000; Chin et al., 2001). The *E. arenarium* epixenosome 16S rRNA gene shares 83–84% sequence similarity with those from other members of subdivision 4 but only 74–78% sequence similarity with members of the other subdivisions (Chin et al., 2001).

Taxonomy

The epixenosomes of *E. arenarium* and *E. itoi* have not been isolated in pure culture and therefore have not been officially named.

Habitat

Epixenosomes reside on the dorsal surface of their ciliate hosts and are arrayed in a well defined cortical band. While only the epixenosomes of *E. arenarium* and *E. itoi* have been studied in detail, symbionts resembling epixenosomes have been observed on the surface of other marine ciliates in the *Euplotidium* genus. *Euplotidium* spp. are prevalent in marine coastal waters.

Life Cycle and Cell Structure

While epixenosomes are not necessary for host cell survival in a noncompetitive environment, they have been shown to influence the morphogenesis of the cortical band region, where they reside. The cortical region of the host differs when epixenosomes are present versus absent. Similarly, epixenosomes have been shown to possess two distinct developmental phases linked to the host cell cycle.

Stage I epixenosomes divide by binary fission and possess a simple bacteria-like ultrastructure. Cells are spherical and measure 1 μm in diameter. Stage II epixenosomes are ovoid and larger in size (2.5 μm × 1.2 μm) and develop by gradually acquiring a more complex internal structure (Fig. 4). Internal structures that are present in stage II epixenosomes but absent from stage I epixenosomes include an electron-dense region composed of DNA and protein, a ribbon-like extrusive apparatus, and a "basket-like" structure composed of regularly arrayed microtubule-like tubules. During the transition from stage I to stage II, the tubules appear randomly distributed throughout the cytoplasm, but once the extrusive apparatus is completely formed they are arranged in a basket-like formation around the extrusome. Cell division ceases once the epixenosomes reach stage II.

Ejection of the extrusive apparatus (Fig. 5) occurs when *Euplotidium* species encounter predatory protozoa and can be stimulated in the laboratory with adrenalin (Rosati et al., 1997). Extrusion is mediated when external signals of unknown origin are detected via membrane receptors on the top of the bacterium. These receptors are involved in signal transduction and activation of the adenylate cyclase-cAMP system (Rosati et al., 1997). The tubular structures surrounding the extrusive apparatus also appear to be directly involved in the ejection process. Experiments in which the tubules are disrupted using either nocodazole or cold treatment (both of which have been shown to cause microtubule depolymerization) result in loss of the ejection capability (Rosati et al., 1993).

"*Candidatus Xiphinematobacter*"

The candidate genus "*Candidatus Xiphinematobacter*" was proposed to incorporate three species of Verrucomicrobia that are obligate endosymbionts of nematodes in the genus *Xiphinema* (Vandekerckhove et al., 2000). These bacteria reside inside the epithelial cells of the nematode ovarial walls, where they are believed to influence the reproductive mechanism of their host.

Phylogeny

"*Candidatus Xiphinematobacter*" strains belong to subdivision 2 of the Verrucomicrobia (Hugenholtz et al., 1998a; Vandekerckhove et al., 2000). Their closest relatives are represented by uncultured clones from a hydrocarbon and chlorinated-solvent containing reactor and from soil and freshwater. The three species of "*Xiphinematobacter*" share a mean 16S rDNA similarity of 93%, but share less than 87% simi-

larity with their nearest relative, aquifer clone WCHD3-88.

Taxonomy

As "*Xiphinematobacter*" are endosymbionts and have never been isolated in pure culture, the genus remains at *Candidatus* status. "*Candidatus Xiphinematobacter*" contains three species: "*Candidatus Xiphinematobacter brevicolli*," the type species, "*Candidatus Xiphinematobacter americanum*" and "*Candidatus Xiphinematobacter rivesi*."

Habitat

"*Xiphinematobacter*" species live as endosymbionts in the ovarial and intestinal epithelium of nematodes in the *Xiphinema americanum* group. These symbioses are host specific, and thus *Xiphinema brevicollum*, *Xiphinema americanum* and *Xiphinema rivesi* are host to "*Xiphinematobacter brevicolli*," "*Xiphinematobacter americanum*" and "*Xiphinematobacter rivesi*," respectively.

"*Xiphinematobacter*" species are maternally inherited via transovarial transmission and therefore their survival is dependent on the host sex ratio. Interestingly, nematodes belonging to the symbiont-containing *Xiphinema americanum* group all reproduce via thelytokous (mother-daughter) parthenogenesis, while *Xiphinema* species that are uninfected can reproduce either sexually or via parthenogenesis, depending on the species. Therefore, it has been suggested that "*Xiphinematobacter*" species may be responsible for manipulating the sex ratio of the host via induction of thelytokous parthenogenesis, in a similar manner to the α-proteobacterium, *Wolbachia pipientis* (Coomans et al., 2000).

Morphology

"*Xiphinematobacter*" species are rod-shaped with rounded ends and are nonmotile, nonprosthecate and nonsporulating. Cell dimensions are 0.7–1.0 μm × 2.1–3.2 μm. Cells stain Gram negative with no peptidoglycan layer discernable in thin-section electronmicroscopy. Instead, a hexagonally arrayed layer of 10-nm proteins, similar to those seen in *Chlamydia* sp., are associated with the inner edge of the outer membrane. As with *Prosthecobacter* species, cell division is unipolar.

Genus *Victivallis*

The genus *Victivallis* contains one species, *V. vadensis* with the type strain Cello^T (named for its ability to utilize cellobiose). This species was isolated from a human fecal sample and is a strict anaerobe. *Victivallis vadensis* is the most recently isolated member of the phylum Verrucomicrobia.

Phylogeny

Victivallis vadensis does not fall into any of the Verrucomicrobia subdivisions proposed by Hugenholtz et al. (1998a) but forms a separate cluster with several uncultured clones obtained from anaerobic digesters (see Fig. 3). Thus, *V. vadensis* represents a seventh subdivision of the Verrucomicrobia, of which it is the only cultured member. Anaerobic digester clones AA08 and vadinHB65 share only 94% sequence similarity with *V. vadensis*, indicating that they form separate species.

Taxonomy

Victivallis vadensis is the only species in the genus *Victivallis* and is represented by a single strain, Cello^T.

Habitat and Isolation

Victivallis vadensis was isolated from a human fecal sample. A density gradient gel electrophoresis (DGGE) profile of the same sample indicated that *V. vadensis* was not a dominant member of the fecal flora (Zoetendal et al., 2003); however, the detection of the 16S rRNA genes of related organisms in an anaerobic digester indicates that these organisms may be ubiquitous.

To isolate *V. vadensis*, a bicarbonate buffered anaerobic medium supplemented with cellobiose is employed (see below). One notable characteristic of *V. vadensis* is its inability to grow on solid media. *Victivallis vadensis* can be cultivated in broth or semisolid (0.75%) agar media.

Bicarbonate-buffered Anaerobic Medium (Stams et al., 1993)

Basal Medium	
$NaHPO_4 \cdot 2H_2O$	0.53 g
KH_2PO_4	0.41 g
NH_4Cl	0.30 g
$CaCl_2 \cdot 2H_2O$	0.11 g
$MgCl_2 \cdot 6H_2O$	0.10 g
NaCl	0.30 g
$NaHCO_3$	4.0 g
$Na_2S \cdot 9H_2O$	0.48 g
Distilled water	1 liter

Dissolve and dilute ingredients up to 1 liter with distilled water. Autoclave the medium and dispense into serum vials with a headspace of N_2/CO_2 or H_2/CO_2 (80:20 [vol/vol]). Add 1 ml each of acid and alkaline trace element solution and 0.2 ml of vitamin solution. Supplement the medium with 10 mM cellobiose (filter sterilized) and 0.7% (vol/vol) sterile clarified rumen fluid.

Acid Trace Element Solution

$FeCl_2$	7.5 mM
H_3BO_4	1.0 mM
$ZnCl_2$	0.5 mM
$CuCl_2$	0.1 mM
$MnCl_2$	0.5 mM
$CoCl_2$	0.5 mM
$NiCl_2$	0.1 mM
HCl	50 mM

Filter sterilize the solution.

Alkaline Trace Element Solution

Na_2SeO_3	0.1 mM
Na_2WO_4	0.1 mM
Na_2MoO_4	0.1 mM
NaOH	10 mM

Filter sterilize the solution.

Vitamin Solution

Biotin	20 mg
Niacin	200 mg
Pyridoxine	500 mg
Riboflavin	100 mg
Thiamine	200 mg
Cyanocobalamin	100 mg
p-Aminobenzoic acid	100 mg
Pantothenic acid	100 mg

Filter sterilize the solution.

Identification

Morphological and Cytological Characteristics *Victivallis vadensis* is a strict anaerobe. Cells are coccoid with diameters of 0.5–1.3 μm and are nonmotile. An extracellular slime layer is produced. Cells stain Gram negative and do not produce spores. Strain Cello^T has a mol% G+C ratio of 59.2%.

Metabolic and Biochemical Properties *Victivallis vadensis* is a strict anaerobe that grows well on a variety of sugars including cellobiose, glucose, fructose, galactose, lactose, lactulose, maltose, maltotriose, mannitol, melibiose, *myo*-inositol, raftilose, rhamnose, ribose, sucrose and xylose. Compounds that do not support growth include cellulose, fucose, gelatin, pectin, raffinose, starch, fumarate, casamino acids, oleate, pyruvate, yeast extract, methanol, ethanol, 1-propanol, 2-propanol, 2-butanol, casein, peptone, tryptone and H_2/CO_2. *Victivallis vadensis* is not able to use sulfate or nitrate as electron acceptor. Glucose is converted to acetate, ethanol, bicarbonate and H_2.

On cellobiose, temperatures between 20°C and 40°C and pHs between 5 and 7.5 support the growth of *V. vadensis*; however, optimal growth occurs at 37°C and pH 6.5, conditions that mimic those of the human intestinal tract.

Literature Cited

Albrecht, W., A. Fischer, J. Smida, and E. Stackebrandt. 1987. Verrucomicrobium spinosum, a eubacterium representing an ancient line of descent. System. Appl. Microbiol. 10:57–62.

Bano, N., and J. T. Hollibaugh. 2002. Phylogenetic composition of bacterioplankton assemblages from the Arctic Ocean. Appl. Environ. Microbiol. 68:505–518.

Bomar, D., and E. Stackebrandt. 1987. 5S rRNA sequences from Nitrobacter winogradskyi, Caulobacter crescentus, Stella humosa and Verrucomicrobium spinosum. Nucleic Acids Res. 25:9597.

Bornemann, J., and E. W. Triplett. 1997. Molecular microbial diversity in soils from eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol. 63:2647–2653.

Brambilla, E., H. Hippe, A. Hagelstein, B. J. Tindall, and E. Stackebrandt. 2001. 16S rDNA diversity of cultured and uncultured prokaryotes of a mat sample from Lake Fryxell, McMurdo Dry Valleys, Antarctica. Extremophiles 5:23–33.

Chin, K.-J., D. Hahn, U. Hengtsmann, W. Liesack, and P. H. Janssen. 1999. Characterization and identification of numerically abundant culturable bacteria from the anoxic bulk soil of rice paddy microcosms. Appl. Environ. Microbiol. 65:5042–5049.

Chin, K.-J., W. Liesack, and P. H. Janssen. 2001. Opitutus terrae gen. nov., sp. nov., to accommodate novel strains of the division "Verrucomicrobia" isolated from rice paddy soil. Int. J. Syst. Bacteriol. 51:1965–1968.

Cho, J.-C., and S.-J. Kim. 2000. Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. Appl. Environ. Microbiol. 66:956–965.

Christner, B. C., B. H. Kvitko 2nd, and J. N. Reeve. 2003. Molecular identification of bacteria and eukarya inhabiting an Antarctic cryoconite hole. Extremophiles 7:177–183.

Cohen-Bazire, G., W. R. Sistrom, and R. Y. Stanier. 1957. Kinetic studies of pigment synthesis by non-sulphur purple bacteria. J. Cell. Comp. Physiol. 49:25–68.

Coomans, A., T. T. M. Vandekerckhove, and M. Claeys. 2000. Transovarial transmission of symbionts in Xiphinema brevicollum (Nematoda: Longidoridae). Nematology 2:455–461.

Crump, B. C., E. V. Armbrust, and J. A. Baross. 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. Appl. Environ. Microbiol. 65:3192–3204.

DeBont, J. A. M., J. T. Staley, and H. S. Pankratz. 1970. Isolation and description of a non-motile, fusiform, stalked bacterium, a representative of a new genus. Ant. v. Leeuwenhoek 36:397–407.

Dojka, M. A., P. Hugenholtz, S. K. Haack, and N. R. Pace. 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64:3869–3877.

Elshahed, M. S, J. M. Senko, F. Z. Najar, S. M. Kenton, B. A. Roe, T. A. Dewers, J. R. Spear, and L. R. Krumholz. 2003. Bacterial diversity and sulfur cycling in a meso-

philic sulfide-rich spring. Appl. Environ. Microbiol. 69:5609–5621.

Felske, A., and A. D. L. Akkermans. 1998. Prominent occurrence of ribosomes from an uncultured bacterium of the Verrucomicrobiales-cluster in grassland soils. Lett. Appl. Microbiol. 26:219–223.

Freitag, T. E., and J. I. Prosser. 2003. Community structure of ammonia-oxidizing bacteria within anoxic marine sediments. Appl. Environ. Microbiol. 69:1359–1371.

Garrity, M. G., and J. G. Holt. 2001. The road map to the manual. *In:* D. R. Boone and R. W. Castenholz. Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 119–162.

Glöckner, F. O., E. Zaichikov, N. Belkova, L. Denissova, J. Pernthaler, A. Pernthaler, and R. Amann. 2002. Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria. Appl. Environ. Microbiol. 66:5053–5065.

Gorlenko, V. M. 1970. A new phototrophic green sulphur bacterium: Prosthecochloris aestuarii nov. gen. nov. spec. Z. Allgem. Mikrobiol. 10:147–149.

Gorlenko, V. M., and E. V. Lebedeva. 1971. New green sulphur bacteria with appendages [in Russian, with English summary]. Mikrobiologya 40:1035–1039.

Gremion, F., A. Chatzinotas, and H. Harms. 2003. Comparative 16S rDNA and 16S rRNA sequence analysis indicates that actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. Environ. Microbiol. 5:896–907.

Hedlund, B. P., J. J. Gosink, and J. T. Staley. 1996. Phylogeny of Prosthecobacter fusiformis, the fusiform caulobacters: Members of a recently discovered division of the Bacteria. Int. J. Syst. Bacteriol. 46:960–966.

Hedlund, B. P., J. J. Gosink, and J. T. Staley. 1997. Verrucomicrobia div. nov., a new division of the Bacteria containing three new species of Prosthecobacter. Ant. v. Leeuwenhoek 72:29–38.

Hengstmann, U., K.-J. Chin, P. H. Janssen, and W. Liesack. 1999. Comparative phylogenetic assignment of environmental sequences of genes encoding 16S rRNA and numerically abundant culturable bacteria from an anoxic rice paddy soil. Appl. Environ. Microbiol. 65: 5050–5058.

Henrici, A. T., and D. E. Johnson. 1935. Studies of freshwater bacteria. II: Stalked bacteria, a new order of Schizomycetes. J. Bacteriol. 30:61–93.

Hiorns, W. D., B. A. Methé, S. A. Nierzwicki-Bauer, and J. P. Zehr. 1997. Bacterial diversity in Adirondack Mountain lakes as revealed by 16S rRNA gene sequences. Appl. Environ. Microbiol. 63:2957–2960.

Hirsch, P., M. Müller, and H. Schlesner. 1977. Aquatic Microbiology. Academic Press. London, UK. Society for Applied Bacteriology Symposium Series 6:107–133.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998a. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180: 4765–4774.

Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace. 1998b. Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. 180:366–376.

Humayoun, S. B., N. Bano, and J. T. Hollibaugh. 2003. Depth distribution of microbial diversity in Mono Lake, a meromictic soda lake in Californa. Appl. Environ. Microbiol. 69:1030–1042.

Janssen, P. H., A. Schumann, E. Mörschel, and F. A. Rainey. 1997. Novel anaerobic ultramicrobacteria belonging to the Verrucomicrobiales lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. Appl. Environ. Microbiol. 63:1382–1388.

Janssen, P. H., P. S. Yates, B. E. Grinton, P. M. Taylor, and M. Sait. 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Appl. Environ. Microbiol. 68: 2391–2396.

Jenkins, C., R. Samudrala, I. Anderson, B. P. Hedlund, G. Petroni, N. Michailova, N. Pinel, R. Overbeek, G. Rosati, and J. T. Staley. 2002. Genes for the cytoskeletal protein tubulin in the bacterial genus Prosthecobacter. PNAS 99:17049–17054.

Juretschko, S., A. Loy, S. Lehner, and M. Wagner. 2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. System. Appl. Microbiol. 25:84–99.

Lee, S.-Y., J. Bollinger, D. Bezdicek, and A. Ogram. 1996. Estimation of the abundance of an uncultured soil bacterial strain by competitive quantitative PCR method. Appl. Environ. Microbiol. 62:3787–3793.

Liesack, W., and E. Stackebrandt. 1992. Occurrence of novel groups of the domain Bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bacteriol. 174:5072–5078.

Lopez-Garcia, P., F. Gaill, and D. Moreira. 2002. Wide bacterial diversity associated with tubes of the vent worm Riftia pachyptila. Environ. Microbiol. 4:204–215.

Lyman, J., and R. H. Fleming. 1940. Composition of sea water. J. Marine Res. (Sears Foundation) 3:134–146.

Madrid, V. M., G. T. Taylor, M. I. Scranton, and A. Y. Chistoserdov. 2001. Phylogenetic diversity of bacterial and archaeal communities in the anoxic zone of the Cariaco Basin. Appl. Environ. Microbiol. 67:1663–1674.

McCaig, A. E., L. A. Glover, and J. I. Prosser. 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Environ. Microbiol. 65:1721–1730.

O'Farrell, K. A., and P. H. Janssen. 1999. Detection of Verrucomicrobia in a pasture soil by PCR-mediated amplification of 16S rRNA genes. Appl. Environ. Microbiol. 65:4280–4284.

Petroni, G., S. Spring, K.-H. Schleifer, F. Verni, and G. Rosati. 2000. Defensive extrusive ectosymbionts of Euplotidium (Ciliophora) that contain microtubule-like structures are bacteria related to Verrucomicrobia. Proc. Natl. Acad. Sci. USA 97:1813–1817.

Poindexter, J. S., and J. T. Staley. 1989. Prosthecobacter. *In:* J. T. Staley, M. Bryant, and N. Pfennig (Eds.) Bergey's Manual of Systematic Bacteriology, 1st ed. Williams and Wilkins. Baltimore, MD. 3:1942–1945.

Ravenschlag, K., K. Sahm, J. Pernthaler, and R. Amann. 1999. High bacterial diversity in permanently cold marine sediments. Appl. Environ. Microbiol. 65:3982–3989.

Rheims, H., F. A. Rainey, and E. Stackebrandt. 1996a. A molecular approach to search for diversity among bacteria in the environment. J. Indust. Microbiol. 17:159–169.

Rheims, H., C. Sproer, F. A. Rainey, and E. Stackebrandt. 1996b. Molecular biological evidence for the occurrence

of uncultured members of the actinomycete line of descent in different environments and geographical locations. Microbiology 142:2863–2870.

Rosati, G., P. Lenzi, and V. Franco. 1993. "Epixenisomes": Peculiar epibionts of the protozoon ciliate Euplotidium itoi. Do their cytoplasmic tubules consist of tubulin? Micron 24:465–471.

Rosati, G., M. A. Giambelluca, M. Grossi, and A. Morelli. 1997. Epixenosomes, peculiar epibionts of the ciliate Euplotidium itoi: involvement of membrane receptors and the adenylate cyclase-cyclic AMP system in the ejecting process. Protoplasma 197:57–63.

Rosati, G., G. Petroni, S. Quochi, L. Modeo, and V. Franco. 1999. Epixenosomes: peculiar epibionts of the hypotrich ciliate Euplotidium itoi defend their host against predators. J. Euk. Microbiol. 46:278–282.

Sakai, T., K. Ishizuka, and I. Kato. 2003. Isolation and characterization of a fucoidan-degrading marine bacterium. Mar. Biotechnol. 5:409–416.

Salzman, N. H., H. de Jong, Y. Paterson, H. J. M. Harmsen, G. W. Welling, and N. A. Bos. 2002. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. Microbiology 148:3651–3660.

Schäfer, H., P. Servais, and G. Muyzer. 2000. Successional changes in the genetic diversity of a marine bacterial assemblage during confinement. Arch. Microbiol. 173:138–145.

Schlesner, H. 1986. Pirella marina sp. nov., a budding, peptidoglycan-less bacterium from brackish water. System. Appl. Microbiol. 8:177–180.

Schlesner, H. 1987. Verrucomicrobium spinosum gen. nov., sp. nov.: a fimbriated prosthecate bacterium. System. Appl. Microbiol. 10:54–56.

Schlesner, H. 1992. The genus verrucomicrobium. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer. The Prokaryotes. Springer-Verlag. New York, NY. IV:3806–3808.

Sittig, M., and H. Schlesner. 1993. Chemotaxonomic investigation of various prosthecate and/or budding bacteria. System. Appl. Microbiol. 16:92–103.

Staley, J. T. 1968. Prosthecomicrobium and Ancalomicrobium: New freshwater prosthecate bacteria. J. Bacteriol. 95:1921–1942.

Staley, J. T., J. A. M. DeBont, and K. DeJonge. 1976. Prosthecobacter fusiformis nov. gen. et sp., the fusiform caulobacter. Ant. v. Leeuwenhoek 42:333–342.

Staley, J. T.. 1992. The genera: prosthecomicrobium, ancalomicrobium and prosthecobacter. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. III:2160–2166.

Stams, A. J. M., J. B. van Dijk, C. Dijkema and C. M. Plugge. 1993. Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl. Environ. Microbiol. 59:1114–1119.

Suau, A., R. Bonnet, M. Sutren, J.-J. Godon, G. R. Gibson, M. D. Collins, and J. Doré. 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. 65:4799–4807.

Sullivan, C. W., and A. C. Palmisano. 1984. Sea ice microbial communities: distribution, abundance, and diversity of ice bacteria in McMurdo Sound, Antarctica, in 1980. Appl. Environ. Microbiol. 47:788–795.

Tanner, M. A., C. L. Everett, W. J. Coleman, M. M. Yang, and D. C. Youvan. 2000. Complex microbial communities inhabiting sulfide-rich black mud from marine coastal environments. Biotechnology 8:1–16.

Ueda, T., Y. Suga, and T. Matsuguchi. 1995. Molecular phylogenetic analysis of a soil microbial community in a soy bean field. Eur. J. Soil Sci. 46:415–421.

Urakawa, H., K. Kita-Tsukamoto, and K. Ohwada. 1999. Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. Microbiology 145:3305–3315.

Vandekerckhove, T. T. M., A. Willems, M. Gillis, and A. Coomans. 2000. Occurrence of novel verrucomicrobial species, endosymbiotic and associated with parthenogenesis in Xiphinema americanum-group species (Nematoda, Longidoridae). J. Syst. Evol. Microbiol. 50:2197–2205.

Vasilyeva, L. V. 1985. Stella, a new genus of soil prosthecobacteria, with proposals for Stella humosa sp. nov. and Stella vacuolata sp. nov. Int. J. Syst. Bacteriol. 35:518–521.

Ward-Rainey, N., F. A. Rainey, H. Schlesner, and E. Stackebrandt. 1995. Assignment of hitherto unidentified 16S rDNA species to a main line of descent within the domain Bacteria. Microbiology 141:3247–3250.

Ward-Rainey, N., F. A. Rainey, and E. Stackebrandt. 1997. The presence of a dnaK (HSP70) multigene family in members of the orders Planctomycetales and Verrucomicrobiales. J. Bacteriol. 179:6360–6366.

Wise, M. G., J. V. McArthur, and L. J. Shimkets. 1997. Bacterial diversity of a Carolina bay as determined by 16S rRNA gene analysis: Confirmation of novel taxa. Appl. Environ. Microbiol. 63:1505–1514.

Zoetendal, E. G., C. M. Plugge, A. D. Akkermans, and W. M. de Vos. 2003. Victivallis vadensis gen. nov., sp. nov., a sugar-fermenting anaerobe from human faeces. Int. J. Syst. Evol. Microbiol. 53:211–215.

Zwart, G., R. Huismans, M. P. van Agterveld, Y. van de Peer, P. De Rijk, H. Eenhoorn, G. Muyzer, E. J. van Hannen, H. J. Gons, and H. J. Laanbroek. 1998. Divergent members of the bacterial division Verrucomicrobiales in a temperate freshwater lake. FEMS Microbiol. Ecol. 25:159–169.

PART 12

Thermotogales

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CHAPTER 12.1

Thermotogales

ROBERT HUBER AND MICHAEL HANNIG

Introduction

Members of the Thermotogales represent a very deep phylogenetic branch within the 16S rRNA gene tree. Within the order, members of the genus *Thermotoga* have an upper temperature border of growth at 90°C and represent, together with members of the order *Aquificales*, the bacteria with the highest growth temperatures known so far. Representatives of the Thermotogales are widespread and cosmopolitan, and they thrive mainly in volcanically or geothermally heated environments. Owing to their strictly organotrophic way of life, they are consumers of microbial biomaterial within high temperature ecosystems.

For the Thermotogales, the following order criteria are characteristic: thermophilic, non-sporeforming, rod-shaped cells with an outer sheath-like envelope ('toga'); Gram-negative, but *meso*-diaminopimelic acid not present in the peptidoglycan; strictly anaerobic, fermentative bacteria; acetate, carbon dioxide, and hydrogen metabolites from glucose fermentation; inhibition of growth by hydrogen; lysozyme-sensitive; unusual long-chain dicarboxylic fatty acids present in the lipids; and G+C content, 29–50 mol%.

Phylogeny

By 16S rRNA gene sequence comparison it was shown first in 1987 that *Thermotoga maritima* represents a slowly evolving lineage and a deep phylogenetic branch within the bacterial domain (Huber et al., 1986; Woese, 1987; Fig. 1). On the basis of this result, a thermophilic origin of the bacterial domain was proposed (Achenbach-Richter et al., 1987). The outstanding phylogenetic placement of *T. maritima* was strengthened by comparative analysis of other macromolecules such as 23S rRNA, elongation factor Tu and G, ß-subunit of the ATPase, *fus* gene and of ferredoxins (Bachleitner et al., 1989; Schleifer and Ludwig, 1989; Tiboni et al., 1991; Ludwig et al., 1993; Blamey et al., 1994; Darimont and Sterner, 1994) and by whole genome-based phylogenetic analysis (Fitz-Gibbon and House, 1999). In contrast, sequence comparisons of bacterial RNA-polymerase large subunits placed *T. maritima* next to the chloroplasts, and the analysis of the bacterial DNA polymerase III, class II, placed *T. maritima* next to *Clostridium acetobutylicum* (Palm et al., 1993). Furthermore, a 16S rRNA phylogenetic tree based on the most conserved positions proposed that the Planctomycetales is the first branching bacterial group and not a hyperthermophilic bacterium (Brochier and Philippe, 2002). On the basis of conserved inserts and deletions found in various proteins, Gupta and Griffith (2002) placed *T. maritima* phylogenetically within the Gram-positive Bacteria. However, owing to biochemical and ultrastructural features, *T. maritima* is considered to be a true Gram-negative bacterium (see also Identification).

Taxonomy

Owing to the outstanding phylogenetic position of *T. maritima* in combination with unique morphological, physiological and biochemical criteria, the order Thermotogales consisting of the single family Thermotogaceae was described (Huber et al., 1992b). Within the Thermotogaceae, the genera *Thermotoga* (Huber et al., 1986; Huber and Stetter, 2001b), *Thermosipho* (Huber et al., 1989a; Ravot et al., 1996b; Huber and Stetter, 2001b), *Fervidobacterium* (Patel et al., 1985a; Huber et al., 1990; Huber and Stetter, 2001b), *Geotoga*, *Petrotoga* (Davey et al., 1993a; Davey et al., 2001a; Davey et al., 2001b) and *Marinitoga* (Wery et al., 2001) have been described. The species belonging to the different genera of the Thermotogales are listed in Table 1; their phylogenetic relationship is shown in Fig. 2. On the basis of 16S rRNA gene sequence analysis, *T. maritima*, *Thermotoga neapolitana*,

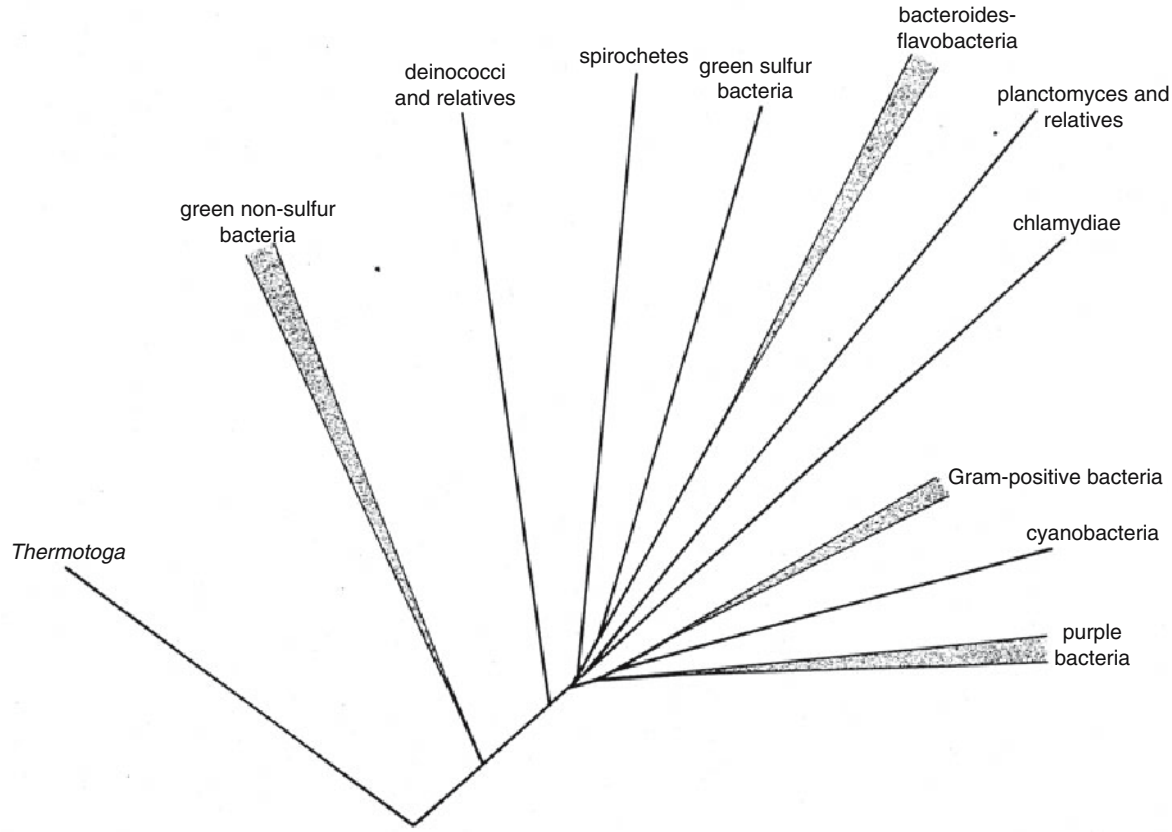

Fig. 1. Phylogenetic tree based upon 16S rRNA gene sequence comparison showing the phylogenetic position of the species *Thermotoga maritima* at the root of bacterial tree. (From Woese, 1987; courtesy of C. W. Woese.)

Thermotoga petrophila and *Thermotoga naphthophila* show a very close relationship (Fig. 2). However, based on specific sequence signatures in the 16S rRNA gene (*Escherichia coli* numbering; Brosius et al., 1981; Woese, 1987), the *T. maritima – T. neapolitana* group (position 1031, C; 1290, G; 1364, U) can be distinguished from the *T. naphthophila – T. petrophila* group (position 1031, U; 1290, A; 1364, C). Recently, the phylum Thermotogae, consisting of the single class Thermotogae was proposed (Reysenbach, 2001).

Habitat

Members of the Thermotogales are widespread and cosmopolitan. They thrive within continental solfatara springs of low salinity, shallow and deep-sea marine hydrothermal systems and high-temperature marine and continental oil fields.

From the genus *Thermotoga*, the type species *T. maritima* had been originally isolated from a geothermally heated, shallow marine sediment at Vulcano, Italy (Huber et al., 1986). The second species of this genus, *T. neapolitana*, was obtained from a submarine thermal vent at Lucrino near Naples, Italy (Belkin et al., 1986; Jannasch et al., 1988). Members of the marine *T. maritima – T. neapolitana* group are widespread within high temperature ecosystems and have also been isolated from shallow submarine hydrothermal systems on Sangeang Island (Indonesia), Ribeira Quente, Sao Miguel Island (the Azores), Kunashir Island (north of Japan) and the Fiji Island (Huber and Stetter, 1992b). Additional isolates were obtained from the Kolbeinsey Ridge north of Iceland in a depth of 106 m and from deep-sea hot sediments in a depth of 2000 m (Guaymas, Mexico; Huber and Stetter, 1992a; Huber and Stetter, 1992b). *Thermotoga thermarum* was originally isolated from continental solfataras with low ionic strength at Lac Abbé, Djibouti, Africa (Windberger et al., 1989). The closest cultivated relative of *T. thermarum* is the so far undescribed new *Thermotoga* species BB13-1-L6A, which thrives in the geothermally heated water (68°C; pH 6.8)

Table 1. List of the described species within the Thermotogales.

Species	Culture collection	Accession number	Effective publication	Validation
Thermotoga maritima MSB8	DSM 3109 and ATCC 43589	M21774	Huber et al., 1986	Stetter and Huber, 1986
Thermotoga neapolitana NS-E	DSM 4359 and ATCC 49049	AB039768	Jannasch et al., 1988	Jannasch et al., 1989
Thermotoga thermarum LA3	DSM 5069	AB039769	Windberger et al., 1989	Windberger et al., 1992
Thermotoga elfii SEBR 6459	DSM 9442 and ATCC 51869	X80790	Ravot et al., 1995	Ravot et al., 1995
Thermotoga subterranea SL1	DSM 9912	U22664	Jeanthon et al., 1995	Jeanthon et al., 2000
Thermotoga hypogea SEBR 7054	DSM 11164	U89768	Fardeau et al., 1997	Fardeau et al., 1997
Thermotoga petrophila RKU-1	DSM 13995, ATCC BAA-488, and JCM 10881	AB027016	Takahata et al., 2001	Takahata et al., 2001
Thermotoga naphthophila RKU-10	DSM 13996, ATCC BAA-489, and JCM 10882	AB027017	Takahata et al., 2001	Takahata et al., 2001
Thermotoga lettingae TMO	DSM 14385 and ATCC BAA-301	AF355615	Balk et al., 2002	Balk et al., 2002
Thermosipho africanus Ob7	DSM5309	M24022	Huber et al., 1989a	Huber et al., 1989b
Thermosipho melanesiensis BI429	DSM 12029 and CIP 104789	Z70248	Antoine et al., 1997	Antoine et al., 1997
Thermosipho japonicus IHB1	DSM 13481 and JCM 10495	AB024932	Takai und Horikoshi, 2000a	Takai and Horikoshi, 2000b
Thermosipho geolei SL31	DSM 13256 and JCM 10986	AJ272022	L'Haridon et al., 2001	L'Haridon et al., 2001
Fervidobacterium nodosum Rt17-B	DSM 5306 and ATCC 35602	M59177	Patel et al., 1985a	Patel et al., 1985b
Fervidobacterium islandicum H21	DSM 5733 and ATCC 49647	M59176	Huber et al., 1990	Huber et al., 1991
Fervidobacterium gondwanense AB39	DSM 13020 and ACM 5017	Z49117	Andrews and Patel, 1996	Andrews and Patel, 1996
Fervidobacterium pennivorans Ven5	DSM 9078		Friedrich and Antranikian, 1996	Friedrich and Antranikian, 1999
Geotoga petraea T5	ATCC 51226	L10658	Davey et al., 1993a	Davey et al., 1993b
Geotoga subterranea CC-1	ATCC 51225	L10659	Davey et al., 1993a	Davey et al., 1993b
Petrotoga miotherma 42-6	DSM 10691 and ATCC 51224	L10657	Davey et al., 1993a	Davey et al., 1993b
Petrotoga mobilis SJ95	DSM 10674	Y15479	Lien et al., 1998	Lien et al., 1998
Petrotoga olearia SL24	DSM 13574 and JCM 11234	AJ311703	L'Haridon et al., 2002	L'Haridon et al., 2002
Petrotoga sibirica SL25	DSM 13575 and JCM 11235	AJ311702	L'Haridon et al., 2002	L'Haridon et al., 2002
Marinitoga camini MV 1075	DSM 13578 and CNCM I-2413	AJ250439	Wery et al., 2001	Wery et al., 2001
Marinitoga piezophila KA3	DSM 14283 and JCM 11233	AF326121	Alain et al., 2002	Alain et al., 2002

Abbreviations: DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; JCM, Japan Collection of Microorganisms, RIKEN, Saitama, Japan; ATCC, American Type Culture Collection, Manassas, VA, United States; CNCM, Collection Nationale de Cultures de Microorganismes, Paris, France; ACM, University of Queensland Microbial Culture Collection, Australia; and CIP, Collection de L'Institut Pasteur, Paris, France.

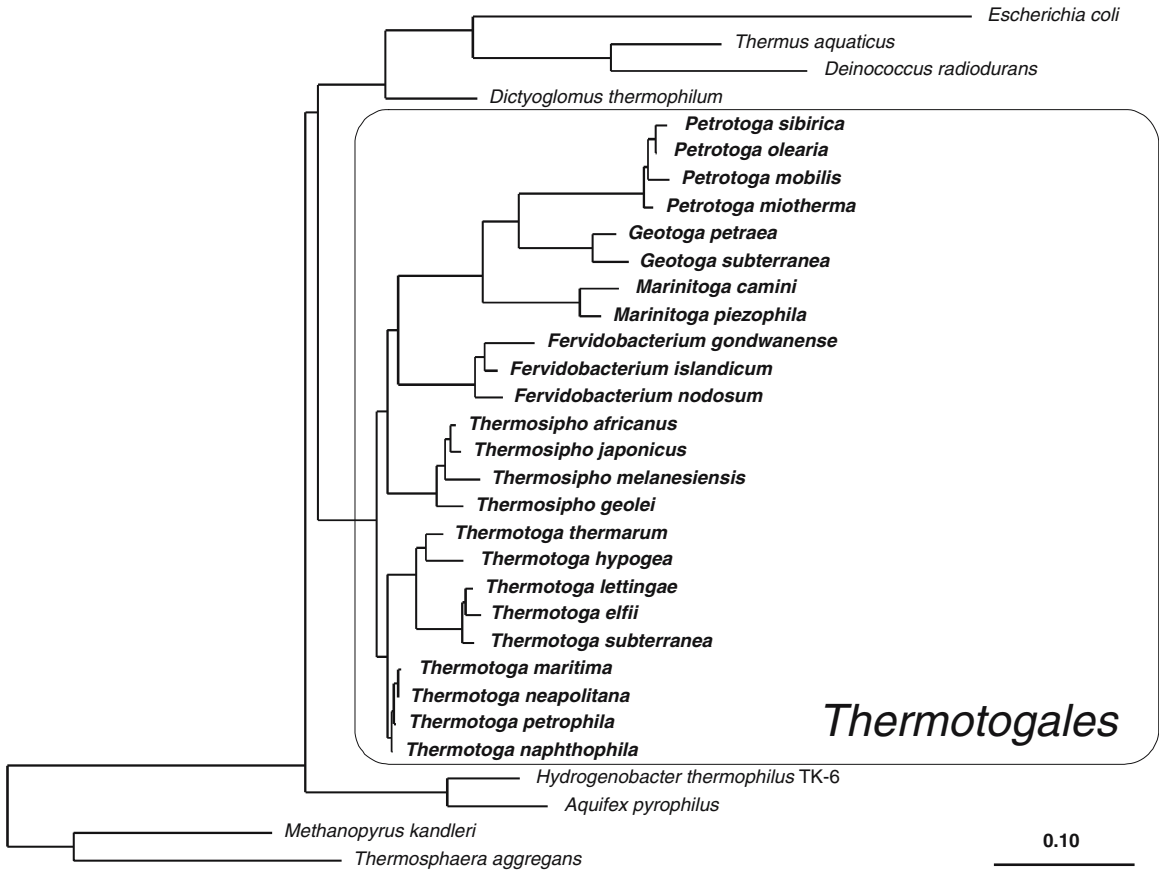

Fig. 2. 16S rRNA-based phylogenetic tree of the Thermotogales. The tree topology is based on the ARB database of 11,800 sequence entries and was reconstructed using the ARB parsimony tool. A filter defining positions that share identical residues in at least 50% of all included sequences from the Thermotogales was used for reconstructing the tree. Reference sequences were chosen to represent the broadest diversity of bacteria. Accession numbers for the sequences are given in Table 1. The scale bar represents 0.10 fixed mutations per nucleotide position.

of the Friedrichstollen in Baden-Baden (Germany; Fig. 3).

Evidence for the presence of Thermotogales relatives in deep-subsurface petroleum reservoirs was reported for the first time by Stetter et al. (1993). *Thermotoga*-related organisms (designated "Pb-isolates") were obtained from seawater flooded oil fields, about 3000 m below the permafrost surface of the North Slope of Alaska (Prudhoe Bay, Endicott and Kuparuk oil fields; Stetter et al., 1993; Stetter and Huber, 2000). By 16S rRNA gene sequence comparison and sequence signature analysis, these Pb-isolates were found to group together with the recently described *T. petrophila* and *T. naphthophila* from the Kubiki oil reservoir in Niigata (Japan; Takahata et al., 2001). Further *Thermotoga* isolates from oil production wells were described as *Thermotoga elfii* (Africa; Ravot et al., 1995), *Thermotoga subterranea* (East Paris Basin; Jeanthon et al., 1995) and *Thermotoga hypogea* (Africa; Fardeau et al., 1997). *Thermotoga*-like organisms have been also reported from a continental oil reservoir (L'Haridon et al., 1995), and Thermotogales relatives were also found during a systematic survey in high temperature oil reservoirs with different salinities (Grassia et al., 1996).

Members of the genus *Fervidobacterium* seem to be restricted to biotopes with low salinity. *Fervidobacterium nodosum* was originally isolated from a hot spring in New Zealand (Patel et al., 1985a). *Fervidobacterium islandicum* was obtained from a continental solfatara field at Hveragerthi (Iceland; Huber et al., 1990) and further, so far undescribed *Fervidobacterium* relatives were isolated from continental hot springs in Tibet (Huber and Stetter, 1992a), from Nadi, Viti Levu (Fiji Island), and from Octopus Spring (Yellowstone National Park, United States). *Fer-*

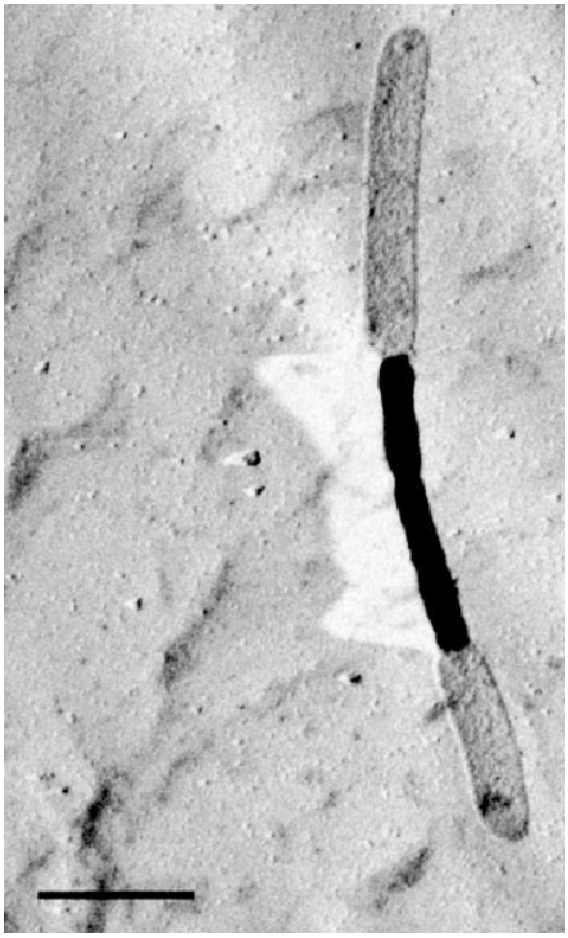

Fig. 3. The new *Thermotoga* species BB13-1-L6A, isolated from geothermally heated water from the Friedrichstollen in Baden-Baden, Germany. The rod-shaped cell is covered by an elongated sheath-like outer membrane ("toga"), overballooning at both ends. Bar, 1 μm.

vidobacterium gondwanense was obtained from a sample taken from a runoff channel of the Great Artesian Basin of Australia (Andrews and Patel, 1996), *Fervidobacterium pennivorans* from a hot spring on San Miguel (the Azores, Portugal; Friedrich and Antranikian, 1996).

From marine hydrothermal springs and sandy sediments of the Gulf of Tadjoura southwest of Obock (Djibouti, Africa), three strains belonging to the genus *Thermosipho* have been isolated. They represented a single species, *Thermosipho africanus* (Huber et al., 1989a). *Thermosipho melanesiensis*, the second species within this genus, was obtained from the gills of *Bathymodiolus brevior*, a deep-sea hydrothermal vent mussel. This invertebrate was collected at the bottom of a black smoker in the Lau Basin (depth 1832 m), Southwest Pacific Ocean (Antoine et al., 1997). Recently, *Thermosipho japonicus* was isolated from a deep-sea hydrothermal vent in the Iheya Basin (Japan; Takai and Horikoshi, 2000a). Members of the genus *Thermosipho* thrive also in hot oil reservoirs. A so far undescribed, new *Thermosipho* species was obtained from reservoir fluids taken at the Thistle oil production platform (East Shetland Basin, North Sea; Stetter et al., 1993; Stetter and Huber, 2000), and recently, *Thermosipho geolei* from a continental petroleum reservoir in Western Siberia was published (Russia; L'Haridon et al., 2001).

From petroleum reservoirs located in Oklahoma and Texas, members of *Geotoga* and *Petrotoga* have been isolated for the first time (Davey et al., 1993a). The isolates have been described as *Geotoga petraea*, *Geotoga subterranea* and *Petrotoga miotherma*. *Petrotoga mobilis* was obtained from a North Sea oil production well (Lien et al., 1998) and two recent isolates were obtained from a continental petroleum reservoir in Western Siberia, namely *Petrotoga olearia* and *Petrotoga sibirica* (L'Haridon et al., 2002).

The type species of *Marinitoga*, *Marinitoga camini*, was isolated from a deep-sea hydrothermal chimney sample on the Mid-Atlantic Ridge (Wery et al., 2001) and *Marinitoga piezophila* was isolated from a chimney rock on the East-Pacific Rise (Alain et al., 2002).

Isolation

Members of the Thermotogales can be enriched in anaerobic culture media with a pH around 7 (Table 2) under a gas phase consisting of 300 kPa N_2 or 300 kPa N_2/CO_2 (80:20; vol/vol). Depending on the salt requirement of the organism, either half strength seawater media or media with low ionic strength are used (Table 2). The media, supplemented with a defined carbon source (e.g., maltose or starch; Table 3) and complex organic material (e.g., yeast extract or peptone; Table 3), are inoculated with original sampling material. Members of the *T. maritima NT. neapolitana* group can be selectively enriched at an incubation temperature of 85°C with starch as the only carbon and energy source. Keratinophilic (feather-degrading) *Fervidobacterium* species might be obtained in yeast extract/tryptone medium with native feathers from chickens, ducks or geese (Leuschner and Antranikian, 1994). For enrichment of *G. petraea*, *G. subterranea* and *P. miotherma*, alpha-glycerophosphate and starch should be added to petroleum reservoir brines (Davey et al., 1993a). *Marinitoga piezophila* can be selectively enriched heterotrophically under a hydrostatic pressure of 30 MPa (Alain et al., 2002).

The enrichment bottles are incubated at the appropriate temperatures (45–85°C; Table 2)

and monitored for growth over a certain time period (e.g., two weeks) by phase contrast microscopy. When rod-shaped cells with an outer sheath-like structure or with a single terminal bleb become visible, the enrichments are serially diluted. The isolates are obtained by plating under anaerobic conditions and at an incubation temperature between 45 and 80°C (Table 2). For plating, a stainless steel anaerobic jar (Balch et al., 1979) and plates, solidified with Gelrite (0.7–1.0%; Huber and Stetter, 2001a) or agar (0.8–3%), can be used. Other methods for isolation are agar shake tubes (2%) or phytagel roll tubes (4%). For *T. neapolitana*, mean plating efficiencies up to 84% on 0.7% Gelrite have been reported. For growth, the plates were incubated at 77°C in a glass canning jar containing a packet of palladium pellets as a catalyst (Childers et al., 1992; Vargas and Noll, 1994). Colonies of the Thermotogales are uniformly round with a diameter of about 1–2 mm. Colonies of the genera *Thermotoga*, *Fervidobacterium*, *Geotoga* and *Petrotoga* appear whitish, while colonies of *Thermosipho* are colorless or brownish.

From liquid cultures, Thermotogales relatives can be also isolated by a plating-independent, newly developed isolation procedure (selected cell cultivation technique) based on optical trapping of single cells using a strongly focused infrared laser beam ("optical tweezers"; Huber et al., 1995; Beck and Huber, 1997; Huber, 1999; Huber and Stetter, 2001a).

In the future, a new isolation strategy might be used to isolate specifically Thermotogales relatives from their biotopes (Huber et al., 1995; Huber et al., 2000). This method combines 16S

Table 2. Physiological properties of the described species within the Thermotogales.

Species	G+C content (mol%)	Temperature optimum (°C)	Temperature range (°C)	pH optimum	pH range	NaCl optimum (%)	NaCl range (%)
Thermotoga maritima	46	80	55–90	6.5	5.5–9.0	2.7	0.25–6.0
Thermotoga neapolitana	41	80	55–90	7.0	5.5–9.0	2.0	0.25–6.0
Thermotoga thermarum	40	70	55–84	7.0	6.0–9.0	0.35	0.2–0.55
Thermotoga elfii	40	66	50–72	7.5	5.5–8.7	1.0	0–2.4
Thermotoga subterranea	40	70	50–75	7.0	6.0–8.5	1.2	0–2.4
Thermotoga hypogea	50	70	56–90	7.3–7.4	6.1–9.1	0.02	0–0.5
Thermotoga petrophila	46.6	80	47–88	7.0	5.2–9.0	1.0	0–5.5
Thermotoga naphthophila	46.1	80	48–86	7.0	5.4–9.0	1.0	0.1–6.0
Thermotoga lettingae	39.2	65	50–75	7.0	6.0–8.5	1.0	0–2.8
Thermosipho africanus	30	75	35–77	7.2	6.0–8.0	n.d.	0.11–3.6
Thermosipho melanesiensis	30.5	70	50–75	6.5–7.5	4.5–8.5	3.0	1.0–6.0
Thermosipho japonicus	31	72	45–80	7.2–7.6	5.3–9.3	4.0	0.66–7.9
Thermosipho geolei	30.0	70	45–75	7.5	6.0–9.4	2–3	0.5–7.0
Fervidobacterium nodosum	33.7	70	41–79	7.0	6.0–8.0	<1.0	n.d.
Fervidobacterium islandicum	40	65	50–80	7.0	6.0–8.0	<0.7	n.d.
Fervidobacterium gondwanense	35	65–68	45–80	7.0	6.0–8.0	<0.2	n.d.
Fervidobacterium pennivorans	40	70	50–80	6.5	5.5–8.0	0.4	0–4
Geotoga petraea	30	50	30–55	6.5	5.5–9.0	3	0.5–10
Geotoga subterranea	30	45	30–66	6.5	5.5–9.0	4	0.5–10
Petrotoga miotherma	40	55	35–65	6.5	5.5–9.0	2	0.5–10
Petrotoga mobilis	31–34	58–60	40–65	6.5–7.0	5.5–8.5	3–4	0.5–9.0
Petrotoga olearia	35	55	37–60	7.5	6.5–8.5	2	0.5–8
Petrotoga sibirica	33	55	37–55	8	6.5–9.4	1	0.5–7.0
Marinitoga camini	29	55	25–65	7.0	5.0–9.0	3.0	1.0–4.5
Marinitoga piezophila	29 (±1)	65	45–70	6.0	5.0–8.0	3.0	1.0–5.0

Abbreviation: n.d., not determined.

Table 3. Substrate specificity of the Thermotogales type strains.

Substrate	Growth on different substrates																																								
	Monosaccharide[a]								Di- and trisaccharide[a]					Polysaccharide[a]							Alcohol[a]						Organic acid[a]							Complex organic substrate							
Species	Arabinose	Fructose	Galactose	Glucose	Mannose	Rhamnose	Ribose	Xylose	Cellobiose	Lactose	Maltose	Sucrose	Raffinose	Cellulose	Chitin	Glycogen	Maltodextrin	Pectin	Starch	Xylan	Buthanol	Ethanol	Glycerol	Mannitol	Propanol	Sorbitol	Acetate	Butyrate	Lactate	Formate	Propionate	Pyruvate	Succinate	Casein	Casoamino acids	Keratin	Peptone	Tryptone	Yeast extract	Bio-Trypcase	Brain heart infusion
Thermotoga maritima	nr	+	+	+	nr	nr	+	+	nr	+	+	+	–	+	nr	+	nr	nr	+	+	nr	–	–	–	nr	nr	–	nr	–	–	–	–	nr	nr	–	nr	+	nr	+	nr	nr
Thermotoga neapolitana	nr	+	+	+	nr	nr	+	+	nr	+	+	+	+	+	nr	+	nr	nr	+	nr	nr	–	–	–	nr	nr	–	nr	–	–	–	–	nr	nr	–	nr	(+)	(+)	+	nr	nr
Thermotoga thermarum	–	–	–	+	–	nr	nr	+	nr	–	+	+	nr	nr	nr	nr	nr	nr	+	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
Thermotoga elfii	+	+	nr	+	+	–	+	+	nd	+	+	+	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	–	nr	nr	–	–	–	nr	–	nr	nr	nr	nr	nr	nr	nr	nr	+	nr
Thermotoga subterranea	nr	nr	nr	+	nr	nr	–	nr	nr	–	+	nr	–	nr	nr	–	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	–	nr	–	nr	nr	nr	nr	+	nr	nr	+	+	+	nr	nr
Thermotoga hypogea	–	+	+	+	+	nr	–	+	nr	+	+	+	nr	nr	nr	nr	nr	nr	nr	+	nr	nr	nr	nr	nr	nr	–	–	–	nr	–	nr	nr	nr	nr	nr	nr	nr	+	+	nr
Thermotoga petrophila	nr	+	+	+	nr	nr	+	–	nr	+	+	+	nr	(+)	–	nr	nr	nr	+	nr	nr	–	nr	–	nr	nr	–	nr	–	–	–	nr	nr	–	–	nr	+	nr	+	nr	nr
Thermotoga naphthophila	nr	+	+	+	nr	nr	+	–	nr	+	+	+	nr	–	–	nr	nr	nr	+	nr	nr	–	nr	+	nr	nr	–	nr	–	–	–	nr	nr	–	–	nr	+	nr	+	nr	nr
Thermotoga lettingae	+	+	+	+	+	+	+	+	+	+	+	+	nr	nr	nr	nr	nr	+	+	+	–	nr	+	–	–	nr	+	nr	+	+[1]	nr	+	–	nr	+	nr	+	nr	+	+	nr
Geotoga petraea	nr	nr	+	+	+	nr	nr	–	nr	+	+	+	–	nr	nr	nr	+	nr	+	nr	nr	–	nr	nr	nr	nr	–	nr	–	–	nr	nr	nr	nr	–	nr	nr	nr	nr	nr	nr
Geotoga subterranea	nr	nr	+	+	+	nr	nr	–	nr	+	+	+	–	nr	nr	nr	+	nr	+	nr	nr	–	nr	nr	nr	nr	–	nr	–	–	nr	nr	nr	nr	–	nr	nr	nr	nr	nr	nr
Petrotoga miotherma	nr	nr	+	+	+	nr	nr	+	nr	+	+	+	–	nr	nr	nr	+	nr	+	–	nr	–	nr	nr	nr	nr	–	nr	–	–	nr	nr	nr	nr	–	nr	nr	nr	nr	nr	nr
Petrotoga mobilis	+	+	+	+	–	+	+	+	+	+	+	+	–	–	nr	nr	+	nr	+	+	–	–	nr	–	–	nr	–	nr	–	–	nr	–	nr	nr	–	nr	nr	nr	nr	nr	nr
Petrotoga olearia	+	+	nr	+	nr	–	+	+	+	nr	+	+	nr	–	nr	nr	nr	nr	+	+	nr	–	nr	–	nr	nr	–	nr	nr	–	nr	+	nr	–	–	nr	+	nr	nr	nr	nr
Petrotoga sibirica	nr	+	+	+	nr	–	+	nr	nr	nr	+	+	nr	–	nr	nr	nr	nr	nr	+	nr	–	nr	–	nr	nr	–	nr	nr	–	nr	+	nr	–	–	nr	+	nr	nr	nr	nr
Thermosipho africanus	nr	nr	nr	–	nr	nr	–	nr	nr	–	–	nr	–	nr	nr	–	nr	nr	nr	nr	nr	–	nr	nr	nr	nr	–	nr	–	nr	–	nr	nr	nr	nr	nr	+	+	+	nr	nr
Thermosipho melanesiensis	nr	–	+	+	nr	nr	nr	–	+	+	+	+	nr	nr	nr	–	nr	nr	+	nr	nr	–	nr	–	nr	–	–	nr	nr	–	nr	nr	nr	nr	–	nr	nr	+	(+)	nr	(+)
Thermosipho japonicus	nr	nr	+	+	nr	nr	+	nr	nr	nr	+	+	nr	nr	–	nr	nr	–	+	–	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	+	–	nr	+	+	+	nr	nr
Thermosipho geolei	–	–	–	+	nr	nr	–	–	nr	nr	–	–	nr	–	nr	nr	nr	nr	–	–	nr	nr	nr	nr	nr	nr	–	nr	nr	–	nr	–	–	–	nr	nr	+	nr	+	nr	nr
Fervidobacterium nodosum	+	+	+	+	+	(+)	–	–	nr	+	+	+	+	–	nr	nr	nr	+	+	nr	nr	nr	+	nr	nr	+	–	nr	–	–	–	(+)	–	nr	nr	nr	nr	nr	nr	nr	nr
Fervidobacterium islandicum	+	+	+	+	+	nr	+	–	nr	–	+	nr	+	+	nr	nr	nr	nr	+	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	+	nr	nr	nr	nr	nr	nr	nr	nr	nr
Fervidobacterium gondwanense	–	+	+	+	+	nr	–	+	+	+	+	nr	–	–	–	nr	nr	nr	+	–	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	+	nr	nr	–	nr	nr	nr	nr	nr	nr
Fervidobacterium pennivorans	–	+	+	+	+	nr	+	+	nr	–	+	nr	–	–	nr	+	nr	nr	+	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	–	–	–	nr	+	nr	nr	nr	nr	nr
Marinitoga camini	–	+	–	+	–	nr	–	–	+	–	+	+	nr	+	+	nr	nr	+	+	–	nr	–	–	nr	nr	nr	–	nr	–	–	–	+	–	nr	–	–	+	+	nr	nr	nr
Marinitoga piezophila	nr	(+)	(+)	(+)	nr	nr	(+)	nr	(+)	nr	(+)	nr	nr	nr	nr	nr	nr	nr	(+)	nr	nr	nr	nr	nr	nr	nr	(+)	nr	nr	nr	nr	nr	nr	+	+	nr	+	+	+	nr	+

Symbols and abbreviations: +, growth; (+), weak or very weak growth; +[1], growth with thiosulfate and H_2/CO_2; –, no growth; and nr, not reported.

[a]For degradation, yeast extract is stimulatory or obligately required.

rRNA gene sequence analysis in situ, specific whole-cell hybridization within enrichments, and the cultivation of cells selected by the use of the laser microscope (Huber et al., 1995).

Identification

Cells of Thermotogales representatives are rod-shaped, about 1.0–50.0 µm long and 0.4–1.0 μm in diameter. They form an outer sheath, ballooning over the ends ('toga'), visible in all phases of growth (Figs. 4 and 5). Usually, they grow singly and in pairs (Fig. 4); members of the genus *Thermosipho* form short chains of up to 12 individuals surrounded by a sheath (Fig. 6). The majority of the *Fervidobacterium* cells form a characteristic terminal bleb ('spheroids') on one end of the cells, which occurs during all stages of growth (diameter 1.0–4.0 µm; Fig. 7). Furthermore, growth in short chains is observed, and *F. islandicum* frequently forms aggregates of up to 50 cells. Besides spheroids, *Fervidobacterium* species form spheres (diameter 5.0–8.0 μm; Fig. 8), membrane-bound structures containing one to seven individual cells. All members of the Thermotogales exhibit a Gram-negative staining reaction, but diamino-pimelic acid, typical for Gram-negative bacteria, is absent.

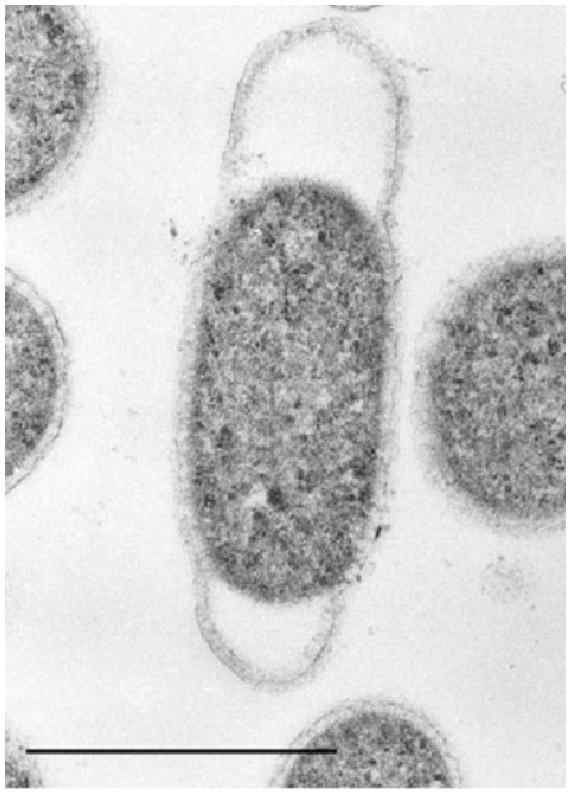

Fig. 5. Ultrathin section of *Thermotoga maritima*. Bar, 1 µm.

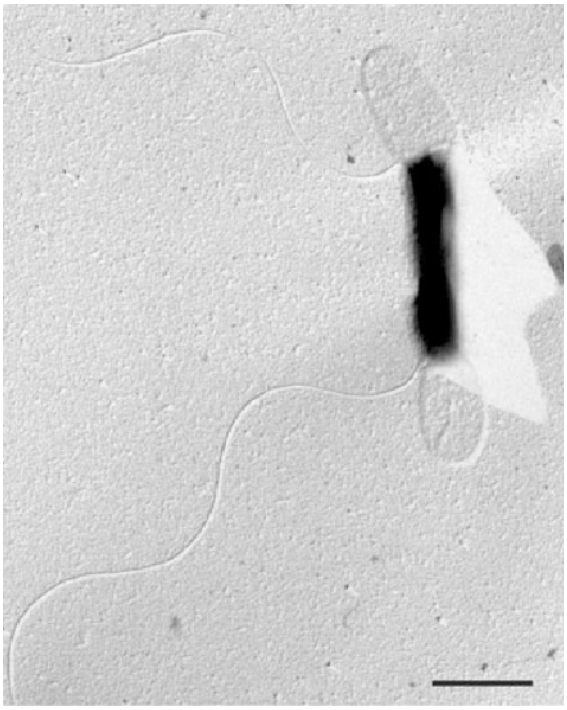

Fig. 4. Platinum-shadowed, flagellated cell of *Thermotoga maritima*. Bar, 1 µm.

Fig. 6. Four rod-shaped cells of *Thermosipho africanus* in a tube-like sheath; platinum-shadowed. Bar, 1 µm.

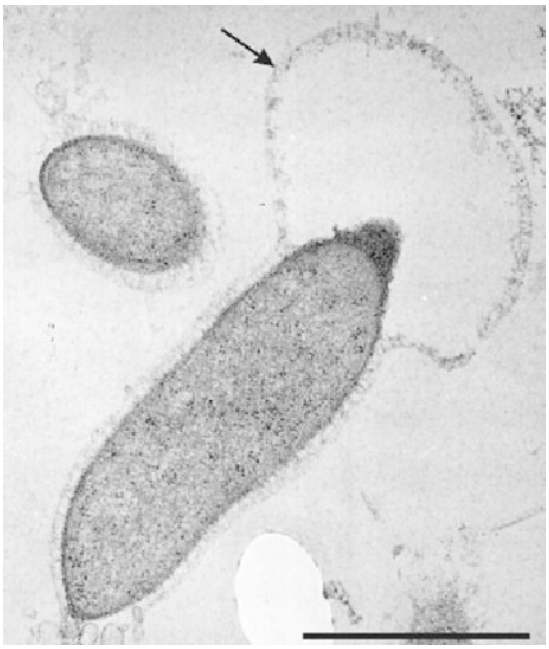

Fig. 7. Ultrathin section of *Fervidobacterium islandicum*. Bar, 1 µm.

Fig. 9. Free-floating biofilm of *Thermotoga* sp. SG1-L2A. Bar, 0.5 cm.

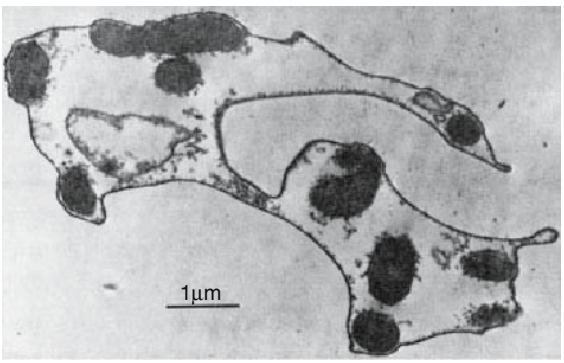

Fig. 8. Ultrathin section of *Fervidobacterium nodosum*. Single sphere, containing several cells. Bar, 1 µm. (Courtesy of H. W. Morgan.)

Thermotogales species either are immotile or possess flagella and are motile (Huber and Stetter, 1999; Huber and Stetter, 2001b; Fig. 4). Motility of *T. maritima* was observed at 60–90°C after heating the microscopic slide. At the optimal growth temperature of 80°C, the maximum speed was approximately 3 mm/min. Furthermore, a thermotactic response to temporal temperature changes was reported for *T. maritima* and thermostable chemotaxis proteins have been identified (Gluch et al., 1995; Swanson et al., 1996). Recently, the crystal structure of CheA, a signal-transducing histidine kinase, and the crystal structures of the middle and C-terminal domain of the flagellar rotor protein FliG from *T. maritima* have been published (Bilwes et al., 1999; Brown et al., 2002).

So far, there is only one report of film formation within the Thermotogales. During continuous cultivation of *T. maritima*, exopolysaccharides were produced under optimal growth conditions. Concomitantly, a thin and white, but not further specified film below the liquid level of the medium was observed (Rinker and Kelly, 2000). Very recently, we found biofilm formation by *Thermotoga* sp. SG1-L2A, produced under physiological growth conditions in batch cultures at the end of the logarithmic growth phase. The cells formed free-floating, white-colored biofilms in the medium with a length of up to 1 cm (Fig. 9).

The ultrastructure of *T. maritima* (Huber et al., 1986) and related species, as determined by transmission electron microscopy by freeze-etching and in ultrathin sections, can only be interpreted as *Thermotoga* being a true Gram-negative bacterium (Rachel et al., 1990). A sheath-like outer membrane and overballooning ends give the cells their characteristic appearance. The porin of the outer membrane has been purified and its specific functional properties characterized after insertion into artificial lipid bilayers (Engel et al., 1993); in addition, its secondary structure contains predominantly beta-sheets and the amino acid composition is characteristic for porins (for a review, see Schirmer,

1998). Outer membrane protein (Omp) alpha is a rod-shaped spacer that spans the periplasm, connecting the outer membrane to the inner body (Engel et al., 1992; Lupas et al., 1995). So far, it is not clear how Omp alpha is connected to the peptidoglycan or the cytoplasmic membrane. It is likely that the COOH-terminal hydrophobic tail of the protein is associated with the porin layer in the "balloon" portion of the cell wall. *Thermotoga* cells have an unusual thin and labile murein layer, presumably owing to a low degree of cross-linking; it is not synthesized in the presence of penicillin G or ampicillin and is sensitive to lysozyme. After addition of lysozyme, the rod-shaped cells of *T. maritima* round up within some minutes, which was also observed for *Ts. africanus* and *F. islandicum*. The murein of *T. maritima* consists of muramic acid, *N*-acetylglucosamine, glutamic acid, alanine and lysine (molar ratio = 0.41:0.69:1.00:1.43:0.89). D- and L-Lysine are present and not found so far in Gram-negative bacteria.

Recently, the crystal structure of the cell division protein FtsA and of the cell division inhibitor MinC was reported from *T. maritima* (van den Ent and Löwe, 2000; Cordell et al., 2001). Biochemical and structural investigations gave evidence that the *T. maritima* MreB protein is the bacterial homolog to the physiological polymer of the eucaryotic F-actin (van den Ent et al., 2001).

Electron microscopic studies showed that the cell envelope of *Fervidobacterium* is composed of two layers. The outer layer protrudes to form spheres (Patel et al., 1985a). In *F. nodosum*, the two layers have an irregular convoluted structure and are connected with regular junctions, and the outer layer is susceptible to lysis by SDS. The cells are able to grow and multiply within the spheres (Patel et al., 1985a).

About 50% of the total polar lipids of *T. maritima* are two amphipathic monopolar glycolipids with a very rare alpha-(1-4) diglucosyl structure (Manca et al., 1992). Unusual long-chain dicarboxylic fatty acids are present in the core lipids of the Thermotogales (Huber et al., 1986; Huber et al., 1989a; Huber et al., 1990; Jeanthon et al., 1995). In *T. maritima*, a total of 37 different fatty acids, including the novel 13,14-dimethyloctacosanedioic acid (Carballeira et al., 1997) and a new ether core lipid, 15,16-dimethyl-30-glyceroloxytriacontanoic acid (DeRosa et al., 1988), have been identified. Comparative analysis showed that this core lipid is characteristic for the genus *Thermotoga* (Huber et al., 1986; DeRosa et al., 1988; Windberger et al., 1989; Jeanthon et al., 1995; Fig. 10).

Members of the Thermotogales differ in their sensitivity to antibiotics (Table 4). For *T. elfii* and *F. pennivorans*, no information on antibiotic sensitivity was reported. Thermotogales representatives are significantly different in their sensitivity to the antibiotic rifampicin, which blocks transcription initiation (Huber and Stetter, 1992a; Table 4). Growth of some species is inhibited by only 1 μg/ml (e.g., *T. thermarum*), while other species (e.g., *T. maritima* and *T. neapolitana*) are resistant even against 100 μg/ml. Unusual for bacteria, the purified RNA polymerase of *T. maritima* is resistant to rifampicin (1 μg/ml), and only 80% of its activity is inhibited by 200 μg/ml (Huber et al., 1986). Growth of *T. maritima* is also not inhibited by 10 μg/ml of the aminoglycoside antibiotics paromomycin, neomycin, streptomycin, gentamicin and kanamycin (Huber et al., 1986). Unprecedented for bacteria, the purified ribosomes of *T. maritima* are resistant to these antibiotics, too (Londei et al., 1988). Another unusual feature of *T. maritima* is the resistance of the elongation factor EF-G to fusidic acid (Huber and Stetter, 1992a).

Cultivation

Owing to the anaerobic nature of the Thermotogales, anaerobic media must be prepared for their cultivation. First, the medium (e.g., 1 liter volume) is flushed with nitrogen for about 20 min to get rid of oxygen. Afterwards, the medium is chemically reduced by the addition of unsterile sodium sulfide and/or cysteine-HCl or after dispension in small portions (see Culture Media) from a sterile, anaerobic stock (see

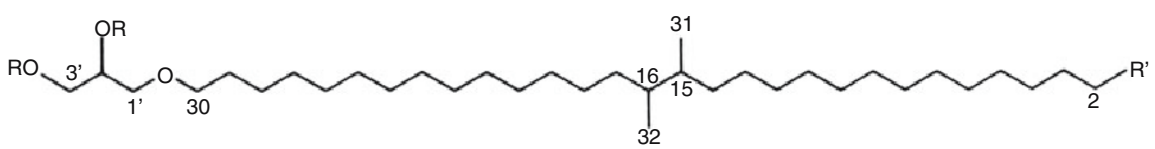

Fig. 10. The new 15,16-dimethyl-30 glyceryloxytriacontanoic acid from *Thermotoga maritima* (courtesy A. Gambacorta).

Table 4. Influence of antibiotics on growth of the Thermotogales species.

Antibiotic (μg/ml)	Ampicillin			Chloramphenicol			Cycloserine		Fusidic acid	Gentamycin		Hygromycin	Kanamycin		Nalidixic acid	Neomycin	Novobiocin	Paromomycin		Penicillin G		Phosphomycin		Polomyxin B	Rifampicin				Spectinomycin	Streptomycin			Tetracyclin	Vancomycin			
Species	10	100	150	10	25	100	10	100	10	10	200	10	10	100	100	10	10	10	10	25	100	10	100	100	1	10	25	100	150	10	25	100	10	10	25	50	100
Thermotoga maritima	n	–	n	n	n	–	n	–	n	n	n	n	n	n	n	n	n	n	n	n	–	n	–	n	n	n	n	+	n	n	n	n	n	n	n	n	–
Thermotoga neapolitana	n	n	n	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	+	n	n	n	–	n	n	n	n	–
Thermotoga thermarum	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	–	n	n	–	n	n	n	n	n	n	n	n	n
Thermotoga elfii	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Thermotoga subterranea	n	n	n	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	+	n	n	n	n	n	n	+	n	n	n	–	n	n	n	n	n
Thermotoga hypogea	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	+	n	n	n	n	n	n	n	n	n
Thermotoga petrophila	n	n	n	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	–	n	n	n	–	n	n	n	n	–
Thermotoga naphthophila	n	n	n	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	–	n	n	n	–	n	n	n	n	–
Thermotoga lettingae	n	n	n	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	+	n	n	n	n	n	n	+	n	n	n	–	n	n	n	n	n
Thermosipho africanus	n	n	n	–	n	–	n	n	n	(–)	n	(–)	(–)	n	n	(–)	n	(–)	n	n	n	–	n	n	n	+	n	–	n	–	n	n	n	–	n	n	–
Thermosipho melanesiensis	n	n	n	–	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	–	n	–	n	n	n	–	n	n	n	(+)
Thermosipho japonicus	n	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	+	–	n	–	n	n	n	–	n	n	n
Thermosipho geolei	n	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	+	n	–	n	n	n	n	n	n	n	n	+
Fervidobacterium islandicum	–	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	–	n	n	n	–	n	n	n	–	n	n	n
Fervidobacterium nodosum	n	n	n	+	n	n	+	+	n	n	n	n	n	n	n	+	n	n	+	n	n	n	n	n	n	n	n	n	n	+	n	n	–	n	n	n	n
Fervidobacterium gondwanense	n	n	n	n	n	+	n	n	n	n	n	n	n	n	n	n	+	n	+	n	n	n	+	n	+	n	n	n	+	n	+	n	+	+	n	n	n
Fervidobacterium pennivorans	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Geotoga petraea	n	n	n	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	–	n	n	n	+	n	n	n	n	–
Geotoga subterranea	n	n	n	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	–	n	n	n	+	n	n	n	n	–
Petrotoga miotherma	n	n	n	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	–	n	n	n	+	n	n	n	n	–
Petrotoga mobilis	–	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	–	n	n	n	n	n	–	n	n	n	–	n	n	n	–	n	n	n
Petrotoga olearia	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	+	n	n	n	+	n	n	n	n	n
Petrotoga sibirica	–	n	n	–	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	–	n	n	n	–	n	n	n	–	n
Marinitoga camini	n	n	n	n	–	n	n	n	n	n	n	n	n	+	n	n	n	n	n	–	n	n	n	n	n	n	n	–	n	n	–	n	n	n	–	n	n
Marinitoga piezophila	n	n	–	–	n	n	n	n	–	n	+	n	n	+	–	n	n	n	n	–	n	n	n	n	n	n	–	n	–	n	–	n	–	n	n	n	n

Symbols and abbreviations: –, growth inhibited; (–), temporary inhibition; (+), slight growth; +, growth not inhibited; and n, not reported.

Culture Media). After pH-adjustment, the media are dispensed in 10–20 ml portions in an anaerobic chamber into the culture bottles. They are closed by rubber stoppers and the gas atmosphere is changed to the desired gas mixture (see Culture Media). Thermotogales relatives grow well using 28-ml serum tubes (borosilicate glass; Schott, Mainz, Germany) or 120-ml soda-lime-silicate bottles (Stute GmbH, Rheinbreitbach, Germany). All media were autoclaved for 20 min at 121°C, and the stock-solutions were filter-sterilized.

At large scale, members of the Thermotogales are routinely grown in batch cultures, using enamel-protected fermentors with an operating volume up to 300 liters (HTE, Bioengineering, Wald, Switzerland; Huber et al., 1986). The inhibitory hydrogen gas produced during growth should be removed by continuously flushing the fermentor with nitrogen or argon gas (Huber et al., 1986; Huber et al., 2000).

Culture Media

MSH-Medium for *T. maritima* and *T. neapolitana*

KH_2PO_4	0.5 g
$(NH_4)_2SO_4$	0.5 g
$NaHCO_3$	0.1 g
Yeast extract (BD Difco™)	0.5 g
Starch	5 g
Trace mineral solution (see Trace Mineral Solution)	10 ml
Resazurin	1 mg
Artificial sea water (see Artificial Sea Water)	250 ml
$Na_2S \cdot 9H_2O$	0.5 g
H_2O, double distilled	700.0 ml

Adjust pH to 7.0 with 25% H_2SO_4 and volume to 1 liter with double-distilled water. The headspace of the culture bottles consisted of N_2 (300 kPa).

Trace Mineral Solution (modified; Balch et al., 1979)

Nitrilotriacetic acid	1.5 g
$MgSO_4 \cdot 7H_2O$	3.0 g
$MnSO_4 \cdot H_2O$	0.5 g
NaCl	1.0 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CoSO_4$	0.1 g
$CaCl_2 \cdot 2H_2O$	0.1 g
$CuSO_4 \cdot 5H_2O$	0.01 g
$AlK(SO_4)_2$	0.01 g
H_3BO_3	0.01 g
$ZnSO_4$	0.1 g
$Na_2WO_4 \cdot 2H_2O$	0.01 g
$Na_2MoO_4 \cdot 2H_2O$	0.01 g
Na_2SeO_4	0.01 g
$(NH_4)_2Ni(SO_4)_2$	0.2 g
H_2O, double distilled	950.0 ml

Adjust pH of the solution to 6.5 with 5N KOH and volume to 1 liter with double-distilled water. Store at 4°C in the dark.

Artificial Sea Water (Huber et al., 1990)

NaCl	27.7 g
$MgSO_4 \cdot 7H_2O$	7.0 g
$MgCl_2 \cdot 6H_2O$	5.5 g
KCl	0.6 g
NaBr	0.1 g
H_3BO_3	0.03 g
$SrCl_2 \cdot 6H_2O$	0.015 g
KI	0.05 mg
$CaCl_2 \cdot 2H_2O$	1.5 g
H_2O, double distilled	950.0 ml

Adjust pH to 5.5–6.0 and volume to 1 liter with double-distilled water.

LA3 Medium for *T. thermarum*

NaCl	3.46 g
$MgSO_4 \cdot 7H_2O$	0.88 g
$MgCl_2 \cdot 6H_2O$	0.69 g
KH_2PO_4	0.5 g
$CaCl_2$	0.09 g
KCl	0.08 g
NaBr	12.50 mg
H_3BO_3	3.75 mg
$SrCl_2 \cdot 6H_2O$	1.9 mg
KI	0.006 mg
$(NH_4)_2Ni(SO_4)_2$	3 mg
Trace mineral solution (see Trace Mineral Solution)	15 ml
EDTA · Na_4-salt	0.9 g
Starch	5 g
Yeast extract (BD Difco™)	0.5 g
Resazurin	1 mg
$Na_2S \cdot 9H_2O$	0.5 g
H_2O, double distilled	950.0 ml

Adjust pH to 7.0 with 25% H_2SO_4 and volume to 1 liter with double-distilled water. The headspace of the culture bottles consisted of N_2 (300 kPa).

Trace Mineral Solution (Balch et al., 1979)

Nitrilotriacetic acid	1.5 g
$MgSO_4 \cdot 7H_2O$	3.0 g
$MnSO_4 \cdot 2H_2O$	0.5 g
NaCl	1.0 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CoCl_2$	0.1 g
$CaCl_2 \cdot 2H_2O$	0.1 g
$ZnSO_4$	0.1 g
$CuSO_4 \cdot 5H_2O$	0.01 g
$AlK(SO_4)_2$	0.01 g
H_3BO_3	0.01 g
$Na_2MoO_4 \cdot 2H_2O$	0.01 g
H_2O, double distilled	950.0 ml

Adjust pH of the solution to 6.5 with 5N KOH and the volume to 1 liter with double-distilled water. Store at 4°C in the dark.

MB Medium for *T. elfii*

NH_4Cl	1.0 g
K_2HPO_4	0.3 g
KH_2PO_4	0.3 g
$MgCl_2 \cdot 6H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	0.1 g
NaCl	10.0 g
KCl	0.1 g
Sodium acetate	0.5 g

Trace mineral solution (see LA3-medium)	10 ml
Yeast extract (BD Difco™)	2.0 g
Bio-Trypcase (bioMérieux)	2.0 g
Resazurin	1 mg
Cysteine · HCl	0.5 g
$Na_2S \cdot 9H_2O$	0.4 g
Na_2CO_3	2.0 g
H_2O, double distilled	950.0 ml

Dissolve all components except the $Na_2S \cdot 9H_2O$ and Na_2CO_3. Adjust pH to 8.0 with 10M KOH and volume to 1 liter with double-distilled water. Autoclave (121°C, 20 min) the medium, then add the $Na_2S \cdot 9H_2O$ and Na_2CO_3 (from sterile, anaerobic stocks). Store or incubate medium in a gas phase of N_2/CO_2 (80:20 v/v; 200 kPa).

Medium for *T. subterranea*

NaCl	12.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
PIPES (piperazine-*N*, *N*′-bis[ethanesulfonic acid])	3.4 g
KCl	2.0 g
NH_4Cl	0.1 g
$CaCl_2 \cdot 2H_2O$	25 mg
K_2HPO_4	20 mg
Trace mineral solution (see LA3 medium)	10 ml
Vitamin solution (see Vitamin Solution	10 ml
Yeast extract	0.5 g
Peptone	1.0 g
Resazurin	1 mg
$Na_2S \cdot 9H_2O$	0.5 g
H_2O, double distilled	950.0 ml

Add the $Na_2S \cdot 9H_2O$ from a sterile, anaerobic stock to the autoclaved (121°C, 20 min) solution of all other components. Adjust the pH to 7.0 with 25% H_2SO_4 and the volume to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of N_2 (100 kPa).

Vitamin Solution (Balch et al., 1979)

Biotin	2 mg
Folic acid	2 mg
Pyridoxine · HCl	10 mg
Thiamine · HCl	5 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
DL-Calcium pantothenate	5 mg
Vitamin B_{12}	0.10 mg
p-Aminobenzoic acid	5 mg
Lipoic acid	5 mg
H_2O, double distilled	950.0 ml

Adjust volume to 1 liter with double-distilled water, filter-sterilize, and store in the dark at 4°C.

Modified MB Medium for *T. hypogea*

NaCl	10.0 g
NH_4Cl	1.0 g
K_2HPO_4	0.3 g
KH_2PO_4	0.3 g
$MgCl_2$	0.5 g
$CaCl_2$	0.1 g
KCl	0.2 g
Trace mineral solution (see LA3 Medium)	10 ml
Yeast extract (BD Difco™)	2.0 g
Bio-Trypcase (bioMérieux)	2.0 g
Xylan	10.0 g
Resazurin	1 mg
Na_2CO_3	2.0 g
$Na_2S_2O_3 \cdot 5H_2O$	20 mM
Cysteine · HCl	0.5 g
$Na_2S \cdot 9H_2O$	0.4 g
H_2O, double distilled	950.0 ml

Add the Na_2CO_3, $Na_2S_2O_3 \cdot 5H_2O$, and $Na_2S \cdot 9H_2O$ (from sterile, anaerobic stocks) to the autoclaved (121°C, 20 min) solution of the remaining components. Adjust pH to 8.0 with 10M KOH and the volume to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of N_2/CO_2 (80:20 v/v; 100 kPa).

Modified DSMZ Medium No. 664 for *T. lettingae*

NH_4Cl	1.0 g
K_2HPO_4	0.3 g
KH_2PO_4	0.3 g
$MgCl_2 \cdot 6H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	0.1 g
KCl	0.1 g
NaCl	10.0 g
Na_2CO_3	2.0 g
$Na_2S_2O_3 \cdot 5H_2O$	5.0 g
Yeast extract	0.5 g
Glucose	4.0 g
Trace mineral solution (see Trace Mineral Solution [DSMZ Medium 141])	10.0 ml
Resazurin	0.5 mg
Cysteine · HCl	0.5 g
$Na_2S \cdot 9H_2O$	0.4 g
$NaHCO_3$	2.0 g
H_2O, double distilled	950.0 ml

Add the Na_2CO_3, $Na_2S_2O_3 \cdot 5H_2O$, glucose, $Na_2S \cdot 9H_2O$, and $NaHCO_3$ (from a sterile, anaerobic stocks) to an autoclaved (121°C, 20 min) solution of the remaining components. Adjust the pH to 7.0 with 10M KOH and the volume to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of N_2/CO_2 (80:20 v/v; 100 kPa).

Trace Mineral Solution (DSMZ Medium 141)

Nitrilotriacetic acid	1.5 g
$MgSO_4 \cdot 7H_2O$	3.0 g
$MnSO_4 \cdot 2H_2O$	0.5 g
NaCl	1.0 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CoSO_4 \cdot 7H_2O$	0.18 g
$CaCl_2 \cdot 2H_2O$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.18 g
$CuSO_4 \cdot 5H_2O$	0.01 g
$AlK(SO_4)_2 \cdot 12H_2O$	0.02 g
H_3BO_3	0.01 g
$Na_2MoO_4 \cdot 2H_2O$	0.01 g
$NiCl_2 \cdot 6H_2O$	0.025 g
$Na_2SeO_3 \cdot 5H_2O$	0.3 g
H_2O, double distilled	950.0 ml

Prior to adding nitrilotriacetic acid to the other solution components, adjust its pH to 6.5 with 5N KOH. Adjust pH to 7.0 with 10 M KOH and volume to 1 liter with double-distilled water.

YE Medium for *T. petrophila* and *T. naphthophila*

Yeast extract	2.0 g
$Na_2S \cdot 9H_2O$	0.4 mM
Artificial sea water (ASW; see Artificial Sea Water)	950.0 ml

Add the $Na_2S \cdot 9H_2O$ (from a sterile, anaerobic stock) to an autoclaved (121°C, 20 min) solution of the remaining components. Adjust pH to 7.0 with 6M HCl and the volume to 1 liter with artificial sea water. Store or incubate medium in an atmosphere of N_2 (100kPa).

Artificial Sea Water (ASW; Jannasch et al., 1995)

NaCl	20.0 g
$MgCl_2 \cdot 6H_2O$	3.0 g
$MgSO_4 \cdot 7H_2O$	6.0 g
$(NH_4)_2SO_4$	1.0 g
$CaCl_2 \cdot 2H_2O$	0.3 g
KH_2PO_4	0.2 g
KCl	0.5 g
NaBr	0.05 g
H_3BO_3	0.025 g
$SrCl_2 \cdot 6H_2O$	0.02 g
Ferric ammonium citrate	0.01 g
Bis-tris-propane	2.25 g
Trace mineral solution (see Trace Mineral Solution)	10 ml
Vitamin solution (see Vitamin Solution)	10 ml
Resazurin	0.6 mg
H_2O, double distilled	950.0 ml

Adjust volume to 1 liter with double-distilled water.

Trace Mineral Solution (Wolin et al., 1963)

Nitrilotriacetic acid	1.5 g
$MgSO_4$	3.0 g
$MnSO_4$	0.5 g
NaCl	1.0 g
$FeSO_4$	0.1 g
$CoCl_2$	0.1 g
$CaCl_2$	0.1 g
$ZnSO_4$	0.1 g
$CuSO_4$	0.01 g
$AlK(SO_4)_2$	0.01 g
H_3BO_3	0.01 g
Na_2MoO_4	0.01 g
H_2O, double distilled	950.0 ml

Adjust volume to 1 liter with double-distilled water.

Vitamin Solution (Bazylinski et al., 1989)

Niacin	10 mg
Calcium pantothenate	10 mg
p-Aminobenzoic acid	10 mg
Thiamine	10 mg
Riboflavin	10 mg
Pyridoxine	10 mg
Cobalamin	10 mg
Thioctic (alpha-lipoic) acid	10 mg
Folic acid	4 mg
Biotin	4 mg
H_2O, double distilled	950.0 ml

Filter sterilize and store solution in the dark at 4°C.

MG Medium for *Ts. africanus*

NaCl	18.0 g
$MgSO_4 \cdot 7H_2O$	3.45 g
$MgCl_2 \cdot 6H_2O$	4.30 g
KCl	0.34 g
NH_4Cl	0.25 g
$CaCl_2 \cdot 2H_2O$	0.14 g
$K_2HPO_4 \cdot 3H_2O$	0.14 g
$(NH_4)_2Fe(SO_4)_2$ (0.2%)	1.0 ml
$(NH_4)_2Ni(SO_4)_2$ (0.2%)	1.0 ml
Sodium acetate $\cdot 3H_2O$	1.0 g
Trace mineral solution (see: MSH Medium)	10 ml
Vitamin solution (see: *T. subterranea* Medium)	10 ml
Yeast extract (BD Difco™)	2.0 g
Peptone (pancreatic digest of casein; Merck) (pankreatisch verdaut verwendet)	2.0 g
Resazurin (0.1%)	0.5 ml
$Na_2S \cdot 9H_2O$	0.5 g
H_2O, double distilled	950.0 ml

Add the trace mineral solution (from a sterile, anaerobic stock) to an autoclaved (121°C, 20 min) solution of the remaining components.Adjust pH to 6.5 with 25% formic acid and volume to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of N_2 (300 kPa).

DSMZ Medium No. 343 for *Ts. melanesiensis*

NaCl	20.0 g
KH_2PO_4	0.5 g
$NiCl_2 \cdot 6H_2O$	2.0 mg
Trace mineral solution (see *T. lettingae* Medium)	15.0 ml
Yeast extract (BD Difco™)	0.5 g
Starch	5.0 g
Resazurin	1.0 mg
$Na_2S \cdot 9H_2O$	0.5 g
Artificial sea water (see: MSH Medium)	250 ml
H_2O, double distilled	700.0 ml

Adjust pH to 6.5 with 25% formic acid and volume to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of N_2 (100 kPa).

MJDYP Medium for *Ts. japonicus*

Yeast extract	2.0 g
Trypticase peptone	2.0 g
Resazurin	1.0 mg
$Na_2S \cdot 9H_2O$	0.5 g
MJD synthetic water (see MJD Synthetic Water)	1000.0 ml

Adjust pH to 7.5 with 6N HCl. Store or incubate medium in an atmosphere of N_2 (200 kPa).

MJD Synthetic Water

NaCl	30.0 g
K_2HPO_4	0.14 g
$CaCl_2 \cdot 2H_2O$	0.14 g
$MgSO_4 \cdot 7H_2O$	3.4 g
$MgCl_2 \cdot 6H_2O$	4.18 g
KCl	0.33 g
$NiCl_2 \cdot 6H_2O$	0.5 mg

$Na_2SeO_3 \cdot 5H_2O$	0.5 mg
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.01 g
Trace mineral solution (see LA3 Medium)	10 ml
DHV mineral solution (see DHV Mineral Solution)	1 ml
H_2O, double distilled	950.0 ml

Adjust volume to 1 liter with double-distilled water.

DHV Mineral Solution

$Na_2SiO_3 \cdot 9H_2O$	2.3 g
$SrCl_2 \cdot 6H_2O$	1.1 g
$CoSO_4 \cdot 7H_2O$	1.1 g
$Na_2MoO_4 \cdot 2H_2O$	0.97 g
$MnSO_4 \cdot 2H_2O$	0.96 g
$NiCl_2 \cdot 6H_2O$	0.95 g
$Na_2WO_4 \cdot 2H_2O$	0.66 g
$ZnSO_4 \cdot 7H_2O$	0.58 g
$CuSO_4 \cdot 5H_2O$	0.50 g
$VSO_4 \cdot xH_2O$	0.25 g
Na_2SeO_3	0.17 g
$LiSO_4 \cdot H_2O$	0.13 g
H_2O, double distilled	950.0 ml

Adjust volume to 1 liter with double-distilled water.

Medium for *Ts. geolei*

NaCl	15.0 g
$MgCl_2 \cdot 6H_2O$	0.5 g
PIPES	3.4 g
KCl	0.2 g
NH_4Cl	1.0 g
$CaCl_2 \cdot 2H_2O$	0.1 g
K_2HPO_4	0.35 g
KH_2PO_4	0.35 g
Sodium acetate $\cdot 3H_2O$	2.72 g
Yeast extract	2.0 g
Bio-Trypcase (bioMérieux)	2.0 g
Resazurin	1.0 mg
Maltose	2.0 g
$Na_2S \cdot 9H_2O$	1.0 g
H_2O, double distilled	950.0 ml

Add the maltose and $Na_2S \cdot 9H_2O$ (from sterile, anaerobic stocks) to an autoclaved (121°C, 20 min) solution of the remaining components. Adjust pH to 7.0 with 5M HCl and volume to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of N_2 (100 kPa).

TYEG Medium for *F. nodosum*, *F. islandicum* and *F. gondwanense*

NH_4Cl	0.9 g
$MgCl_2 \cdot 6H_2O$	0.2 g
KH_2PO_4	0.75 g
K_2HPO_4	1.5 g
$FeSO_4 \cdot 7H_2O$ (10%)	0.03 ml
Trace mineral solution (see Trace Mineral Solution)	9.0 ml
Vitamin solution (see *T. subterranea* Medium)	5.0 ml
Yeast extract (BD Difco™)	3.0 g
Peptone (from tryptically digested meat; Merck)	10.0 g
Glucose	5.0 g
Resazurin (0.2%)	1.0 ml
$Na_2S \cdot 9H_2O$	1.0 g
H_2O, double distilled	950.0 ml

Add the vitamin solution and glucose (from a sterile, anaerobic stock) to an autoclaved (121°C, 20 min) solution of the remaining components. Adjust pH to 7.2 with 25% H_2SO_4 and the volume up to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of N_2 (300 kPa).

Trace Mineral Solution (Patel et al., 1985a)

Nitrilotriacetic acid	12.5 g
$FeCl_3 \cdot 4H_2O$	0.2 g
$MnCl_2 \cdot 4H_2O$	0.1 g
$CoCl_2 \cdot 6H_2O$	0.017 g
$CaCl_2 \cdot 2H_2O$	0.1 g
$ZnCl_2$	0.1 g
$CuCl_2$	0.02 g
H_3BO_3	0.01 g
$Na_2MoO_4 \cdot 2H_2O$	0.01 g
NaCl	1.0 g
Na_2SeO_3	0.02 g
H_2O, double distilled	950.0 ml

Prior to adding to the trace mineral solution, adjust pH of the nitrilotriacetic acid to 6.5 with 5N KOH. Adjust pH of the trace mineral solution to 6.5 with 5N KOH and the volume of the trace mineral solution to 1 liter with double-distilled water. Store medium at 4°C in the dark.

TF Medium for *F. pennivorans*

NH_4Cl	0.50 g
$MgSO_4 \cdot 7H_2O$	0.16 g
K_2HPO_4	1.6 g
$NaH_2PO_4 \cdot H_2O$	1.0 g
$CaCl_2 \cdot 2H_2O$	0.06 g
Trace mineral solution (see: LA3 Medium)	10 ml
Vitamin solution (see *T. subterranea* Medium)	10 ml
Yeast extract	2.0 g
Trypticase	2.0 g
Resazurin	1 mg
$Na_2S \cdot 9H_2O$	0.3 g
Cysteine $\cdot$ HCl	0.3 g
Glucose or starch	3.0 g
H_2O, double distilled	950.0 ml

Add the $CaCl_2 \cdot 2H_2O$ (from a sterile, anaerobic stock) to an autoclaved (121°C, 20 min) solution of the remaining components. Adjust pH to 6.8 with 10N KOH and the volume up to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of N_2.

MSH Medium for *P. miotherma*, *G. petraea* and *G. subterranea*

See MSH Medium for *T. maritima* and *T. neapolitana*

Medium for *P. mobilis*

NaCl	30.0 g
$MgSO_4 \cdot 7H_2O$	7.0 g
KCl	0.34 g
NH_4Cl	0.25 g
$CaCl_2 \cdot 2H_2O$	0.14 g
KH_2PO_4	0.14 g
Yeast extract (BD Difco™)*	0.2 g
Trace element solution SL-10 (see Trace Element Solution SL-10)	1.0 ml
Vitamin solution (see *T. subterranea* Medium)*	10 ml

Resazurin (0.02%)	0.5 ml
$Na_2S \cdot 9H_2O$ (0.5 M)	4 ml
H_2O, double distilled	950.0 ml

Add the yeast extract and the vitamin solution (from a sterile, anaerobic stock) to an autoclaved (121°C, 20 min) solution of the remaining components. Adjust pH to 6.5 with 6M HCl and the volume up to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of argon (200 kPa).

Trace Element Solution SL-10 (Widdel et al., 1983)

HCl (25% w/w, 7.7 M)	10 ml
$FeCl_2 \cdot 4H_2O$	1.5 g
$CoCl_2 \cdot 6H_2O$	190 mg
$MnCl_2 \cdot 4H_2O$	100 mg
$ZnCl_2$	70 mg
H_3BO_3	6 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$CuCl_2 \cdot 2H_2O$	2 mg
H_2O, double distilled	950.0 ml

Adjust volume up to 1 liter with double-distilled water.

Medium for *P. olearia* and *P. sibirica*

NaCl	25.0 g
$MgCl_2 \cdot 6H_2O$	0.5 g
PIPES	3.4 g
KCl	0.2 g
NH_4Cl	1.0 g
$CaCl_2 \cdot 2H_2O$	0.1 g
K_2HPO_4	0.35 g
KH_2PO_4	0.35 g
Peptone	1.5 g
Tryptone	1.5 g
Yeast extract	1.5 g
Maltose	2.0 g
Resazurin	1.0 mg
$Na_2S \cdot 9H_2O$	1.0 g
H_2O, double distilled	950.0 ml

Add the maltose and $Na_2S \cdot 9H_2O$ (from sterile, anaerobic stocks) to an autoclaved (121°C, 20 min) solution of the remaining components. Adjust pH to 7.0 with 5M HCl and the volume up to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of N_2 (100 kPa).

YPCS Medium for *M. camini*

Yeast extract (BD Difco™)	0.5 g
Peptone (BD Difco™)	1.0 g
Cellobiose (Sigma-Aldrich)	5.0 g
Sea salt (Sigma-Aldrich)	30.0 g
PIPES buffer (Sigma-Aldrich)	6.05 g
Resazurin	1.0 mg
$Na_2S \cdot 9H_2O$	0.5 g
H_2O, double distilled	950.0 ml

Adjust pH to 7.5 with 5M HCl and the volume up to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of $N_2/H_2/CO_2$ (90:5:5 v/v/v, 100 kPa).

RC+ Medium for *M. piezophila*

NaCl	30.0 g
MES (2-[*N*-morpholino]ethanesulfonic acid); Sigma-Aldrich)	10 mM
NH_4Cl	1.0 g
K_2HPO_4	0.3 g
$MgCl_2 \cdot 6H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	0.1 g
KCl	0.1 g
Sodium acetate $\cdot 3H_2O$	0.83 g
Yeast extract (BD Difco™)	5.0 g
Bio-Trypcase (BD Difco™)	5.0 g
Maltose (Sigma-Aldrich)	20 mM
L-Cystine	12.0 g
Resazurin	1.0 mg
$Na_2S \cdot 9H_2O$	0.5 g
H_2O, double distilled	950.0 ml

Add the L-cystine and $Na_2S \cdot 9H_2O$ (from sterile, anaerobic stocks) to an autoclaved (121°C, 20 min) solution of the remaining components. Adjust pH to 6.0 with 5M HCl and the volume up to 1 liter with double-distilled water. Store or incubate medium in an atmosphere of $N_2/H_2/CO_2$ (90:5:5 v/v/v, 100 kPa up to 40 MPa).

Preservation

Under anaerobic conditions, members of the Thermotogales can be stored for several months at 4°C. For long-term preservation, storage at –140°C in liquid nitrogen in the presence of 5% dimethylsulfoxide is recommended (Malik, 1999). No loss of cell viability of *Thermotoga*, *Thermosipho* and *Fervidobacterium* was observed after storage over a period of more than ten years. *Thermotoga subterranea* was stored at –80°C in the same medium containing 20% (w/v) glycerol (Jeanthon et al., 1995).

Physiology

Members of the Thermotogales are strictly anaerobic, extremely thermophilic or hyperthermophilic bacteria with optimal growth in the neutral pH range (Huber and Stetter, 1999; Table 2). With an optimal growth temperature around 80°C and a maximum growth temperature of 90°C, the hyperthermophilic species *T. maritima* and *T. neaplitana* represent the organisms with the highest growth temperatures within the order. Growth of some Thermotogales species is restricted to low salinity, while other species exhibit a broad salt tolerance, reflecting their adaptation to the natural biotope (Huber and Stetter, 1992a; Huber and Stetter, 1999; Table 2). Detailed physiological properties of the Thermotogales are listed in Table 2.

All members of the Thermotogales are strict organotrophs, fermenting preferentially simple and complex carbohydrates or complex organic matter (Table 3). When grown on defined carbon sources, the addition of yeast extract and/or peptone/bio-trypticase to the medium is often stimulatory or required for growth (Table 3; see

also Culture Media). In addition, cell homogenates of bacteria (e.g., *Lactobacillus bavaricus*) and archaea (e.g., *Pyrodictium brockii*) can be used as substrates (Huber et al., 1986). Furthermore, *T. lettingae* is able to degrade methanol, but only in the presence of yeast extract, which is required for growth (Balk et al., 2002).

With glucose as growth substrate, Thermotogales representatives form L(+)-lactate, acetate, ethanol, L-alanine, carbon dioxide, and hydrogen as major final products. Some of the isolates form traces of isovaleric acid, isobutyric acid, alpha-aminobutyrate, hydroxy-phenylacetate and phenylacetate in addition. The L-alanine production of members of the Thermotogales is a trait in common with members of the archaeal order Thermococcales. Therefore, it has been proposed that L-alanine production from sugar fermentation is a remnant of an ancestral metabolism (Ravot et al., 1996a). The pathway of arginine synthesis is the only biosynthetic reaction studied in the Thermotogales so far. *Thermotoga maritima* generates arginine from glutamate via *N*-acetylated intermediates in an eight-step pathway, as in mesophilic bacteria (Van de Casteele et al., 1990).

Thermotoga maritima degrades glucose mainly via the Embden-Meyerhof glycolytic pathway and, to a lesser extent, via the Entner-Doudoroff pathway (Schröder et al., 1994; Selig et al., 1997). In *T. neapolitana*, it was shown that D-glucose is taken up via an active transport system, energized by an ion gradient. This gradient is generated by ATP, derived from substrate-level phosphorylation (Galperin et al., 1996). The role of this gradient is not known since glucose (and maltose) periplasmic binding proteins appear to be involved in transport (Nanavati et al., 2002).

Hydrogen, which accumulates during fermentation processes, can be a potent inhibitor of growth of the Thermotogales. This inhibition can be overcome by the addition of sulfur or inorganic sulfur-containing compounds, depending on the species. Under these culture conditions, H_2S is formed as final product. Therefore, H_2S formation may be a kind of detoxification reaction of H_2 (Huber et al., 1986; Huber et al., 1992a). A general trait of different Thermotogales is the production of H_2S when grown in the presence of thiosulfate, except for *Ts. geolei*, *M. piezophila*, *P. olearia* and *P. sibirica* (Ravot et al., 1995; L'Haridon et al., 2001; L'Haridon et al., 2002; Alain et al., 2002). In addition to removal by sulfurous compounds, hydrogen can be removed by gassing the media with nitrogen or argon (Huber et al., 1986; Huber and Stetter, 1999) or by interspecies hydrogen transfer during cocultivation of the Thermotogales with hydrogen-consuming hyperthermophiles (e.g., *Methanococcus*, *Methanopyrus*, *Archaeoglobus* or *Ferroglobus*; Huber et al., 2000).

Recently, respiratory growth of *T. maritima* had been reported, with hydrogen as electron donor and Fe(III) as the electron acceptor, forming Fe(II) as final product (Vargas et al., 1998). In contrast, growth of *T. maritima* with similar doubling times and final cell densities, in the absence of both hydrogen and Fe(III) in the same culture medium, was observed (Huber and Stetter, 2001a). Furthermore, when *T. maritima* was cultivated in the presence of hydrogen (100 kPa) and Fe(III), Fe(II) was formed without obvious growth stimulation (Huber and Stetter, 2001a). These results indicate that *T. maritima* may not gain energy by iron-respiration but may use Fe(III) in place of sulfur as an additional electron sink to get rid of inhibitory hydrogen during fermentation (Huber et al., 1986; Schröder et al., 1994).

Different low-molecular-weight organic compounds, serving as compatible solutes, are present in the different members of the Thermotogales (Martins et al., 1996; Ramakrishnan et al., 1997). However, the accumulation of solutes in response to either salt stress or supraoptimal temperatures is restricted to Thermotogales species that grow in saline media containing over 0.4% sodium chloride (Martins et al., 1996).

An enormous variety of enzymes (and proteins) from the order Thermotogales, especially *T. maritima*, has been isolated and intensively studied since the late 1980s. In general, these enzymes exhibit a high thermostability, with denaturation temperatures often above the optimum growth temperature of the bacterium. Despite the strict anaerobic nature of *T. maritima*, these enzymes are apparently not oxygen sensitive. A comprehensive overview of the biochemistry and biophysics of these "hyperthermophilic enzymes and proteins" was published recently in volumes 330, 331 and 334 of *Methods in Enzymology* (2001).

Genetics

So far, the only plasmids discovered in the Thermotogales are in *Thermotoga* sp. RQ7, an isolate obtained from the hot sea-floor of Ribeira Quente (the Azores), and in *Thermotoga maritima* MC24, an isolate from hydrothermal vents near the Kuril Islands (Huber et al., 1986; Harriott et al., 1994; Akimkina et al., 1999). These small cryptic miniplasmids, pRQ7 and pMC24, are very closely related and were shown to be the smallest natural replicon so far described (846 bp). Both were shown to be negatively supercoiled (Harriott et al., 1994; Akimkina et al., 1999). The plasmid pRQ7 replicates by the

rolling-circle mechanism (Yu and Noll, 1997). With pRQ7, a vector system was developed and a genetic transformation of *T. maritima* and *T. neapolitana* spheroplasts was achieved using cationic liposomes (Yu et al., 2001).

For genetic studies, auxotrophic and antimetabolite-resistant mutants of *T. neapolitana* have been isolated by the use of mutagenic agents (Vargas and Noll, 1994).

The rather small, 1.8-Mb genome from *T. maritima* has been sequenced at The Institute for Genomic Research (TIGR; Rockville, MD, USA; Nelson et al., 1999). It is a single circular chromosome and contains 1877 predicted coding regions. Fifty-four percent of these coding regions have functional assignments, while 46% are of unknown function (Nelson et al., 1999; Nelson et al., 2001). Genome analysis has identified metabolic pathways involved in the degradation of sugar and plant polysaccharides. About 7% of the predicted codon sequences in the genome are involved in the metabolism of simple and complex sugars, a percentage more than twice that seen in the genomes of other bacteria or archaea (Nelson et al., 2001). The metabolism and transport in *T. maritima* (Fig. 11) were elucidated from the genome sequence data.

Comparison of total genome sequences of different organisms showed that about 24% of the *T. maritima* genes are of archaeal origin (Nelson et al., 1999). Therefore, the authors have claimed that hyperthermophilic archaea and bacteria have often exchanged genes, particularly by lateral gene transfer (Aravind et al., 1998; Nelson et al., 1999; see chapter on Aquificales in this Volume). On the other hand, it was argued that if *Thermotoga* (and *Aquifex*; see chapter on Aquificales in this Volume) are truly deep branching, it is equally parsimonious to suppose that some of the genes are primitive features shared by these hyperthermophilic bacteria with archaea. They have retained ancestral genes that have since been lost in most bacteria as they adapted to mesophilic environments (Kyrpides and Olson, 1999; Logsdon and Faguy, 1999). On the basis of recent phylogenetic analyses of archaeal genes in different bacteria, it was hypothesized that lateral gene transfer in hyper-

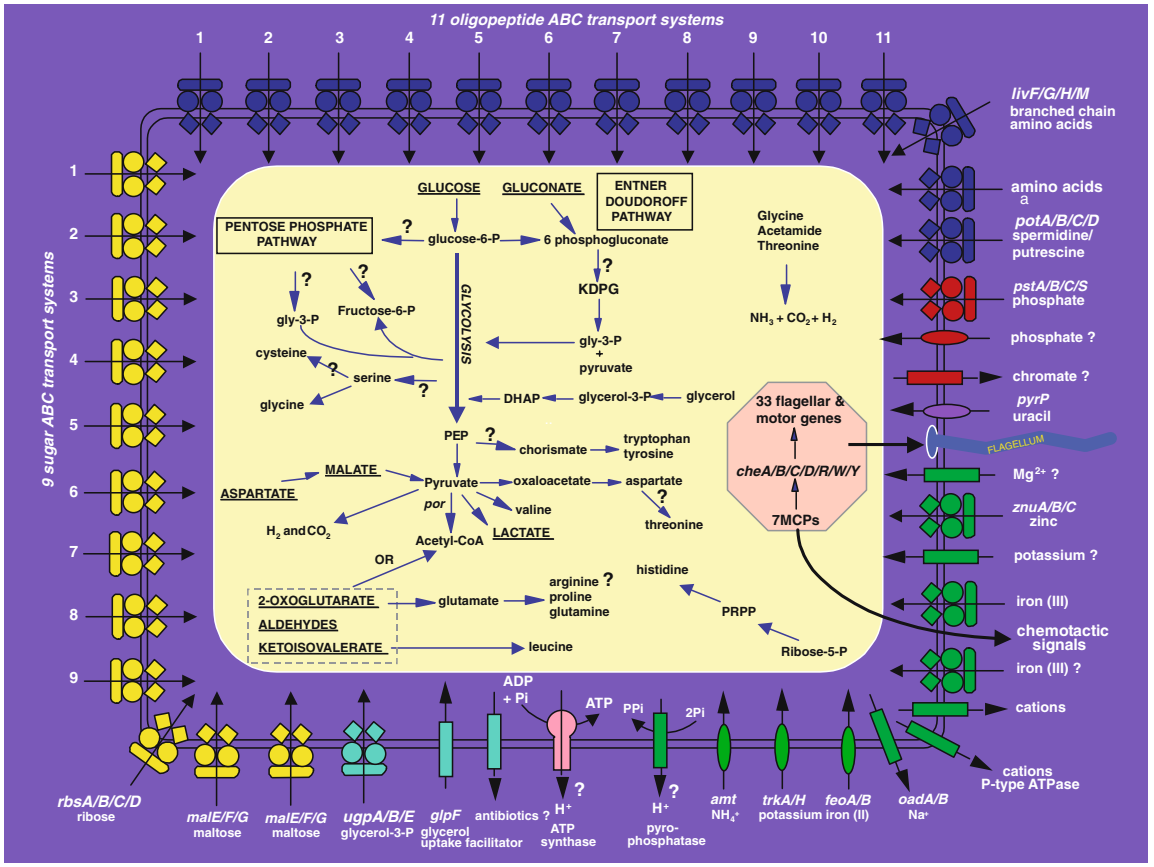

Fig. 11. Overview of metabolism and transport in *Thermotoga maritima*. Pathways for energy production and the metabolism of organic compounds, acids and aldehydes are shown. (From Nelson et al., 1999, with permission from Nature, http://www.nature.com.)

thermophiles may be as much the consequence as the cause of adaptation to hyperthermophily (Nesbø et al., 2001). By using suppressive subtractive hybridization, extensive genomic diversity in *T. maritima* was reported recently and frequent lateral gene transfer within the order Thermotogales was suggested (Nesbø et al., 2002).

Very recently, a high-throughput structural genomics pipeline and its application to the proteome of *T. maritima* were designed and implemented. By using this pipeline, 1376 of the predicted 1877 genes (73%) were successfully cloned, their expression was attempted, and the crystallization conditions for 432 proteins (23% of the *T. maritima* proteome) were identified (Lesley et al., 2002).

Biotechnology

Enzymes active at high temperatures are sought for industrial processes. Intra- and extracellular enzymes of Thermotogales are highly thermostable and might be of great interest as biocatalysts, e.g., in the chemical or food industry. A recombinant xylanase from *T. maritima* was shown to be active for several hours at 100°C and efficient in releasing lignin from kraft pulp (Chen et al., 1997). Therefore, such xylanases have a high potential for use in the pulp and paper industry. Highly thermostable amylases might be used in starch processing and high-temperature glucose isomerases in the production of corn syrup. From *T. maritima*, the recombinant Ultma™ DNA polymerase with proofreading activities is commercially available. The 70-kDa enzyme is highly thermostable and has a half-life of 40–50 min at 97.5°C. In addition, the sequencing of the total genome of *T. maritima* makes available the use of its genes as tools for a variety of biotechnological applications (Nelson et al., 2001).

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Literature Cited

Achenbach-Richter, L., R. Gupta, K. O. Stetter, and C. R. Woese. 1987. Were the original eubacteria thermophiles? Syst. Appl. Microbiol. 9:34–39.

Akimkina, T., P. Ivanov, S. Kostrov, T. Sokolova, E. Bonch-Osmolovskaya, K. Firman, C. F. Dutta, and J. A. McClellan. 1999. A highly conserved plasmid from the extreme thermophile Thermotoga maritima MC24 is a member of a family of plasmids distributed worldwide. Plasmid 42:236–240.

Alain, K., V. T. Marteinsson, M. L. Miroshnichenko, E. A. Bonch-Osmolovskaya, D. Prieur, and J. L. Birrien. 2002. Marinitoga piezophila sp. nov., a rod-shaped, thermopiezophilic bacterium isolated under high hydrostatic pressure from a deep-sea hydrothermal vent. Int. J. Syst. Evol. Bacteriol. 52:1331–1339.

Andrews, K. T., and B. K. C. Patel. 1996. Fervidobacterium gondwanense sp. nov., a new thermophilic anaerobic bacterium isolated from nonvolcanically heated geothermal waters of the Great Artesian Basin of Australia. Int. J. Syst. Bacteriol. 46:265–269.

Antoine, E., V. Cilia, J. R. Meunier, J. Guezennec, F. Lesongeur, and G. Barbier. 1997. Thermosipho melanesiensis sp. nov., a new thermophilic anaerobic bacterium belonging to the order Thermotogales, isolated from deep-sea hydrothermal vents in the southwestern pacific ocean. Int. J. Syst. Bacteriol. 47:1118–1123.

Aravind, L., R. L. Tatusov, Y. I. Wolf, D. R. Walker, and E. V. Koonin. 1998. Evidence for massive gene exchange between archaeal and bacterial hyperthermophiles. Trends Genet. 14:442–444.

Bachleitner, M., W. Ludwig, K. O. Stetter, and K.-H. Schleifer. 1989. Nucleotide sequence of the gene coding for the elongation factor Tu from the extremely thermophilic eubacterium Thermotoga maritima. FEMS Microbiol. Lett. 48:115–120.

Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. 43:260–296.

Balk, M., J. Weijma, and J. M. Stams-Alfons. 2002. Thermotoga lettingae sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int. J. Syst. Evol. Bacteriol. 52:1361–1368.

Bazylinski, D. A., C. O. Wirsen, and H. W. Jannasch. 1989. Microbial utilization of naturally occuring hydrocarbons at the Guaymas Basin hydrothermal vent site. Appl. Environ. Microbiol. 55:2832–2836.

Beck, P., and R. Huber. 1997. Detection of cell viability in cultures of hyperthermophiles. FEMS Microbiol. Lett. 147:11–14.

Belkin, S., C. O. Wirsen, and H. W. Jannasch. 1986. A new sulfur-reducing, extremely thermophilic eubacterium from a submarine thermal vent. Appl. Environ. Microbiol. 51:1180–1185.

Bilwes, A. M., L. A. Alex, B. R. Crane, and M. I. Simon. 1999. Structure of CheA, a signal-transducing histidine kinase. Cell 96:131–141.

Blamey, J., S. Mukund, and M. W. W. Adams. 1994. Properties of a thermostable 4Fe-ferredoxin from the hyperthermophilic bacterium Thermotoga maritima. FEMS Microbiol. Lett. 121:165–170.

Brochier, C., and H. Phillipe. 2002. A non-hyperthermophilic ancestor for bacteria. Nature 417:244.

Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller. 1981. Gene organization and primary structure of a ribosomal RNA operon from Escherichia coli. J. Molec. Biol. 148: 107–127.

Brown, P. N., C. P. Hill, and D. F. Blair. 2002. Crystal structure of the middle and C-terminal domains of the flagellar rotor protein FliG. EMBO J. 21:3225–3234.

Carballeira, N. M., M. Reyes, A. Sostre, H. Huang, M. F. J. M. Verhagen, and M. W. W. Adams. 1997. Unusual fatty acid compositions of the hyperthermophilic archaeon Pyrococcus furiosus and the bacterium Thermotoga maritima. J. Bacteriol. 179:2766–2768.

Chen, C.-C., R. Adolphson, J. F. D. Dean, K.-E. L. Eriksson, M. W. W. Adams, and J. Westpheling. 1997. Release of lignin from kraft pulp by a hyperthermophilic xylanase from Thermotoga maritima. Enz. Microb. Technol. 20:39–45.

Childers, S. E., M. Vargas, and K. M. Noll. 1992. Improved methods for cultivation of the extremely thermophilic bacterium Thermotoga neapolitana. Appl. Environ. Microbiol. 58:3949–3953.

Cordell, C., R. E. Anderson, and J. Löwe. 2001. Crystal structure of the bacterial cell division inhibitor MinC. EMBO J. 20:2454–2461.

Darimont, B., and R. Sterner. 1994. Sequence, assembly and evolution of a primordial ferredoxin from Thermotoga maritima. EMBO J. 13:1772–1781.

Davey, M. E., W. A. Wood, R. Key, K. Nakamura, and D. A. Stahl. 1993a. Isolation of three species of Geotoga and Petrotoga: Two new genera, representing a new lineage in the bacterial line of descent distantly related to the "Thermotogales." Syst. Appl. Microbiol. 16:191–200.

Davey, M. E., W. A. Wood, R. Key, K. Nakamura, and D. A. Stahl. 1993b. Validation of the publication of new names and new combinations previously effectively published outside the IJSB: List No. 47. Int. J. Syst. Bacteriol. 43:864–865.

Davey, M. E., B. J. MacGregor, and D. A. Stahl. 2001a. Genus III: Geotoga. *In:* D. R. Boone and R. W.Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:377–381.

Davey, M. E., B. J. MacGregor and D. A. Stahl. 2001b. Genus IV: Petrotoga. *In:* D. R. Boone and R. W.Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:372–385.

De Rosa, M., A. Gambacorta, R. Huber, V. Lanzotti, B. Nicolaus, K. O. Stetter, and A. Trincone. 1988. A new 15,16-dimethyl-30-glyceroloxytriacontanoic acid from lipids of Thermotoga maritima. J. Chem. Soc. Chem. Commun. 1300–1301.

Engel, A. M., Z. Cejka, A. Lupas, F. Lottspeich, and W. Baumeister. 1992. Isolation and cloning of Omp alpha, a coiled-coil protein spanning the periplasmic space of the ancestral eubacterium Thermotoga maritima. EMBO J. 11:4369–4378.

Engel, A. M., M. Brunen, and W. Baumeister. 1993. The functional properties of Omp beta, the regularely arrayed porin of the hyperthermophilic bacterium Thermotoga maritima. FEMS Microbiol. Lett. 109: 231–236.

Fardeau, M. L., B. Ollivier, B. K. C. Patel, M. Magot, P. Thomas, A. Rimbault, F. Rocchiccioli, and J. L. Garcia. 1997. Thermotoga hypogea sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. Int. J. Syst. Bacteriol. 47:1013–1019.

Fitz-Gibbon, S. T., and C. H. House. 1999. Whole genome-based phylogenetic analysis of free-living microorganisms. Nucleic Acids Res. 27:4218–4222.

Friedrich, A. B., and G. Antranikian. 1996. Keratin degradation by Fervidobacterium pennavorans, a novel thermophilic anaerobic species of the order Thermotogales. Appl. Environ. Microbiol. 62:2875–2882.

Friedrich, A. B., and G. Antranikian. 1999. Validation of the publication of new names and new combinations previously effectively published outside the IJSB: List No. 68. Int. J. Syst. Bacteriol. 49:1–3.

Galperin, M. Y., K. M. Noll, and A. H. Romano. 1996. The glucose transport system of the hyperthermophilic anaerobic bacterium Thermotoga neapolitana. Appl. Environ. Microbiol. 62:2915–2918.

Gluch, M. F., D. Typke, and W. Baumeister. 1995. Motility and thermotactic responses of Thermotoga maritima. J. Bacteriol. 177:5473–5479.

Grassia, G. S., K. M. McLean, P. Glenat, J. Bauld, and A. J. Sheehy. 1996. A systematic survey for thermophilic fermentative bacteria and archaea in high temperature petroleum reservoirs. FEMS Microbiol. Ecol. 21:47–58.

Gupta, R. S., and E. Griffiths. 2002. Critical issues in bacterial phylogeny. Theor. Pop. Biol. 61:423–434.

Harriott, O. T., R. Huber, K. O. Stetter, P. W. Betts, and K. M. Noll. 1994. A cryptic miniplasmid from the hyperthermophilic bacterium Thermotoga sp. strain RQ7. J. Bacteriol. 176:2759–2762.

Huber, R., T. A. Langworthy, H. König, M. Thomm, C. R. Woese, U. B. Sleytr, and K. O. Stetter. 1986. Thermotoga maritima sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch. Microbiol. 144:324–333.

Huber, R., C. R. Woese, T. A. Langworthy, H. Fricke, and K. O. Stetter. 1989a. Thermosipho africanus gen. nov., represents a new genus of thermophilic eubacteria within the "Thermotogales." Syst. Appl. Microbiol. 12:32–37.

Huber, R., C. R. Woese, T. A. Langworth, H. Fricke, and K. O. Stetter. 1989b. Validation of the publication of new names and new combinations previously effectively published outside the IJSB: List No. 31. Int. J. Syst. Bacteriol. 39:495–497.

Huber, R., C. R. Woese, T. A. Langworthy, J. K. Kristjansson, and K. O. Stetter. 1990. Fervidobacterium islandicum sp. nov., a new extremely thermophilic eubacterium belonging to the "Thermotogales." Arch. Microbiol. 154:105–111.

Huber, R., C. R. Woese, T. A. Langworth, J. K. Kristjansson, and K. O. Stetter. 1991. Validation of the publication of new names and new combinations previously effectively published outside the IJSB: List No. 36. Int. J. Syst. Bacteriol. 41:178–179.

Huber, R., and K. O. Stetter. 1992a. Hyperthermophilic and extremely thermophilic bacteria. *In:* J. K. Kristjansson (Ed.) Thermophilic Bacteria. CRC Press. Boca Raton, FL. 185–194.

Huber, R., and K. O. Stetter. 1992b. The order Thermotogales. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. 3809–3815.

Huber, R., S. Burggraf, T. Mayer, S. M. Barns, P. Rossnagel, and K. O. Stetter. 1995. Isolation of a hyperthermophilic archaeum predicted by in situ RNA analysis. Nature 376:57–58.

Huber, R. 1999. Die Laserpinzette als Basis für Einzelzellkultivierungen. BIOspektrum 5:289–291.

Huber, R. and K. O. Stetter. 1999. Thermotogales. Embryonic Encyclopedia of Life Sciences (http://www.els.net). Nature Publishing Group. London, UK.

Huber, R., H. Huber, and K. O. Stetter. 2000. Towards the ecology of hyperthermophiles: Biotopes, new isolation strategies and novel metabolic properties. FEMS Microbiol. Rev. 24:615–623.

Huber, R., and K. O. Stetter. 2001a. Discovery of hyperthermophilic microorganisms. *In:* M. W. W. Adams and R. M. Kelly (Eds.) Methods in Enzymology. Academic Press. San Diego, CA. 11–24.

Huber, R., and K. O. Stetter. 2001b. Thermotogales. *In:* D. R. Boone and R.W.Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 1:369–387.

Jannasch, H. W., R. Huber, S. Belkin, and K. O. Stetter. 1988. Thermotoga neapolitana sp. nov. of the extremely thermophilic, eubacterial genus Thermotoga. Arch. Microbiol. 150:103–104.

Jannasch, H. W., R. Huber, S. Belkin, and K. O. Stetter. 1989. Validation of the publication of new names and new combinations previously effectively published outside the IJSB: List No. 28. Int. J. Syst. Bacteriol. 39:93–94.

Jannasch, H. W., C. O. Wirsen, and T. Hoaki. 1995. Isolation and cultivation of heterotrophic hyperthermophiles from deep-sea hydrothermal vents. *In:* F.R. Robb and A.R. Place (eds.) Archaea: A Laboratory Manual, Protocol 1. Cold Spring Harbor Laboratory Press. US. 9–13.

Jeanthon, C., A. L. Reysenbach, S. L'Haridon, A. Gambacorta, N. R. Pace, P. Glénat, and D. Prieur. 1995. Thermotoga subterranea sp. nov., a new thermophilic bacterium isolated from a continental oil reservoir. Arch. Microbiol. 164:91–97.

Jeanthon, C., A.-L. Reysenbach, S. L'Haridon, A. Gabacorta, N. R. Pace, P. Glénat, and D. Prieur. 2000. Validation of the publication of new names and new combinations previously effectively published outside the IJSB: List No. 76. Int. J. Syst. Evol. Bacteriol. 50:1699–1700.

Kyrpides, N. C., and G. J. Olsen. 1999. Archaeal and bacterial hyperthermophiles: Horizontal gene exchange or common ancestry? Trends Genet. 15:298–299.

Lesley, S. A., P. Kuhn, A. Godzik, A. M. Deacon, I. Mathews, A. Kreusch, G. Spraggon, H. E. Klock, D. McMullan, T. Shin, J. Vincent, A. Robb, L. S. Brinen, M. D. Miller, T. M. McPhillips, M. A. Miller, D. Scheibe, J. M. Canaves, C. Guda, L. Jaroszewski, T. L. Selby, M. A. Elsliger, J. Wooley, S. S. Taylor, K. O. Hodgson, I. A. Wilson, P. G. Schultz, and R. C. Stevens. 2002. Structural genomics of the Thermotoga maritima proteome implemented in a high-throughput structure determination pipeline. Proc. Natl. Acad. Sci. USA 99:11664–11669.

Leuschner, C., and G. Antranikian. 1994. Heat-stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. World J. Microbiol. Biotechnol. 11:95–114.

L'Haridon S., A. L. Reysenbach, P. Glenat, D. Prieur, and C. Jeanthon. 1995. Hot subterranean biosphere in a continental oil reservoir. Nature 377:223–224.

L'Haridon S., M. L. Miroshnichenko, H. Hippe, M. L. Fardeau, O. E. Bonch, E. Stackebrandt, and C. Jeanthon. 2001. Thermosipho geolei sp. nov., a thermophilic bacterium isolated from a continental petroleum reservoir in Western Siberia. Int. J. Syst. Evol. Bacteriol. 51:1327–1334.

L'Haridon S., M. L. Miroshnichenko, H. Hippe, M. L. Fardeau, E. A. Bonch-Osmolovskaya, E. Stackebrandt, and C. Jeanthon. 2002. Petrotoga olearia sp. nov. and Petrotoga sibirica sp. nov., two thermophilic bacteria isolated from a continental petroleum reservoir in West Siberia. Int. J. Syst. Evol. Bacteriol. 52:1715–1722.

Lien, T., M. Madsen, and F. A. Rainey. 1998. Petrotoga mobilis sp. nov., from a North Sea oil-production well. Int. J. Syst. Bacteriol. 48:1007–1013.

Logsdon, J. M., and D. M. Faguy. 1999. Thermotoga heats up lateral gene transfer. Curr. Biol. 9:R747–R751.

Londei, P., S. Altamura, R. Huber, K. O. Stetter, and P. Cammarano. 1988. Ribosomes of the extremely thermophilic eubacterium Thermotoga maritima are uniquely insensitive to the miscoding-inducing action of aminoglycoside antibiotics. J. Bacteriol. 170:4353–4360.

Ludwig, W., J. Neumaier, N. Klugbauer, E. Brockmann, C. Roller, S. Jilg, K. Reetz, I. Schachtner, A. Ludvigsen, M. Bachleitner, U. Fischer, and K.-H. Schleifer. 1993. Phlogenetic relationships of Bacteria based on comparative sequence analysis of elongation factor Tu and ATP-synthase beta-subunit genes. Ant. v. Leeuwenhoek 64:285–305.

Lupas, A., S. Müller, K. Goldie, A. M. Engel, A. Engel, and W. Baumeister. 1995. Model structure of the Omp alpha rod, a parallel four-stranded coiled coil from the hyperthermophilic eubacterium Thermotoga maritima. J. Molec. Biol. 248:180–189.

Malik, K. A. 1999. Preservation of some extremely thermophilic chemolithoautotrophic bacteria by deep-freezing and liquid-drying methods. J. Microbiol. Meth. 35:177–182.

Manca, M. C., B. Nicolaus, V. Lanzotti, A. Trincone, A. Gambacorta, J. Peter Katalinic, H. Egge, R. Huber, and K. O. Stetter. 1992. Glycolipids from Thermotoga maritima, a hyperthermophilic microorganism belonging to Bacteria domain. Biochim. Biophys. Acta 1124:249–252.

Martins, L. O., L. S. Carreto, M. S. Da Costa, and H. Santos. 1996. New compatible solutes related to di-myo-inositol-phosphate in members of the order Thermotogales. J. Bacteriol. 178:5644–5651.

Nanavati, D., Noll, K. M., and A. H Romano. 2002. Periplasmic maltose- and glucose-binding protein activities in cell-free extracts of Thermotoga maritima. Microbiology 148:3531–3537.

Nelson, K. E., R. A. Clayton, S. R. Gill, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, W. C. Nelson, K. A. Ketchum, L. McDonald, T. R. Utterback, J. A. Malek, K. D. Linher, M. M. Garrett, A. M. Stewart, M. D. Cotton, M. S. Pratt, C. A. Phillips, D. Richardson, J. Heidelberg, G. G. Sutton, R. D. Fleischmann, J. A. Eisen, O. White, S. L. Salzberg, H. O. Smith, J. C. Venter, and C. M. Fraser. 1999. Evidence for lateral gene transfer between Archaea and Bacteria from genome sequence of Thermotoga maritima. Nature 399:323–329.

Nelson, K. E., J. A. Eisen, and C. M. Fraser. 2001. Genome of Thermotoga maritima MSB8. *In:* M. W. W. Adams and R. M.Kelly (Eds.) Methods in Enzymology. Academic Press. San Diego, CA. 169–180.

Nesbø, C. L., S. L'Haridon, K. O. Stetter, and W. F. Doolittle. 2001. Phylogenetic analyses of two "archaeal" genes in Thermotoga maritima reveal multiple transfers between Archaea and Bacteria. Molec. Biol. Evol. 18:362–375.

Nesbø, C. L., K. E. Nelson, and W. F. Doolittle. 2002. Suppressive subtractive hybridization detects extensive genomic diversity in Thermotoga maritima. J. Bacteriol. 184:4475–4488.

Palm, P., C. Schleper, I. Arnold-Ammer, I. Holz, T. Meier, F. Lottspeich, and W. Zillig. 1993. The DNA-dependent RNA-polymerase of Thermotoga maritima; characterization of the enzyme and the DNA-sequence of the genes for the large subunits. Nucleic Acids Res. 21:4904–4908.

Patel, B. K. C., H. W. Morgan, and R. M. Daniel. 1985a. Fervidobacterium nodosum gen. nov. and spec. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch. Microbiol. 141:121–127.

Patel, B. K. C., H. W. Morgan, and R. M. Daniel. 1985b. Validation of the publication of new names and new combinations previously effectively published outside the IJSB: List No. 19. Int. J. Syst. Bacteriol. 35:535–535.

Rachel, R., A. M. Engel, R. Huber, K. O. Stetter, and W. Baumeister. 1990. A porin-type like protein is the major constituent of the cell envelope of the ancestral eubacterium Thermotoga maritima. FEBS Lett. 262:64–68.

Ramakrishnan, V., M. F. J. M. Verhagen, and M. W. W. Adams. 1997. Characterization of di-myo-inositol-1,1′-phospate in the hyperthermophilic bacterium Thermotoga maritima. Appl. Environ. Microbiol. 63:347–350.

Ravot, G., M. Magot, M. L. Fardeau, B. K. C. Patel, G. Prensier, A. Egan, J. L. Garcia, and B. Ollivier. 1995. Thermotoga elfii sp. nov., a novel thermophilic bacterium from an african oil-producing well. Int. J. Syst. Bacteriol. 45:308–314.

Ravot, G., B. Ollivier, M. L. Fardeau, B. K. C. Patel, K. T. Andrews, M. Magot, and J. L. Garcia. 1996a. L-alanine production from glucose fermentation by hyperthermophilic members of the domains Bacteria and Archaea: A remnant of an ancestral metabolism? Appl. Environ. Microbiol. 62:2657–2659.

Ravot, G., B. Ollivier, B. K. C. Patel, M. Magot, and J. L. Garcia. 1996b. Emended description of Thermosipho africanus as a carbohydrate-fermenting species using thiosulfate as an electron acceptor. Int. J. Syst. Bacteriol. 46:321–323.

Reysenbach, A.-L. 2001. Phylum BII: Thermotogae phy. nov. *In:* D. R. Boone and R. W.Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY. 369.

Rinker, K. D., and R. M. Kelly. 2000. Effect of carbon and nitrogen sources on growth dynamics and exopolysaccharide production for the hyperthermophilic archaeon Thermococcus litoralis and bacterium Thermotoga maritima. Biotechnol. Bioengin. 69:537–547.

Schirmer, T. 1998. General and specific porins form bacterial outer membranes. J. Struct. Biol. 121:101–109.

Schleifer, K.-H., and W. Ludwig. 1989. Phylogenetic relationships among bacteria. *In:* B. Fernholm, K. Bremer, and H. Jörnvall (Eds.) The Hierarchy of Life. Elsevier Science Publishers. Amsterdam, The Netherlands. 103–117.

Schröder, C., M. Selig, and P. Schönheit. 1994. Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium Thermotoga maritima: Involvement of the Embden-Meyerhof pathway. Arch. Microbiol. 161:460–470.

Selig, M., K. B. Xavier, and H. Santos. 1997. Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium Thermotoga. Arch. Microbiol. 167:217–232.

Stetter, K. O., and R. Huber. 1986. Validation of the publication of new names and new combinations previously effectively published outside the IJSB: List No. 22. Int. J. Syst. Bacteriol. 36:573–576.

Stetter, K. O., R. Huber, E. Blöchl, M. Kurr, R. D. Eden, M. Fiedler, H. Cash, and I. Vance. 1993. Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. Nature 365:743–745.

Stetter, K. O., and R. Huber. 2000. The role of hyperthermophilic prokaryotes in oil fields. *In:* C.R. Bell, M. Brylinsky, P. Johnson-Green, (eds.) Micobial biosystems: New Frontiers Proceedings 8th ASM. Proceedings of the 8th international symposium on microbial ecology. Halifax, Canada. 369–375.

Swanson, R. V., M. G. Sanna, and M. I. Simon. 1996. Thermostable chemotaxis proteins from the hyperthermophilic bacterium Thermotoga maritima. J. Bacteriol. 178:484–489.

Takahata, Y., M. Nishijima, T. Hoaki, and T. Maruyama. 2001. Thermotoga petrophila sp. nov. and Thermotoga naphthophila sp. nov., two hyperthermophilic bacteria from the Kubiki oil reservoir in Niigata, Japan. Int. J. Syst. Evol. Bacteriol. 51:1901–1909.

Takai, K., and K. Horikoshi. 2000a. Thermosipho japonicus sp. nov., an extremely thermophilic bacterium isolated from a deep-sea hydrothermal vent in Japan. Extremophiles 4:9–17.

Takai, K., and K. Horikoshi. 2000b. Validation of the publication of new names and new combinations previously effectively published outside the IJSB: List No. 76. Int. J. Syst. Evol. Bacteriol. 50:1699–1700.

Tiboni, O., R. Cantoni, R. Creti, P. Cammarano, and A. M. Sanangelantoni. 1991. Phylogenetic depth of Thermotoga maritima inferred from analysis of the fus gene: Amino acid sequence of elongation factor G and organization of the Thermotoga str operon. J. Molec. Evol. 33:142–151.

Van de Casteele, M., M. Demarez, C. Legrain, N. Glansdorff, and A. Piérard. 1990. Pathways of arginine biosynthesis in extreme thermophilic archaeo- and eubacteria. J. Gen. Microbiol. 136:1177–1183.

Van den Ent, F., and J. Löwe. 2000. Crystal structure of the cell division protein FtsA from Thermotoga maritima. EMBO J. 19:5300–5307.

Van den Ent, F., L. A. Amos, and J. Löwe. 2001. Prokaryotic origin of the actin cytoskeleton. Nature 413:39–44.

Vargas, M., and K. M. Noll. 1994. Isolation of auxotrophic and antimetabolite-resistant mutants of the hyperthermophilic bacterium Thermotoga neapolitana. Arch. Microbiol. 162:357–361.

Vargas, M., K. Kashefi, E. L. Blunt-Harris, and D. R. Lovley. 1998. Microbiological evidence for Fe(III) reduction on early Earth. Nature 395:65–67.

Wery, N., F. Lesongeur, P. Pignet, V. Derennes, M. A. Cambon-Bonavita, A. Godfroy, and G. Barbier. 2001. Marinitoga camini gen. nov., sp. nov., a rod-shaped bacterium belonging to the order Thermotogales, isolated from a deep-sea hydrothermal vent. Int. J. Syst. Evol. Bacteriol. 51:495–504.

Widdel, F., G. W. Kohringen, and F. Mayer. 1983. Studies of dissimilatory sulfate-reducing bacteria that dexompose fatty acids. III: Characterization of the filamentours gliding Desulfonema limicola gen. nov. and sp. nov. and Desulfonema magnum sp. nov. Arch. Microbiol. 129:286–294.

Windberger, E., R. Huber, A. Trincone, H. Fricke, and K. O. Stetter. 1989. Thermotoga thermarum and Thermotoga neapolitana occurring in African continental solfataric springs. Arch. Microbiol. 151:506–512.

Windberger, E., R. Huber, and K. O. Stetter. 1992. Validation of the publication of new names and new combinations previously effectively published outside the IJSB: List No. 41. Int. J. Syst. Bacteriol. 42:327–329.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.

Wolin, E. A., M. J. Wolin, and R. S. Wolfe. 1963. Formation of methane by bacterial extracts. J. Biol. Chem. 238:2882–2886.

Yu, J.-S., and K. M. Noll. 1997. Plasmid pRQ7 from the hyperthermophilic bacterium Thermotoga species strain RQ7 replicates by rolling-circle mechanism. J. Bacteriol. 179:7161–7164.

Yu, J.-S., M. Vargas, C. Mityas, and K. M. Noll. 2001. Liposome-mediated DNA uptake and transient expression in Thermotoga. Extremophiles 5:53–60.

PART 13

Aquificales

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CHAPTER 13.1

Aquificales

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Introduction

Members of the order Aquificales represent a very deep phylogenetic branch within the 16S rRNA tree of the domain Bacteria. Within the order, members of the genus *Aquifex* have an upper temperature border of growth at 95°C and represent the bacteria with the highest growth temperatures known so far. Aquificales representatives are widespread and cosmopolitan, and they thrive mainly in volcanically or geothermally heated environments. Owing to their chemolithoautotrophic way of life, they are primary producers of bacterial biomass within high temperature ecosystems. In their energy-yielding reactions, they preferentially perform the "Knallgas" reaction, the oxidation of hydrogen and reduction of oxygen.

Phylogeny

By 16S rRNA gene sequence comparisons, it was shown that the Aquificales represent one of the deepest and earliest branching groups within the phylogenetic tree (Burggraf et al., 1992; Huber et al., 1992b; Fig. 1) along with the separate phylogenetic branch of the Thermotogales order (Huber and Stetter, 1992a; Huber and Stetter, 1999b; see also Thermotogales in this Volume). However, phylogenetic analysis of other gene and protein sequences of the genus *Aquifex* showed that placement with respect to other bacterial groups was variable (Acca et al., 1994; Klenk et al., 1994; Wetmur et al., 1994; Bocchetta et al., 1995; Bocchetta et al., 2000; Brown and Doolittle, 1995; Baldauf et al., 1996; Pennisi, 1998; Pennisi, 1999; Klenk et al., 1999; Snel et al., 1999; Schütz et al., 2000). Also the analysis of different genes from the completely sequenced genome of "*Aquifex aeolicus*" gave no consistant picture of the Aquificales phylogeny (Deckert et al., 1998).

Taxonomy

Recently, the phylum Aquificae, consisting of a single class and the single order Aquificales, was created (Reysenbach, 2001). Based on 16S rRNA gene sequence comparisons, the Aquificales are composed of the Aquificaceae and the "Hydrogenothermaceae" (Fig. 1). Within the "Hydrogenothermaceae," the only cultivated representatives are isolate EX-H1 and *Hydrogenothermus marinus* VM1 (*Ht. marinus*; Reysenbach et al., 2000a; Eder and Huber, 2002; Stöhr et al., 2001; Fig. 1). In addition, the new family contains environmental sequences from different locations on earth (Hugenholtz et al., 1998; Yamamoto et al., 1998; Reysenbach et al., 2000b; Reysenbach et al., 2000c; Skirnisdottir et al., 2000; Graber et al., 2001). Within the Aquificaceae, the genera *Calderobacterium* (Kryukov et al., 1983), *Hydrogenobacter* (Kawasumi et al., 1984), *Aquifex* (Huber et al., 1992b), *Hydrogenobaculum* (Stöhr et al., 2001) and *Thermocrinis* (Huber et al., 1998) have been described. *Calderobacterium* is very similar to *Hydrogenobacter* in phylogenetic, phenotypic and biochemical properties, which resulted in the transfer of *C. hydrogenophilum* to *Hydrogenobacter hydrogenophilus* comb. nov. (Stöhr et al., 2001), as previously proposed with the "*Hydrogenobacter-Calderobacterium*" group (Aragno, 1992b; Pitulle et al., 1994). Within the Aquificaceae, the acidophilic *Hydrogenobacter acidophilus* forms a separate lineage (Shima et al., 1994; Fig. 1). Owing to its phylogenetic position and its low pH optimum for growth, *Hydrogenobacter acidophilus* was recently reclassified and named *Hydrogenobaculum acidophilum* (*Hb. acidophilum*; Stöhr et al., 2001). One genus, *Desulfurobacterium*, was provisionally placed within the phylum Aquificae, but may represent another, currently undefined phylum (Reysenbach, 2001). In Table 1, representatives belonging to the different genera within the Aquificales are listed.

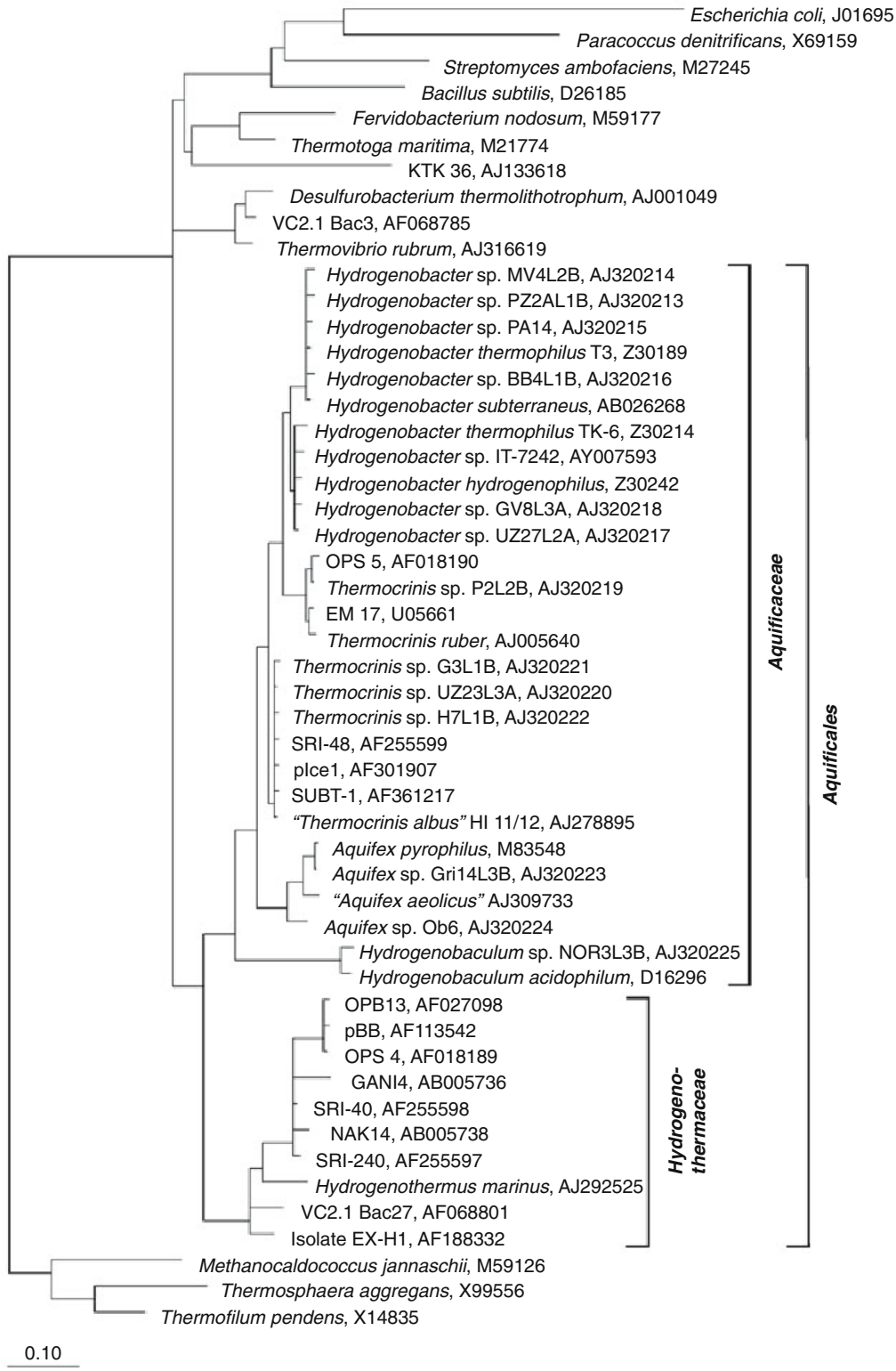

Fig. 1. 16S rRNA-based phylogenetic tree of the Aquificales. The tree topology is based on the ARB database (http://www.arb-home.de) of 11,000 sequence entries and was reconstructed using the ARB parsimony tool. A filter defining positions that share identical residues in at least 50% of all included sequences from the Aquificales was used for reconstructing the tree. Multifurications indicate that a (statistically) significant relative branching order could not be determined or is not supported by other treeing methods. Reference sequences were chosen to represent the broadest diversity of bacteria. Accession numbers for the sequences are indicated. The scale bar represents 0.10 fixed mutations per nucleotide position.

Table 1. List of the different representatives within the Aquificales.

Species	Culture collection	16S rDNA accession number	References
Aquifex pyrophilus Kol5a^T	DSM 6858	M83548	Huber et al., 1992
"Aquifex aeolicus" VF5	—	AJ309733	Huber and Stetter, 2001
Hydrogenobacter thermophilus TK-6^T	DSM 6534; IAM 12695	Z30214	Kawasumi et al., 1984
Hydrogenobacter thermophilus T3	—	Z30189	Bonjour and Aragno, 1986 Pitulle et al., 1994
Hydrogenobacter subterraneus HGP1^T	JCM 10560	AB026268	Takai et al., 2001
Hydrogenobacter halophilus TH-112	—	—	Nishihara et al., 1990
Hydrogenobacter hydrogenophilus Z-829^T (formerly: *Calderobacterium hydrogenophilum*)	DSM 2913; JCM 8158	Z30242	Kryukov et al., 1983 Stöhr et al., 2001
Hydrogenobaculum acidophilum 3H-1^T (formerly: *Hydrogenobacter acidophilus*)	DSM 11251; JCM 8795	D16296	Shima and Suzuki, 1993 Stöhr et al., 2001
Hydrogenothermus marinus VM1^T	DSM 12046; JCM 10974	AJ292525	Stöhr et al., 2001
Thermocrinis ruber OC 1/4^T	DSM 12173	AJ005640	Huber et al., 1998
Thermocrinis albus HI 11/12	DSM 14484; JCM 11386	AJ278895	Eder and Huber, 2002

Abbreviations: ^{T}, type strain; DSM(Z), Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Deutschland; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; and JCM, Japan Collection of Microorganisms, RIKEN, Saitama, Japan.

Habitat

Almost all cultivated members of the Aquificales have been found in geothermally and volcanically heated environments. *Hydrogenobacter* and *Thermocrinis* relatives have been isolated mainly from continental solfatara fields with low ionic strength. In contrast, members of the genus *Aquifex* have been obtained exclusively from marine hydrothermal systems (Huber and Stetter, 1999b; Eder and Huber, 2002).

Hydrogenobacter thermophilus TK-6 was originally isolated from a hot spring in Japan, whereas *H. hydrogenophilus* was obtained from a thermal spring in the Geyser Valley, Kamchatka, Russia (Kryukov et al., 1983; Kawasumi et al., 1984). Organisms similar to *Hydrogenobacter* were subsequently isolated from other geothermal areas, located in Iceland, Italy, Japan, the Azores, the United States and New Zealand (Kawasumi et al., 1984; Kristjansson et al., 1985; Bonjour and Aragno, 1986; Aragno, 1992b; Skirnisdottir et al., 2001). *Hydrogenobacter thermophilus* T3 was obtained from a geothermal spring in Tuscany, Italy (Bonjour and Aragno, 1986; Pitulle et al., 1994). Recently, new *Hydrogenobacter* isolates were reported, which were isolated from Pemba (Lake Tanganyika), Kamchatka (Russia), Pozzuoli (Italy) and Baden-Baden (Germany; Eder and Huber, 2002). From a deep subsurface geothermal water pool (depth: 1,500 m), the new species *H. subterraneus* was isolated recently (Takai et al., 2001). The only halophilic strain within the genus *Hydrogenobacter*, *H. halophilus*, was obtained from a seaside saline hot spring in Izu Peninsula, Japan (Nishihara et al., 1990). Further, *Hydrogenobacter* relatives were cultivated from hot compost (Beffa et al., 1996).

Hydrogenobaculum acidophilum was originally isolated from a solfataric field in Tsumagoi, Japan (Shima and Suzuki, 1993). Recently, another acidophilic *Hydrogenobaculum* isolate was isolated from an acidic hot spring of Norris Geyser Basin, Yellowstone National Park, Wyoming, United States. This indicates that acidophilic Aquificales form a phylogenetically tight group and are widely distributed (Eder and Huber, 2002; Fig. 1).

In nature, members of the genus *Thermocrinis* grow in large, streamer-like cell accumulations, macroscopically visible as bacterial masses thriving in the hot water current. From biomass of pink filamentous streamers ("pink filaments") occurring in the upper outflow channel (temperature: 82–88°C) of Octopus Spring, Yellowstone National Park, Wyoming (Brock, 1967; Brock, 1978; Brock, 1998; Marler, 1973), the type species *Thermocrinis ruber* (*T. ruber*) was isolated (Huber et al., 1998). A second species was obtained from grayish filaments present in the volcanic Hveragerthi area (temperature: up to 88°C), Iceland, and described as *Thermocrinis albus* (Huber et al., 1998; Eder and Huber, 2002; Fig. 1). Further, so far undescribed *Thermocrinis*

isolates have been obtained from grayish-colored cell masses taken in the Uzon Valley (temperature: 75–80°C), Kamchatka, Russia, and from whitish filaments of a hot spring (temperature: 72°C) in Graendalur, Iceland (Eder and Huber, 2002). Moreover, environmental 16S rRNA gene sequences related to *Thermocrinis* were derived from water samples and different filamentous streamers of hot springs (Reysenbach et al., 1994; Skirnisdottir et al., 2000; Graber et al., 2001; Marteinsson et al., 2001a; Marteinsson et al., 2001b; Takacs et al., 2001). However, Thermocrinis relatives were also frequently found in usual hot ponds of Iceland and in Yellowstone National Park (United States), not containing visible streamers. This indicates that these organisms, in addition to their ability to form streamers, may play in general an important role in high temperature ecosystems (Huber et al, 1998; Huber et al, 2000b; Eder and Huber, 2002).

Within the marine genus *Aquifex*, the type species *Aquifex pyrophilus* (*A. pyrophilus*) was originally isolated from a submarine hydrothermal vent system (depth: 106 m) at the Kolbeinsey Ridge north of Iceland (Huber et al., 1992b). A further species, "*A. aeolicus*," was obtained from a shallow submarine hydrothermal system at Vulcano, Italy (Huber and Stetter, 2000a). Novel *Aquifex* relatives were isolated from white Anhydrite Smokers with temperatures up to 250°C, detected at a depth of 400 m near Grimsey Island, north of Iceland, and from a coastal hot spring in Obock, Gulf of Tadjoura, Djibouti (Huber and Stetter, 1999b; Eder and Huber, 2002).

From a shallow marine hydrothermal area of Vulcano Island, Italy, *Hydrogenothermus marinus* was isolated, whereas isolate EX-H1 was obtained from sulfide chimneys, taken in a deep-sea hydrothermal system at the Mid-Atlantic Ridge (Reysenbach et al., 2000a; Stöhr et al., 2001).

Isolation

To enrich cultures for Aquificales, inorganic, liquid media and a pH around 7 (3.5 for *Hb. acidophilum*) are recommended. Depending on the environment sampled, either media with low ionic strength or sea water media can be used (Table 2; for details, see Culture Media). Owing to the microaerophilic nature of most Aquificales, the enrichment cultures should contain a gas mixture with reduced oxygen content (for details, see Culture Media). For the enrichment of a particular member of the Aquificales, an incubation temperature close to the optimal growth temperature is recommended (Table 2). Owing to the high incubation temperature and the use of inorganic media, the enrichments are selective and Aquificales should be obtained. Alternatively, *Thermocrinis* species might be selectively enriched in a medium at 85°C, containing formamide or formate as single carbon and energy sources (Huber et al., 1998; Eder and Huber, 2002). The enrichment media are incubated with original sampling material (e.g., water, sediment, mud, and microbial streamer biomass) and monitored for growth over a certain period (e.g., two weeks) by phase contrast microscopy. When rod-shaped cells become visible in the enrichments, the isolates are obtained by plating or by the use of "optical tweezers."

For plating, a stainless steel anaerobic jar and microaerophilic incubation conditions might be used (Balch et al., 1976). The media are solidified with 1–1.5% Gelrite (Roth, Karlsruhe, Germany; Kang et al., 1982; Huber et al., 2000c), and the plates are incubated at the corresponding temperature. Colony formation of *Hydrogenobacter thermophilus* TK-6 with very low plating efficiencies have been reported (Ishii et al., 1987). As an alternative, *Hydrogenobacter* relatives can be isolated on 2.5% agar plates and thiosulfate, incubated either under a H_2-containing gas mixture or under air (Alfredsson et al., 1986; Aragno, 1992a). *Aquifex* species and *Thermocrinis ruber* grow well on Gelrite plates, and with "*Aquifex aeolicus*," plating efficiences up to 100% were obtained. Aquificales colonies are round with a diameter of about 1 mm. Colonies of the *Aquifex* species are brownish yellow, of *T. ruber* brownish red, while colonies of *Hydrogenobacter* relatives appear bright yellow. *Hydrogenothermus marinus* was obtained by serial dilutions; no plating procedure for isolation was reported (Stöhr et al., 2001).

From liquid cultures, Aquificales also can be isolated by a plating-independent isolation procedure (selected cell cultivation technique; Beck and Huber, 1997; Huber et al., 1998; Eder and Huber, 2002). This method is based on optical trapping of single cells by the use of a strongly focused infrared laser beam ("optical tweezers"; Huber et al., 1995; Huber et al., 2000c; Huber, 1999a; see Thermoproteales in Volume 3).

Identification

Members of the Aquificales are nonspore-forming, Gram-negative rods. Cells of *Hydrogenobacter* are about 0.3–0.9 × 2.0–8.0 μm (Reysenbach, 2001; Takai et al., 2001), while the *Aquifex* cells have a length between 2.0 and 6.0 μm and a diameter of 0.4–0.5 μm (Huber and Stetter, 2000a; Huber et al., 2000b). Members of *Hydrogenobacter*, *Hydrogenobaculum*, *Ther-*

Table 2. Physiological properties of different representatives within the Aquificales.

Species	G+C content (mol%)	Temperature optimum (°C)	Temperature range (°C)	pH optimum	pH range	NaCl optimum (%)	NaCl range (%)
Aquifex pyrophilus Kol5a^T	47[a]	85	67–95	6.8	5.4–7.5	3	1–5
"Aquifex aeolicus" VF5	43.4[b]	85–90	58–95	6.5–7.0	5.5–8.0	3	n.d.
Hydrogenobacter thermophilus TK-6^T	43.5–43.9 38.3[c]	70–75	50–78	7.0	Neutral	n.d.	n.d.
Hydrogenobacter thermophilus T3	39.9[c]	75	n.d-85	7.0	5.0–8.0	n.d.	n.d.
Hydrogenobacter subterraneus HGP1	44.7	78	60–85	7.5	5.5–9.0	n.d.	n.d.
Hydrogenobacter halophilus TH-112	46	70	60–75	7.0–7.5	n.d.	1.75–2.9	<5.8
Hydrogenobacter hydrogenophilus Z-829	39–41 37.5	74–76	50–82	6.0–7.0	n.d.	n.d.	n.d.
Hydrogenobaculum acidophilum 3H-1^T	35	65	50–70	3.0–4.0	2.0–6.0	n.d.	n.d.
Hydrogenothermus marinus VM1^T	45	65	45–80	n.d.	5–7	2–3	0.5–6
Thermocrinis ruber OC 1/4^T	47.5	80	44–89	n.d.	Neutral/ slightly alkaline	n.d.	<0.4
Thermocrinis albus HI 11/12	49.6	n.d.	55–89	n.d.	Neutral	n.d.	<0.8

Abbreviations: ^{T}, type strain; and n.d. = not determined.
[a]Huber and Stetter, 2000.
[b]Deckert et al., 1998.
[c]Pitulle et al., 1994.

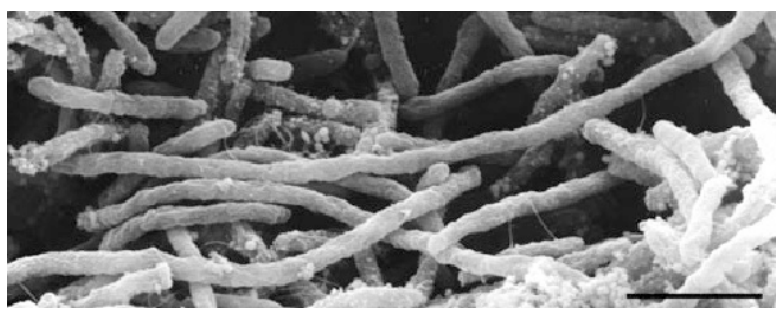

Fig. 2. Scanning electron micrograph of long filaments of *Thermocrinis ruber* from a pinkish streamer network formed at the outflow of a growth chamber during growth in a permanent flow of medium under exposure to air. Bar, 2 µm.

mocrinis, *Aquifex* and *Hydrogenothermus* usually grow singly and in pairs (Kryukov et al., 1983; Kawasumi et al., 1984; Kristjansson et al., 1985; Bonjour and Aragno, 1986; Nishihara et al., 1990; Aragno, 1992a; Huber et al, 1992b; Shima and Suzuki, 1993; Beffa et al., 1996; Huber et al., 1998; Eder and Huber, 2002; Stöhr et al., 2001; Takai et al., 2001). In addition, *Aquifex* and *Thermocrinis* species form large cell aggregates, containing up to about 100 individual cells. Growth in aggregates was also reported for *Hydrogenothermus marinus* (Stöhr et al., 2001). In the exponential growth phase, the *Aquifex* species form wedge-shaped polar or central refractile areas, which disappear in the stationary growth phase (Huber et al., 1992b). Within a permanent flow of medium under exposure to air, the *Thermocrinis* species grow in streamer-like cell masses predominantly composed of long filaments (Fig. 2). *Thermocrinis ruber* forms pink, and *T. albus* white, cell masses, which is in agreement with their growth behavior in the environment (see also Habitat; Huber et al., 1998; Eder and Huber, 2002). The pink-colored cell masses of *Thermocrinis ruber* formed in batch culture also form in a growth chamber under a perma-

nent flow of medium (Huber et al., 1998). The formation of the pink color is independent of the light regime, the oxygen concentration or the electron donor used (hydrogen, sulfur, thiosulfate or formate; Eder and Huber, 2002). *Thermocrinis albus* builds up filaments up to 60 micrometer under a permanent flow of medium (Eder and Huber, 2002).

Motility has been observed for *Hydrogenobacter subterraneus*, *Hydrogenobaculum acidophilum* and *Hydrogenothermus marinus*, which are all polarly flagellated (Shima and Suzuki, 1993; Stöhr et al., 2001; Takai et al., 2001). Some *Hydrogenobacter* isolates from hot composts were also motile, whereas most *Hydrogenobacter* isolates were reported to be immotile (Beffa et al., 1996; Reysenbach, 2001; Ishii et al., 2001). In contrast, the *Aquifex* species and single cells of the *Thermocrinis* species are polarly flagellated and highly motile (Fig. 3a, b). Cultures of *Aquifex pyrophilus* even remain motile when stored for 2 weeks at 4°C. The 19-nm-diameter flagellar filaments of *A. pyrophilus* are composed of a 54-kDa flagellin monomer, which exhibits high thermostability (Behammer et al., 1995). In the genome of "*A. aeolicus*," more than 25 genes involved in flagellar structure and biosynthesis are present. However, no homologs of the bacterial chemotaxis system were identified (Deckert et al., 1998; Swanson, 2001).

Diaminopimelic acid is present in *Aquifex* and *Thermocrinis* species. In contrast, diaminopimelic acid is absent in *Hydrogenobacter* (Huber et al., 1992b; Huber et al., 1998; Eder and Huber, 2002). A regular surface layer was identified in *A. pyrophilus*, in *Hydrogenobacter hydrogenophilus* and in different *Hydrogenobacter* isolates, but is absent in *T. ruber* and *T. albus* (Kristjansson et al., 1985; Huber et al., 1992b; Huber et al., 1998; Ludvik et al., 1994; Eder and Huber, 2002). *Aquifex pyrophilus* contains a complex cell envelope consisting of a peptidoglycan layer (murein type A1 gamma), an outer membrane, and a surface layer protein (Huber et al., 1992b).

The fatty acid composition of *Hydrogenobacter thermophilus* TK-6, *H. subterraneus*, *H. halophilus*, *Hydrogenobaculum acidophilum* and *Hydrogenothermus marinus* is quite similar (Kawasumi et al., 1984; Nishihara et al., 1990; Shima and Suzuki, 1993; Stöhr et al., 2001; Takai et al., 2001). A very similar distribution of fatty acids was found in *Hydrogenobacter thermophilus* TK-6 and in the *Thermocrinis* and *Aquifex* species (Jahnke et al., 2001; Table 3). The presence of glycerol monoethers is a characteristic feature of *Hydrogenobacter thermophilus* TK-6 and the *Thermocrinis* species under autotrophic culture conditions, whereas the lipids of the *Aquifex* species are composed of alkyl glycerol

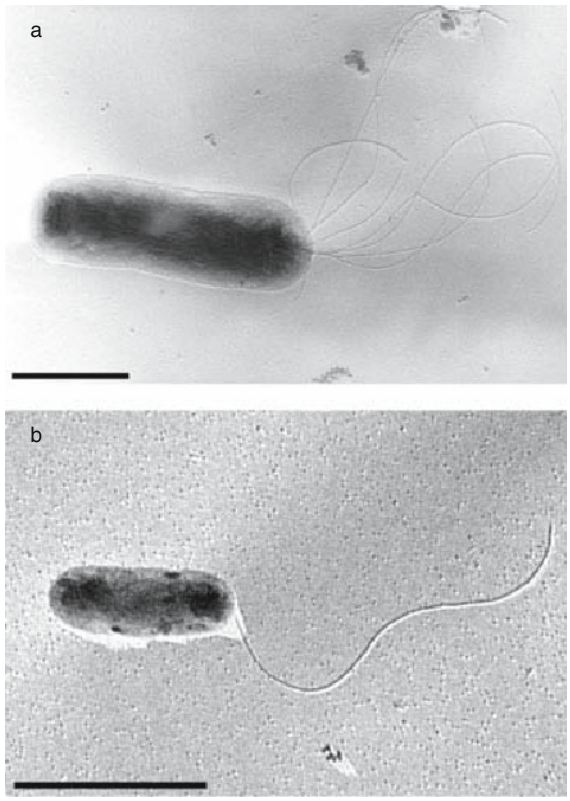

Fig. 3 a) Transmission electron micrograph of a platinum-shadowed, multi-flagellated cell of *Aquifex pyrophilus*. Bar, 1 µm. b) Transmission electron micrograph of a platinum-shadowed, single-flagellated cell of "*Thermocrinis albus*" (static culture). Bar, 1 µm.

diethers and glycerol monoethers (Huber and Stetter, 1992a; Jahnke et al., 2001; Table 3). The detailed fatty acid analysis of different Aquificaceae representatives verified that the Aquificaceae are a monophyletic group within the bacteria, which is in line with the 16S rRNA data. Also, *n*-$C_{20:0}$, *n*-$C_{20:1}$, *n*-$C_{21:0}$, *cy*-C_{21}, *n*-$C_{22:0}$ and *n*-$C_{22:1}$ fatty acids were identified as signature lipids for the Aquificales, which could be used as biomarkers (Jahnke et al., 2001).

A novel type of lipid A, indicative of lipopolysaccharide, was identified in *Aquifex pyrophilus*. This lipid A contains D-galacturonic acid, which replaces phosphate residues (Plötz et al., 2000).

The DNA-dependent polymerase of *Hydrogenobacter hydrogenophilus* exhibits a typical bacterial subunit pattern, is rifampicin resistant and is thermostable (no loss of activity at 100°C for 40 min; Andera et al., 1991). In addition, a reverse gyrase (optimal reaction temperature: 90°C) was isolated from *H. hydrogenophilus* (Andera et al., 1993). In the genome of *A. aeolicus*, two genes for reverse gyrase were detected (Deckert et al., 1998). From *Hydrogenobacter*

Table 3. Comparison of ester-linked fatty acid and glycerol ether composition of the Aquificales species.[a]

Compound	*Thermocrinis ruber* C-source: CO_2	*Thermocrinis ruber* C-source: formate	*Thermocrinis albus*	*Aquifex pyrophilus*	*"Aquifex aeolicus"*	*Hydrogenobacter thermophilus* TK-6
Fatty acid[a]						
14:0	<0.1	<0.1	<0.1	0.3	0.1	<0.1
i-15:0	nd	nd	nd	nd	nd	nd
15:0	nd	<0.1	<0.1	0.1	<0.1	<0.1
i-16:0	nd	nd	nd	nd	nd	nd
16:1*c*Δ7	0.2	0.8	0.3	0.5	0.4	0.2
16:1*c*Δ9	nd	nd	<0.1	0.1	0.1	<0.1
16:0	0.3	0.8	0.5	2.3	1.0	0.4
i-17:0	nd	nd	nd	nd	nd	nd
ai-17:0	nd	nd	nd	nd	nd	nd
17:0	<0.1	<0.1	<0.1	0.2	nd	<0.1
cy-17	nd	nd	nd	nd	nd	nd
i-18:0	nd	nd	nd	nd	nd	nd
18:1*c*Δ9	2.3	9.0	2.4	4.0	3.4	3.5
18:1*c*Δ11	2.4	5.1	1.1	1.8	2.2	2.5
18:1*t*Δ9	0.4	tr	0.6	0.7	nd	0.2
18:0	12.1	12.6	14.1	27.3	14.0	16.5
i-19:0	nd	nd	nd	nd	nd	nd
ai-19:0	nd	nd	<0.1	0.1	nd	nd
cy-19 (2 isomers)	2.2	1.4	2.9	2.6	1.8	0.6
19:0	<0.1	<0.1	0.1	0.2	nd	<0.1
i-20:0	nd	nd	nd	nd	nd	nd
20:1*c*Δ11	23.1	50.9	10.7	18.4	17.5	49.4
20:1*c*Δ13	14.3	3.1	8.2	1.3	3.7	11.3
20:1*t*Δ11	9.4	nd	5.4	5.4	11.1	nd
20:1*t*Δ13	3.8	nd	3.5	nd	nd	nd
20:0	3.9	1.9	4.6	5.6	3.4	3.2
i-20:0	nd	nd	nd	nd	nd	nd
cy-21 (2 isomers)	24.7	14.2	42.4	29.8	40.3	11.6
21:0	<0.1	nd	0.1	0.1	0.1	nd
22:1*c*Δ13	0.7	0.2	0.8	0.4	0.3	0.3
22:0	0.3	<0.1	<0.1	0.1	<0.1	<0.1
μmol FA/g dwt	92.2	89.2	103.5	13.8	35.3	38.8
Glycerol ether						
GME-18:1	1.8	10.7	tr	3.9	2.2	2.7
GME-18:0	75.2	38.5	78.5	57.3	73.0	82.7
GME-19:0	nd	0.6	tr	2.5[c]	0.2[c]	nd
GME-20:1	13.2	37.5	17.6	14.4	13.9	11.1
GME-20:0	4.1	4.1	2.0	13.8	10.3	3.5
GME-21:1	3.8	5.7	1.9	8.1	0.5	nd
μmol GME/g dwt	5.9	1.6	0.9	3.1	19.3	1.2
GDE-17:0,17:0	nd	nd	nd	8.9	nd	nd
GDE-17:0,18:0	nd	nd	nd	2.1	nd	nd
GDE-18:0,18:0	nd	73.2[c]	nd	34.5[c]	32.2	nd
GDE-18:0,19:0	nd	nd	nd	10.0	2.5	nd
GDE-18:0,20:0	nd	26.8[c]	nd	13.5	9.1	nd
GDE-18:0,21:1	nd	nd	nd	23.4	44.9	nd
GDE-20:0,20:0	nd	nd	nd	7.7	11.2	nd
GDE-19:0,21:1	nd	nd	nd	nd	nd	nd
μmol GDE/g dwt	nd	0.1	nd	1.1	0.6	nd

Abbreviations: FA, fatty acid; nd, not determined; tr, trace; dwt, dry weight; GME, glycerol monoether; and GDE, glycerol diether.

[a]Lipid distribution is given in % wt.

[b]Acyl-alkyl chain nomenclature designates carbon number as saturated (:0) or monounsaturated (:1) with double bond of *cis* (*c*) or *trans* (*t*) configuration and position relative to carboxyl end (Δ) or cyclopropyl ring (*cy*), with *iso*- (*i*) or *anteiso*- (*ai*) methyl branching.

[c]Designates presence of multiple GDE isomers with saturated and/or unsaturated alkyl chains (:1 or *cy*). Adapted from Jahnke et al. (2001); for a more detailed analysis of *T. ruber* lipids and methods, see Jahnke et al. (2001).

thermophilus TK-6, an ATP-citrate lyase and a membrane-bound hydrogenase were identified (Ishii et al., 1989). A variety of enzymes from *Aquifex* have been cloned, expressed and characterized, including superoxide dismutase, glutamate racemase, a [2Fe-2S] protein, a serine-type protease, glutamine phosphoribosylpyrophosphate, glycinamide ribonucleotide synthase, an alternative sigma factor, a *NifA*-like protein, and a DNA ligase (Lim et al., 1997; Kim et al., 1999; Chatelet et al., 1999; Choi et al., 1999; Yoo et al., 1999; Bera et al., 2000; Studholme and Buck, 2000a; Studholme et al., 2000b; Lim et al., 2001).

Cytochromes *b*, *c* and *o*, but no cytochrome *a*/*a*3 have been identified in strains of *Hydrogenobacter* (Aragno, 1992b). The terminal oxidase is an *o*-type cytochrome. The cytochrome c_{552} from *H. thermophilus* TK-6 is extremely thermostable and can restore its conformation even after being autoclaved for 10 min. at 121°C. Type *b* and *c* cytochromes and methionaquinone are reduced by a membrane-bound hydrogenase activity (Ishii et al., 2001). For oxygen respiration, the following enzymes were identified in "*Aquifex aeolicus*": ubiquinol cytochrome *c* oxidoreductase (bc_1 complex), cytochrome *c* and cytochrome *c* oxidase. The alternative system with cytochrome *bd* ubiquinol oxidase was also detected (Deckert et al., 1998). The cytochrome *bc* complex in "*A. aeolicus*" was characterized in detail and belongs to a group of (low-potential) menaquinol-oxidizing enzymes (Schütz et al., 2000). The sulfide:quinone oxidoreductase of "*A. aeolicus*" belongs to the glutathione reductase family of flavoproteins. The electron transport from sulfide to oxygen employs the cytochrome *bc* complex via the quinone pool (Nübel et al., 2000).

In *Hydrogenobacter thermophilus* TK-6 and *H. halophilus*, the main quinonic component is an unusual sulfur-containing quinone, 2-methylthio-3-VI, VII-tetrahydromultiprenyl-1,4-naphthoquinone (i.e., methionaquinone; Aragno, 1992a; Ishii et al., 2001). *Hydrogenothermus marinus* appears to contain a new menathioquinone, the structure of which is currently not known (Stöhr et al., 2001).

Cultivation

Aquificales grow well using 120-ml soda-lime-silicate glass (Stute GmbH, Rheinbreitbach, Germany) or 28-ml serum tubes (borosilicate glass, Schott, Mainz, Germany) for cultivation. For growth of more oxygen-tolerant Aquificales, the medium can be dispensed in air. The medium for more oxygen-sensitive Aquificales (e.g., *Aquifex pyrophilus*) should be flushed with nitrogen for 20 min. to get rid of oxygen. Afterwards, the medium is dispensed in 10–20 ml portions in an anaerobic chamber. The bottles are closed by rubber stoppers and the gas atmosphere is changed to the desired gas mixture (see Culture Media). If not mentioned otherwise, the atmosphere in the bottles consists of 300 kPa gas.

On a large scale, growth of members of the Aquificales are routinely in batch cultures, using enamel-protected fermentors with an operating volume of up to 300 liters (HTE, Bioengineering, Wald, Switzerland; Huber et al., 1992b; Huber et al., 1998; Jahnke et al., 2001). Furthermore, continuous cultivation of *Hydrogenobacter thermophilus* ITI 553 at low partial hydrogen pressures was reported (Manelius et al., 1997).

Culture Media

SME* Medium for *Aquifex pyrophilus* and "*A. aeolicus*"

NaCl	30.0 g
$MgSO_4 \cdot 7H_2O$	7.0 g
$MgCl_2 \cdot 6H_2O$	5.5 g
$NaHCO_3$	2.0 g
KCl	0.65 g
$CaCl_2 \cdot 2H_2O$	0.50 g
$K_2HPO_4 \cdot 3H_2O$	0.15 g
NH_4Cl	0.15 g
NaBr	0.10 g

Trace mineral solution *Aquifex*, 10 × 1.0 ml

H_2O (double-distilled, add up to the total volume) 1000.0 ml

Gas phase ($H_2/CO_2/O_2$) should be 79:20:1 (v/v/v; 300 kPa) and pH is adjusted to 6.5 with H_2SO_4. For the growth of "*A. aeolicus*," the medium should be supplemented with 0.1% thiosulfate.

Trace Mineral Solution *Aquifex*, 10 ×

Titriplex 1 (Nitrilotriacetic acid)	15.0 g
$MgSO_4 \cdot 7H_2O$	30.0 g
$MnSO_4 \cdot H_2O$	5.0 g
NaCl	10.0 g
$FeSO_4 \cdot 7H_2O$	1.0 g
$CoSO_4 \cdot 7H_2O$	1.8 g
$CaCl_2 \cdot 2H_2O$	1.0 g
$ZnSO_4 \cdot 7H_2O$	1.8 g
$CuSO_4 \cdot 5H_2O$	0.1 g
$KAl(SO_4)_2 \cdot 12H_2O$	0.18 g
H_3BO_3	0.1 g
$Na_2MoO_4 \cdot 2H_2O$	0.1 g
$(NH_4)_2Ni(SO_4)_2 \cdot 6H_2O$	28.0 g
$Na_2WO_4 \cdot 2H_2O$	0.5 g
Na_2SeO_4	0.5 g
H_2O (double-distilled, add up to the total volume)	1000.0 ml

Adjust pH to 6.5 with 5N KOH and store at 4°C in the dark.

OS Medium for *Thermocrinis ruber* and *T. albus*

$NaHCO_3$	1.0 g
NaCl	256.0 mg
Na_2SO_4	23.0 mg
KCl	15.0 mg
H_3BO_3	10.3 mg
$(NH_4)_2SO_4$	10.0 mg
KH_2PO_4	1.7 mg
$CaCl_2 \cdot 2H_2O$	0.8 mg
$NaNO_3$	0.3 mg
$FeCl_3 \cdot 6H_2O$ (0.05%)	0.2 ml
$MnSO_4 \cdot H_2O$ (0.03%)	0.2 ml
Thiosulfate	1.0 g

Trace mineral solution, modified, 10 × 1.0 ml

H_2O (double-distilled, add up to the total volume) 1000.0 ml

Gas phase ($N_2/H_2/O_2$) should be 96:3:1 (v/v/v; 300 kPa), and pH is adjusted to 6.5–7.0 with H_2SO_4.

Trace Mineral Solution, Modified, 10 ×

Titriplex 1 (Nitrilotriacetic acid)	15.0 g
$MgSO_4 \cdot 7H_2O$	30.0 g
$MnSO_4 \cdot H_2O$	5.0 g
NaCl	10.0 g
$FeSO_4 \cdot 7H_2O$	1.0 g
$CoSO_4 \cdot 7H_2O$	1.8 g
$CaCl_2 \cdot 2H_2O$	1.0 g
$ZnSO_4 \cdot 7H_2O$	1.8 g
$CuSO_4 \cdot 5H_2O$	0.1 g
$KAL(SO_4)_2 \cdot 12H_2O$	0.18 g
H_3BO_3	0.1 g
$Na_2MoO_4 \cdot 2H_2O$	0.1 g
$(NH_4)_2Ni(SO_4)_2 \cdot 6H_2O$	2.80 g
$Na_2WO_4 \cdot 2H_2O$	0.1 g
Na_2SeO_4	0.1 g
H_2O (double-distilled, add up to the total volume)	1000.0 ml

Adjust pH to 6.5 with 5N KOH and store at 4°C in the dark.

TK-6 Medium for *Hydrogenobacter thermophilus*

TK-6

$(NH_4)_2SO_4$	3.0 g
$K_2HPO_4 \cdot 3H_2O$	2.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NaCl	0.25 g
$CaCl_2 \cdot 2H_2O$	0.03 g
$FeSO_4 \cdot 7H_2O$	0.014 g

Trace mineral solution, modified, 10 × (see: OS medium) 0.75 ml

H_2O (double-distilled, add up to the total volume) 1000.0 ml

Gas phase ($H_2/CO_2/O_2$) should be 79:20:1 (v/v/v; 300 kPa) and pH is adjusted to 7.0 with H_2SO_4.

Medium for *Hydrogenobacter halophilus*

$(NH_4)_2SO_4$	2.0 g
K_2HPO_4	2.5 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	29.3 g
$CaCl_2 \cdot 2H_2O$	10.0 mg
$FeSO_4 \cdot 7H_2O$	10.0 mg
$NiSO_4 \cdot 7H_2O$	0.6 mg

Trace element solution 2.0 ml

H_2O (double-distilled, add up to the total volume) 1000.0 ml

Gas phase ($H_2/CO_2/O_2$) should be 7:1:1 (v/v/v; 100 kPa) and pH is adjusted to 7–7.5.

Trace Element Solution

$MnSO_4 \cdot 5H_2O$	1.0 mg
$CoCl_2 \cdot 6H_2O$	1.0 mg
$ZnSO_4 \cdot 7H_2O$	7.0 mg
$CuSO_4 \cdot 5H_2O$	0.5 mg
H_3BO_3	1.0 mg
MoO_3	1.0 mg
H_2O (double-distilled, add up to the total volume)	1000.0 ml

Medium for *Hydrogenobaculum acidophilum*

$(NH_4)_2SO_4$	1.0 g
K_2HPO_4	1.0 g
Sulfur	5.0 g
$MgSO_4 \cdot 7H_2O$	0.3 g
NaCl	1.0 g
$CaCl_2$	1.0 mg
$FeSO_4 \cdot 7H_2O$	1.0 mg
Trace element solution; see medium for *H. halophilus*	2.0 ml
H_2O (double-distilled, add up to the total volume)	1000.0 ml

Gas phase ($H_2/CO_2/O_2$) should be 8:1:1 (v/v/v, 100 kPa) and pH adjusted to 3.0 with HCl.

mjYPGS Medium for *Hydrogenobacter subterraneus*

NaCl	3.0 g
$MgSO_4 \cdot 7H_2O$	0.34 g
$MgCl_2 \cdot 6H_2O$	0.42 g
$Na_2SiO_3 \cdot 9H_2O$	1.0 g
$Na_2S_2O_3 \cdot 5H_2O$	2.48 g
KCl	33 mg
$CaCl_2 \cdot 2H_2O$	14.0 mg
K_2HPO_4	14.0 mg
$NiCl_2 \cdot 6H_2O$	0.05 mg
$Na_2SeO_3 \cdot 5H_2O$	0.05 mg
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.001 mg
Sodium succinate	0.2 g
Yeast extract	1.0 g
Peptone	1.0 g
Glucose	0.2 g
Trace mineral solution, 10 ×	1.0 ml
H_2O (double-distilled, add up to the total volume)	1000.0 ml

Gas phase should be air and pH is adjusted to 7.5 with H_2SO_4.

Trace Mineral Solution, 10 ×

Titriplex 1 (Nitrilotriacetic acid)	15.0 g
$MgSO_4 \cdot 7H_2O$	30.0 g
$MnSO_4 \cdot 2H_2O$	5.0 g
NaCl	10.0 g
$FeSO_4 \cdot 7H_2O$	1.0 g
$CoSO_4 \cdot 7H_2O$	1.8 g
$CaCl_2 \cdot 2H_2O$	1.0 g
$ZnSO_4 \cdot 7H_2O$	1.8 g
$CuSO_4 \cdot 5H_2O$	0.1 g

$KAl(SO_4)_2 \cdot 12H_2O$	0.18 g
H_3BO_3	0.1 g
$Na_2MoO_4 \cdot 2H_2O$	0.1 g
H_2O (double-distilled, add up to the total volume)	1000.0 ml

Adjust pH to 6.5 with 5N KOH and store at 4°C in the dark.

Medium for *Hydrogenothermus marinus*

NaCl	19.4 g
$MgCl_2 \cdot 6H_2O$	12.6 g
$NaHCO_3$	0.16 g
KCl	0.56 g
Na_2SO_4	3.24 g
Sulfur	0.5 g
NH_4Cl	0.30 g
$CaCl_2 \cdot 2H_2O$	2.38 g
Resazurin (1%)	1.0 ml

Trace element solution (10 ×; see medium for *H. halophilus*) 10.0 ml

H_2O (double-distilled, add up to the total volume) 1000.0 ml

Gas phase (H_2/CO_2) should be 80:20 (v/v; 300 kPa) with 20 ml of air and pH adjusted to 7 with H_2SO_4.

Preservation

Hydrogenobacter strains can be preserved by lyophilization using 0.1 M phosphate buffer (pH 7) containing 3% sodium glutamate as the suspending medium (Ishii et al., 2001). For short-time storage, Aquificaceae relatives can be stored at 4°C for several months. For long-time storage, preservation in liquid nitrogen at –140°C in the presence of 5% dimethylsulfoxide is recommended. No loss of cell viability of *Aquifex* and *Hydrogenobacter* strains was observed after a storage over a period of six years. A cryopreservation method for Aquificales representatives was described by Malik (1999).

Physiology

Members of the order Aquificales are extremely thermophilic or hyperthermophilic bacteria with optimal growth in the neutral pH range (except *Hydrogenobaculum acidophilum* and *Hydrogenobaculum* sp. NOR3L3B; Eder and Huber, 2002; Table 2). With optimal growth temperature around 85°C and a maximum growth temperature of 95°C, the hyperthermophilic species *Aquifex pyrophilus* and "*A. aeolicus*" represent the organisms with the highest growth temperatures within the domain Bacteria. The physiological properties of the Aquificales are listed in detail in Table 2.

Most Aquificales can grow with hydrogen as sole electron donor and oxygen as electron acceptor, performing the "Knallgas" reaction, the reduction of O_2 with H_2:

$$2H_2 + O_2 \rightarrow 2H_2O$$

The final product of this metabolic reaction is the formation of water (*Aquifex* meaning "water-maker"). Instead of hydrogen, most Aquificales can use thiosulfate or sulfur as a single energy source, and sulfuric acid is formed as the final product. *Aquifex*- and *Thermocrinis* species produce H_2S in addition to sulfuric acid in the presence of sulfur. Hydrogen-dependent growth was first reported for "*A. aeolicus*" (Huber and Stetter, 1999b) and was recently also described for *Hydrogenothermus marinus*, which requires sulfur for growth in addition (Stöhr et al., 2001). *Hydrogenobaculum acidophilum* and *Hydrogenobacter* sp. IT-7242 grow only when sulfur or thiosulfate is present in the medium (Shima and Suzuki, 1993; Skirnisdottir et al., 2001). Most of the Aquificales are strictly aerobic, growing preferentially under microaerophilic culture conditions. Interestingly, *Aquifex pyrophilus* can grow by using oxygen as low as 7.5 ppm (Deckert et al., 1998). *Aquifex pyrophilus* was the first representative of the Aquificales shown to grow anaerobically by nitrate reduction, forming N_2 as final product (denitrification; Huber et al., 1992b). Recently, growth of *Hydrogenobacter thermophilus* TK-6 on nitrate was reported (Suzuki et al., 2001). This is also true for *Hydrogenobacter* sp. PA14, isolated from Lake Tanganyika (Eder and Huber, 2002).

Most of the Aquificales are obligate autotrophs, using carbon dioxide as carbon source. It has been shown for *Hydrogenobacter thermophilus* TK-6 and the *Aquifex* species that carbon dioxide is fixed via the reductive citric acid cycle (Shiba et al., 1985; Beh et al., 1993; Deckert et al., 1998). More recently, the same CO_2 fixation pathway was proposed for *Thermocrinis ruber* (Jahnke et al., 2001).

Reports on heterotrophic growth of Aquificales are very rare (Eder and Huber, 2002). *Thermocrinis ruber* was the first organism shown to grow chemoorganoheterotrophically on formate and formamide under microaerophilic growth conditions. Both compounds were used simultaneously as carbon and energy sources (Huber et al., 1998). Recently, the ability to grow on formate and formamide was also reported for *Hydrogenobacter thermophilus* TK-6 (Eder and Huber, 2002). Furthermore, it was shown that *H. subterraneus*, a strictly aerobic heterotroph, is capable of utilizing a number of substrates such as yeast extract, peptone, tryptone, carbohydrates, sugars, amino acids and organic acids. As an electron donor for growth, sulfur, thiosulfate, sulfide or cysteine-HCl is required (Takai et al., 2001).

Genetics

A physical map was established from genomic DNA from *Aquifex pyrophilus*, and a random sequence analysis was performed (Shao et al., 1994; Choi et al., 1997). The complete genome sequence of "*A. aeolicus*" was determined (Deckert et al, 1998). It is a relatively small genome for a free-living bacterium, with a genome length of only 1.55 million base pairs. The most striking feature of the genome is the organizational relationship of the genes relative to one another. The majority of the genes appear to be organized within operons. Some genes are clustered in operons of common function. However, many pathway components are scattered throughout the genome or appear in novel operons (Deckert et al., 1998; Swanson, 2001). In addition, a single extrachromosomal element present at roughly twice the copy number of the chromosome was identified. The element has a length of 39,456 base pairs and a protein-coding region of 53% (Deckert et al, 1998). No introns or inteins were detected in the genome (Deckert et al., 1998). However, the intein Aae RIR2 was identified in the genome of "*A. aeolicus*" recently and deposited in the New England Biolabs Intein Database. Comparison of total genome sequences of different organisms showed that about 16% of the "*A. aeolicus*" genes are of archaeal origin (Aravind et al., 1998). Owing to this result, the authors have claimed that hyperthermophilic bacteria and archaea have exchanged genes particularly often by lateral gene transfer (Aravind et al., 1998; Nelson et al., 1999; see also Thermotogales in the second edition). On the other hand, it was argued that if *Aquifex* and *Thermotoga* (see also Thermotogales in the second edition) are truly deep branching, it is equally parsimonious to suppose that some of the features specified by the shared genes of these hyperthermophilic bacteria and archaea are primitive. They have retained ancestral genes which have since been lost in most bacteria as they have adapted to mesophilic environments (Kyrpides and Olson, 1999; Logsdon and Faguy, 1999). Based on recent phylogenetic analyses of archaeal genes in different bacteria, it was hypothesized that lateral gene transfer in hyperthermophiles may be as much a consequence as the cause of adaptation to hyperthermophily (Nesbø et al., 2001).

Biotechnology

Enzymes that are active at high temperatures are sought for industrial processes. Therefore, the highly thermostable enzymes of the Aquificales might be of great interest as biocatalysts, e.g., in the food or chemical industry. In addition, the sequencing of the total genome of "*A. aeolicus*" offers now the opportunity to use its genes as tools for a variety of biotechnological applications.

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Literature Cited

Acca, M., M. Bocchetta, E. Ceccarelli, R. Creti, K. O. Stetter, and P. Cammarano. 1994. Updating mass and composition of archaeal and bacterial ribosomes: Archaeal-like features of ribosomes from the deep-branching bacterium Aquifex pyrophilus. Syst. Appl. Microbiol. 16:629–637.

Alfredsson, G. A., A. Ingason, and J. K. Kristjansson. 1986. Growth of thermophilic obligately autotrophic hydrogen-oxidizing bacteria on thiosulfate. Lett. Appl. Microbiol. 2:21–23.

Andera, L., K. Mikulik, P. Branny, and A. Puscheva. 1991. DNA-dependent RNA polymerase from an extremely thermophilic hydrogen-oxidizing bacterium Calderobacterium hydrogenophilum. Biochem. Biophys. Res. Commun. 175:949–954.

Andera, L., K. Mikulik, and N. D. Savelyeva. 1993. Characterization of a reverse gyrase from the extremely thermophilic hydrogen-oxidizing eubacterium Calderobacterium hydrogenophilum. FEMS Microbiol. Lett. 110:107–112.

Aragno, M. 1992a. Aerobic, chemolithoautotrophic, thermophilic bacteria. *In:* J. K. Kristjansson (Ed.) Thermophilic Bacteria. CRC Press. Boca Raton, FL. 77–103.

Aragno, M. 1992b. Thermophilic, aerobic, hydrogenoxidizing (Knallgas) bacteria. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (Eds.) The Prokaryotes (2nd ed.). Springer-Verlag. New York, NY. 3917–3933.

Aravind, L., R. L. Tatusov, Y. I. Wolf, D. R. Walker, and E. V. Koonin. 1998. Evidence for massive gene exchange between archaeal and bacterial hyperthermophiles. TIG 14:442–444.

Balch, W. E., and R. S. Wolfe. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of Methanobacterium ruminantium in a pressurized atmosphere. Appl. Environ. Microbiol. 32:781–791.

Baldauf, S. L., J. D. Palmer, and W. F. Doolittle. 1996. The root of the universal tree and the origin of eukaryotes based on elongation factor phylogeny. Proc. Natl. Acad. Sci. USA 93:7749–7754.

Beck, P., and R. Huber. 1997. Detection of cell viability in cultures of hyperthermophiles. FEMS Microbiol. Lett. 147:11–14.

Beffa, T., M. Blanc, and M. Aragno. 1996. Obligately and facultatively autotrophic, sulfur- and hydrogen-oxidizing

thermophilic bacteria isolated from hot composts. Arch. Microbiol. 165:34–40.

Beh, M., G. Strauss, R. Huber, K. O. Stetter, and G. Fuchs. 1993. Enzymes of the reductive citric acid cycle in the autotrophic eubacterium Aquifex pyrophilus and in the archaebacterium Thermoproteus neutrophilus. Arch. Microbiol. 160:306–311.

Behammer, W., Z. Shao, W. Mages, R. Rachel, K. O. Stetter, and R. Schmitt. 1995. Flagellar structure and hyperthermophily: Analysis of a single flagellin gene and its product in Aquifex pyrophilus. J. Bacteriol. 177:6630–6637.

Bera, A. K., S. Chen, J. L. Smith, and H. Zalkin. 2000. Temperature-dependent function of the glutamine phosphoribosylpyrophosphate amidotransferase ammonia channel and coupling with glycinamide ribonucleotide synthetase in a hyperthermophile. J. Bacteriol. 182: 3734–3739.

Bocchetta, M., E. Ceccarelli, R. Creti, A. M. Sanangelantoni, O. Tiboni, and P. Cammarano. 1995. Arrangement and nucleotide sequence of the gene (fus) encoding elongation factor G (EF-G) from the hyperthermophilic bacterium Aquifex pyrophilus: Phylogenetic depth of hyperthermophilic bacteria inferred from analysis of the EF-G/fus sequences. J. Molec. Evol. 41:803–812.

Bocchetta, M., S. Gribaldo, A. Sanangelantoni, and P. Cammarano. 2000. Phylogenetic depth of the bacterial genera Aquifex and Thermotoga inferred from analysis of ribosomal protein, elongation factor and RNA polymerase subunit sequences. J. Molec. Evol. 50:366–380.

Bonjour, F., and M. Aragno. 1986. Growth of thermophilic, obligatorily chemolithoautotrophic hydrogen-oxidizing bacteria related to Hydrogenobacter with thiosulfate and elemental sulfur as electron and energy source. FEMS Microbiol. Lett. 35:11–15.

Brock, T. D. 1967. Life at high temperatures. Science 158:1012–1019.

Brock, T. D. 1978. Thermophilic microorganisms and life at high temperatures. Springer-Verlag. New York, NY.

Brock, T. D. 1998. Early days in Yellowstone microbiology. ASM News 64:137–140.

Brown, J. R., and W. F. Doolittle. 1995. Root of the universal tree of life based on ancient aminoacyl-tRNA synthetase gene duplications. Proc. Natl. Acad. Sci. USA 92:2441–2445.

Burggraf, S., G. J. Olsen, K. O. Stetter, and C. R. Woese. 1992. A phylogenetic analysis of Aquifex pyrophilus. Syst. Appl. Microbiol. 15:352–356.

Chatelet, C., J. Gaillard, Y. Petillot, M. Louwagie, and J. Meyer. 1999. A [2Fe-2S] protein from the hyperthermophilic bacterium Aquifex aeolicus. Biochem. Biophys. Res. Commun. 261:885–889.

Choi, I. G., S. S. Kim, J.-R. Ryu, Y. S. Han, W. G. Bang, S. H. Kim, and Y. G. Yu. 1997. Random sequence analysis of genomic DNA of a hyperthermophile: Aquifex pyrophilus. Extremophiles 1:125–134.

Choi, I. G., W. G. Bang, S. H. Kim, and Y. G. Yu. 1999. Extremely thermostable serine-type protease from Aquifex pyrophilus. J. Biol. Chem. 274:881–888.

Deckert, G., P. V. Warren, T. Gaasterland, W. G. Young, A. L. Lenox, D. E. Graham, R. Overbeek, M. A. Snead, M. Keller, M. Aujay, R. Huber, R. A. Feldman, J. M. Short, G. J. Olsen, and R. V. Swanson. 1998. The complete genome of the hyperthermophilic bacterium Aquifex aeolicus. Nature 392:353–358.

Eder, W., and R. Huber. 2002. New isolates and physiological properties of the Aquificales and description of Thermocrinis albus sp. nov. Extremophiles 6(4):309–318.

Graber, J. R., J. Kirshtein, M. Speck, and A.-L. Reysenbach. 2001. Community structure along a thermal gradient in a stream near Obsidian Pool, Yellowstone National Park. *In:* A.-L. Reysenbach, M. Voytek, and R. Mancinelli (Eds.) Thermophiles: Biodiversity, Ecology, and Evolution. Kluwer Academic/Plenum Publishers. New York, NY. 81–91.

Huber, R., and K. O. Stetter. 1992a. The order Thermotogales. *In:* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (Eds.) The Prokaryotes. Springer-Verlag. New York, NY. 3809–3815.

Huber, R., T. Wilharm, D. Huber, A. Trincone, S. Burggraf, H. König, R. Rachel, I. Rockinger, H. Fricke, and K. O. Stetter. 1992b. Aquifex pyrophilus gen.nov., sp.nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. Syst. Appl. Microbiol. 15:340–351.

Huber, R., S. Burggraf, T. Mayer, S. M. Barns, P. Rossnagel, and K. O. Stetter. 1995. Isolation of a hyperthermophilic archaeum predicted by in situ RNA analysis. Nature 376:57–58.

Huber, R., W. Eder, S. Heldwein, G. Wanner, H. Huber, R. Rachel, and K. O. Stetter. 1998. Thermocrinis ruber gen. nov., sp. nov., a pink-filament-forming hyperthermophilic bacterium isolated from Yellowstone National Park. Appl. Environ. Microbiol. 64:3576–3583.

Huber, R. 1999a. Die Laserpinzette als Basis für Einzelzellkultivierungen. BIOspektrum 5:289–291.

Huber, R., and K. O. Stetter. 1999b. Thermotogales. Encyclopedia of Life Sciences (http://www.els.net). Nature Publishing Group. London, England.

Huber, R., and K. O. Stetter. 2000a. Genus Aquifex. *In:* G. M. Garrity, D. R. Boone, and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology. Springer-Verlag. New York, NY. 1:360–362.

Huber, R., W. Eder, and K. O. Stetter. 2000b. Genus Thermocrinis. *In:* G. M. Garrity, D. R. Boone, and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology. Springer-Verlag. New York, NY. 1:364–366.

Huber, R., H. Huber, and K. O. Stetter. 2000c. Towards the ecology of hyperthermophiles: Biotopes, new isolation strategies and novel metabolic properties. FEMS Microbiol. Rev. 24:615–623.

Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace. 1998. Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. 180:366–376.

Ishii, M., Y. Igarashi, and T. Kodama. 1987. Purification and characterization of ATP: Citrate Lyase from Hydrogenobacter thermophilus TK-6. J. Bacteriol. 171:1788–1792.

Ishii, M., Y. Igarashi, and T. Kodama. 1989. Colony formation of Hydrogenobacter thermophilus on a plate solidified with Gelrite. Agric. Biol. Chem. 51:3139–3141.

Ishii, M., T. Kawasumi, Y. Igarashi, and A.-L. Reysenbach. 2001. Genus Hydrogenobacter. *In:* G. M. Garrity, D. R. Boone, and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology. Springer-Verlag. New York, NY. 1:363–364.

Jahnke, L. L., W. Eder, R. Huber, J. M. Hope, K.-U. Hinrichs, J. M. Hayes, D. J. Des Marais, S. L. Cady, and R. E. Summons. 2001. Composition of hydrothermal vent

microbial communities as revealed by analyses of signature lipids, stable carbon isotopes and Aquificales cultures. Appl. Environ. Microbiol. 67:5179–5189.

Kang, K. K., G. T. Veeder, P. J. Mirrasoul, T. Kaneko, and I. W. Cottrell. 1982. Agar-like polysaccharide produced by a Pseudomonas species: Production and basic properties. Appl. Environ. Microbiol. 43:1086–1091.

Kawasumi, T., Y. Igarashi, T. Kodama, and Y. Minoda. 1984. Hydrogenobacter thermophilus gen. nov., sp. nov., an extremely thermophilic, aerobic, hydrogen-oxidizing bacterium. Int. J. Syst. Bacteriol. 34:5–10.

Kim, S. S., I. G. Choi, S. H. Kim, and Y. G. Yu. 1999. Molecular cloning, expression, and characterization of a thermostable glutamate racemase from a hyperthermophilic bacterium, Aquifex pyrophilus. Extremophiles 3:175–183.

Klenk, H.-P., P. Palm, and W. Zillig. 1994. DNA-dependent RNA polymerases as phylogenetic marker molecules. Syst. Appl. Microbiol. 16:638–647.

Klenk, H.-P., T.-D. Meier, P. Durovic, V. Schwass, F. Lottspeich, P. P. Dennis, and W. Zillig. 1999. RNA polymerase of Aquifex pyrophilus: Implications for the evolution of the bacterial rpoBC operon and extremely thermophilic bacteria. J. Molec. Evol. 48:528–541.

Kristjansson, K. J., A. Ingason, and G. A. Alfredsson. 1985. Isolation of thermophilic obligately autotrophic hydrogen-oxidizing bacteria, similar to Hydrogenobacter thermophilus, from Icelandic hot springs. Arch. Microbiol. 140:321–325.

Kryukov, V. R., N. D. Savel'eva, and M. A. Pusheva. 1983. Calderobacterium hydrogenophilum nov. gen., sp. nov., an extremely thermophilic hydrogen bacterium and its hydrogenase activity. Microbiology 52:611–618.

Kyrpides, N. C. and G. J. Olsen. 1999. Archaeal and bacterial hyperthermophiles: Horizontal gene exchange or common ancestry? Trends Genet. 15:298–299.

Lim, J. H., Y. G. Yu, I. G. Choi, J.-R. Ryu, B.-Y. Ahn, S. H. Kim, and Y. S. Han. 1997. Cloning and expression of superoxide dismutase from Aquifex pyrophilus, a hyperthermophilic bacterium. FEBS Lett. 406:142–146.

Lim, J. H., J. Choi, S. J. Han, S. H. Kim, H. Z. Hwang, D. K. Jin, B. Y. Ahn, and Y. S. Han. 2001. Molecular cloning and characterization of thermostable DNA ligase from Aquifex pyrophilus, a hyperthermophilic bacterium. Extremophiles 5:161–168.

Logsdon, J. M. and D. M. Faguy. 1999. Thermotoga heats up lateral gene transfer. Curr. Biol. 9:R747–R751.

Ludvik, J., O. Benada, and K. Mikulik. 1994. Ultrastructure of an extreme thermophilic hydrogen-oxidizing bacterium Calderobacterium hydrogenophilum. Arch. Microbiol. 162:267–271.

Malik, K. A. 1999. Preservation of some extremely thermophilic chemolithoautotrophic bacteria by deep-freezing and liquid-drying methods. J. Microb. Meth. 35:177–182.

Manelius, A., O. Holst, G. O. Hreggvidsson, J. K. Kristjansson, N. Raven, and R. Sharp. 1997. Continous cultivation of Hydrogenobacter thermophilus at low partial pressures of H_2. Biotechnol.Techniques 11:177–181.

Marler, G. D. 1973. Inventory of Thermal Features of the Firehole River Geyser Basins and Other Selected Areas of Yellowstone National Park. US Dept. of Commerce, National Technical Information Service. Springfield, IL. PB-221289.

Marteinsson, V. T., S. Hauksdottir, C. F. V. Hobel, H. Kristmannsdottir, G. O. Hreggvidsson, and J. K. Kristjansson. 2001a. Phylogenetic diversity analysis of subterranean hot springs in Iceland. Appl. Environ. Microbiol. 67:4242–4248.

Marteinsson, V. T., J. K. Kristjansson, H. Kristmannsdottir, M. Dahlkvist, K. Saemundsson, M. Hannigton, S. K. Petursdottir, A. Geptner, and P. Stoffers. 2001b. Discovery and description of giant submarine smectite cones on the seafloor in Eyjafjordur, northern Iceland, and a novel thermal habitat. Appl. Environ. Microbiol. 67:827–833.

Nelson, K. E., R. A. Clayton, S. R. Gill, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, W. C. Nelson, K. A. Ketchum, L. McDonald, T. R. Utterback, J. A. Malek, K. D. Linher, M. M. Garrett, A. M. Stewart, M. D. Cotton, M. S. Pratt, C. A. Phillips, D. Richardson, J. Heidelberg, G. G. Sutton, R. D. Fleischmann, J. A. Eisen, O. White, S. L. Salzberg, H. O. Smith, J. C. Venter, and C. M. Fraser. 1999. Evidence for lateral gene transfer between Archaea and Bacteria from genome sequence of Thermotoga maritima. Nature 399:323–329.

Nesbo, C. L., S. L'Haridon, K. O. Stetter, and W. F. Doolittle. 2001. Phylogenetic analyses of two "archaeal" genes in Thermotoga maritima reveal multiple transfers between Archaea and Bacteria. Molec. Biol. Evol. 18:362–375.

Nishihara, H., Y. Igarashi, and T. Kodama. 1990. A new isolate of Hydrogenobacter, an obligately chemolithoautotrophic, thermophilic, halophilic and aerobic hydrogen-oxidizing bacterium from seaside saline hot spring. Arch. Microbiol. 153:294–298.

Nübel, T., C. Klughammer, R. Huber, G. Hauska, and M. Schütz. 2000. Sulfide:quinone oxidoreductase in membranes of the hyperthermophilic bacterium Aquifex aeolicus (VF5). Arch. Microbiol. 173:233–244.

Pennisi, E. 1998. Genome data shake the tree of life. Science 280:672–674.

Pennisi, E. 1999. Is it time to uproot the tree of life? Science 284:1305–1307.

Pitulle, C., Y. Yinqing, M. Marchiani, E. R. B. Moore, J. L. Siefert, M. Aragno, P. Jurtshuk, and G. E. Fox. 1994. Phylogenetic position of the genus Hydrogenobacter. Int. J. Syst. Bacteriol. 44:620–626.

Plötz, B. M., B. Lindner, K. O. Stetter, and O. Holst. 2000. Characterization of a novel lipid A containing D-galacturonic acid that replaces phosphate residues. J. Biol. Chem. 275:11222–11228.

Reysenbach, A.-L., G. S. Whickham, and N. R. Pace. 1994. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl. Environ. Microbiol. 60:2113–2119.

Reysenbach, A.-L., A. B. Banta, D. R. Boone, S. C. Cary, and G. W. Luther. 2000a. Microbial essentials at hydrothermal vents. Nature 404:835.

Reysenbach, A.-L., M. Ehringer, and K. Hershberger. 2000b. Microbial diversity at 83°C in Calcite Springs, Yellowstone National Park: Another environment where the Aquificales and "Korarchaeota" coexist. Extremophiles 4:61–67.

Reysenbach, A.-L., K. Longnecker, and J. Kirshtein. 2000c. Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic ridge hydrothermal vent. Appl. Environ. Microbiol. 66:3798–3806.

Reysenbach, A.-L. 2001. Class I: Aquificae class. nov. *In:* G. M. Garrity, D. R. Boone, and R. W. Castenholz (Eds.) Bergey's Manual of Systematic Bacteriology. Springer-Verlag. New York, NY. 1:359.

Schütz, M., M. Brugna, E. Lebrun, F. Baymann, R. Huber, K. O. Stetter, G. Hauska, R. Toci, D. Lemesle-Meunier, P. Tron, C. Schmidt, and W. Nitschke. 2000. Early evolution of cytochrome bc complexes. J. Molec. Biol. 300:663–675.

Shao, Z., W. Mages, and R. Schmitt. 1994. A physical map of the hyperthermophilic bacterium Aquifex pyrophilus chromosome. J. Bacteriol. 176:6776–6780.

Shiba, H., T. Kawasumi, Y. Igarashi, T. Kodama, and Y. Minoda. 1985. The CO_2 assimilation via the reductive tricarboxylic acid cycle in an obligately autotrophic, aerobic hydrogen-oxidzing bacterium, Hydrogenobacter thermophilus. Arch. Microbiol. 141:198–203.

Shima, S. and K. I. Suzuki. 1993. Hydrogenobacter acidophilus sp. nov., a thermoacidophilic, aerobic, hydrogen-oxidizing bacterium requiring elemental sulfur for growth. Int. J. Syst. Bacteriol. 43:703–708.

Shima, S., M. Yanagi, and H. Saiki. 1994. The phylogenetic position of Hydrogenobacter acidophilus based on 16S rRNA sequence analysis. FEMS Microbiol. Lett. 119: 119–122.

Skirnisdottir, S., G. O. Hreggvidsson, S. Hjörleifsdottir, V. T. Marteinsson, S. K. Petursdottir, O. Holst, and J. K. Kristjansson. 2000. Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats. Appl. Environ. Microbiol. 66:2835–2841.

Skirnisdottir, S., G. O. Hreggvidsson, O. Holst, and J. K. Kristjansson. 2001. A new ecological adaptation to high sulfide by a Hydrogenobacter sp. growing on sulfur compounds but not on hydrogen. Microbiol. Res. 156:41–47.

Snel, B., P. Bork, and M. A. Huynen. 1999. Genome phylogeny based on gene content. Nature Genet. 21:108–110.

Stöhr, R., A. Waberski, H. Völker, B. J. Tindall, and M. Thomm. 2001. Hydrogenothermus marinus gen. nov., sp. nov., a novel thermophilic hydrogen-oxidizing bacterium, recognition of Calderobacterium hydrogenophilum as a member of the genus Hydrogenobacter and proposal of the reclassification of Hydrogenobacter acidophilus as Hydrogenobaculum acidophilum gen. nov., comb. nov., in the phylum "Hydrogenobacter/Aquifex." Int. J. Syst. Evol. Microbiol. 51:1853–1862.

Studholme, D. J. and M. Buck. 2000a. The alternative sigma factor σ^{28} of the extreme thermophile Aquifex aeolicus restores motility to an Escherichia coli fliA mutant. FEMS Microbiol. Lett. 191:103–107.

Studholme, D. J., S. R. Wigneshwereraraj, M.-T. Gallegos, and M. Buck. 2000b. Functionality of purified σ^{N} (σ^{54}) and a NifA-like protein from the hyperthermophile Aquifex aeolicus. J. Bacteriol. 182:1616–1623.

Suzuki, M., Z. J. Cui, M. Ishii, and Y. Igarashi. 2001. Nitrate respiratory metabolism in an obligately autotrophic hydrogen-oxidizing bacterium, Hydrogenobacter thermophilus TK-6. Arch. Microbiol. 175:75–78.

Swanson, R. V. 2001. Genome of Aquifex aeolicus. *In:* M. W. W. Adams and R. M. Kelly (Eds.) Methods in Enzymology. Academic Press. San Diego, CA. 158–169.

Takacs, C. D., M. Ehringer, R. Favre, M. Cermola, G. Eggertsson, A. Palsdottir, and A.-L. Reysenbach. 2001. Phylogenetic characterization of the blue filamentous bacterial community from an Icelandic geothermal spring. FEMS Microbiol. Ecol. 35:123–128.

Takai, K., T. Komatsu, and K. Horikoshi. 2001. Hydrogenobacter subterraneus sp. nov., an extremely thermophilic, heterotrophic bacterium unable to grow on hydrogen gas, from deep subsurface geothermal water. Int. J. Syst. Evol. Microbiol. 51:1425–1435.

Wetmur, J. G., D. M. Wong, B. Ortiz, J. Tong, F. Reichert, and D. H. Gelfand. 1994. Cloning, sequencing, and expression of RecA proteins from three distantly related thermophilic eubacteria. J. Biol. Chem. 269:25928–25935.

Yamamoto, H., A. Hiraishi, K. Kato, H. X. Chiura, Y. Maki, and A. Shimizu. 1998. Phylogenetic evidence for the existence of novel thermophilic bacteria in hot spring sulfur-turf microbial mats in Japan. Appl. Environ. Microbiol. 64:1680–1687.

Yoo, S. J., J. Meyer, and E. Münck. 1999. Mössbauer evidence for a diferrous [2Fe-2S] cluster in a ferredoxin from Aquifex aeolicus. J. Am. Chem. Soc. 121:10450–10451.

PART 14

Phylogenetically Unaffiliated Bacteria

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CHAPTER 14.1

Morphologically Conspicuous Sulfur-Oxidizing Eubacteria

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This chapter deals with the genera *Achromatium*, *Macromonas*, *Thiobacterium*, *Thiospira*, and *Thiovulum* (see Fig. 1). They all belong to chemotrophic microbial populations generally encountered in natural habitats that are characterized by the simultaneous presence of H_2S and O_2, i.e., at the border between aerobic and anaerobic zones in surface waters and in the outflows of H_2S-bearing springs. It will be useful to introduce this discussion with some general remarks on the special nature of this ecological niche to help explain the embarassing paucity of knowledge we possess about its inhabitants.

In nature, the coexistence of H_2S and O_2 can only be sustained in systems subject to continuous inputs of both substances, because H_2S is not stable in the presence of O_2. H_2S can be rapidly oxidized without the intervention of living organisms, the rate of oxidation depending on pH, temperature, and the presence of catalysts and/or inhibitors (Chen and Morris, 1972a, 1972b); reaction products range from sulfur and polysulfides to thiosulfate, sulfite, and sulfate. Thus, this habitat contains an array of reduced sulfur compounds that are all potential substrates for chemolithotrophic oxidation but that differ greatly in their stability in the presence of O_2.

The chemotrophic segment of the microbial population occupying this special niche includes—in addition to accidental "interlopers" such as H_2S-tolerant microaerophilic heterotrophs—a group of organisms called *colorless sulfur bacteria,* which appear to interact directly with the reduced-sulfur compounds characterizing this habitat.

These colorless sulfur bacteria include in the first place *Sulfolobus, Thiobacillus*, and *Thiomicrospira* (see The Order Thermoproteales and The Genera *Thiobacillus*, *Thiomicrospira* and *Thiosphaera* both in the second edition), which have been shown to possess the capacity to chemolithotrophically oxidize reduced-sulfur compounds. Secondly, the group includes the five genera to be discussed here—*Achromatium*, *Macromonas*, *Thiobacterium*, *Thiospira*, and *Thiovulum*—as well as *Beggiatoa*, *Thiothrix,* and *Thioploca* (see 16 and 166). These eight genera are included among the colorless sulfur bacteria because the observed appearance and disappearance of sulfur inclusions suggest the possession of at least the capacity to oxidize sulfide and sulfur. The nutritional status of these genera is by no means certain and their relationship to reduced-sulfur compounds may range from obligate chemolithotrophy to protective, detoxifying sulfide oxidation or to merely gratuitous sulfide oxidation. Another argument for inclusion of this group is the observed absence of these genera in habitats devoid of H_2S. Finally, there are an indefinite number of colorless sulfur bacteria that are not recognized in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974) or discussed in this Handbook because they have been described poorly or no more than once. The works of the old masters, such as Gicklhorn, Hinze, Kolkwitz, Lauterborn, Molisch, Nadson, Warming, and Winogradsky, offer clear indications that several genera and even more species of colorless sulfur bacteria exist that cannot now be recognized usefully.

Thus the 11 genera discussed in this book represent only the most accessible part of the inhabitants of this ecological niche; even so, only five of them exist at the moment in pure culture: *Thiobacillus*, *Sulfolobus,* and *Thiomicrospira*, which can be cultivated with the stable compounds thiosulfate and/or sulfur as oxidizable substrates, *Macromonas*, of which only heterotrophic strains exist in pure culture, and *Beggiatoa*, from marine and freshwater habitats. Only the marine *Beggiatoa* strains seem to be facultative autotrophs (Nelson, 1989). The other six genera have not yielded pure cultures most probably because they are obligate sulfide-oxidizers, which, in view of the autoxidizability of H_2S, renders the use of solid media impossible and that of liquid media extremely cumbersome, since continuous inputs of H_2S and O_2 have to be maintained. Thus, for the moment, these six

This chapter was taken unchanged from the second edition.

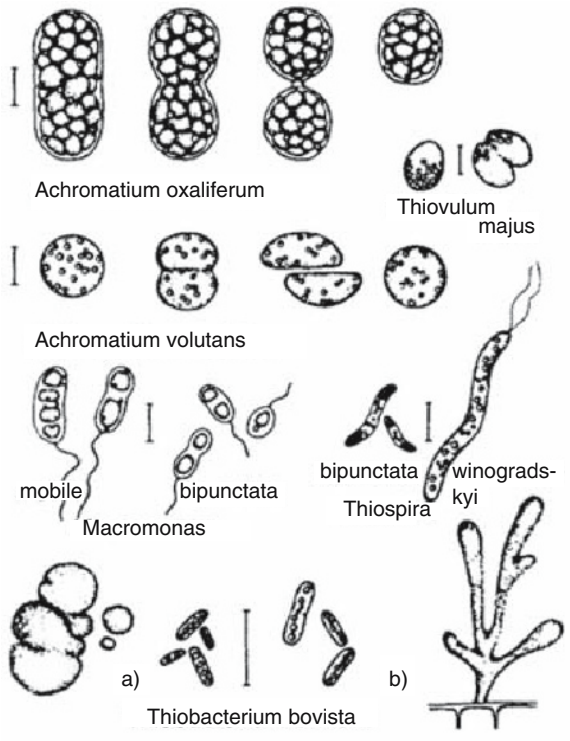


Fig. 1. Composite drawing of the described organisms, redrawn from the original literature. Bars = 10 µm—except for the typical colonies of the *Thiobacterium* species: (a) "puffball" shape, (b) dendroid shape, Bars = 5 µm.

genera exist only by virtue of their morphological recognizability. The morphological characteristics are the only basis for the division into different genera.

In nature, quite often the predominance of one or a few colorless sulfur bacteria can be observed. Knowledge of the factors determining such predominance would obviously be of great help in isolation studies, but so far we have no clue as to the determinants of competition and survival of these diverse organisms, which have to compete not only among one another, but very likely also against the chemical oxidation of H_2S.

Thus we are faced with a vicious circle: lack of knowledge of the physiology of these organisms impedes the design of effective isolation procedures, which, in turn, are the very requirement for obtaining such physiological knowledge through pure culture study. The riddle they pose is further complicated by the complexity of their natural habitat. In addition to natural aeration, when exposed to light, the medium also receives O_2 from photosynthesis, subject to diurnal fluctuations. Furthermore, H_2S can originate from groundwaters poor in organic matter, as well as from sulfate reduction in mud layers, in which case it is invariably accompanied by organic compounds. The mineral contents of the water also appear to play an important role. A relatively high Ca^{2+} concentration or the presence of solid $CaCO_3$ characterizes many of the habitats in which the organisms are found; the relationship is most obvious for *Achromatium* and *Macromonas*, which both can have $CaCO_3$ inclusions.

Enrichment and Cultivation Methods

Imitation of the natural conditions in the laboratory appears to be the best approach to enrichment, cultivation, and isolation of *Achromatium*, *Macromonas*, *Thiobacterium*, *Thiospira*, and *Thiovulum,* the five genera under discussion in this chapter. The enrichment methods consist essentially of using Winogradsky columns (Winogradsky, 1888) that are kept in the dark and that contain sediments in which H_2S is generated through sulfate reduction. Hence, adequate, i.e., slowly decaying, organic material—and in the case of freshwater enrichments, $CaSO_4$—has to be included in the sediment; in seawater enrichments, a considerable amount of sulfate is already present in the water phase. Besides stationary columns, flow-through variations have been used in which the water phase is continuously and slowly replenished, which provides a means of controlling the position of the H_2S/O_2 border zone and of eliminating contaminants. This method has led to reproducible enrichment procedures for *Thiovulum,* while a more classical enrichment method could be used for the heterotrophic *Macromonas bipunctata*. For the other three genera, enrichment is still extremely difficult and at best capricious.

In the design of effective enrichment and cultivation methods, some guidelines can be further derived from specific ecological attributes of the organisms themselves:

1. The presence of elemental sulfur inclusions, a stored energy source, implies that the culture can survive periods of absence of H_2S but not of O_2 (all genera).
2. Possession of strong chemotactic properties introduces the possibility of exerting selection through water flow (*Thiovulum, Thiospira*).
3. Limited motility, or its absence in some genera, calls for stability of the position of the H_2S/O_2 border zone in the place where the organism is expected to accumulate (*Achromatium*: at the bottom; *Thiobacterium*: at the surface).
4. Capacity for attachment to solid surfaces permits selection by water flow, provided the water contains the proper mix of H_2S and O_2 (*Thiobacterium*, *Thiothrix).*

5. The organism permitting, application of low temperatures (10–17°C) appears favorable, as this slows down chemical H_2S oxidation and thus leads to greater stability.

In conjunction with these methods, purification of cell material has been undertaken by taking advantage of chemotaxis *(Thiovulum)* and of selective sedimentation based on high specific gravity (*Achromatium*). Suspending the cells in sterile solutions followed by transfer to fresh sterile solution after separation had taken place could be carried out repeatedly, the "washing efficiency" depending on the initial degree of contamination and the endurance of the cells to repeated washings.

Such purified material, derived from nature or enrichment cultures, can then be subjected to cultivation in aseptic, controlled systems in which constant inputs of H_2S and O_2 are maintained. A great deal of ingenuity has gone into the design of such systems, based, for instance, upon the use of gas mixtures, semipermeable membranes, controlled pumping of sterile nutrient solutions, and solid/liquid phase systems in which the solid phase provides H_2S. So far, the use of sulfide-charged ion-exchangers or badly soluble sulfides as a constant source of H_2S has not been found practicable, nor has the use of inhibitors of chemical H_2S oxidation. Important examples of devices successfully used in specific instances are presented by Devidé (1954), Keil (1912), la Rivière (1963, 1965), and Perfil'ev and Gabe (1961). Wirsen and Jannasch (1978) described a variety of cultivation systems for *Thiovulum* (see below) in which full advantage was taken of modern materials and equipment. It is likely that these systems are also suitable for cultivation of other recalcitrant sulfur bacteria.

Presently, however, our knowledge is still restricted to such data as can be obtained from observations on natural populations and on enrichment cultures in the laboratory, both macro- and microscopically. In addition, laboratory cultures have in several cases produced sufficient quantities of cell material, sufficiently free from contamination, to make electron microscopy of whole cells and thin sections possible. Similarly, such cell material has permitted the determination of sometimes important physiological data. The main target of studies in complex culture systems remains, however, the acquirement of knowledge about the organism that will lead to the design of simpler cultivation methods. After all, one should keep in mind that one successful petri dish culture offers more promise for isolation and subsequent work than any of the culture systems mentioned above.

Significance of the Colorless Sulfur Bacteria

This group of organisms poses the question of how such a great morphological diversity has evolved in a very specialized and restricted niche in competition with chemical sulfide oxidation. One might well speculate that this niche must have been much larger at the time when, during the evolution of the present biosphere, the first oxygen appeared on an initially anaerobic stage and that, for a long time since, a much smaller O_2 concentration prevailed in the atmosphere than at present. However this may be, it is certain that microbial activity, including that of colorless sulfur bacteria, has played and is still playing an important role in the biogeochemical cycle of sulfur (Jørgensen, 1989; Kuenen, 1975; SCOPE 19, 1983; SCOPE 39, 1989; Kuenen and Bos, 1989; la Rivière, 1966). It is, for instance, generally accepted that the sulfur deposits that are mined today are biogenic and have been formed by sulfate reduction followed by an oxidation step that may have involved colorless sulfur bacteria. The well-known *sulfuretum,* in which sulfur is still accumulating today, offers an opportunity for determining the additional contributions of chemical and photosynthetic sulfide oxidation. Furthermore, sulfide oxidation to sulfate is an important step in the regeneration of oxidizing power within water bodies where sulfate plays a role in anaerobic mineralization. Also, the release of H_2S into the atmosphere from oceanic, marshy, and estuarine areas is at the moment a matter of concern on a global scale in judging the impact of artificial SO_2 emissions that are superimposed on it (Svensson and Söderlund, 1976). The magnitude of the biogenic H_2S emissions is presently not well known, but it is certain that the colorless sulfur bacteria codetermine it to a large extent. Finally, biological sulfide oxidation is responsible for the generation of certain types of acid soils and the formation of acid mine effluents. In the mining industry, so-called microbial leaching is presently being practiced for improving the yield of various metals from sulfide ores. For this purpose, *Thiobacillus* species are frequently being used (see The Genus *Thermoplasma* in the second edition). Furthermore, the microbiological methods for removing sulfur from coal, and removing hydrogen sulfide from biogas, generated by anaerobic digestion, are growing in industrial importance.

In the situation as outlined in this introduction, more detailed presentation of available information that follows can offer no more than some springboards and roadsigns for those who want to penetrate further into a fascinating and as yet unconquered territory of the microbial kingdom. Such endeavors also offer the rare

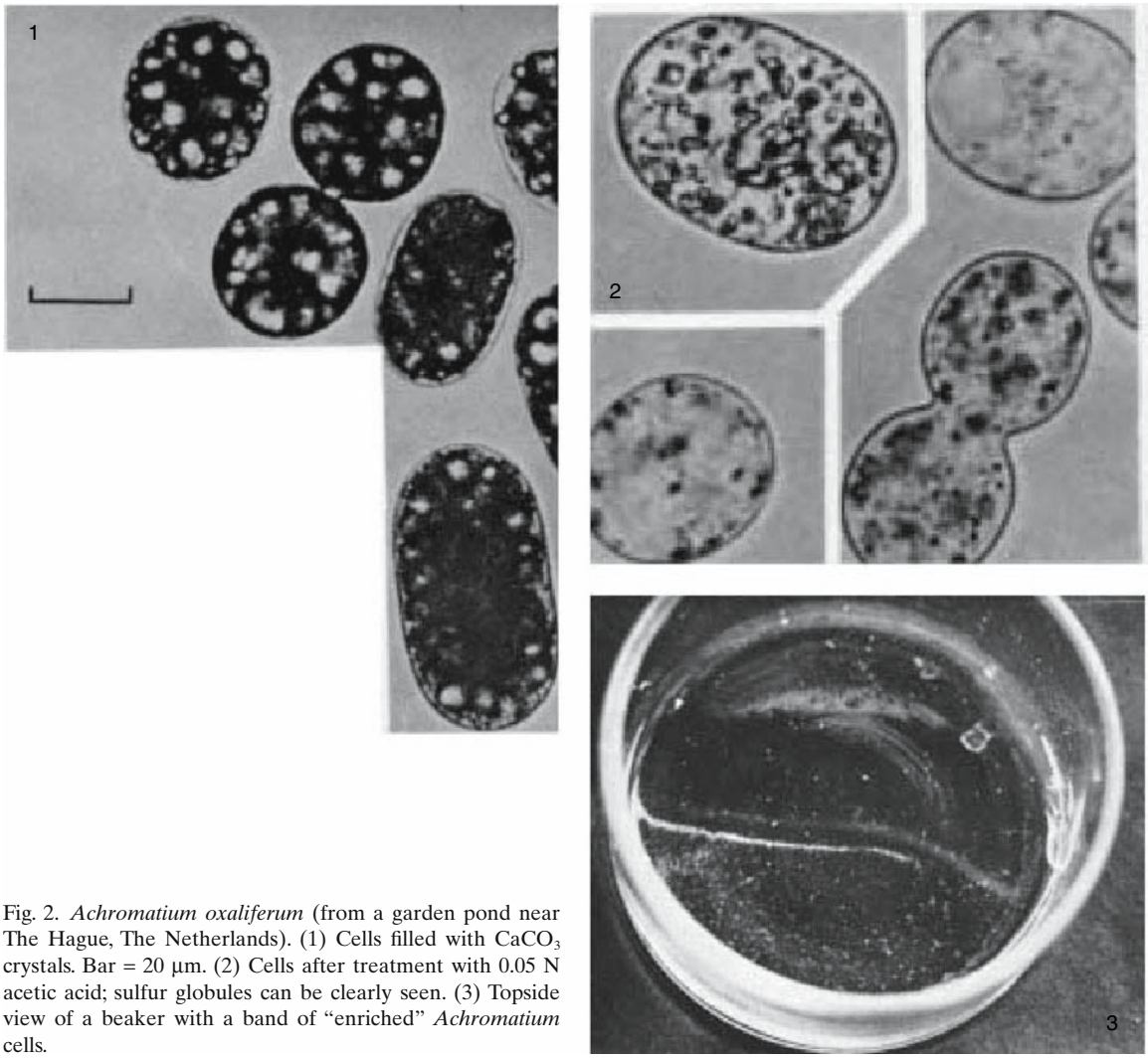


Fig. 2. *Achromatium oxaliferum* (from a garden pond near The Hague, The Netherlands). (1) Cells filled with $CaCO_3$ crystals. Bar = 20 µm. (2) Cells after treatment with 0.05 N acetic acid; sulfur globules can be clearly seen. (3) Topside view of a beaker with a band of "enriched" *Achromatium* cells.

sensation of reading the works of the old masters as valuable guidebooks of undiminished contemporary relevance. In order to facilitate such reading, pertinent references as well as earlier used names of organisms have been included.

The Genus *Achromatium*

Two species of Achromatium are known (La Rivière and Schmidt, 1989): *Achromatium oxaliferum*, which occurs in fresh waters and marine environments, and *Achromatium volutans*, which is less well known and has been found in marine muds only.

Achromatium oxaliferum

Achromatium oxaliferum (Figs. 1 and 2) was described for the first time by Schewiakoff (1893). Since then the organism has temporarily also carried the names *Hillhousia mirabilis* (West and Griffiths, 1909), *Hillhousia palustris* (West and Griffiths, 1913), and *Achromatium gigas* (Nadson, 1913). Its cells are conspicuous because of their massive $CaCO_3$ inclusions occurring together with much smaller sulfur globules. In addition, their large size immediately attracts attention: the cells are spherical, ovoid, or cylindrical with a minimum length of the small axis of 5 µm and a maximum length of the long axis of 100 µm. Division is by constriction. The cells are slowly motile on solid surfaces only. It is, therefore, not surprising that the organism has tantalized numerous observers, none of whom, however, has so far succeeded in developing an effective enrichment method.

Ecology. In all cases, the cells were encountered in or on the bottom mud of fresh and saline waters, as is to be expected from the high specific weight of these organisms, which prevents them from moving vertically in the water. Since they are not at all commonly found among the macroscopically recognizable white masses that usu-

ally guide the investigator most effectively to natural populations of colorless sulfur bacteria, it appears useful to list here some of the habitats in which the organism was found, all the more so because no useful common characteristic of these habitats has as yet emerged.

Gicklhorn (1920), Bersa (1920), and Bavendamm (1924) found *Achromatium* in ponds in the botanical garden in Graz where the alga *Chara* also occurred in large numbers. The bottom mud of Swedish lakes was the source for Skuja (1948), and that of Lake Windermere was the source for Skerman (Starr and Skerman, 1965). Lackey and Lackey (1961) found the organism in fresh and brackish waters in Florida as well as in salt marshes at Woods Hole. It was also found in a small concrete garden pond in Pacific Grove, California, and in 2 out of 14 garden ponds examined near The Hague (J. la Rivière, unpublished). These were made of concrete, had a depth of 40 cm, and contained very loosely packed mud with much organic matter as well as cells of algae and cyanobacteria. Nadson and Visloukh (1923) found *Achromatium* in marine muds. Some of these waters were high, others low, in Ca^{2+} concentration.

Since all authors agree that the organism is an obligate aerobe and the high weight of its cells restricts it to the bottom habitat, it seems obvious that in all of the above-mentioned locations the borderzone between H_2S and O_2 must have resided in the mud or on top of it, indicating that the H_2S generation in the sediment must have been relatively weak, or, alternatively, aeration consistently strong. This appears to make *Achromatium oxaliferum* the counterpart to *Thiobacterium*, which (as a "puffball" type) floats at the surface. There is, however, one important difference in that *Achromatium* possesses some means of locomotion while *Thiobacterium* does not. This motility is very slow and restricted to jerky rolling movements on solid surfaces, most probably effected by means of peritrichous filaments moving about in the slime layer that surrounds the cells (de Boer et al., 1971). In this way, the cells are capable of migrating chemotactically through the mud, where, depending on the distribution of localized microsources of H_2S, concentration gradients exist over short distances that are manageable by the organisms.

Lauterborn (1915) noted that cells were packed with $CaCO_3$ and had few sulfur inclusions when encountered on top of the mud, while cells below the surface contained little or no $CaCO_3$ and much more sulfur. This suggests that the restricted capacity for chemotaxis is somewhat complemented by the storage of sulfur but does not explain the significance that $CaCO_3$ storage could have for the organism beyond keeping it in the bottom layers by sheer weight. In this respect, any possible interaction with surrounding photosynthetic organisms appears important, as these exert a considerable influence not only on the O_2 concentration but also on the $CO_2/CaCO_3$ equilibrium, which are both subject to diurnal changes. In addition, $CaCO_3$ might exert a buffering effect with respect to H_2SO_4 formed by sulfur oxidation.

Enrichment and Cultivation. Most observations on the organism have been done on material taken directly from nature. No effective laboratory enrichment culture methods are available; at best the natural populations can be kept alive in the laboratory for 6–10 months. Schewiakoff (1893) used a watch glass containing mud, *Achromatium* cells, and some supernatant water from the natural habitat. After placing it in sunlight he observed "energetic multiplication" (perhaps chemotactic accumulation?) and formation of white agglomerations of 50–100 cells each. West and Griffiths (1913) placed the mud with overlaying tap water in small cylinders (15 cm wide, 5 cm high) and stirred the mud from time to time; in this way they kept the organisms alive for 9 months. They stated that the cells had a doubling time of 24–48 h under these conditions and that death resulting from O_2 deficiency was accompanied by the loss of the $CaCO_3$ inclusions. De Boer et al. (1971) maintained natural populations for 10 months by placing the loosely packed mud from a pond in closed plastic bottles at 5°C. The overlaying pond water column was about four times higher than the mud layer. The same authors used a very simple method, suggested to them by C. B. van Niel, for concentrating the cells in such a mud-water system. It is based on the heavy weight and white color of the *Achromatium* cells:

Collecting and Concentrating *Achromatium* Cells Found in Nature (de Boer et al., 1971)

> Material from the upper part of the sediment was collected. Lightweight particles were removed by repeated decanting in tap water. Layers of heavier mud particles, approximately 0.5 cm thick, were subjected to gentle swirling in a tilted beaker that at the same time was rotated slowly along its vertical axis (Fig. 2). This treatment led to further fractionation of the particles according to their specific gravity and the heavy Achromatium cells soon became visible as a narrow white band very close to that of the sand-particle fraction. The cells were pipetted off and transferred to a smaller beaker and further purified by the same method. This procedure was repeated several times until a dense mass of Achromatium cells was obtained. This no longer contained cells of algae, protozoa, or sand particles, and the number of smaller bacteria present had been reduced to a very small minority, as shown by microscopic examination.
>
> The method was obviously only successful for *Achromatium* cells that actually contained appreciable quantities of $CaCO_3$ inclusions.

At least 50% of the cells obtained in this way were viable, as shown by their motility observed

under the microscope. They proved to be quite suitable for electron microscopy and some elementary physiological tests.

Further Characteristics. The organism is very easy to identify by the morphological characteristics already mentioned. Healthy cells are almost completely filled with highly refractile $CaCO_3$ crystals, which makes it difficult to observe the sulfur globules. These are best seen after selective removal of the $CaCO_3$ by treating the cells with 0.05 N acetic acid (de Boer et al., 1971) (Fig. 2).

Cells collected by the method described above remained contaminated by heterotrophs even after repeated washings with sterile water. Nevertheless, indications were obtained that the cells moved and multiplied more actively in the presence of organic acids, casamino acids, or both. The experiments, however, could not be pursued further because of overgrowth by more rapidly multiplying heterotrophs (K. Schmidt, unpublished). The cells could be lysed by lysozyme and proved to be Gram-negative and catalase-negative.

Achromatium volutans

Achromatium volutans (Fig. 1 and 3) was first described by Hinze (1903) under the name of *Thiophysa volutans* and later by Nadson (1913, 1914) under the name of *Thiophysa macrophysa.* The organism was found in saline waters only: in association with mud and decaying seaweeds from the Bay of Naples (Hinze, 1903, 1913), in the saline sulfur spring "Solgraben von Artern" (Kolkwitz, 1918), and in a saline spring in Florida (Lackey and Lackey, 1961). The best description is probably that of Hinze (1903), who obtained his cell material from marine mud placed in a flat dish; within 1 h, many cells were found at the mud surface, concentrated through chemotaxis into a thin white layer.

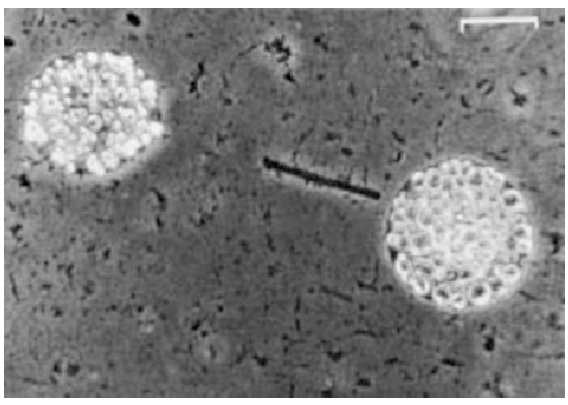


Fig. 3. *Achromatium* (= *Thiophysa*) *volutans* (from Solgraben in Artern, Germany). Bar = 20 μm.

The organism was very similar to *Achromatium oxaliferum* with respect to cell shape, motility, and mode of division. A striking difference was the total lack of $CaCO_3$ inclusions and the presence of far more and larger sulfur globules. Also the cells were consistently smaller, ranging from spheres with a diameter of 5 μm, when young, to ovals up to 40 μm in length. Right after division cells were flat at one end, which was never observed in *Achromatium oxaliferum* (Fig. 1).

The observations of Hinze (1903) suggest that this organism is more sensitive to environmental conditions like H_2S and O_2 concentrations than is *Achromatium oxaliferum*. This appears plausible because its lower specific gravity is likely to permit more rapid chemotactic response.

The Genus *Macromonas*

Cells of *Macromonas* are often found in habitats where *Achromatium oxaliferum* also occurs (Bavendamm, 1924; Gicklhorn, 1920; Lauterborn, 1915; Skuja, 1956). To the knowledge of the authors, only one attempt at enrichment of these organisms has been described (Dubinina and Grabovich, 1984). Hence, most available data originate from observations on material directly taken from natural sources.

The cells are colorless, cylindrical or bean shaped and sluggishly motile with one polar flagellum. Since flagellation was observed in the light microscope, presence of a tuft of flagella is more likely. Their typical characteristic is the presence of several large refractile spherules, which have been thought to consist of $CaCO_3$; definite proof for this is lacking, however. In addition, small sulfur globules may be present.

Two species have been recognized (la Rivière and Dubinina, 1989).

Of the two species *Macromonas mobilis* (Fig. 1) was first described by Lauterborn (1915) under the name *Achromatium mobile* and later by Gicklhorn (1920) as *Microspira vacillans.* The cells are ellipsoidal or cylindrical and slightly curved, measuring usually 9 by 20 μm. Smaller cells are often present and are believed to be daughter cells (Gicklhorn, 1920). The single polar locomotor organ (tuft of flagella?) measures 20–40 μm and can be observed by light microscopy. It provides for motility at a rate of 800 μm/min. Besides small sulfur inclusions, usually between 5 and 15, one to four large refractile bodies occur, which closely resemble the $CaCO_3$ inclusions of *Achromatium oxaliferum* and, therefore, are provisionally considered to consist of $CaCO_3$. No methods for isolation are known.

Macromonas bipunctata (Fig. 1) was first described by Gicklhorn (1920) as *Pseudomonas bipunctata* and renamed *Macromonas bipunctata* by Utermöhl and Koppe (Koppe, 1923). Dubinina and Grabovich (1984) reported the isolation of heterotrophic bacteria answering the description of *Macromonas bipunctata* by these authors. The cells are single or in pairs, pear-shaped, cylindrical or curved; they measure 2.2–4 × 3.3–6.5 μm. They are motile by a polar tuft of flagella, Gram-negative, strictly aerobic and catalase-positive. Sulfur inclusions are produced when the cells are grown in media containing sulfide, which is oxidized by hydrogen peroxide and thus offers no useful energy to the cell.

While recognizing the valid description of *Macromonas bipunctata* by Dubinina and Grabovich, la Rivière and Dubinina (1989) still maintain *Macromonas mobilis* as type species because "… now that the way has been paved, it is felt that isolation of Macromonas mobilis may take place in the near future and thus complete the taxonomy of the genus with a minimum of alterations."

Enrichment and Isolation of *Macromonas bipunctata* (Dubinina and Grabovich, 1984)

> The white mat found on the surface of bottom sediments in a sewage treatment plant was used for inoculating 10 ml portions of the following semisolid medium contained in test tubes:
>
> | | |
> |---|---|
> | Sodium acetate | 1 g/l |
> | Calcium chloride | 0.1 g/l |
> | Casein hydrolysate (Difco) | 0.1 g/l |
> | Yeast extract (Difco) | 0.1 g/l |
> | Agar (Difco) | 1 g/l |
>
> After sterilization, a mixture of vitamins and of trace-elements (Pfennig and Lippert, 1966) was added, as well as 0.2 mg freshly prepared FeS per 10 ml medium as source of sulfide. The pH was adjusted to 7.2–7.4.
>
> After two to three days at 28°C, a white surface film appeared, which contained a large number of typical Macromonas cells. Streaks were made from a suspension of this film on agar plates containing the medium described above solidified with 1 g agar/l. After two to three days flat Macromonas colonies appeared with a diameter of 1 to 4 mm and a finegrained structure. These colonies were used for subsequent purification by re-streaking on solid medium.

The Genus *Thiobacterium*

The organisms of this genus are nonmotile rods that form sulfur inclusions. The cells are embedded in gelatinous masses that are either "puffball"-shaped or dendroid. Spherical, "puffball"-shaped gelatinous masses may be found freely floating in stagnant, H_2S-bearing waters (Fig. 1; *Thiobacterium* a). Dendroid gelatinous masses may be found attached to solid surfaces that are exposed to flowing, H_2S-containing waters (Fig. 1; *Thiobacterium* b).

Their lack of motility prevents these organisms from following chemotactically the spatial fluctuations of the zone of optimal H_2S and O_2 concentrations. This suggests that they are capable of growing under a wider range of H_2S and O_2 concentrations than acceptable to most of the motile colorless sulfur bacteria, or, alternatively, that they are confined to growth in locations where their optimal conditions more or less continuously prevail. The latter alternative appears to be supported by the fact that they are found floating at water-air interfaces or attached to solid surfaces. The first case is likely to occur when H_2S generation is consistently so high that the zone of optimal conditions is kept automatically at the top of the anaerobic water column. The second case arises when a water stream with more or less constant H_2S and O_2 concentrations is maintained for longer periods of time; this is the situation one finds in certain sections of the outflows of sulfur springs.

In *Bergey's Manual* (Buchanan and Gibbons, 1974; la Rivière and Kuenen, 1989a), only one species, *Thiobacterium bovista*, was recognized. It comprised the bacteria embedded in spherical, gelatinous masses, first described by Molisch (1912) under the name of *Bacterium bovista,* as well as those embedded in dendroid structures, first described by Lackey and Lackey (1961) under the name of *Thiodendron mucosum*. Observations in nature of *Thiobacterium bovista* have been reported by Scheminzky et al. (1972) and by Caldwell and Caldwell (1974).

Molisch obtained the spherical colony type (Fig. 1) of *Thiobacterium* at the surface of Winogradsky column enrichments prepared with seawater plus a sediment consisting of black mud mixed with decaying algae, all ingredients originating from the harbor of Trieste, in which the organism was also directly observed. The diameter of the spherical structures ranged from microscopic dimensions to about 4 mm and the structures were sometimes clustered together. The lengths of the cells were between 2 and 5 μm, their width between 0.6 and 1.5 μm. They usually contained four sulfur globules per cell. These cells were embedded in the gelatinous skin of the spherical masses, the inside of which was filled with water. The nature of the mucoid matrix was not elucidated.

The dendroid forms (Fig. 1) were found by Lackey and Lackey (1961) in saline, H_2S-bearing waters in Florida at temperatures between 13 and 30°C. The dendroid matrix, of as yet unidentified material, had the shape of branching filaments with a varying thickness of up to 20μm, individual branches sometimes being clubshaped. Thus, the colonies acquired treelike

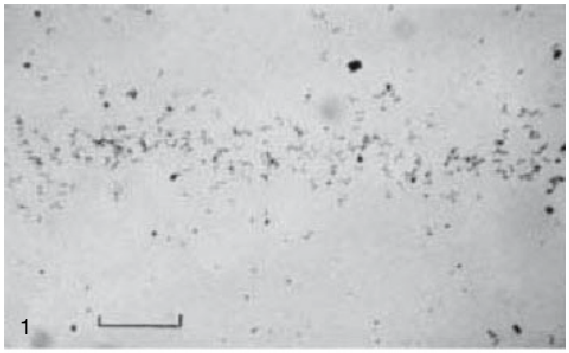
structures, reaching a width of up to 3 mm. Most of the colonies were found attached to algal and cyanobacterial cells (generally of the genus *Lyngbya*), which raises the question of a possible interaction involving O_2.

The cells were randomly arranged throughout the branches and consisted of rods, normally 3–5 μm and 1.5 μm wide; in the swollen ends of the branches, however, cells three times that size were found. Most cells contained three to eight sulfur globules arranged on a longitudinal axis.

The organism could not be grown in enrichments in the laboratory but was kept alive for 3 months in jars with water of the original habitat at 20°C.

The Genus *Thiospira*

Thiospira cells consist of typical colorless spirilla with sulfur inclusions. Their polar flagella are in some cases united in a tuft, visible in the light microscope. The cells are chemotactic with respect to O_2 and H_2S. They can arrange themselves in characteristic agglomerations located in regions of optimal H_2S and O_2 concentrations, forming "Bakterienplatten" in liquid columns and "Atmungsfiguren" in covered slide preparations (Fig. 4). The members of the genus have been found in fresh waters and marine environments in which H_2S was present.



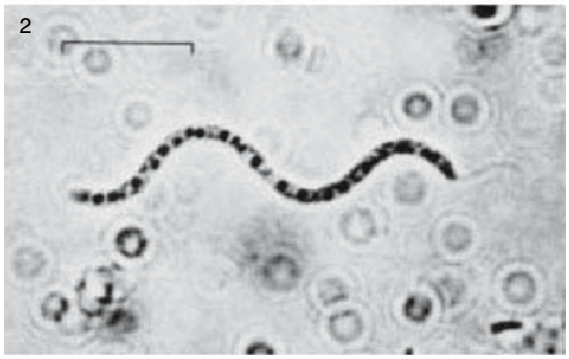


Fig. 4. *Thiospira* sp. (from upwelling groundwater near Delft, The Netherlands). (Courtesy of J. Klein, IHE, Delft.) (1) Cells accumulating at the optimum O_2/H_2S-zone. Bar = 100 μm. (2) Dividing cell with a flagellum. Bar = 10 μm.

At the moment, two species are recognized (la Rivière and Kuenen, 1989b).

Thiospira bipunctata (Fig. 1) was first described by Molisch (1912) and at that time named *Spirillum bipunctatum*. Its cells are slightly twisted rods forming short spirilla with a width of 1.7–2.4 μm and a length of 6.6–14 μm. They are most probably polarly flagellated. Each end of the cell typically contains a large granule of some storage material, described as volutin, leaving a clear space in the middle normally filled with two or three sulfur globules. The latter are sometimes also found at the ends.

Thiospira winogradskyi (Fig. 1) was first described by Omelianski (1905) and at that time named *Thiospirillum winogradskii.* It is a colorless spirillum containing numerous sulfur globules that may disappear when the H_2S in the medium is exhausted. It is 2–2.5 μm wide and can reach a length of 50 μm. "Volutin" bodies are absent. The cells have polar flagella and are strongly chemotactic.

Dubinina and Grabovich (1983) reported the isolation of heterotrophic spirilla under the name of *Thiospira bipunctata*. These were morphologically similar to *Thiospira bipunctata* as described by Molisch since they formed sulfur inclusions when grown in media containing sulfide. The sulfur was formed by nonspecific sulfide oxidation by metabolically produced hydrogen peroxide. Since other heterotrophs have also been found to form sulfur inclusions in sulfide-containing media (Skerman et al., 1957), further study is required for determining the proper taxonomic status of these isolates, which may well prove to be identical to existing *Spirillum* species.

Enrichment Procedures

All enrichments for *Thiospira* have been carried out in Winogradsky columns (Winogradsky, 1888) in which H_2S was released from a sediment.

Molisch (1912) enriched *Thiospira bipunctata* from mud from a Black Sea estuary (a liman) in cylinders (5–15 × 10–30 cm) filled with seawater and a sediment of mud mixed with dying or dried algae. The columns were kept for several weeks at room temperature in the dark or under dim light.

Omelianski (1905) enriched *Thiospira winogradskyi* using tap water and a mixture of liman mud, $CaSO_4$, and fragmented rhizomes in cylinders 7 cm wide and 40 cm high. After some months' incubation at room temperature in the dark, "Bakterienplatten" appeared in the lower part of the columns, which moved up and down

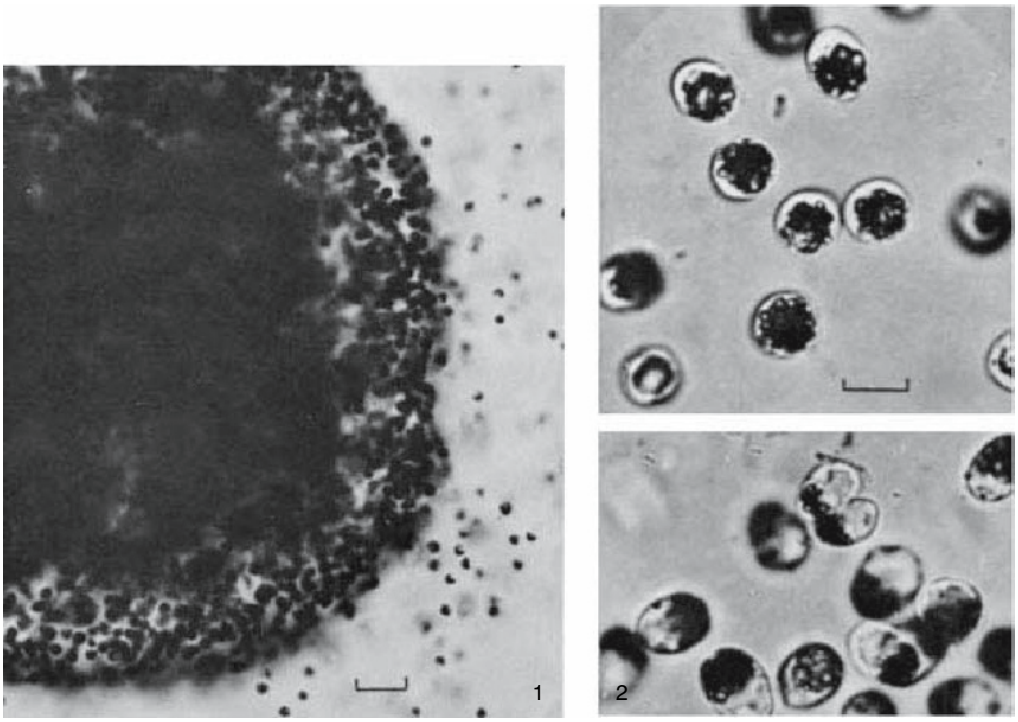


Fig. 5. *Thiovulum majus* (from Bergen op Zoom, The Netherlands). (1) Periphery of a veil of *Thiovulum majus.* Bar = 50 μm. (2) Swarming cells. Bar = 20 μm. (From Schlegel, 1976.)

with the prevailing H_2S concentration. When the cells had settled to the bottom, the sediment was stirred up, which apparently led to renewed H_2S production and prolongation of the lifetime of the culture, which in this way could be kept for 2 years. The present authors had occasion to observe a *Thiospira* species among the colorless sulfur bacteria at 7°C in the outflow of an upwelling, H_2S-bearing groundwater near Delft, The Netherlands (Fig. 4). The cells formed "Atmungsfiguren" in slide preparations and could be kept viable for at least 6 weeks at 4°C in the dark, in cylinders containing groundwater and some mud from the location of origin.

The Genus *Thiovulum*

The cells of this spectacular genus (Figs. 1 and 5) are ovoid and measure 5–25 μm in length. They normally contain sulfur inclusions, often concentrated at one end. The cells multiply by longitudinal fission preceded by constriction. The cells have peritrichous flagella that provide strong chemotactic motility, the cells rotating around an axis coinciding with the path of travel; they never reverse direction and concentrate at places of optimum O_2 and H_2S concentrations in sharply defined characteristic veils or webs consisting of separate, ever moving, individual cells, to some extent held together by a very loose slime matrix (Fig. 5).

Because of this strong chemotaxis, the development of methods for enrichment and cultivation has been more successful for *Thiovulum* than for the other genera discussed in this chapter.

Only one species, *Thiovulum majus,* is recognized (la Rivière and Kuenen, 1989c). This includes the former species *Thiovulum minus* (Hinze, 1913). Other obsolete names are *Monas muelleri* (Warming, 1875) and *Thiovulum muelleri* (Lauterborn, 1915).

Ecology

Thiovulum has been observed in marine environments and saline springs by Warming (1875), Hinze (1903, 1913), Molisch (1912), Bavendamm (1924), Fauré-Fremiet and Rouiller (1958), Lackey and Lackey (1961), and la Rivière (1963). Freshwater forms were reported by Lauterborn (1915) and Lackey and Lackey (1961). In all instances, *Thiovulum* is found in very sharply localized white masses in situations where H_2S meets with O_2. Since it appears restricted to low concentrations of each substance for optimal growth, the patterns it forms provide a macroscopic visualization of this borderline, even in slowly flowing waters, as the cells

rapidly follow changes of the concentration gradient. This makes it sometimes possible to identify *Thiovulum* accumulations in nature by their characteristic white patterns observed above black, sulfide-releasing muds in otherwise clear water. Similarly, such veils may be observed over decaying organic matter in flowthrough marine aquaria.

Measurements with microelectrodes in natural and laboratory systems (Jørgensen and Revsbech, 1983) have established that these veils are about 100 µm thick and are formed at the interface of the oxygen and sulfide zones. The veils create an unstirred boundary layer preventing mixing of oxygen and sulfide and thus also preventing chemical sulfide oxidation. The veils thus create a situation in which the *Thiovulum* cells are fed by diffusion of both substrates, which each penetrate over a distance of 50 µm into the veil. In oxygen-rich water the veils take the shape of small spheres which are made anaerobic in the center by the rapid oxygen utilization of the cells, which thus create the gradient required for optimum growth. Jørgensen and Revsbech (1983) provide further fascinating evidence showing that the chemotactic behavior of *Thiovulum* is extremely sensitive to environmental influences and is an important instrument, by "concerted action" of many cells, in the creation of the precarious niche in which *Thiovulum* lives.

Enrichment

Unpublished work by van Niel, Wijler, and Lascelles (van Niel, 1955) led to the development of a simple enrichment method, described by la Rivière (1963, 1965). The method has been successfully used in Pacific Grove (California), in Delft (The Netherlands), and also in Woods Hole (Massachusetts) (Wirsen and Jannasch, 1978).

Enrichment of *Thiovulum* (Wirsen and Jannasch, 1978)

A layer of decaying seaweed (Ulva) mixed with some marine mud is placed on the bottom of a jar of 1–10 liters filled with seawater, and the thickness of the compressed algal layer is kept to less than half the height of the jar (Fig. 6A). A continuous, slow flow of seawater is introduced near the surface of the sediment, and the jar is allowed to overflow. The layer of Ulva via sulfate reduction acts as a source of H_2S while the flowing seawater provides a continuous supply of O_2. By means of chemotaxis, the cells of Thiovulum seek out the regions of optimal concentration, where growth takes place in characteristic veils. The flow of seawater has also the essential functions of supplying the sediment with SO_4^{2-} as a source of H_2S and of flushing out contaminating organisms that are not chemotactic; as a rule the Thiovulum veils are surrounded by clear water.

When fresh *Ulva* is used, one should wait ca. 2 weeks before starting the seawater flow; a thick film of bacteria usually has developed at the surface at that time. After the flow of seawater is started, growth of *Beggiatoa* and/or *Thiothrix* may be observed in the upper part of the jar. In that case, the flow rate should be increased somewhat; the filamentous bacteria move to lower regions and eventually to the bottom while *Thiovulum* appears in the top layer usually 2–3 weeks after the start of the experiment. This indicates that the optimum sulfide concentration for *Thiovulum* is definitely lower than that for *Beggiatoa* and *Thiotrix*. When the flow rate is further increased, the veils of *Thiovulum* move further downwards.

At all times the culture should be kept in the dark, and relatively low temperatures (15°C) appear to be favor-

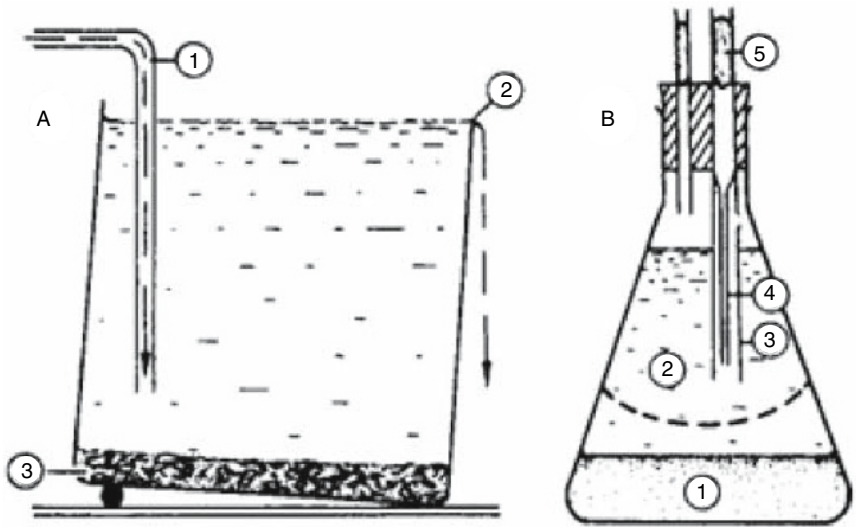


Fig. 6. Culture vessels for enrichment of *Thiovulum:* (A) by Wijler's method and (B) for stationary cultivation of *Thiovulum.* (A): 1, inflow of seawater; 2, overflow; and 3, layer of decaying *Ulva.* (B): 1, sulfide-containing agar layer; 2, seawater; 3, protective tube for localized aeration; 4, Pasteur pipette; and 5, cotton plug. The broken line indicates the zone of growth of *Thiovulum.*

able. The layer of *Ulva* can be replaced by decaying marine animals like starfishes and sea urchins; a layer of fishmeal has also given positive results.

Wirsen and Jannasch (1978) found that an initial addition of neutralized Na_2S led to an earlier appearance of *Thiovulum* veils and to larger enriched populations.

Enrichment cultures of *Thiovulum* can be used as a source of crude cell material for several weeks until the H_2S-generating power of the sediment is exhausted. Lateral illumination with a flashlight greatly facilitates observation and harvesting of the *Thiovulum* veils.

Purification of Crude Cell Material

Besides filtration, methods based upon the chemotaxis of *Thiovulum* have been used, singly or in combination. la Rivière (1963, 1965) used the following method:

Purification of *Thiovulum* (la Rivière, 1963, 1965)

A total of 50–100 ml of a cell suspension carefully harvested by pipetting from an enrichment culture is rapidly filtered through a thin layer of cotton or of cheesecloth into a 100-ml glass cylinder. After 5–10 min, the cells settle chemotactically near the bottom, from where they are transferred by means of a pipette in as small a volume as possible into a test tube containing 15 ml sterile, aerobic seawater, cooled to about 15°C, in which they again form a veil near the bottom in 5–15 min. Repeated transfers in a similar manner to fresh tubes with sterile seawater lead to highly purified cell suspensions, as the initial filtration removes debris, protozoa, and other larger organisms while subsequent "washings" decrease the number of smaller contaminants. Plating on media for Thiobacillus and for heterotrophs permits following the effectiveness of the purification steps.

The success of the method depends entirely on the degree of initial contamination of the raw material and the capacity of the cells to survive successive purification steps. In experiments in Pacific Grove, "washing" could be performed 6–10 times in succession, leading to suspensions that were consistently free from contaminants. However, similar pure suspensions were not obtained in experiments in The Netherlands (la Rivière, 1965) or in Woods Hole (Wirsen and Jannsch, 1978), which is probably caused by differences in vigor between the enriched populations and in properties of the seawater used, such as temperature, degree of pollution, and microbial contamination.

Wirsen and Jannasch (1978) used the following modifications of the method described above:

1. Repeated chemotactic "washing" by pipetting or decantation in sterile aerobic seawater without an initial filtration step; this could be done only when freshly formed veils, relatively free from larger organisms, were available in the enrichments.

2. Chemotactic "washing" in deoxygenated sterile seawater, in which case the cells migrate to the top layer.

3. Concentration and washing of the cells by means of a membrane filter with a porosity of 8 μm. Initial cell suspensions are concentrated to about 2 ml by suction, whereupon fresh sterile seawater is added, followed again by concentration by suction, *Thiovulum* cells being retained by the filter while contaminants are passed through it. Three to five such "washings" are possible.

Application of these methods combined with subsequent cultivation procedures provided Wirsen and Jannasch (1978) with highly purified cell suspensions suitable for electron microscopy and for the performance of some important physiological experiments. The various treatments, however, could not be repeated a sufficient number of times to obtain suspensions that were entirely free from contaminants.

Cultivation of Purified *Thiovulum* Populations

Stationary Cultivation of Thiovulum Suspensions free from Contaminants (La Rivière, 1963, 1965). In a 500-ml Erlenmeyer flask, 250 ml of the following sterile agar medium is sterilized at 120°C for 10 min: 250 ml seawater, 2% agar (Difco), 0.01% NH_4Cl, 0.01% KH_2PO_4. Just prior to solidification of the agar, 2.5 ml of a separately heat-sterilized solution of 1% Na_2CO_3 in seawater is added, followed by 8 ml of sterile seawater previously saturated by gaseous H_2S. The pH does not need adjustment; it should be around 8.0. After setting of the agar, 250 ml of seawater are added and the flask is closed with a steam-sterilized stopper allowing for local aeration by means of a Pasteur pipette surrounded by a vertical glass tube which is open at the lower end (Fig. 6B). In this way, aeration is restricted to the inside of the protective glass tube, the dissolved oxygen slowly diffusing downwards to the bulk of the seawater layer. Thus, a gradient between the tube and the agar block is established which can be easily demonstrated with a redox dye; e.g., thionine. Without aeration, the dye is in its reduced form throughout the liquid; with conventional aeration without protection tube, the turbulence distributes the oxygen uniformly and the dye is in its oxidized form throughout the liquid. With localized aeration we obtain a sharp boundary between a "reduced" and an "oxidized" zone, which can be maintained for weeks.

The flask is placed at a temperature of 10–15°C and kept under constant aeration with cotton-filtered air. After equilibration overnight, the flask is ready for inoculation with purified cell material, and soon the individual cells of the inoculum can be seen to settle in one plane somewhere between the agar surface and the aeration tube.

Within days, the cells increase in number until a sharply defined web is formed at about 1 cm above the agar surface in an otherwise clear liquid. On prolonged incubation, the web rises slowly because of increasing O_2 demand; later on the web moves downward as H_2S becomes exhausted. By this time, signs of decay become apparent: White strands consisting of dead cells adhering to each other hang down from the sides of the flask. The life of the culture lasts for about 3 weeks.

This procedure has been successfully used only in Pacific Grove, where purification of the inoculum could be pursued to the point where all contamination had been eliminated. It was also used—with some modification—by Pringsheim and Kowallik (1964) in their study of H_2S oxidation by *Beggiatoa*.

It should be pointed out that the method is unsuitable when impurities are present, as *Thiovulum* is rapidly overgrown in any closed stationary system. It should further be realized that even when successful the method yields only very small amounts of cell material.

Cultivation in Open, Flow-through Systems. Such systems are based upon the same principle that underlies the enrichment culture method. Besides the use of sterilized, aerated seawater fed into the culture vessel by controlled pumping, different sources for input of H_2S have been applied. These include periodical addition of H_2S or portions of saturated solutions of H_2S once or twice daily (la Rivière, 1963, 1965). Wirsen and Jannasch (1978) developed several reliable devices in which continuous H_2S provision was assured from different sources:

1. Decaying *Ulva* kept separately from the culture vessel by a nylon membrane of 0.45-µm porosity.
2. A pure culture of *Desulfovibrio aestuarii* separated from the culture vessel in the same way, H_2S generation being kept up by periodic addition (every 2–4 weeks) of fresh sodium sulfate/lactate medium. This system (Fig. 7) provided the best results, permitting the maintenance of active Thiovulum populations for 220 days without reinoculation; during this time cells were frequently harvested, leading to rejuvenation of the veils.

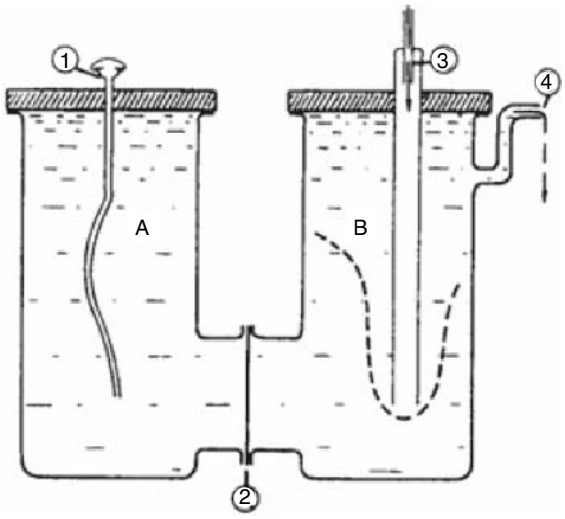


Fig. 7. Double culture vessel for the maintenance of purified cell suspensions of *Thiovulum* sp. (after Jannasch and Mateles, 1974; and Wirsen and Jannasch, 1978). (A) Culture vessel with *Desulfovibrio estuarii*. (B) Culture vessel for *Thiovulum*. 1, lactate supply; 2, semipermeable membrane; 3, inflow of air-saturated seawater; 4, overflow. The broken line indicates the zone of growth of *Thiovulum*.

3. A neutralized solution of Na_2S in sterile seawater separated from the culture vessel by a dialysis membrane.
4. A coil of silicone tubing, permeable to H_2S, through which a gas mixture of N_2 and H_2S (1%) was kept circulating. The coil was placed on the bottom of the culture vessel.

The same authors, furthermore, used closed-flow systems based upon recirculation rather than open systems with overflow. In the closed system, no *Thiovulum* cells are lost, but contaminants are not flushed away; hence they are only useful for specific short-term experiments.

Finally, it should be pointed out that none of the authors who studied *Thiovulum* has been successful in using solid media. Also H_2S could not be replaced by thiosulfate, nor could nitrate be substituted for O_2.

State of Present Knowledge on *Thiovulum*

Besides the properties mentioned earlier, further characteristics of the organism have been determined by study of cell material derived from enrichments and from mass cultivation of purified material.

Thiovulum is Gram-negative and catalase-negative (la Rivière, 1965); the cells die quickly at high O_2 concentrations, as can be easily seen when they are trapped at the edges of a slide preparation. They are also immediately killed by anaerobic conditions. Its internal structure

has been studied by electron microscopy by Fauré-Fremiet and Rouiller (1958), de Boer et al. (1961), Remsen and Watson (1972), and Wirsen and Jannasch (1978). Among its most striking features are Fauré-Fremiet's polar fibrillar organelle, the thin cell wall, and the slime excretion, which plays a role in structuring and maintaining the veils in which, nevertheless, the cells are in continuous motion. A discussion of the earlier results is presented by Starr and Skerman (1965).

Wirsen and Jannasch (1978) performed uptake experiments with CO_2 and some organic compounds labeled with $^{14}CO_2$ on highly purified cell suspensions, which permitted correction for uptake by contaminants. They showed substantial CO_2 uptake in *Thiovulum* which was optimal at H_2S concentrations of 1 mM in initially air-saturated seawater. No significant carbon uptake was registered for casamino acids, acetate, glutamate, mannitol, and some vitamins. These results provide strong evidence for the chemolithotrophic nature of the *Thiovulum* strains examined.

Literature Cited

Bavendamm, W. 1924. Die farblosen und roten Schwefelbakterien des Süβ-und Salzwassers. 7–156. Kolkwitz, R. (ed.) Pflanzenforschung. Jena, Gustav Fischer Verlag.

Bersa, E. 1920. Über das Vorkommen von kohlensaurem Kalk in einer Gruppe von Schwefelbakterien. Akademie der Wissenschaften Wien, Sitzungsbericht, Abt. 1 129:231–259.

Buchanan, R. E., Gibbons, N. E. (ed.). 1974. Bergey's manual of determination bacteriology 8th ed.

Caldwell, D. E., Caldwell, S. J. 1974. The response of littoral communities of bacteria to variations in sulfide and thiosulfate. Abstracts Ann. Meeting Am. Soc. Microbiol. 74:59.

Chen, K. Y., Morris, J. C. 1972a. Kinetics of oxidation of aqueous sulfide by O_2. Environmental Science and Technology 6:529–537.

Chen, K. Y., Morris, J. C. 1972b. Oxidation of aqueous sulfide by O_2. Jenkins, S. H. (ed.)Advances in water pollution research. New York, NY. Pergamon Press. III:32/1–32/17.

De Boer, W. E., la Rivière, J. W. M., Houwink, A. L. 1961. Observations on the morphology of *Thiovulum majus* Hinze. Antonie van Leeuwenhoek Journal of Microbiology and Serology 27:447–456.

De Boer, W. E., la Rivière, J. W. M., Schmidt, K. 1971. Some properties of *Achromatium oxaliferum*. Antonie van Leeuwenhoek Journal of Microbiology and Serology 37:553–563.

Devidé, Z. 1954. Istrazˇivanja o stanici bezbojnih sumpornih bacterija "Studies on the cells of colorless sulfur bacteria." Acta Pharmaceutica Jugoslawia 4:147–176.

Dubinina, G. A., Grabovich, M. Yu. 1983. Isolation of pure *Thiospira* cultures and investigation of their sulfur metabolism. Microbiologiya 52:5–12.

Dubinina, G. A., Grabovich, M. Yu. 1984. Isolation, cultivation and characteristics of *Macromonas bipunctata*. Microbiologiya 53:748–755.

Fauré-Fremiet, E., Rouiller, C. 1958. Étude au microscope électronique d'une bactérie sulfureuse, *Thiovulum majus* Hinze. Experimental Cell Research 14:29–46.

Gicklhorn, J. 1920. Über neue farblose Schwefelbakterien. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 2 50:415–427.

Hinze, G. 1903. *Thiophysa volutans,* ein neues Schwefelbakterium. Berichte der Deutschen Botanischen Gesellschaft 21:309–316.

Hinze, G. 1913. Beiträge zur Kenntnis der farblosen Schwefelbakterien. Berichte der Deutschen Botansichen Gesellschaft 31:189–202.

Jannasch, H. W., Mateles, R. J. 1974. Experimental bacterial ecology in continuous culture. Advances in Microbial Physiology 11:165–212.

Jørgensen, B. B. 1989. Biogeochemistry of chemoautotrophic bacteria. 117–146. H. G. Schlegel and B. Bowien (ed.) Autotrophic bacteria. Science Tech Publishers. Madison, WI.

Jørgensen, B. B., Revsbech, N. P. 1983. Colorless Sulfur Bacteria, *Beggiatoa* spp. and *Thiovulum* spp., in O_2 and H_2S microgradients. Applied and Environmental Microbiology 45:1261–1270.

Keil, F. 1912. Beiträge zur Physiologie der farblosen Schwefelbakterien. Beiträge zur Biologie der Pflanzen 11:335–372.

Kolkwitz, R. 1918. Über die Schwefelbakterienflora des Solgrabens von Artern. Berichte der Deutschen Botanischen Gesellschaft 36:218–224.

Koppe, F. 1923. Die Schlammflora der ostholsteinischen Seen und des Bodensees. Archiv für Hydrobiologie 14:619–672.

Kuenen, J. G. 1975. Colorless sulfur bacteria and their role in the sulfur cycle. Plant and Soil 43:49–76.

Kuenen, J. G., Bos, R. 1989. Habitats and ecological niches of chemolitho (auto) trophic bacteria. 53–80. H. G. Schlegel and B. Bowien, (ed.) Autotrophic Bacteria. Science Tech Publishers. Madison, WI.

Lackey, J. B., Lackey, E. W. 1961. The habital and description of a new genus of sulphur bacterium. Journal of General Microbiology 26:29–39.

la Rivière, J. W. M. 1963. Cultivation and properties of *Thiovulum majus* Hinze. 61–72. Oppenheimer, C. H. (ed.) Symposium on marine microbiology.. Springfield, Illinois. Charles C. Thomas.

la Rivière, J. W. M. 1965. Enrichment of colorless sulfur bacteria. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 1 Suppl. 1:17–27.

la Rivière, J. W. M. 1966. The microbial sulfur cycle and some of its implications for the geochemistry of sulfur isotopes. Geologische Rundschau 55:568–582.

la Rivière, J. W. M., Dubinina, G. A. 1989. Genus *Macromonas*. 1838–1840. Staley, J. (ed.) Bergey's manual of systematic bacteriology, vol 3. Baltimore, Williams & Wilkins.

la Rivière, J. W. M., Kuenen, J. G. 1989a. Genus *Thiobacterium*. 1838. Staley, J. (ed.) Bergey's manual of systematic bacteriology, vol 3. Baltimore, Williams & Wilkins.

la Rivière, J. W. M., Kuenen, J. G. 1989b. Genus *Thiospira*. 1840–1841. Staley, J. (ed.) Bergey's manual of systematic bacteriology, vol 3. Baltimore, Williams & Wilkins.

la Rivière, J. W. M., Kuenen, J. G. 1989c. Genus *Thiovulum*. 1841–1842. Staley, J. (ed.) Bergey's manual of systematic bacteriology, vol 3. Baltimore, Williams & Wilkins.

la Rivière, J. W. M., Schmidt, K. 1989. Genus *Achromatium*. 2131–2133. Staley, J. (ed.) Bergey's manual of systematic bacteriology, vol 3. Baltimore, Williams & Wilkins.

Lauterborn, H. 1915. Die sapropelische Lebewelt. Ein Beitrag zur Biologie des Faulschlamms natürlicher Gewässer. Verhandlungen des Naturhistorischen-Medizinischen Vereins zu Heidelberg 13:395–481.

Molisch, H. 1912. Neue farblose Schwefelbakterien. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 2 33:55–62.

Nadson, G. A. 1913. Über Schwefelmikroorganismen des Haspaler Meerbusens. Bulletin du Jardin Impériale Botanique de St.-Pétersbourg T 13:106–112.

Nadson, G. A. 1914. Über die Schwefelbakterien: *Thiophysa* und *Thiosphaerella*. Zeitschrift für Mikrobiologie, St. Petersburg 1:52–72.

Nadson, G. A., Visloukh, S. M. 1923. La structure et la vie de la bactérie géante *Achromatium oxaliferum* Schew. Bulletin du Jardin Impériale Botanique de St.-Pétersbourg, Suppl. 1 22:1–37.

Nelson, D. C. 1989. Physiology and biochemistry of filamentous sulfur bacteria. 219–238. H. G. Schlegel and B. Bowien (ed.) Autotrophic Bacteria. Science Tech Publishers. Madison, WI.

Omelianski, W. 1905. Über eine neue Art farbloser Thiospirillen. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 2 14:769–772.

Perfil'ev, B. V., Gabe, D. R. 1961. Capillary methods of investigating micro-organisms. "In Russian" Moscow: Izdatel'stvo Akademii Nauk. Shewan, J. M., translator. 1969. Toronto, University of Toronto Press.

Pfennig, N., Lippert, R. D. 1966. Über das Vitamin B12-Bedürfnis phototropher Schwefelbakterien. Archiv für Mikrobiologie 55:245–256.

Pringsheim, E. G., Kowallik, U. 1964. Ist *Beggiatoa* chemoautotroph? Naturwissenschaften 51:492.

Remsen, C. C., Watson, S. W. 1972. Freeze-etching of bacteria. International Reviews of Cytology 33:253–296.

Scheminzky, F., Klas, Z., Job, C. 1972. Über das Vorkommen von *Thiobacterium bovista* in Thermalwässern. Int. Revue ges. Hydrobiol. 57:801–813.

Schewiakoff, W. 1893. Über einen neuen bakterienähnlichen Organismus des Süßwassers. Heidelberg, Habilitationsschrift.

Schlegel, H. G. 1976. Allgemeine Mikrobiologie, 4th ed. Stuttgart, Thieme.

Ivanov, M. V. Freney (ed.), J. R. 1983. The global biogeochemical sulphur cycle. Wiley and Sons Ltd. Chichester, Sussex. UK.

Brimblecombe, P. Lein (ed.), A. Y. 1989. Evolution of the Global Biogeochemical sulphur cycle. Wiley and Sons Ltd. Chichester, Sussex. UK.

Skerman, V. B. D., Dementjeva, G., Carey, B. J. 1957. Intracellular deposition of sulfur by Sphaerotilus natans. Journal of Bacteriology 73:504–512.

Skuja, H. 1948. Taxonomie des Phytoplanktons einiger Seen in Uppland. Symbolae Botanicae Upsalienses 9:1–399.

Skuja, H. 1956. Taxonomische und biologische Studien über das Phytoplankton Schwedischer Binnengewässer. Nova Acta Regiae Societatis Scientiarum Upsalienses, Serie IV 16:1–404.

Staley, J. T., Bryant, M. R., Pfennig, N., Holt, J. G. (ed.). 1989. Bergey's manual of systematic bacteriology, vol. 3. Baltimore, Williams and Wilkins.

Starr, M. P., Skerman, V. B. D. 1965. Bacterial diversity: The natural history of selected morphologically unusual bacteria. Annual Reviews of Microbiology 19:420–422.

Svensson, B. H., Söderlund, R. (ed.). 1976. Nitrogen, phosphorus and sulphur—global cycles. SCOPE Report No. 7 and Ecological Bulletin No. 22. Stockholm, Swedish Natural Research Council.

van Niel, C. B. 1955. Natural selection in the microbial world. Journal of General Microbiology 13:201–217.

Warming, E. 1875. Om nogle ved Danmarks kyster levende bakterier. Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening; Khobenhaven 20–28:1–116.

West, G. S., Griffiths, B. M. 1909. *Hillhousia mirabilis,* a giant sulphur bacterium. Proceedings of the Royal Society of London, Series B 81:389–409.

West, G. S., Griffiths, B. M. 1913. The lime-sulphur bacteria of the genus *Hillhousia*. Annals of Botany 27:83–91.

Winogradsky, S. 1888. Beiträge zur Morphologie und Physiologie der Bakterien. 1. Zur Morphologie und Physiologie der Schwefelbakterien. Leipzig, Felix.

Wirsen, C. O., Jannasch, H. W. 1978. Physiological and morphological observations on *Thiovulum* sp. Journal of Bacteriology 136:765–774.

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CHAPTER 14.2

The Genus *Propionigenium*

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The genus *Propionigenium* consists so far of two species, *P. modestum* and *P. maris*. *Propionigenium modestum* comprises four strains of physiologically and morphologically similar isolates from various origins (Schink and Pfennig, 1982). This genus was created to house strictly anaerobic bacteria that are able to grow by decarboxylation of succinate to propionate. Enrichment cultures, which were set up originally to enrich for syntrophic succinate degraders from marine and freshwater sediments, developed unexpectedly fast growth of small, coccoid bacteria that did not depend on cooperation with hydrogen-scavenging partners and formed propionate as the sole organic fermentation product. Pure cultures could be obtained only with enrichment cultures from marine sources; the freshwater enrichments grew much slower, and pure cultures were finally isolated when the sodium chloride concentration of the medium was enhanced to 100–150 mM. This finding gave the first hint of sodium dependence of this new type of energy conservation. A further species, *P. maris*, was created later to comprise bacteria similar to *P. modestum* which, however, are metabolically much more versatile and are able to ferment, beyond succinate, also carbohydrates, amino acids, and other organic acids (Janssen and Liesack, 1995).

Succinate is formed in large amounts in the rumen of cows and sheep, but the animal host cannot take up this valuable fermentation product (Wolin, 1979). Conversion of succinate to propionate is therefore an important function of the rumen microbiota, and succinate decarboxylation appears to be a rather common capacity of several propionate-forming fermenting bacteria, e.g., *Selenomonas* (Scheifinger and Wolin, 1973), *Veillonella* (Yousten and Delwiche, 1961), or certain *Propionibacterium* spp. (Yousten and Delwiche, 1961). Initially these conversions were regarded as "cometabolic" activities, which did not provide any advantage to the bacteria. Many years later, it was shown that the methylmalonyl-CoA decarboxylase of *Veillonella alcalescens* is a membrane-bound enzyme that couples the decarboxylation reaction with the transfer of sodium ions across the cytoplasmic membrane (Hilpert and Dimroth, 1982). The sodium gradient thus established helps to provide energy for transport of dicarboxylic acids across the membranes, but it does not allow growth of these bacteria with succinate. It was quite a surprise, therefore, when a bacterium was isolated from sediments that can run its entire energy metabolism for growth on this decarboxylation reaction. This unusual capacity explains why these isolates were placed in a new taxonomic unit, apart from other propionate-forming bacteria.

Today several other bacteria able to grow by conversion of succinate to propionate are known. Among these are Gram-negative mesophiles and also thermophiles (Denger and Schink, 1990; C. Guangsheng and A. Stams, unpublished observations). The coupling between sodium-extruding decarboxylation and ATP synthesis varies to some extent between these isolates, and the direct sodium coupling between a methylmalonyl CoA decarboxylase and a sodium-dependent ATPase as found in *Propionigenium modestum* (see below) is not the only solution to this problem. Later we could show that also *Veillonella parvula* (Mays et al., 1982; Delwiche et al., 1985) harnesses energy from succinate decarboxylation leading to enhanced growth yields in the presence of other substrates, but it cannot convert succinate decarboxylation directly into ATP formation and thus is unable to grow with succinate as sole substrate (Denger and Schink, 1992).

Habitats

Propionigenium modestum was originally isolated from a black, anoxic, marine sediment sample taken from the Canal Grande in Venice, Italy, where it made up 100 cells/ml of sediment (Schink and Pfennig, 1982). Similar strains were isolated later from many other marine habitats and also from human saliva. Enrichments from freshwater sediments sometimes produced cells of similar morphology as *P. modestum* and could be cultivated only in media with enhanced (100–

150 mM) sodium chloride concentrations. Later enrichment experiments with freshwater sediments from many different sites in Germany, Italy and the United States yielded different, vibrioid, Gram-negative bacteria (Denger and Schink, 1990). Also *P. maris* was isolated from marine sediments (Janssen and Liesack, 1995). No isolates able to grow with succinate as the sole energy source have yet been obtained from rumen ecosystems.

It has to be assumed that anoxic marine sediments are the typical habitats of these bacteria. Their energy metabolism is based on sodium ions as coupling ions in energy conservation. With this ability, they are well adapted to a marine environment. Several marine bacteria have been found to use sodium ions as energy couplers in various functions, e.g., respiration (Skulachev, 1985; Dibrov et al., 1986b) or motility (Brown et al., 1983; Dibrov et al., 1986a).

Propionigenium maris-like bacteria were isolated also from burrows of bromophenol-producing marine infauna, where they obviously are involved in reductive debromination of bromophenols (Watson et al., 2000). They probably used organic excretions of the infauna as electron donor for this reductive reaction.

Isolation

Medium for Isolation and Cultivation

A strictly anoxic, sulfide-reduced mineral medium with 20 mM succinate as the sole organic carbon and energy source, and incubation at 27–30°C, has proved to be highly selective for the enrichment of *P. modestum* if marine sediment samples of about 5-ml volume are used as the inoculum. The carbonate-buffered standard medium used for enrichment and isolation is described below (Widdel and Pfennig, 1981; Schink and Pfennig, 1982).

Bicarbonate-Buffered Standard Medium

KH_2PO_4	0.2 g
NH_4Cl	0.5 g
NaCl	20.0 g
$MgCl_2 \cdot 6H_2O$	3.0 g
KCl	0.5 g
$CaCl_2 \cdot 2H_2O$	0.15 g

Dissolve ingredients in 1 liter of distilled water. Autoclave the complete mineral medium in a vessel equipped with a filter inlet to allow flushing of the headspace with sterile oxygen-free gas and use screw cap inlets for addition of thermally unstable additives after autoclaving. Connect silicon tubing from the bottom of the vessel to a dispensing tap (if possible with a protecting bell) for sterile dispensing of the medium (do not use latex tubing; it releases compounds that are toxic to many anaerobes). A stirring bar is also required. After autoclaving, connect the vessel with the still hot medium to a line of oxygen-free nitrogen: carbon dioxide mixture (90% N_2:10% CO_2) at low pressure (<100 mbar). Flush the headspace and cool the medium under this atmosphere to room temperature, perhaps with the help of a cooling water bath.

The mineral medium is amended with the following additions from stock solutions that have been sterilized separately (amounts per liter of medium): a) 30 ml of 1 M $NaHCO_3$ solution (autoclaved in a *tightly closed* screw cap bottle with about 30% headspace. The bottle should be autoclaved in a further protecting vessel, e.g., a polypropylene beaker, to avoid spills of carbonate if the bottle breaks in the autoclave); b) 2 ml of 0.5 M $Na_2S \cdot 9H_2O$ solution (autoclaved separately under oxygen-free gas atmosphere as above); c) 1 ml of trace element solution, e.g., SL 10 (Widdel et al., 1983); d) 0.5 ml of 10-fold concentrated, filter-sterilized vitamin solution (Pfennig, 1978); and e) adequate amounts of sterile 1 M HCl or 1 M Na_2CO_3 to adjust the pH to 7.1–7.3.

The complete medium is dispensed into either screw-cap bottles or screw-cap tubes which are filled to the top, leaving a lentil-sized air bubble for pressure equilibration. Enrichment cultures usually produce gas in the first enrichment stages and are better cultivated in half-filled 50- to 100-ml serum bottles under a headspace of nitrogen: carbon dioxide mixture (90% N_2:10% CO_2). Not all strains require this vitamin mixture.

For mass cultivation of *P. modestum* strain GraSucc2 in fermentors, a phosphate-buffered medium is easier to handle. The following medium has been applied successfully (Hilpert et al., 1984).

Phosphate-Buffered Medium

Distilled water	940 ml
KH_2PO_4	4.2 g
NH_4Cl	0.5 g
NaCl	20.0 g
$MgCl_2 \cdot 6H_2O$	3.0 g
$CaCl_2 \cdot 2H_2O$	0.15 g

Add sulfide, trace elements, and vitamins as above, as well as 60 ml of a neutralized 1 M sodium succinate solution, and adjust the pH to 7.0 before inoculation.

Isolation of Pure Cultures

After two to three transfers in liquid medium, gas should no longer be formed by the enrichment cultures, and a dominant population of short, coccoid rods should be established. These bacteria can be isolated in anoxic agar deep dilution series (Pfennig, 1978) or in roll tubes (Balch et al., 1979). Streaking Petri dishes in an anoxic glove box has not yet been tried with these bacteria. Preparation of pure cultures requires two subsequent dilution series; purity should be checked after growth in selective mineral

medium and in complex medium, e.g., AC medium (Difco Laboratories, Ann Arbor, MI, USA). AC medium prepared according to the original recipe appears to inhibit several sediment bacteria. Therefore, a 1:10 or 1:5 diluted variation (in seawater as well as in freshwater medium, with adjusted agar concentration) of this medium should be used to ensure that the cultures are pure.

Propionigenium maris requires yeast extract (0.1% [w/v]) for growth in pure culture. Addition of yeast extract is not recommended in the liquid enrichment cultures but is needed in the purification step.

Identification and Further Properties

In phase contrast microscopy, cells of *P. modestum* appear as short, coccoid rods (0.5–0.6 × 0.5–2.0 μm), often in short chains (Fig. 1). They are Gram negative and do not form spores. The G+C content of the DNA is in the range of 32–36 mol%.

Propionigenium modestum is strictly anaerobic and does not withstand increased oxygen tensions. The optimum temperature for growth is 30–33°C, and under these conditions, doubling times of 2.5–4.5/h are obtained, depending on whether yeast extract (0.1% [w/v]) is provided as source for assimilatory metabolism. Yeast extract is not required and does not support the energy metabolism, but it enhances the growth yields by about 35%.

In addition to succinate, fumarate and pyruvate also support growth and are fermented stoichiometrically to acetate and propionate. No sugars, alcohols, or any other organic substrates are utilized. No cytochromes have been detected, which is consistent with the absence of electron transport phosphorylation. The growth yields with fumarate and pyruvate are small (3.8 and 6.2 g/mol of fumarate and pyruvate, respectively).

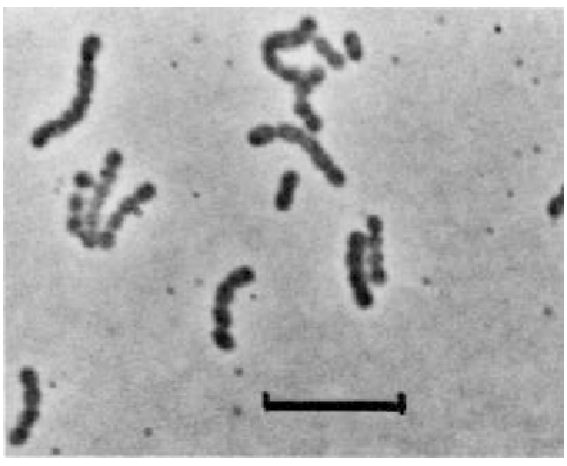

Fig. 1. Phase contrast photomicrograph of cells of *Propionigenium modestum* strain GraSucc2 (type strain) after growth with 20 mM succinate. Bar = 5 μm.

Also *P. maris* forms coccoid to ovoid cells or short rod-like cells of similar size as *P. modestum* cells. It is metabolically much more versatile than *P. modestum* but depends on yeast extract as an obligate medium constituent. In the presence of yeast extract, it ferments succinate, fumarate, pyruvate, citrate, 3-hydroxybutyrate, glucose, fructose, maltose, aspartate, lysine, threonine, glutamate and cysteine; the typical organic fermentation products include formate, acetate, propionate and butyrate. Succinate is fermented to propionate, carbohydrates are fermented to formate, acetate, ethanol and lactate. Hydrogen is produced from carbohydrates and yeast extract, ammonia from amino acids, and sulfide from cysteine. Sulfate, sulfur, thiosulfate and nitrate are not reduced; catalase is not present, and indole is formed from L-tryptophan. *Propionigenium maris* requires anoxic growth conditions. The temperature optimum is at 34–37°C and the pH optimum, at 6.9–7.7. Growth requires at least 5 g/liter of NaCl, at maximum 55 g/liter of NaCl. Cytochromes were not detected. The DNA base ratio is 40 mol% G+C.

Early 16S rRNA gene sequence analyses revealed that *P. maris* and *P. modestum* are closely related and form a distinct lineage within a phylogenetically coherent group characterized by *Fusobacterium nucleatum* and other *Fusobacterium* species, together with *Clostridium rectum*, *Leptotrichia buccalis* and *Sebaldella termitidis* (Both et al., 1991; Janssen and Liesack, 1995). This group has been elevated to the rank of a phylum, *Fusobacteria*, embracing besides *Propionigenium* the genera *Fusobacterium*, *Ilyobacter*, *Leptothrichia*, *Sebaldella*, *Streptobacillus* and *Sneathia* (Garrity et al., 2002).

The biochemical basis of energy conservation with succinate as sole substrate has been studied in detail with *P. modestum* (Hilpert et al., 1984). Succinate exchanges in the cell with propionyl-CoA to form succinyl-CoA and free propionate, which leaves the cell. Succinyl-CoA is rearranged to methylmalonyl-CoA and is decarboxylated by a membrane-bound decarboxylase that couples this reaction with the transport of sodium ions across the cytoplasmic membrane. The sodium ion gradient thus established drives ATP synthesis via a membrane-bound, sodium-dependent ATP synthase.

This unusual coupling of decarboxylation and ATP synthesis was the first case of an energy metabolism that was based entirely on sodium as coupling ions and which did not involve classical

substrate-linked phosphorylation or electron transport phosphorylation steps. Today we know of several more cases of bacteria whose total energy metabolism is based on decarboxylation reactions (Dimroth and Schink, 1998). The biochemical basis for energy conservation may be quite different in each case: e.g., anaerobic bacteria that ferment oxalate to formate and carbon dioxide employ a decarboxylase enzyme which is soluble in the cytoplasm and does not act as an ion pump, and energy conservation occurs via substrate import and product export by an antiporter system (Anantharam et al., 1989).

The ATPase of *P. modestum* has been characterized in more detail (Laubinger and Dimroth, 1987; Laubinger and Dimroth, 1988; Dimroth, 2000). It is composed of an F_1 and an F_0 moiety, both of which have subunits comparable to those of a classical bacterial proton ATPase. The enzyme of *P. modestum* can also pump protons across the membrane, but only if the sodium concentration is very low (<0.1 mM). Under physiological conditions, ATP synthesis is driven entirely by sodium ions. The Na^+ translocating ATP synthase of *P. modestum* became a model system for the study of ion translocation in ATPase enzymes in general. The specificity for Na^+ translocation is localized in the F_0 unit, as could be shown elegantly in transport experiments with hybrid ATPases composed of F_1 units from *Escherichia coli* and F_0 units of *P. modestum* (Laubinger et al., 1990). The specificity for sodium ions could be changed by site-directed mutagenesis (Kaim and Dimroth, 1995; Wehrle et al., 2002). The enzyme has been cloned and formed an active complex after homologous recombination in *E. coli* (Kaim and Dimroth, 1993). Detailed analysis of the rotation process by which ATP is synthesized by this enzyme has shown that voltage is the determining factor, together with the chemiosmotic effect of Na^+ (Kaim and Dimroth, 1998). Today, understanding of the ATP synthesis apparatus of *P. modestum* is very detailed (Dimroth, 2000) and at the same level as the corresponding proton-pumping enzyme of *E. coli*. It has become one of the two favorite ATPase systems studied in depth to understand the coupling of F_1 rotation in the F_0 stator system, and it may be the most promising one to finally elucidate the quantitative relationship between ion transport and ATP formation.

Energy conservation by decarboxylation is of special interest because the free energy change of such reactions is small and yields only about 20–25 kJ/mol. If such reactions drive ATP synthesis (the free energy expense is 70–75 kJ/mol of ATP; Thauer et al., 1977), several decarboxylation reactions have to be coupled with one ATP synthesis reaction. Although the exact stoichiometries of sodium ion translocation by the methylmalonyl-CoA decarboxylase and the ATP synthase have not yet been determined, it is obvious from these free energy calculations that at least three decarboxylations are necessary to allow synthesis of one ATP. This calculation agrees well with the cell yield obtained with *P. modestum* (2.0–2.5 g/mol of succinate). Whole energy metabolism based on a decarboxylation reaction is the most "modest" form of energy conservation, i.e., it produces the lowest amount of energy that can be converted into ATP by a living cell in pure culture (Thauer and Morris, 1984). Partners in syntrophic cocultures active in methanogenic degradation of, e.g., fatty acids, have to operate in the same range of energy yields (see the chapter Syntrophism among Prokaryotes in Volume 2).

Applications

So far there is no commercial application for the *Propionigenium* species. The fact that their unique energy conservation mechanism can also be reversed and allows carboxylation reactions without the direct involvement of ATP (Dimroth and Hilpert, 1984) can possibly render such systems interesting for new ways of biochemical syntheses. The debrominating activity of *P. maris* (Watson et al., 2000) might be of interest in the cleanup of halogenated contaminants in marine ecosystems.

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Literature Cited

Anantharam, V., M. J. Allison, and P. C. Maloney. 1989. Oxalate:formate exchange: The basis for energy coupling in Oxalobacter. J. Biol. Chem. 264:7244–7250.

Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe. 1979. Methanogens: Reevaluation of a unique biological group. Microbiol. Rev. 43:260–296.

Both, B., G. Kaim, J. Wolters, K. H. Schleifer, E. Stackebrandt, and W. Ludwig. 1991. Propionigenium modestum: A separate line of descent within the eubacteria. FEMS Microbiol. Lett. 62:53–8.

Brown, I. I., M. J. Galperin, A. N. Glagolev, and V. P Skulachev. 1983. Utilization of energy stored in the form of Na^+ and K^+ ion gradients by bacterial cells. Eur. J. Biochem. 134:345–349.

Delwiche, E. A., J. J. Pestka, and M. L. Tortorello. 1985. The Veillonellae: Gram-negative cocci with a unique physiology. Ann. Rev. Microbiol. 39:175–193.

Denger, K., and B. Schink. 1990. New motile anaerobic bacteria growing by succinate decarboxylation to propionate. Arch. Microbiol. 154:550–555.

Denger, K., and B. Schink. 1992. Energy conservation by succinate decarboxylation in Veillonella parvula. J. Gen. Microbiol. 138:967–971.

Dibrov, P. A., V. A. Kostyrko, R. L. Lazarova, V. P. Skulachev, and I. A. Smirnova. 1986a. The sodium cycle. I: Na^+-dependent motility and modes of membrane energetization in the marine alkalotolerant Vibrio alginolyticus. Biochim. Biophys. Acta 850:449–457.

Dibrov, P. A., R. L. Lazarova, V. P. Skulachev, and M. L. Verkhovskaya. 1986b. The sodium cycle. II: Na^+-coupled oxidative phosphorylation in Vibrio alginolyticus cells. Biochim. Biophys. Acta 850:458–465.

Dimroth, P., and W. Hilpert. 1984. Carboxylation of pyruvate and acetyl coenzyme A by reversal of the Na^+ pumps oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase. Biochemistry 23:5360–5371.

Dimroth, P., and B. Schink. 1998. Energy conservation in the decarboxylation of dicarboxylic acids by fermenting bacteria. Arch. Microbiol. 170:69–77.

Dimroth, P. 2000. Operation of the F_O motor of the ATP synthase. Biochim. Biophys. Acta 1458:374–386.

Garrity, G. M., K. L. Johnson, J. A. Bell, and D. B. Searles. 2002. Taxonomic outline of the Procaryotes. *In:* Bergey's Manual of Systematic Bacteriology, 2nd ed. Springer-Verlag. New York, NY.

Hilpert, W., and P. Dimroth. 1982. Conversion of the chemical energy of methylmalonyl-CoA decarboxylation into a Na^+ gradient. Nature 296:584–585.

Hilpert, W., B. Schink, and P. Dimroth. 1984. Life by a new energy conservation mechanism with Na Na^+ as coupling ion. EMBO J. 3:1665–1670.

Janssen, P. H., and W. Liesack. 1995. Succinate decarboxylation by Propionigenium maris sp. nov., a new anaerobic bacterium from an estuarine sediment. Arch. Microbiol. 164:29–35.

Kaim, G., and P. Dimroth. 1993. Formation of a functionally active sodium-translocating hybrid F_1F_O ATPase in Escherichia coli by homologous recombination. Eur. J. Biochem. 218:937–944.

Kaim, G., and P. Dimroth. 1995. A double mutation in subunit c of the Na^+-specific F1FO -ATPase of Propionigenium modestum results in a switch from Na^+ to H^+-coupled ATP synthesis in the Escherichia coli host cells. J. Molec. Biol. 253:726–738.

Kaim, G., and P. Dimroth. 1998. Voltage-generated torque drives the motor of the ATP synthase. EMBO J. 17:5887–5895.

Laubinger, W., and P. Dimroth. 1987. Characterization of the Na^+-stimulated ATPase of Propionigenium modestum as an enzyme of the F_1F_O type. Eur. J. Biochem. 168:475–480.

Laubinger, W., and P. Dimroth. 1988. Characterization of the ATP synthase of Propionigenium modestum as a primary sodium pump. Biochemistry 27:7531–7537.

Laubinger, W., G. Deckers-Hebestreit, K. Altendorf, and P. Dimroth. 1990. A hybrid adenosinetriphosphatase composed of F_1 of Escherichia coli and F_O of Propionigenium modestum is a functional sodium ion pump. Biochemistry 29:5458–5463.

Mays, T. D., L. V. Holdeman, W. E. C. Moore, M. Rogosa, and J. L. Johnson. 1982. Taxonomy of the genus Veillonella Prévot. Int. J. Syst. Bacteriol. 32:28–36.

Pfennig, N. 1978. Rhodocyclus purpureus gen. nov. and sp. nov., a ring-shaped, vitamin B_{12}-requiring member of the family Rhodospirillaceae. Int. J. Syst. Bacteriol. 28:283–288.

Scheifinger, C. C., and M. J. Wolin. 1973. Propionate formation from cellulose and soluble sugars by combined cultures of Bacteroides succinogenes and Selenomonas ruminantium. Appl. Microbiol. 6:789–795.

Schink, B., and N. Pfennig. 1982. Propionigenium modestum gen. nov. sp. nov., a new strictly anaerobic, nonsporing bacterium growing an succinate. Arch. Microbiol. 133:209–216.

Skulachev, V. P. 1985. Membrane-linked energy transductions. Bioenergetic functions of sodium: H^+ is not unique as a coupling ion. Eur. J. Biochem. 151:199–208.

Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41:100–180.

Thauer, R. K., and J. G. Morris. 1984. Metabolism of chemotrophic anaerobes: Old views and new aspects. *In:* D. P. Kelly and N. G. Carr (Eds.) The Microbe 1984. Part II: Prokaryotes and Eukaryotes. Cambridge University Press. Cambridge, UK. Soc. Gen. Microbiol. Symp. vol. 36:123–168.

Watson, J., G. Y. Matsui, A. Leaphart, J. Wiegel, F. A. Rainey, and C. R. Lovell. 2000. Reductively debrominating strains of Propionigenium maris from burrows of bromophenol-producing marine infauna. Int. J. Syst. Evol. Microbiol. 50:1035–1040.

Wehrle, F., Y. Appoldt, G. Kaim, and P. Dimroth. 2002. Reconstitution of FO of the sodium ion translocating ATP synthase of Propionigenium modestum from its heterologously expressed and purified subunits. Eur. J. Biochem. 269:2567–2573.

Widdel, F., and N. Pfennig. 1981. Studies an dissimilatory sulfate-reducing bacteria that decompose fatty acids. I: Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of Desulfobacter postgatei gen. nov. sp. nov. Arch. Microbiol. 129:395–400.

Widdel, F., G. W. Kohring, and F. Mayer. 1983. Studies an dissimilatory sulfate-reducing bacteria that decompose fatty acids. III: Characterization of the filamentous gliding Desulfonema limicola gen. nov. sp. nov. and Desulfonema magnum sp. nov. Arch. Microbiol. 134:286–294.

Wolin, M. J. 1979. The rumen fermentation: A model for microbial interactions in anaerobic systems. Adv. Microb. Ecol. 3:49–77.

Yousten, A. A., and E. A. Delwiche. 1961. Biotin and vitamin B_{12} coenzymes in succinate decarboxylation by Propionibacterium pentosaceum and Veillonella alcalescens. Bacteriol. Proc. 61:175.

Prokaryotes (2006) 7:960–970
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CHAPTER 14.3

The Genus *Zoogloea*

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Bacteria of the genus *Zoogloea* have historically been considered members of the family Pseudomonadaceae but have been differentiated from other obligately aerobic, Gram-negative, nonsporeforming, rod-shaped bacteria that grow in aquatic habitats on the basis of their production of a characteristic gelatinous matrix. The "zoogloeal matrix" surrounds clumps of cells found in natural aquatic habitats or when grown in unshaken liquid culture in the laboratory (Butterfield, 1935; Butterfield et al., 1937; Wattie, 1943). The name *Zoogloea*, which was derived from the Greek word meaning animal glue, refers to the primary trait, the zoogloeal matrix, that is used to distinguish *Zoogloea* from other metabolically similar bacteria.

The two species of *Zoogloea*, *Z. ramigera* and *Z. filipendula*, have been isolated from sewage or from sewage-treatment systems that employ oxidative methods, i.e., activated sludge systems, trickling filters, or oxidation ponds (Butterfield et al., 1937; Butterfield and Wattie, 1941; McKinney and Horwood, 1952; McKinney and Weichlein, 1953). Both species produce branched, finger-like projections or outgrowths from the floc (i.e., clumps of cells that grow in an aggregated form that settles or "flocs" from aqueous suspension) when grown under quiescent culture conditions but have been differentiated from each other on the basis of cell size, biochemical reactions, and appearance of the zoogloeal matrix.

Characteristics of the Zoogloeal Matrix

The zoogloeal matrix is a capsular envelopment that surrounds several cells and commonly results in a flocculent growth habit in liquid media. That is, cell flocs settle from suspension, leaving a relatively clear supernatant. Unz and coworkers (Unz, 1974; Farrah and Unz, 1975; Unz and Farrah, 1976a) have convincingly demonstrated that floc formers of the *Zoogloea* type grow in an aggregated state and that the individual cells are entrapped within the zoogloeal matrix while cell division occurs. Floc formation of the zoogloeal type implies cell growth in an aggregated form (Friedman and Dugan, 1968b), whereas the term "flocculation" (Peter and Wuhrmann, 1971) has been defined as the aggregation of suspended bacterial cells after growth of cells has occurred, i.e., the "flocculation" of colloidal material. Biologically, the two processes are considerably different although the same physical and chemical laws apply to both.

Although there can be confusion relative to the distinction between zoogloea formation and flocculent growth, there is a consensus that microbial floc formation results from the presence of adherent extracellular fibrils (Busch and Stumm, 1968; Deinema and Zevenhuizen, 1971; Finstein, 1967; Friedman and Dugan, 1968b; Friedman et al., 1968, 1969; Tago and Aida, 1977). The presence of polysaccharide and the ability to flocculate have been demonstrated with mutants lacking polysaccharide and which do not exhibit flocculent growth; recombinant bacteria with restored polysaccharide production also simultaneously show restored floc formation (Easson et al., 1987a, 1987b). All floc-forming bacteria appear to posses extracellular fibrillar strands, but not all Gram-negative floc-forming bacteria produce the characteristic zoogloeal matrix, such as that for *Zoogloea ramigera* strain 115 seen in Figs. 1 and 2.

The polysaccharide of *Zoogloea* may remain as a loose slime layer in the vicinity of the cells, a well-defined capsule, or a zoogloeal matrix around the cells which synthesized it or it may be dispersed in the medium as a colloidal suspension or in solution. Increased viscosity of the surrounding medium may result from extensive polymer synthesis by the cells. The key feature in bioflocculation appears to be the synthesis of relatively insoluble extracellular polymer strands that remain within the vicinity of cells and do not disperse. The photographs shown in Figs. 3, 4, 5, 6 and 7 show the insoluble polysaccharide strands found around flocs of these bacteria. The polymer strands shown in Fig. 6 were photographed under ultraviolet illumination after the flocs were stained with the fluorescent dye Paper White-BP as previously reported (Dugan and Pickrum, 1973; Friedman et al., 1969).

The chemical composition and structure of the extracellular polysaccharides determine its physical and chemical properties. Polysaccharides in solution or suspension vary in water solubility,

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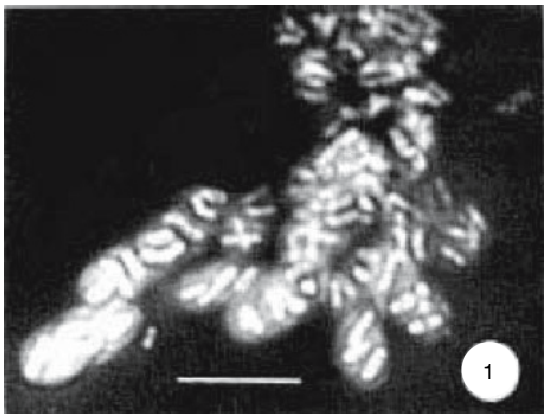

Fig. 1. Photomicrograph of *Zoogloea ramigera* strain 115 floc negatively stained by the Maneval method and photographed through the light microscope. The cells are shown embedded within the zoogleal matrix and with the characteristic finger-link projections. Bar = 10 μm.

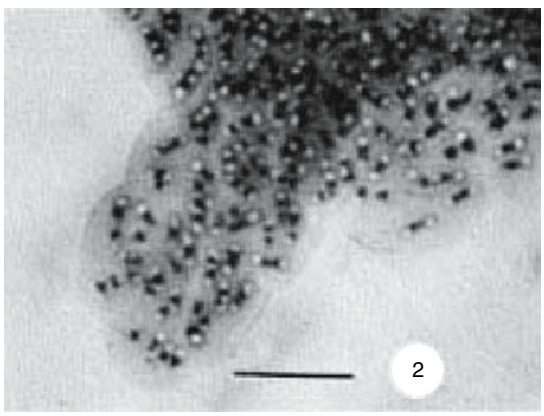

Fig. 2. Photomicrograph of *Zoogloea ramigera* 115 floc stained with 1% aqueous crystal violet. The gelatinous zoogloeal matrix in which cells are embedded is shown. The variable staining appearance is due to polybetahydroxy butyric acid (PHB) granules that accumulate in the cells. Bar = 10 μm.

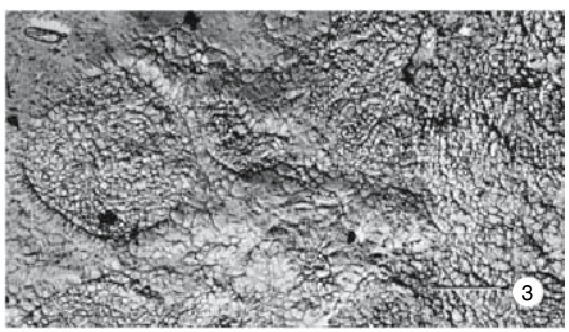

Fig. 3. Electron micrograph of a freeze-fractured specimen of *Zoogloea ramigera* 115. The fibrillar network of extracellular polysaccharide strands from which the zoogloeal matrix is formed is shown. In the living specimen, water is bound in the interstices of the network, which resembles a ball of chicken wire. Bar = 10 μm.

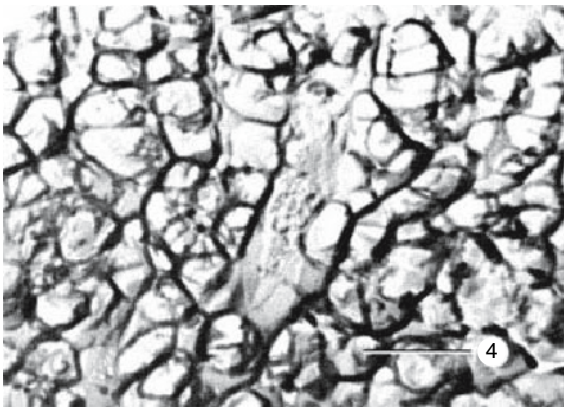

Fig. 4. Enlargement of the floc shown in Fig. 3. A cell embedded within the zoogloeal matrix is shown. Bar = 10 μm.

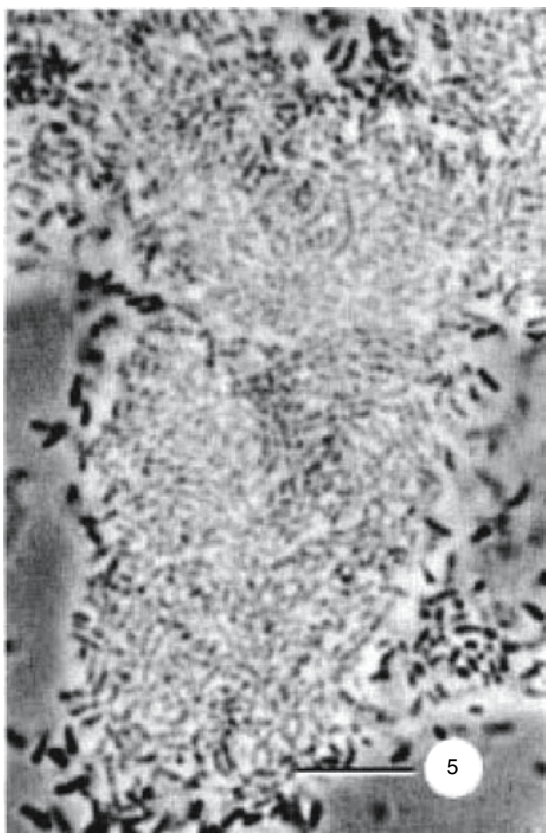

Fig. 5. Photomicrograph of a floc of the *Zoogloea* isolate designated CT-2 originally isolated by K. Crabtree. The floc was stained by a water-clear solution of the fluorescent dye Paper White-BP and photographed under phase-contrast optics. Some of the bacteria are in the focal plane whereas others are not. Bar = 10 μm.

rheological properties (e.g., viscosity, viscoelasticity), chemical and thermal stability, surfactant properties, the ability to bind water, etc. *Zoogloea* strains vary in the composition of the polysaccharide (Table 1), but information concerning the structure of some isolates is limited.

The polysaccharide of *Z. ramigera* 115 has been examined in detail. This weakly acidic polysaccharide is slightly soluble in water, is stable in a pH range of 3 to 10, and is not precipitated in the presence of salt (Stauffer et al., 1980). Stauffer et al. (1980) reported that the polysacchride of *Z. ramigera* 115 is stable to temperature cycling from –15° to 90°C. However, Norberg and Enfors (1982) reported that the first temperature cycle from 25° to 90°C did increase the viscosity of the polymer at the lower temperature. The polysaccharide of *Z. ramigera* 115 is highly viscous and lowers the surface tension of water. The polysaccharide exhibits Newtonian flow at lower concentrations and, as the concentration increases, the polymer becomes increasingly pseudoplastic and viscoelastic (Stauffer et al., 1980). Similar flow behavior was observed in relation to growth and polysaccharide production in a fermentor (Norberg and Enfors, 1982).

The polysaccharide of *Z. ramigera* 115 contains glucose, galactose, and pyruvic acid, the ratios of which may vary with culture conditions and growth phase of the culture (Norberg and Enfors, 1982; Franzen and Norberg, 1984; Ikeda et al., 1982). Growth on different sugar substrates did not alter the composition of sugars in

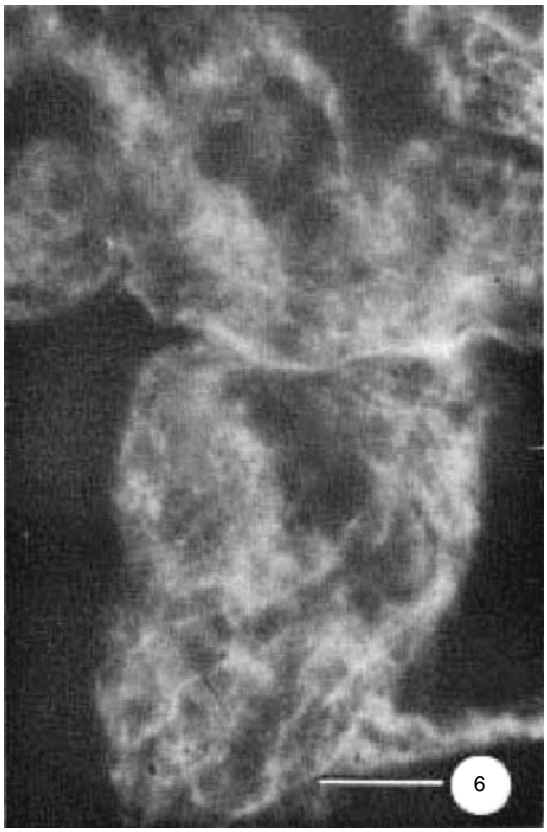

Fig. 6. Photomicrograph taken under ultraviolet (UV) illumination of the identical field shown in Fig. 5. Only material stained by the polysaccharide-specific fluorescent stain Paper White-BP can be seen under UV illumination. This procedure demonstrates that the cell floc shown in Fig. 5 was interspersed by a network of fibrous polysaccharide strands analogous to a "cobweb" which appears to hold the floc intact. Bar = 10 μm.

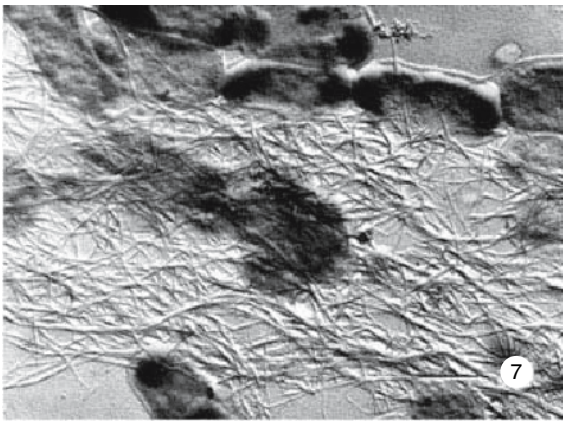

Fig. 7. Shadow-cast electron micrograph of *Zoogloea ramigera* isolate I-16-M after treatment with 1 N NaOH for 24 h at 28°C. Extracellular fibrillar strands and cell ghosts are shown. I-16-M produces flocs but no observable gelatinous zoogloeal matrix. Bar = 10 μm.

Table 1. Composition of purified extracellular polysaccharides of *Zoogloea*.

Strain	Composition	Reference
Z. ramigera 115	Glucose, galactose, pyruvic acid	Ikeda e al., 1982; Franzen and Norberg, 1984
Z. ramigera I-16-M	Glucose, galactose, mannose	Pickrum, 1972
Z. ramigera MP6	Glucosamine, methylpentose amine, hexose sugars, uronic acids	Farrah and Unz, 1976
Z. ramigera NRRL B-3669M	Glucose, galactose, mannose	Wallen and Davis, 1972
Zoogloea species NRRL B-3793	Rhamnose, mannose, galactose	Wallen and Davis, 1972
Zoogloea species C-1	Glucose, galactose, mannose, arabinose, rhamnose	Pickrum, 1972
Zoogloea species	Glucosamine, fucosamine	Tezuka, 1973
Z. filipendula P-8-4	Glucose, galactose, mannose	Pickrum, 1972
Zoogloea isolate P-95-5[a]	Glucose, galactose, mannose	Crabtree et al., 1965; Crabtree and McCoy, 1967

[a]The isolate is probably actually *Pseudomonas denitrificans*.

the polymers (Parsons and Dugan, 1971). A ratio of glucose:galactose of 2:1 with trace amounts of terminal pyruvylated glucose (Franzen and Norberg, 1984; Friedman et al., 1969; Parsons and Dugan, 1971; Sinskey et al., 1986) as well as a ratio of 11.0:3.1:1.5 for glucose:galactose:pyruvic acid (Ikeda et al., 1982) has been determined. Uronic acids, pentose, or amino sugars have not been detected in the polysaccharide of *Z. ramigera* 115 (Franzen and Norberg, 1984; Friedman et al., 1969; Ikeda et al., 1982). The polysaccharide of *Z. ramigera* 115 is a highly branched structure with β-1–4-linkages and a molecular weight of approximately 10^5 (Friedman et al., 1968, 1969; Ikeda et al., 1982). A structure with repeating units has been proposed (Ikeda et al., 1982), although there is conflicting evidence that the polysaccharide of *Z. ramigera* 115 has an irregular structure (Franzen and Norberg, 1984).

A variety of sugars have been identified as components of the polysaccharides of *Zoogloea* cultures (Table 1). Polymer from *Z. ramigera* MP6 contains the amino sugars, glucosamine, and possibly methyl-pentose amine, hexose sugars, and uronic acid (Farrah and Unz, 1976). Tezuka (1973) identified glucosamine and possibly fucosamine as components of the polymer of a *Zoogloea* culture isolated from activated sludge, but did not detect neutral sugars or uronic acids. Polymer of *Z. ramigera* NRRL B-3669M contains glucose, mannose, and galactose, while isolate NRRL B-3793 contained rhamnose, mannose, and possibly galactose (Wallen and Davis, 1972). *Z. filipendula* P-8–4 and *Z. ramigera* contain glucose, galactose, and mannose in the polysaccharide, while an unidentified *Zoogloea* species designated C-1 contained glucose, galactose, mannose, arabinose, and rhamnose (Pickrum, 1972). It is possible that those isolates that have been shown to produce extracellular polysaccharides with only glucose, galactose, and mannose are more closely related to *Pseudomonas dentrificans* than to the *Z. ramigera* 115 isolate (see Table 1). Research on *Zoogloea* isolates has increased significantly because of their potential use in applications for removal of hazardous metal contaminants from waste streams.

Habitats

Zoogloea species have been isolated from organically enriched oxygenated water, particularly domestic sewage and aerobic sewage-treatment systems, such as trickling filters, activated sludge tanks, or oxidation ponds (Amin and Ganapati, 1967; Dugan and Lundgren, 1960; Unz and Farrah, 1972). These are all continuous-flow systems that are rich in dissolved or particulate oxidizable organic materials, which provide for the enrichment of *Zoogloea* and closely related bacteria. Zoogloeal masses may also be found adhering to solid objects suspended or floating in lakes and ponds. For example, 19 of 36 Gram-negative bacteria isolated from Lake Erie formed flocs in the laboratory, and they all clustered with previously identified isolates of *Zoogloea* when subjected to a computer taxonomic program. Therefore, *Zoogloea* appears to be ubiquitous in aquatic environments.

Isolation

Zoogloeal masses obtained from organically rich natural aquatic systems, sewage treatment plants, wastewater, etc. may be used in isolation procedures to obtain pure cultures of *Zoogloea*. These organically rich sources may also be used to inoculate enrichment media from which strains of *Zoogloea* may be isolated. Both the zoogloeal matrix found around the characteristic isolates of *Zoogloea* and the nongelatinous fibrillar strands associated with the nonzoogloeal *Zoogloea* isolates are a deterrent to pure-culture isolation because other bacteria adhere to or become entangled within the floc. Successful isolation of *Zoogloea* has been achieved with methods employing ultrasonic methods to disrupt zoogloeal masses, micromanipulation to manually separate individual cells from the zooglea matrix, the inclusion of inhibitory agents in culture media to prevent the growth of unwanted microorganism and the use of carbon growth substrates which may enrich for *Zoogloea*. These methods are used in conjunction with conventional isolation precedures to obtain isolates of *Zoogloea*.

Enrichment

Several methods can be used to establish enrichments of zoogloeal bacteria. The simplest method is the incubation of raw sewage, sewage sludge, treated sewage effluent, etc. in containers at room temperature until a surface film or pellicle is formed (Amin and Ganapati, 1967). Alternatively, mineral salts solution overlaying a nutritive agar plug may be inoculated and incubated until a surface pellicle develops (Unz, 1984).

Mineral Salts Solution

> The overlay medium is prepared from stock solutions and adjusted to pH 8.5 with 0.5 N NaOH. The mineral salts solution contains:
>
> | | |
> |---|---|
> | $(NH_4)_2SO_4$ | 0.3 g |
> | NaCl | 5.85 g |
> | $CaCl_2 \cdot 2H_2O$ | 0.2 g |
> | K_2HPO_4 | 0.1 g |
> | $MgSO_4 \cdot 7H_2O$ | 0.14 g |

$FeSO_4 \cdot 7H_2O$	0.3 mg
$MnCl_2 \cdot 4H_2O$	6.3 mg
$CoSO_4 \cdot 7H_2O$	0.11 mg
H_3BO_4	0.6 mg
$ZnCl_2$	0.22 mg
$CuSO_4 \cdot 5H_2O$	0.08 mg
Distilled water	1 liter

Nutrient-Enriched Agar

The nutritive agar contains 20 g agar per liter of mineral salts solution. The carbon source is any one of the following added per liter of mineral salts.

Starch	2.4 g
m-Toluic acid	1.35 g (neutralized)
n-Butanol	1.5 ml
Lactic acid (85%)	1.35 g
Ethanol (95%)	1.5 ml
Glucose	2.4 g

After the addition of the carbon source, the pH is adjusted to 8.5 with 0.5 N NaOH.

Isolation

Isolations may be made from surfaces covered with zoogloeal masses and/or effluents taken from organically polluted water. Material on surfaces is scraped off and placed into 0.05% proteose peptone-yeast extract broth (PPYE). The gelatinous material is disrupted by ultrasound (20 kc output, Branson) in sterile PPYE, and samples are serially diluted and incubated in PPYE (28°C). After incubation for 3 days, the highest dilutions showing growth (10^{-7} to 10^{-10}) are streaked onto PPYE agar and tryptone-glucose extract agar (TGE, Difco). When sufficient growth has occurred, colonies of different morphological types are transferred to duplicate tubes of PPYE broth. Tubes are incubated with and without agitation. After incubation, tubes containing a pellicle (stationary) or a floc (shaken) are selected, and the entire isolation procedure is repeated until pure cultures are obtained. In general, isolations can be most readily achieved with the use of organically dilute media.

Micromanipulation may be used to separate bacterial cells from zoogloeal matrices in order to obtain pure cultures of *Zoogloea* (Unz and Dondero, 1967a). In this method, flocs or slimes from enrichment cultures are dispersed in liquid medium and observed at 100× magnification. To remove debris and microorganisms, fingered zoogloeal projections are transferred individually by micropipettes through successive drops of medium. Washed zoogloea are placed upon nutritive agar films coated on the underside of coverslips and individual cells dissected away from the zoogloeal matrix with microneedles. Slides are incubated and the developed microcolonies transferred with sterile micropipettes to broth medium. Alternatively, washed zoogloea may be collected and briefly sonicated prior to streaking on growth medium (Unz, 1984). Media appropriate for the above methods are casitone-yeast (CY) broth (see below), nutrient broth, and the respective agar media (Unz, 1984; Unz and Dondero, 1967a).

CY Medium

CY medium contains per liter of distilled water:

Casitone	5.0 g
Yeast autolysate	1.0 g

The addition of 0.001% crystal violet to isolation media, either broth or agar, has also been used successfully to inhibit many undesirable bacteria that tend to adhere to the flocs. Agar plates that contain crystal violet in the growth medium also have differential value. On these plates, the isolates listed in Table 1 can be identified as follows: *Z. ramigera* 115 decolorizes and degrades crystal violet and forms straw-colored colonies; isolate C-1 fails to develop colonies; and isolates I-16-M, P-8–4, and P-95–5 absorb the dye and produce violet colonies. Since isolate C-1 is different from the known *Zoogloea* isolates, the use of the crystal violet is considered to have value in the identification of *Zoogloea* species, but such use has not been well studied (Friedman and Dugan, 1968b; Dugan, 1975).

Aromatic compounds utilized for growth may be used for the isolation of some *Zoogloea* from environmental samples (Unz and Farrah, 1972). *Zooglea* isolates may be obtained from activated sludge using solid basal medium with 1% *m*-toluate as the carbon source by streaking sludge or other inocula directly on the media and incubating. Solid samples, i.e., soils and feces, may be suspended with equal volumes of sterile distilled water and then streaked onto media. Developed colonies are transferred to CY medium (see above), disrupted by agitation, and streaked for isolation on *m*-toluate medium. *Zoogloea* grown on *m*-toluate medium form large colonies with yellow-green coloration indicative of *meta* cleavage products. Colonies are usually difficult to remove from the surface of the plates. This procedure will not isolate *Zoogloea* that do not utilize *m*-toluate as a growth substrate.

Zoogloea colonies on agar plates have a glistening, viscous appearance (Fig. 8). Colonies are straw-colored on media containing yeast extract or protcose-peptone and white on mineral salts media supplemented with alanine and argenine as the carbon source. The color differences are also observed in liquid media. Colonies have a leathery consistency, and the entire colony can frequently be removed with an inoculating loop (Dugan and Lundgren, 1960; Friedman and Dugan, 1968b). This distinctive characteristic is probably related to the formation of extracellular polysaccharide. Growth of *Zoogloea* in shaken liquid culture is often flocculent but flocs

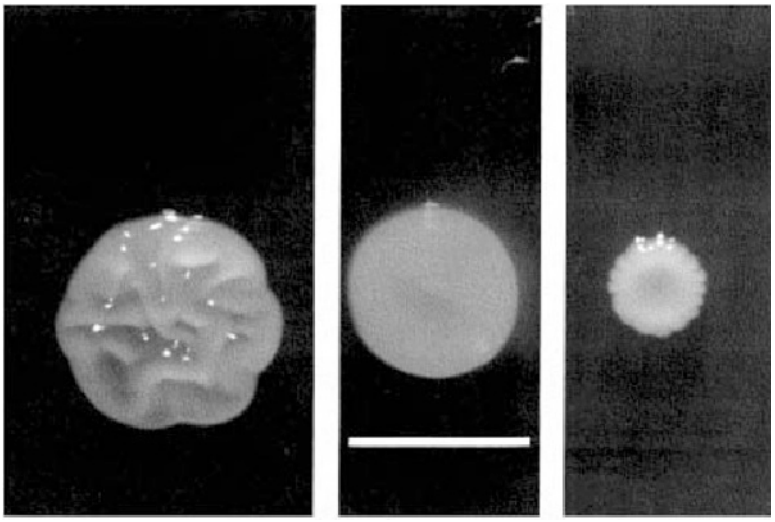

Fig. 8. Colonies of *Zoogloea ramigera* 115 vary in overall appearance and size. Variation is due to culture conditions and age of culture. Bar = 5 mm.

are tight and do not have a typical zoogloeal matrix.

Identification

As the name of the genus implies, *Zoogloea* species have been identified primarily on the basis of the presence of the characteristic extracellular, capsular, or zoogloeal matrix. However, matrix formation and its morphological appearance may be controlled nutritionally by the carbon and nitrogen sources, as well as by the carbon to nitrogen ratio and by the turbulence of the growth medium (Parsons and Dugan, 1971; Unz and Farrah, 1976b). Therefore, the presence or absence of a zoogloeal matrix is not a trait upon which identification can be reliably made. Although the presence of finger-like projections and a zoogloeal matrix is distinctive and a positive indicator, the absence of the zoogloeal matrix does not necessarily indicate that the isolate is not a *Zoogloea* species.

Isolates of *Zoogloea* are Gram-negative, polarly flagellated, rod-shaped bacteria that flocculate and accumulate poly-β-hydroxybutyrate (PHB) or polyalkanoic granules when cultivated in media that are rich in carbon. Cell size ranges from 0.5 to 1.3 μm in diameter and 1.0 to 3.6 μm in length. *Zoogloea* species are oxidative in metabolism and are able to utilize a variety of sugars, sugar alcohols, alcohols, amino acids, and proteins for growth. The inclusion of B vitamins, purines, pyrimidines, and nucleotides in culture media, although not required for growth, decreases the growth lag and increases biomass formation of cultures (Dugan and Lundgren, 1960; Friedman and Dugan, 1968b). The metabolic characteristics of the organisms are not sufficiently distinct to be the basis of identification for the genus *Zoogloea* (Crabtree and McCoy, 1967; Crabtree et al., 1965; Dugan, 1975; Dugan and Lundgren, 1960; Friedman and Dugan, 1968b; Ganapati et al., 1967; Unz, 1974; Unz and Dondero, 1967a, 1967b). However, the overall ability of *Zoogloea* species to oxidatively metabolize many sugars, sugar alcohols, and alcohols, with the formation of esters that often result in the typical fruity odor of esters in culture media, can be of use in restricting the options in identification (Joyce and Dugan, 1972). The strong capability of *Z. ramigera* 115 to form esters from a variety of organic acids and alcohols may be responsible for the addition of pyruvic acid to alcoholic groups on the extracellular zoogloeal polymer and may explain why some investigators were unable to observe pyruvate (Friedman et. al., 1969), whereas others observed traces (Norberg, 1984) and others identified a significant amount (Ikeda et al. 1982).

The taxonomy of the genus *Zoogloea* is uncertain with respect to differentiation from other genera within the family Pseudomonadaceae. Also uncertain is whether *Z. filipendula*, a species historically considered as a member of the genus *Zoogloea*, validly fits in the genus. The designation of the neotype strain of *Zoogloea ramigera* has also been questionable (Unz, 1984). Some *Zoogloea* isolates resemble species of the genera *Pseudomonas* (particularly *P. denitrificans*), *Gluconobacter*, and *Acetobacter. Zoogloea* differ from *Gluconobacter* and *Acetobacter* in the inability to grow at pH 4.5.

Antigenic relationships among some of the *Zoogloea* have been reported (Chorpenning et al., 1978). *Z. ramigera* 115 and I-16-M did not closely relate to *Z. filipendula* P-8–4 on a serologic basis. *Z. ramigera* 115 had greater antigenic relatedness to *Gluconobacter oxydans* subspe-

cies *suboxydans* (ATCC 621) than to either *Z. ramigera* I-16-M or *Z. filipendula* P-8-4. These data indicated that *Z. ramigera* 115 and I-16-M are distinctly different serovars, an observation which is also supported by the difference in composition of their extracellular polysaccharides.

The GC content of the DNA of *Zoogloea* ranged from 60.1 to 64.8 mol% (Pickrum, 1972; Unz, 1984). The data, although not conclusive, reinforce the view that several of the floc-forming isolates are similar organisms that are sufficiently distinctive to allow differentiation from other Gram-negative rods. However, at present there is no definitive description of either the genus *Zoogloea* or its species, other than the gelatinous matrix of finger-like projections, but this matrix is not always present.

Genetics

Because of the potential application of *Zoogloea* in several microbially based technologies, there is a need to isolate and identify genes for polysaccharide and PHB production, determine the biosynthetic pathways, and examine the enzymatic and genetic control mechanisms.

The exopolysaccharides of *Zoogloea* are involved with flocculation (Easson et al., 1987a, 1987b; Friedman et al., 1969) and adsorption of heavy metals (Dugan, 1987; Dugan and Pickrum, 1972; Ikeda et al., 1982). As with other polymers, the structure and physical and chemical properties of polysaccharides define its flocculation and adsorption characteristics. Therefore, manipulation of the structure of the polysaccharide is of importance in developing flocculants and bioadsorbents with desired characteristics that may be applicable to microbially based technologies for waste water treatment or metal reclamation. Genetic control of the biosynthesis of polysaccharides by microorganisms is a means by which the structure of the polymers may be manipulated and production of the polysaccharides increased.

Mutants generated by the insertion of the Tn5 transposon were used to examine the genetics of polysaccharide production in *Z. ramigera* I-16-M (Easson et al., 1987a, 1987b). There is an inherent instability in the chromosome in which DNA sequences spontaneously delete within the polysaccharide gene region, and this results in the loss of polysaccharide production. The frequency of chromosomal deletions within this gene region was enhanced by the presence of Tn5 transposon. Polysaccharide production was restored in the mutants by complementation with a gene library constructed with a broad-host-range cosmid vector. The restoration of polysaccharide production in a number of mutants by a single plasmid vector suggests that the genes for polysaccharide production may be clustered on the chromosome.

The genetics involved with PHB synthesis is of interest because of the potential use of PHB and other polydroxyalkanoates in the plastics industry. PHB metabolism in *Z. ramigera* I-16-M is a cyclic process (Tomita et al., 1983). The biosynthesis of PHB involves the condensation and reduction of acetyl CoA to form D(−)-3-hydroxybutyryl CoA followed by incorporation into PHB granules. Degradation of PHB granules proceeds via the production of D(−)-3-hydroxybutyrate oligomers and hydrolysis and oxidation to acetoacetate. The cycle is completed with the activation of acetoacetate to acetoacetyl CoA.

Studies examining the structure and function of enzymes in PHB synthesis (Davis et al., 1987a, 1987b; Fukui et al., 1976; Nishimura et al., 1978; Saito et al., 1977) and genetic experiments to determine the sequence and location of genes (Peoples et al., 1987) have focused on the enzyme β-ketothiolase, which mediates the first step in the biosynthesis of PHB, the condensation of two acetyl CoA units to form acetoacetyl CoA. A recombinant DNA clone designated LDBK1 expresses the complete thiolase gene sequence in *Escherichia coli* (Peoples et al., 1987). The encoded protein has thiolase enzyme activity and co-migrates with native enzyme in Western blotting and immunodetection experiments. The expression of thiolase-coding sequences is under transcriptional control of the *lac* promoter region contained in the ;glgtll expression vector that was used to generate the recombinant library of *Z. ramigera* DNA. The structural gene for the thiolase enzyme is 1,173 nucleotides long and codes for a protein of 391 amino acids. The calculated molecular weight and the predicted amino acid sequence of the gene product are in agreement with the molecular weight and amino acid sequence determined experimentally for the native thiolase. The high GC content of the complete sequence (66.2 mol%) is in agreement with values obtained for the *Z. ramigera* genome (64.5 mol%). The position of the active site peptide, a potential ribosome binding site, and start codons within the DNA sequence have all been identified.

The major DNA-dependent deoxyribonucleic acid polymerase from *Z. ramigera* isolate I-16-M has been partially purified and biochemically characterized (Pickrum, 1975). The polymerase activity was eluted as a single peak at 0.1 M potassium phosphate from a DEAE-cellulose column. The partially purified enzyme showed one major, one intermediate, and five minor protein bands after polyacrylamide gel electro-

phoresis. The DNA polymerase activity was detected in the acrylamide gels by an in situ assay and coincided with the major protein band. Nuclease activity could not be detected in the preparation after electrophoresis.

The DNA polymerase resembles the *Micrococcus luteus* enzyme in many characteristics, and its properties seem to place it as an intermediate between the polymerase I and polymerase II of *Escherichia coli* and *Bacillus subtilus* (Pickrum, 1975). It appears that the enzyme requires all four deoxynucleoside-5′-triphosphates, a divalent cation, 2-mercaptoethanol, and denatured DNA as a template-primer for maximal activity. Dithiothreitol is capable of replacing 2-mercaptoethanol in the reaction. The enzyme replicated, with varying efficiencies, the DNAs isolated from *Bacillus subtilus*, *Escherichia coli*, calf thymus, salmon, and soft fish roe. Treatment of DNA with pancreatic deoxyribonuclease improved its priming ability, the effectiveness of which depended on the extent of treatment with deoxyribonuclease.

The divalent metal ion requirement of the polymerase could be satisfied with either Mg^{2+} or Mn^{2+}. A slight but observable synthesis was obtained in the presence of 20 mM Co^{2+} or 10 mM Cd^{2+}. The enzyme was unstable in solutions of ionic strength below 0.2 M potassium phosphate or protein concentrations below 1 mg/ml. Enzyme solutions could be stabilized by the addition of glycerol or by increasing the salt or protein concentrations and storing at –20°C.

A synthesis-dependent exonuclease activity, interpreted as a 5′ to 3′ exonuclease, was found, at a low level, with the most purified DNA polymerase preparation; however, this activity was not characterized. Rates were stimulated in the presence of deoxynucleoside triphosphates. The most purified fraction contained no detectable endonuclease activity.

The polymerase enzyme is sensitive to the sulfhydryl-blocking reagents N-ethyl-maleimide, *para*-(chloro)mercuribenzoate, and *para*-(chloro) mercuriphenylsulfonic acid. Of these, *para*-(chloro) mercuribenzoate is the most effective sulfhydryl reagent.

Applications

Z. ramigera is important in inducing flocculation (Unz, 1974) and reducing biological oxygen demand (BOD) in sewage-waste water treatment (Butterfield, 1935; Joyce and Dugan, 1970). The metal-adsorbing characteristic of *Z. ramigera* has been investigated with application to the treatment of heavy metals and transuranic waste streams (Dugan, 1970, 1987; Dugan and Pickrum, 1973; Friedman and Dugan, 1986a; Kuhn and Pfister, 1989; Norberg and Persson, 1984). Production of PHB or other polyhydroxyalkanoates by *Zoogloea* may be useful for the development of copolymers for commercially useful biodegradable plastics.

Waste Water Treatment

An early and continued use of *Zoogloea* in a treatment process has been in the area of domestic waste water treatment. Although zoogloeal bacteria are not the only microorganisms responsible for a successful waste water treatment process, they are important for the degradation of organic carbon and for the flocculation required for settling. *Zoogloea* bacteria are highly active oxidizers of organic compounds which significantly reduce the biological oxygen demand (BOD) in waste water. Butterfield et al. (1937) reported that 50% of the BOD from sewage could be removed in 5 hours by the aerobic, Gram-negative, floc-forming bacteria indigenous to aerobic waste water system, and 68% could be removed in 3 hr by a natural mixed population of bacteria. *Zoogloea ramigera* converts excess carbon to PHB, extracellular polysaccharide, and esterified organic acids (Joyce and Dugan, 1970). It is the interaction of extracellular microbial polymer by adsorption and bridging that aggregates microorganisms and produces flocs (Busch and Stumm, 1968).

Poly-β-hydroxybutyrate Production

A potential use of *Zoogloea* involves the production of PHB, a highly polymerized lipid reserve material that is usually synthesized by the bacteria in response to nitrogen limitation in the presence of excess carbon source. The production, enzymology, and genetic control of PHB metabolism in *Zoogloea* is of interest because of the application of PHB and other polyhydroxyalkanoates in the biodegradable plastics industry. PHB produced by some *Alcaligenes* species is currently being marketed as a biodegradable plastic under the trade name Biopol (Holmes, 1985).

Biosorption of Metals

A developing technology is the use of *Zoogloea* to adsorb and concentrate metals and transuranic elements from contaminated waste streams. The significance of extracellular microbial polymers as agents for relatively nonspecific adsorption of dissolved or suspended organic, particulate, and ionic substances has been established with polysaccharides produced by Gram-negative floc-forming bacteria (Dugan, 1970;

Dugan et al., 1971; Friedman and Dugan, 1968a). The adsorptive characteristics of the whole cell or purified polymer can be exploited for commercial waste water treatment to remove heavy metal and transuranic ions and also may be useful in mineral separation processes, where the use of other flocculating agents of biological origin are currently being investigated.

Zoogloea biomass can remove a variety of metallic cations from solution (Dugan and Pickrum, 1973; Friedman and Dugan, 1968a; Norberg and Persson, 1984; Norberg and Rydin, 1984; Sag and Kutsai, 1989a, 1989b). The removal of aluminum, calcium, cobalt, iron, magnesium, manganese, nickel, and silicon from acidic mine water samples demonstrates the effective metal binding characteristic of *Zooglea* and *Zoogloea*-derived polymer that is applicable to the treatment of complex, relatively undefined aqueous metal containing systems (Dugan, 1987; Dugan and Pickrum, 1973). Presumably, microorganisms are partially responsible for the accumulation of metals within the solids generated by sewage treatment and the reduction in metal concentration in effluent supernatant as compared to the metal concentration of the influent and the supernatant from primary settling (Dugan et al., 1971).

Chemically, the purified polysaccharide isolated from *Z. ramigera* 115 interacts with monovalent cations to form soluble salts, binds but does not appear to form cross-linkages with divalent cations, and forms insoluble precipitates with trivalent cations (Ikeda et al., 1982; Stauffer et al., 1980). Successive removal of uranium, copper, and cadmium from solution by *Zoogloea* biomass indicates selective binding among cations in mixed solutions (Norberg and Persson, 1984).

With the potential use of *Zoogloea* for the treatment of metal-bearing waste waters demonstrated, a microbially based technology for the treatment of these wastes appears to be developing. *Z. ramigera* 115 cells, immobilized in calcium alginate beads, have been used in air-bubbled column reactors to remove mixtures of cadmium, copper, lead, manganese, strontium, and zinc from aqueous solutions (Kuhn and Pfister, 1989,1990). Although calcium alginate alone sorbed metals, the use of immobilized *Z. ramigera* 115 enhanced metal binding significantly. A batch method was used to study the removal of cadmium, copper, and uranium from solution using biomass of *Z. ramigera* 115 (Norberg and Persson, 1984). A bench-scale continuous process was demonstrated with *Zoogloea* biomass to adsorb copper from solution (Norberg and Rydin, 1984). Affecting adsorption was the concentration of biomass and copper in solution and the operating pH.

Important to the costs of a treatment process is the ability to recycle biomass for multiple treatment cycles. Acid treatment re-leases cadmium, copper, and uranium adsorbed to *Zoogloea* biomass, thus enabling multiple cycling for the complete removal of metals from solution (Norberg and Persson, 1984). In experiments with cadmium it was shown that the sorbed cadmium could be eluted by exposing the beads to nutrient solutions whereupon more cadmium could be sorbed (Kuhn and Pfister, 1989, 1990). Alternatively, the release of cadmium from calcium alginate immobilized *Z. ramigera* 115 was achieved by use of a 0.04% aqueous nitrilotriacetate solution (Kuhn and Pfister, 1989, 1990).

Literature Cited

Amin, P. M., S. V. Ganapati. 1967. Occurrence of *Zoogloea* colonies and protozoans at different stages of sewage purification. Appl. Microbiol. 15:17–21.

Busch, P. L., W. Stumm. 1968. Chemical interactions in the aggregation of bacteria bioflocculation in waste treatment. Environ. Sci. Technol. 2:49–53.

Butterfield, C. T. 1935. Studies of sewage purification. II. A zoogloea-forming organism found in activated sludge. U.S. Pub. Health Rep. 50:671–684.

Butterfield, C. T., C. C. Ruchhoft, P. D. McNamee. 1937. Studies of sewage purification. VI. Biochemical oxidation by sludges developed by pure cultures of bacteria isolated from activated sludge. U.S. Pub. Health Rep. 52:387–391.

Butterfield, C. T., E. Wattie. 1941. Studies of sewage purification. XV. Effective bacteria in purification by trickling filters. U.S. Pub. Health Rep. 56:2445–2464.

Chorpenning, F. W., D. H. Schmidt, H. B. Stamper, P. R. Dugan. 1978. Antigenic relationships among floc-forming Pseudomonadaceae. Ohio J. of Science 78:29–33.

Crabtree, K., E. McCoy. 1967. *Zoogloea ramigera* Itsigsohn, identification and description. Request for an opinion as to the status of the generic name Zoogloea. International Journal of Systematic Bacteriology 17:1–10.

Crabtree, K., E. McCoy, W. C. Boyle, G. A. Rohlich. 1965. Isolation, identification and metabolic role of the sudanophilic granules of *Zoogloea ramigera*. Appl. Microbiol. 13:218–226.

Davis, J. T., H. H. Chen, R. Moore, Y. Nishitani, S. Masamune, A. J. Sinskey, C. T. Walsh. 1987b. Biosynthetic thiolase from *Zoogloea ramigera*. II. Inactivation with halacetyl CoA analogs. J. Biol. Chem. 262:90–96.

Davis, J. T., R. N. Moore, B. Imperiali, A. J. Pratt, K. Kobayashi, S. Masamune, A. J. Sinskey, C. T. Walsh, T. Fukui, K. Tomita. 1987a. Biosynthetic thiolase from *Zoogloea ramigera*. I. Preliminary characterization and analysis of proton transfer reaction. J. Biol. Chem. 262:82–89.

Deinema, M. H., L. F. T. M. Zevenhuizen. 1971. Formation of cellulose fibrils by Gram-negative bacteria and their role in bacterial flocculation. Arch. Microbiol. 78:42–57.

Dugan, P. R. 1970. Removal of mine water ions by microbial polymers. 279–283. Proc. 3rd Symp. Coal Mine Drainage Research. Mellon Institute. Pittsburgh, PA.

Dugan, P. R. 1975. Bioflocculation, and the accumulation of chemicals by floc-forming organisms. Cincinnati, Ohio. U.S. Environmental Protection Agency Office of Research and Development. Report EPA-600:2–75–032.

Dugan, P. R. 1987. The function of microbial polysaccharides in bioflocculation and biosorption of mineral ions. 337–350.Y. A. Attia (ed.) Flocculation in biotechnology and separation systems. Proc. Int. Symp. Floccul. Biotech. Separ. Sys. San Francisco, CA, July 28–August 1, 1986. Elsevier. New York.

Dugan, P. R., D. G. Lundgren. 1960. Isolation of the floc-forming organism *Zoogloea ramigera* and its culture in complex and synthetic media. Appl. Microbiol. 8:357–361.

Dugan, P. R., R. M. Pfister, J. I. Frea. 1971. Implications of microbial polymer synthesis in waste water treatment and lake eutrophication. Proceedings of the 5th International Water Pollution Research Conference III. Pergamon Press. New York, NY. 20/1–20/10.

Dugan, P. R., H. M. Pickrum. 1973. Removal of mineral ions from water by microbially produced polymers. 1019–1038. Proc. 27th Ann. Purdue Ind. Waste Conf., West Lafayette, Indiana. Engineering Bulletin of Purdue University.

Easson, D. D., Jr., O. P. Peoples, C. K. Rha, A. J. Sinskey. 1987a. Engineering of biopolymer flocculants: A recombinant DNA approach. 369–381.Y. Attia (ed.) Flocculation in biotechnology and separation systems. Elsevier Science Publishers Amsterdam.

Easson, D. D., Jr., A. J. Sinskey, O. P. Peoples. 1987b. Isolation of *Zoogloea ramigera* I-16-M exopolysaccharide biosynthetic genes and evidence for instability within this region. J. Bact. 169:4518–4524.

Farrah, S. R., R. F. Unz. 1975. Fluorescent-antibody study of natural finger-like zoogloea. Appl. Microbiol. 30:132–139.

Farrah, S. R., R. F. Unz. 1976. Isolation of exocellular polymer from *Zoogloea* strains MP6 and 106 and from activated sludge. Appl. Environ. Microbiol. 32:33–37.

Finstein, M. S. 1967. Growth and flocculation in a *Zoogloea* culture. Appl. Microbiol. 15:962–963.

Franzen, L.-E., A. B. Noberg. 1984. Structural investigation of the acidic polysaccharide secreted by *Zoogloea ramigera* 115. Carbohydrate Res. 128:111–117.

Friedman, B. A., P. R. Dugan. 1968a. Concentration and accumulation of metallic ions by the bacterium *Zoogloea*. Develop. Industrial Microbiol. 9:381–388.

Friedman, B. A., P. R. Dugan. 1968b. Identification of *Zoogloea* species and the relationship to zoogloeal matrix and floc formation. J. Bacteriol. 95:1903–1909.

Friedman, B. A., P. R. Dugan, R. M. Pfister, C. C. Remsen. 1968. Fine structure and composition of the zoogloeal matrix surrounding *Zoogloea ramigera*. J. Bacteriol. 96:2144–2153.

Friedman, B. A., P. R. Dugan, R. M. Pfister, C. C. Remsen. 1969. Structure of exocellular polymers and their relationship to bacterial flocculation. J. Bacteriol. 98:1328–1334.

Fukui, T., A. Yoshimoto, M. Matsumota, S. Hosokawa, T. Saito, H. Nishikawa, K. Tomita. 1976. Enzymatic synthesis of poly-β-hydroxybutyrate in *Zoogloea ramigera*. Arch. Microbiol. 110:149–156.

Ganapati, S. V., P. M. Amin, D. J. Parika. 1967. Studies on *Zoogloea* colonies from stored raw sewage. Water and Sewage Works 114:389–392.

Holmes, P. A. 1985. Applications of PHB—A microbially produced biodegradable thermoplastic. Phys. Technol. 16:32–36.

Ikeda, F., H. Shuto, T. Saito, T. Fukui, K. Tomita. 1982. An extracellular polysaccharide produced by *Zoogloea ramigera* 115. Eur. J. Biochem. 123:437–445.

Joyce, G. H., P. R. Dugan. 1970. The role of floc-forming bacteria in BOD removal from waste water. Develop. Indus. Microbiol. 11:377–386.

Joyce, G., P. R. Dugan. 1972. Ester synthesis by *Zoogloea* ramigera 115 grown in the presence of ethanol. Appl. Microbiol. 23:547–552.

Kuhn, S, R. M. Pfister. 1989. Adsorption of mixed metals and cadmium by calcium-alginate immobilized *Zoogloea ramigera*. Applied Microbiology and Biotechnology 31:619–625.

Kuhn, S., R. M. Pfister. 1990. Accumulation of cadmium by immobilized *Zoogloea ramigera*. Jour. of Industrial Microbiol. 115:31.

McKinney, R. E., M. P. Horwood. 1952. Fundamental approach to the activated sludge process. Sewage and Industrial Wastes 24:117–560.

McKinney, R. E., R. G. Weichlein. 1953. Isolation of floc-producing bacteria from activated sludge. Appl. Microbiol. 1:259–261.

Nishimura, T., T. Saito, K. Tomita. 1978. Purification and properties of β-ketothiolase from *Zoogloea ramigera*. Arch. Microbiol. 116:21–27.

Norberg, A. B., S.-V. Enfors. 1982. Production of extracellular polysaccharide by *Zoogloea ramigera*. Appl. Environ. Microbiol. 44:1231–1237.

Norberg, A. B., H. Persson. 1984. Accumulation of heavy-metal ions by *Zoogloea ramigera*. Biotech. Bioeng. 26:239–246.

Norberg, A., S. Rydin. 1984. Development of a continuous process for metal accumulation by *Zoogloea ramigera*. Biotech. Bioeng. 26:265–268.

Parsons, A., P. R. Dugan. 1971. Production of extracellular polysaccharide matrix by *Zoogloea ramigera*. Appl. Microbiol. 21:657–661.

Peoples, O. P., S. Masamune, C. T. Walsh, A. J. Sinskey. 1987. Biosynthetic thiolase from *Zoogloea ramigera* III. Isolation and characterization of the structural gene. J. Biol. Chem. 262:97–102.

Peter, G., K. Wuhrmann. 1971. Contribution to the problem of bioflocculation in the activated sludge process. Proc. 5th Int. Water Poll. Res. Conf. II:1/1–1/9.

Pickrum, H. M. 1972. Removal of heavy metal ions from water by floc-forming microorganisms. M.S. Thesis. Ohio State University. Columbus, Ohio.

Pickrum, H. M. 1975. Isolation, purification and characterization studies in a deoxyribonucleic acid polymerase from the floc-forming bacterium *Zoogloea ramigera* isolate I-16-M. Ph.D. Dissertation. Ohio State University. Columbus, Ohio.

Sag, Y., T. Kutsai. 1989a. Application of adsorption isotherms to chromium adsorption on Z. ramigera. Biotechnology Lett. 11:141–144.

Sag, Y., T. Kutsai. 1989b. The use of Zoogloea ramigera in waste water treatment containing Cr(VI) and Cd(II) ions. Biotechnology Lett. 11:145–148.

Saito, T., T. Fukui, F. Ikeda, Y. Tanaka, K. Tomita. 1977. An NADP-linked acetacetyl CoA reductase from *Zoogloea ramigera*. Arch. Microbiol. 114:211–217.

Schildkraut, C. L., J. Marmur, P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid form

its buoyant density in CsCl. Journal of Molecular Biology 4:430–443.

Sinskey, A., S. Jamas, D. Easson Jr., C. Rha. 1986. Biopolymers and modified polysachharides. 73–114. S. K. Harlander and T. P. Labuza (ed.) Biotechnology in food processing Noyes Publication. New Jersey.

Stauffer, K. R., J. G. Leeder, S. S. Wang. 1980. Characterization of zooglan 115, an exocellular glycan of *Zoogloea ramigera*-115. J. Food Science 45:946–952.

Tago, Y., K. Aida. 1977. Exocellular mucopolysaccharide closely related to bacterial floc-formation. Appl. and Environ. Microbiol. 34:308–314.

Tezuka, Y. 1973. A *Zoogloea* bacterium with gelatinous mucopolysaccharide matrix. J. Water Pollut. Control Fed. 45:531–536.

Tomita, K., T. Saito, T. Fukui. 1983. Bacterial metabolism of poly-β-hydroxybutyrate. 353–366. D. L. F. Lennon, F. W. Stratman, and R. N. Zahlten (ed.) Biochemistry of metabolic processes. Elsevier. New York.

Unz, R. F. 1974. Bacterial *Zoogloea* formation. Cincinnati, OH. U.S. Environmental Protection Agency. Report EPA-670:2–74–018.

Unz, R. F. 1984. Genus IV. *Zoogloea* Itzigoshn 1868,30. 214–219. N. R. Krieg and J. G. Holt (ed.) Bergey's manual of determinative bacteriology. Williams and Wilkins. Baltimore.

Unz, R. F., N. C. Dondero. 1967a. The predominant bacteria in natural zoogloeal colonies. I. Isolation and identification. Can. J. Microbiol. 13:1671–1682.

Unz, R. F., N. C. Dondero. 1967b. The predominant bacteria in natural zoogloeal colonies. II. Physiology and nutrition. Can. J. Microbiol. 13:1683–1691.

Unz, R. F., S. R. Farrah. 1972. Use of aromatic compounds for growth and isolation of *Zoogloea*. Appl. Microbiol. 23:524–530.

Unz, R. F., S. R. Farrah. 1976a. Observations on the formation of wastewater zoogloeae. Water Res. 10:665–671.

Unz, R. F., S. R. Farrah. 1976b. Exopolymer production and flocculation by *Zoogloea* MP6. Appl. Environ. Microbiol. 31:623–626.

Wallen, L. L., E. N. Davis. 1972. Biopolymers of activated sludge. Environ. Sci. Technol. 6:161–164.

Wattie, E. 1943. Cultural characteristics of zoogloeal-forming bacteria isolated from activated sludge and trickling filters. Sewage Works Journal 15:476–479.

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CHAPTER 14.4

Large Symbiotic Spirochetes: *Clevelandina*, *Cristispira*, *Diplocalyx*, *Hollandina* and *Pillotina*

LYNN MARGULIS AND GREGORY HINKLE

The genera *Cristispira*, *Clevelandina*, *Diplocalyx*, *Hollandina,* and *Pillotina* are morphologically complex, Gram-negative, motile, spirochetes (helical bacteria) in which the flagella are always entirely periplasmic (i.e., located between the inner "plasma" and the outer membrane typical of Gram-negative bacteria) (Fig. 1). (For a general discussion of the morphology of spirochetes, see Free-Living Saccharolytic Spirochetes: The Genus *Spirochaeta* in this Volume). All are obligate symbionts in the digestive system of mollusks or arthropods. These morphologically complex spirochetes have greater than 10 and sometimes as many as 300 flagella inserted at both ends of the cell and overlapping in the middle. If n is the number of flagella at one end of the cell and 2n the number of overlapping flagella in the middle of the cell, then the characteristic array is n:2n:n (e.g., 10:20:10 or 300:600:300). The coated membranes, distinctive cytoplasmic structures (including the sillon, a cell-length invagination or groove of the outer membrane in contact with the inner membrane), and relative proportions that distinguish these genera are depicted in Fig. 2, based on the morphometric analyses summarized in Table 1.

The habitats of these organisms are predictable (e.g., the crystalline style of bivalve mollusks for *Cristispira* and the intestine of dry wood-eating cockroaches and termites for the others). None has been grown axenically. As molecular biological data are not yet available, species have been determined morphologically. Five species of large, symbiotic spirochetes have been described in the modern bacteriological literature and reverified, revised, or named as: *Clevelandina reticulitermitidis*, *Cristispira pectinis*, *Diplocalyx calotermitidis*, *Hollandina pterotermitidis,* and *Pillotina calotermitidis*. (For genera description, including an explanation of the morphometric analysis of spirochetes, see Bermudes et al., 1988.)

Although often classified on the basis of size and light microscopic morphology in the family Spirochaetaceae (e.g., Bermudes et al., 1988), these spirochetes are ultrastructurally distinct from all other members of the Spirochaetaceae (see Free-Living Saccharolytic Spirochetes: The Genus *Spirochaeta* in this Volume). Therefore, we classify the large symbiotic spirochetes in the family Pillotinaceae. (This family was first suggested by Hollande and Gharagozlou, 1967, who used the incorrect Latin derivative "Pillotaceae.") The spirochetes most similar morphologically to any member of the Pillotinaceae are the tick-borne symbionts, e.g., *Borrelia persica*, (flagella formula 25:50:25) (Karimi et al., 1979); the free-living microbial mat spirochete *Mobilifilum chasei* (10:20:10) (Margulis et al., 1990a); and the pectinolytic rumen spirochete *Treponema saccharophilum* strain PB, which is reported to have approximately 32 flagella (whether 32:64:32 or 16:32:16 is not clear) (Paster and Canale-Parola, 1985). All these spirochetes are significantly smaller than any member of the Pillotinaceae. No free-living *Pillotina*-like spirochete has ever been reported—none, at least, larger than 0.5 μm in diameter bearing at least 30 flagella.

A summary of the characteristics of the large, symbiotic spirochetes is presented in Table 1. Although only five genera are in the formal taxonomic literature, hundreds of symbiotic spirochetes from a large number of animals have been reported. For a more extensive discourse on *Cristispira* species in a variety of mollusks, see Kuhn (1981) and Breznak (1984a). Large spirochetes in the hindguts of termites and wood-eating cockroaches are more thoroughly described by To et al. (1980).

Cristispira pectinis (Gross) was first described as the trypanosome *Trypanosoma balbiani* (a eukaryote) by Certes (1882). Thought to have a multicellular, chambered body, *Cristispira* was renamed by Gross (1910), the name *Cristispira* being derived from the unusually prominent flagellar bundle or "crest." The modern understanding of the organism comes from Noguchi (1921), who, detecting them in oysters (*Crassostrea*), clams (*Venus*), and mussels (*Modiola*), recognized *Cristispira* as a spirochete bacterium. This identification was confirmed when the crest

This chapter was taken unchanged from the second edition.

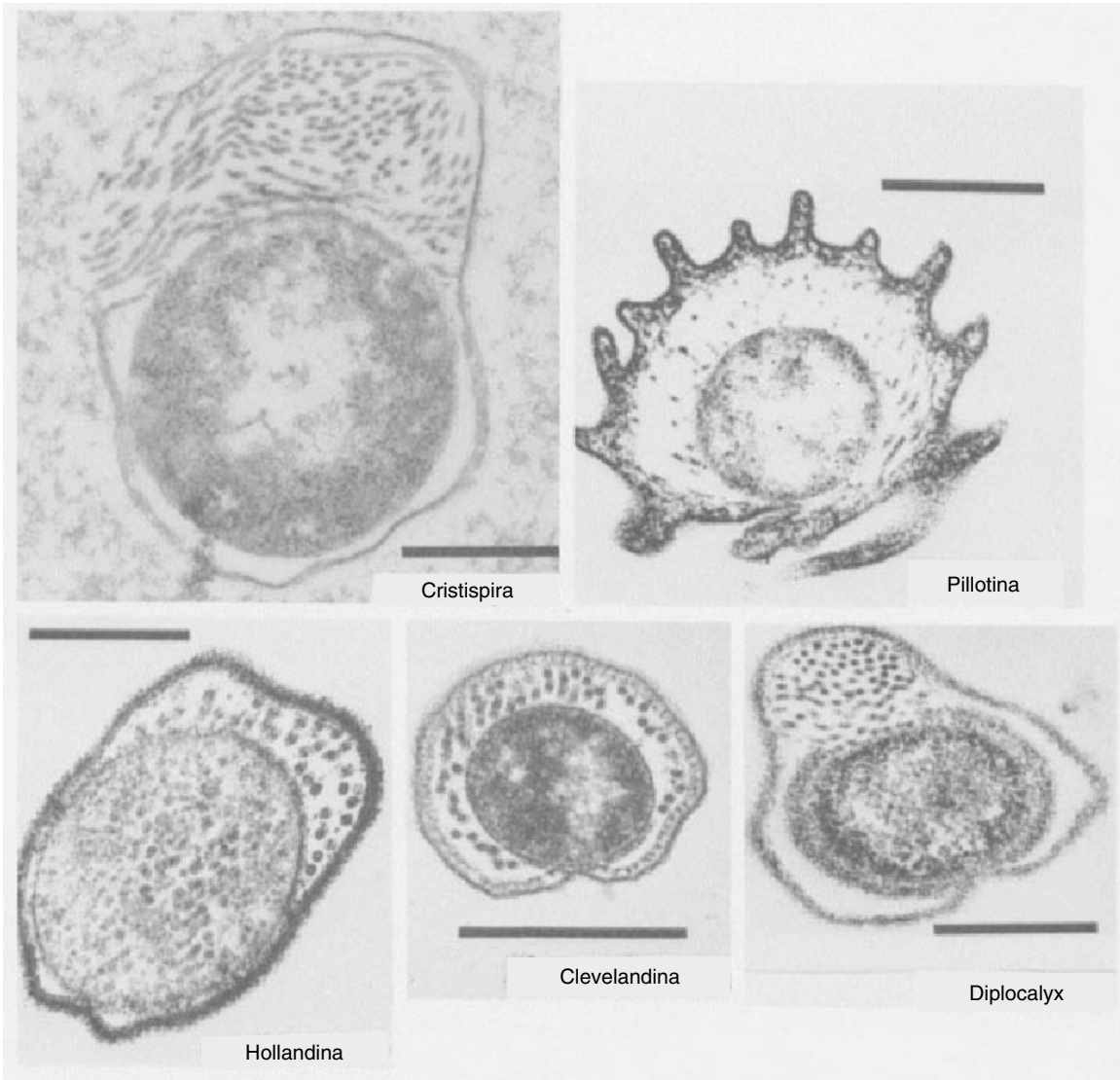
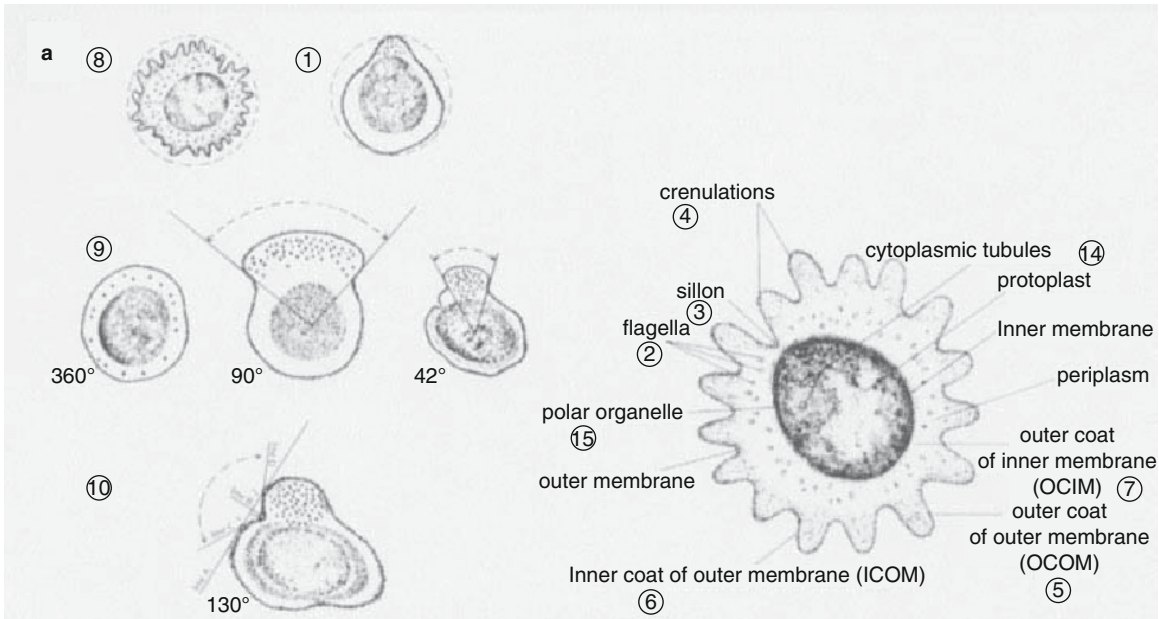
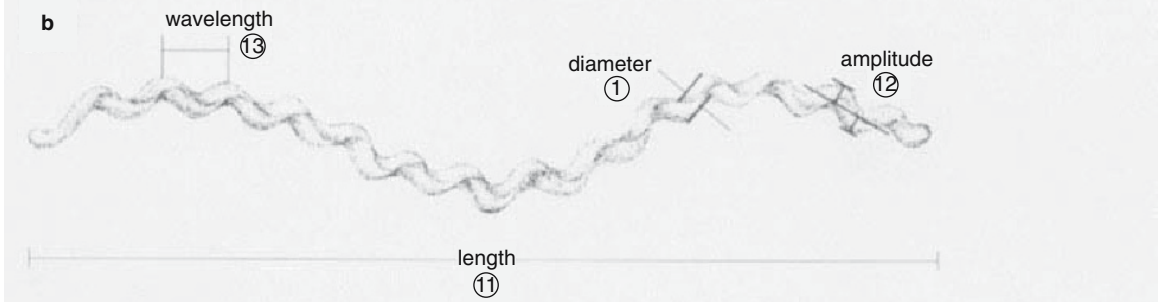


Fig. 1. Large symbiotic spirochetes as shown in transverse electron microscopic sections: *Cristispira* from the East Coast oyster. (Courtesy of J. M. Sieburth.) *Pillotina* from *Reticulitermes flavipes.* (Courtesy of R. Bloodgood.) *Hollandina* from the Sonoran desert termite. (Courtesy of L. P. To.) *Clevelandina* from *Zootermopsis.* (Courtesy of J. Breznak and H. Pankratz.) *Diplocalyx* from the Madeiran termite. Numerous flagella are seen in cross-section in the region between the outer and the plasma membrane. (Bars = 0.5 μm.)

was shown to be a flagellar bundle by Ryter and Pillot (1965).

Leidy (1850) first described spiral-shaped microbes from termite intestines and called them *Spirillum undula*, later renaming them *Vibrio termites* (Leidy, 1881). Dobell (1912) recognized these organisms as spirochetes; he called the larger ones *Treponema termites* and the smaller ones *Treponema minor*. The larger spirochetes were transferred to the genus *Cristipira* by Hollande (1922). They were further described by Damon (1925), Duboscq and Grassé (1929), and Kirby (1941). In an early electron microscopic study, Grimstone (1967) described a large unnamed spirochete from the wood-eating cockroach *Cryptocercus punctulatus.* Electron microscopy and the use of glutaraldehyde as a fixative reinvigorated the study of the termite hindgut microbiota by providing a means of distinguishing morphologically the uncultivable symbiotic bacteria. After the genus *Pillotina* and the family Pillotinaceae were proposed by Hollande and Gharagozlou (1967) for large spirochetes from the Madeiran termite *Incisitermes praecox,* large insect gut spirochetes were informally called "pillotinas." The discovery of a separate spirochete morphotype from the termite *Incisitermes flavicollis* led Gharagozlou (1968) to establish the genus *Diplocalyx*. To et al. (1978) described a third genus, *Hollandina,* from the hindgut of the Sonoran desert termite *Pterotermes occidentis.*





Encircled number	Morphometric criterion
1	Diameter
2	Number of flagella
3	Presence of sillon
4	Presence of crenulations
5	Ratio of thickness of outer coat of outer membrane to outer membrane thickness
6	Ratio of thickness of inner coat of outer membrane to outer membrane thickness
7	Ratio of the thickness of outer coat of inner membrane to inner membrane thickness
8	Ratio of diameter of protoplasmic cylinder to cell diameter
9	Angle of protoplasmic cylinder subtended by flagella
10	Presence of flagellar bundle (angle of concavity CD < 180°)
11	Length
12	Amplitude
13	Wavelength
14	Presence of cytoplasmic tubules[a]
15	Presence of polar organelle
16	Presence of rosettes[b]

[a]Cytoplasmic tubules range from 15 to 25 nm in diameter.
[b]The rosettes are not shown in Fig. 2.
Adapted from Bermudes et al. (1988).

Fig. 2. Spirochete structures used in morphometric analyses. (a) Structures discernible by electron microscopy. (b) Structures discernible by light microscopy and a list of the 16 morphometric criteria. Encircled numbers refer to the criteria given in the table in part 2b. (Drawings by C. Lyons.)

Table 1. Criteria used to identify the spirochete genera.

Criterion	*Cristispira*	*Pillotina*	*Hollandina*	*Diplocalyx*	*Clevelandina*
Primary					
Diameter (μm)	0.5–3.0	0.6–1.5	0.4–1.0	0.7–0.9	0.4–0.8
Approximate number of flagella	≥100	30–70	30–60	40–60	30–45
Presence of sillon	–	+	+, –	+	+
Presence of crenulations	–	+	–	–	–
OCOM/OM[a]	ND	0	2.4–8.0	0.6–2.0	0–2.8
ICOM/OM[b]	ND	3.3–11.9	0–1.3	0.9–2.1	2.8–5.0 (chambered)
OCIM/IM[c]	ND	0	1.0–2.5	6.4–7.7	0–3.6
PC/diameter[d]	0.90	0.56–0.67	0.63–0.90	0.47–0.81	0.60–0.81
Angle subtended by flagella (°)	90–160	190–350	105–330	50–100	140–330
Presence of flagellar bundles	+	–	+, –	+	+, –
Auxiliary					
Length (μm)	30–180	ND	ND	ND	ND
Amplitude (μm)	4–6	ND	ND	ND	ND
Wavelength (μm)	10–20	ND	ND	ND	ND
Presence of cytoplasmic tubules	NDT	+	+	+	NDT
Presence of polar organelle	+	+	+	+	NDT
Presence of rosettes	+	–	–	–	–

Symbols: +, present; –, absent; ND, no data; NDT, not detected.
[a]Ratio of thickness of outer coat of outer membrane to outer membrane thickness.
[b]Ratio of thickness of inner coat of outer membrane to outer membrane thickness.
[c]Ratio of thickness of outer coat of inner membrane to inner membrane thickness.
[d]Ratio of diameter of protoplasmic cylinder to cell diameter.
Adapted from Bermudes et al. (1988).

Known informally from the micrographs of D. G. Chase, *Clevelandina* was fixed in the microbiological literature by Bermudes et al. (1988).

Habitat

Cristispira develops especially dense populations in and on the crystalline style of many marine (and a few freshwater) mollusks. Styles are noncellular, gelatinous, cellulase-containing rods that extend into the stomach of many bivalve mollusks. Their presence, appearance, and consistency differ from species to species and even from individual to individual. In actively feeding animals, the style is rotated by epithelial cilia and, much like a pestle, one end is pushed against the stomach wall. The rotation likely aids in the mixing of food particles and digestive enzymes within the mollusk stomach. Some styles dissolve and reform with a tidal rhythm; when the style dissolves, the *Cristispira* disappear. Other smaller bacteria, including *Spirillum ostrea* (Margulis et al., 1991), an unidentified Gram-negative rod, and a smaller spirochete, have also been found in mollusk styles (Kuhn, 1981). Though they do not have a style, gastropods (single-valve mollusks) have been reported to harbor *Cristispira* as well (Morton, 1952; Orton, 1922; Terasaki, 1960). A partial list of the distribution of *Cristispira* in marine mollusks is given in Table 2.

The four other genera of large spirochetes are insect symbionts; they swim freely in the

Table 2. Genera of mollusks harboring *Cristispira.* From Kuhn (1981).

Marine bivalve genera	
Cardium papillosum	*Penitella ovoidea*
Chama gryphoides	*Pinna nobilis*
Chama pellucida	*Pinna squamosa*
Chama sinistrorsa	*Platyodon cancellatus*
Chione fluctifrage	*Protothaca staminea*
Chione succincta	*Protothaca tenerrima*
Clinocardium nuttallii	*Saxicava arctica*
Crassostrea gigas	*Saxidomus giganteus*
Cryptomya californica	*Saxidomus nuttalli*
Cuminga californica	*Scrobicularia piperata*
Diplodonta orbella	*Siliqua patula*
Entodesma saxicola	*Solen ensis*
Gastrochaena dubia	*Soletellina acuminata*
Gryphaea angulata	*Sphaerium corneum*
Lampsilis anodontoides	*Strophitus* sp.
Lima hians	*Tapes aureus*
Lima inflata	*Tapes decussatus*
Lyonsia pugetensis	*Tapes laeta*
Macoma secta	*Tapes philippinarum*
Mactra sulcataria	*Tapes pullastra*
Modiola barbata	*Taras orbella*
Modiola modiolus	*Tivela stultorum*
Mytilus edulis	*Tresus capax*
Ostrea angulata	*Tresus (Schizothaerus) nuttallii*
Ostrea edulis	*Venerupis japonica*
Ostrea lurida	*Venreupis philippinarum*
Ostrea talienwhaneensis	*Ventricolaria* sp.
Ostrea virginiana	*Venus casta*
Panope generosa	*Venus mercenaria*
Paphia staminea	*Venus verrucosa*
Pecten jacobaeus	*Zirfaea pilsbryi*

From Kuhn (1981).

Table 3. Geographic distribution of insect-spirochete symbioses.

Insect family	Insect genus	Location	Spirochete genus
Order Blattaria			
Cryptocercidae (Protoblattidae; woodeating cockroaches)	*Cryptocercus punctulatus*	United States	*Hollandina*
Order Isoptera			
Hodotermitidae (damp-wood termites)	*Porotermes adamsoni*	Australia	—[a]
Kalotermitidae (dry-wood termites)	*Bifiditermes condonensis*	Australia	—
	Ceratokalotermes apoliator	Australia	—
	Cryptotermes brevis	United States	—
	Cryptotermes cavifrons	United States	—
	Cryptotermes gearyi	Australia	—
	Glyptotermes iridipennis (Kalotermes iridipennis)	Australia	—
	Glyptotermes neotuberculatus	Australia	—
	Kalotermes approximatus[b]	United States	—
	Kalotermes banksiae	Australia	—
	Calotermes flavicollis[b]	France, Spain	*Diplocalyx*
	Kaoltermes jouteli (Neotermes jouteli)	United States	—
	Kalotermes minor (Incisitermes minor)	United States	*Hollandina*
	Kalotermes schwarzi (Incisitermes schwarzi)	United States	*Hollandina* *Pillotina*
	Kalotermes snyderi[b]	United States	*Hollandina*
	Incisitermes milleri	United States	—
	Marginitermes hubbardi (Kalotermes hubbardi)	United States	*Diplocalyx*
	Neotermes insularis	Australia	—
	Paraneotermes simplicicornis	United States	—
	Postelectrotermes praecox (Calotermes praecax)[b]	Madeira, Portugal	*Pillotina*
	Pterotermes occidentis	Mexico, United States	*Hollandina*
Mastotermitidae	*Mastotermes darwiniensis*	Australia	*Hollandina*
Rhinotermitidae (subterranean termites)	*Coptotermes aginaciformis*	Australia	—
	Coptotermes formosanus	Hawaii	*Hollandina*
	Heterotermes aureus	United States	—
	Reticulitermes flavipes	United States	*Clevelandina* *Pillotina*
	Reticulitermes hesperus	United States	*Clevelandina* *Hollandina* *Pillotina*
	Reticulitermes tibialis	United States	*Clevelandina* *Hollandina*

[a]—, There is no ultrastructural information on which identification could be based.
[b]Originally published nomenclature used (*Calotermes* is equivalent to *Kalotermes*).
Adapted from Bermudes et al. (1988).

lumen of the distal portion of the intestine, actually a hypertrophied hindgut or paunch. The paunch, which has less oxygen relative to air (Breznak, 1984b), is easily identified and dissected in any of the wood-eating cockroaches or so-called "lower" termites. The geographical distribution of these spirochetes is listed in Table 3. Their abundance is greatest in insects that attack and ingest dry rather than damp or wet wood. As a rule, Pillotinaceae spirochetes are very abundant in both kalotermitid (dry wood) and rhinotermitid (subterranean) termites. In rhinotermitids they tend to be most abundant just posterior of the midgut/anterior hindgut junction. These spirochetes are apparently absent from the damp-wood-eating nasutitermitids and other "higher" termites. In "higher" termites, a bacterial or bacterial/fungal community rather than a protistan/bacterial community has evolved. (Spirochetes 0.5 μm in diameter with approximately 40–50 flagella have been reported in the hindgut of the higher termite *Nasutitermes exitiosus*; Czolij et al., 1985.) The presence of Pillotinaceae spirochetes is easily determined by light microscopy on the basis of habitat, size, flexibility, and motility, but genera and species are not identifiable without ultrastructural analysis.

Morphology

Given ecological information and high resolution ultrastructural analysis, a single transverse thin section suffices to identify one of these large symbiotic spirochetes to genus. Ultrastructural analysis requires morphometrics (Bermudes et al., 1988). Sixteen criteria are useful when sufficient information is available (Fig. 2b). Diameters (criterion 1) in oblong cells are measured at the narrowest and widest points enclosed by the membrane. In crenulated cells, measurements are made at the tips of the crenulations. The number of flagella (criterion 2) is reported as the number inserted at each end. The number of flagella observed ranges from zero (in sections taken beyond the most distal insertion) to twice the number of flagella inserted at one end, where, toward the center, flagella inserted at one end overlap with the flagella from the other end. The sillon or groove (criterion 3) is a contact or pronounced invagination running the length of the cell of the outer membrane toward the inner membrane (Fig. 2). This structure provides a reference point for the description of other structures. Crenulations (criterion 4) are conspicuous folds or ruffles in the outer membrane (Fig. 2); to date they are limited to the genus *Pillotina*. Criteria 5, 6, and 7 deal with the presence of coatings on the inner and outer membranes of the Gram-negative cell walls (i.e., the glycocalyx or sheath). Criterion 8 is the ratio of the diameter of the protoplasmic cylinder to the cell diameter. Criterion 9, the angle subtended by the flagella, is a measurement of the distribution of the flagella in the periplasm. Flagellar bundles (criterion 10) are deemed present if the angle made by lines C and D is less than 180° (Fig. 2a). Criteria 11, 12, and 13 are the length, amplitude, and wavelength of the spirochete (Fig. 2b). Cytoplasmic tubules (criterion 14) are small, hollow structures within the protoplasmic cylinder as observed by negative stain or transmission electron microscopy (Fig. 2 and 6). They vary from 15 to 25 nm in diameter. The polar organelle (synonym, polar membrane; criterion 15) is an electron-dense lamina located on the inside of the inner (plasma) membrane toward the distal ends of the cell (Fig. 2 and 5). Rosettes (criterion 16) are linearly aligned, peripheral structures of the protoplasmic cylinder (Fig. 5); they have only been seen in *Cristispira*.

The largest spirochete in the microbiological literature, *Cristispira* is usually the only spirochete seen in mollusk styles. Healthy termites, however, may contain from one to more than five different morphotypes of pillotina spirochetes difficult to distinguish from each other with a light microscope (Fig. 3, 7, 8, and 9). Length (from 12 to 100 μm), amplitude (from 1.5 to 6.0 μm), and wavelengths (from 6 to 20 μm) can thus only be represented in approximate terms. The combination of large diameter (0.4 to greater than 1.0 μm), great length, and long wavelength distinguishes pillotinas from the many other smaller, hindgut spirochetes (Figs. 7 and 8). Idealized drawings based on electron micrographs of transverse sections of all five genera are shown in Fig. 4.

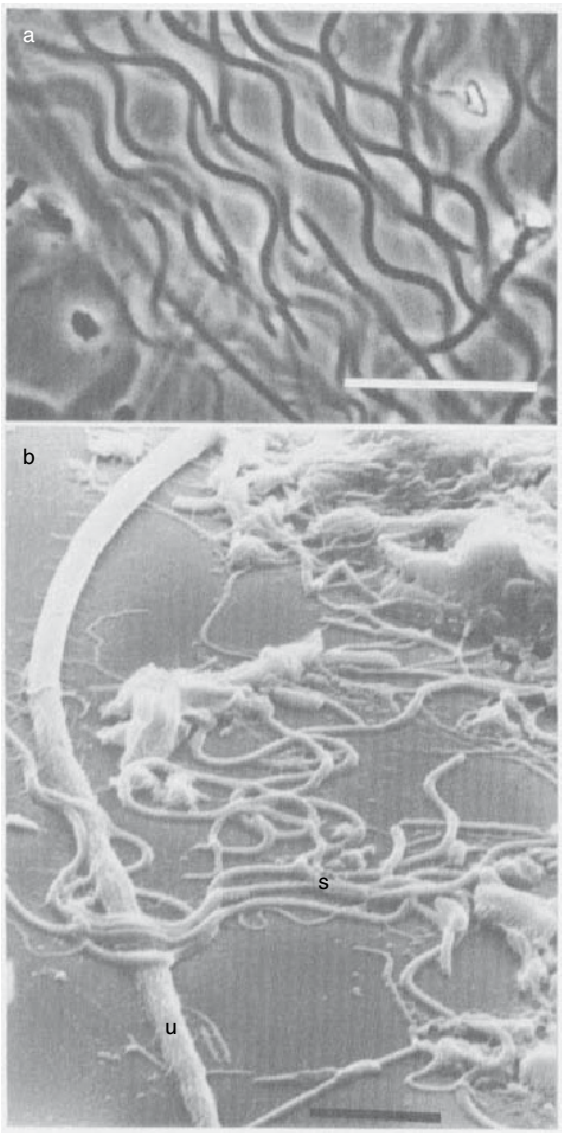


Fig. 3. (a) Live *Hollandina* spirochetes from *Pterotermes occidentis;* light micrograph. (b) *Hollandina pterotermitis* (S) and a huge, unidentified bacterium (U); scanning electron micrograph. (Bar = 5 μm.)

Cristispira

Cristispira cells can be greater than 3 μm in diameter and over 150 μm in length. The enormous number of periplasmic flagella (>100) gives *Cris-*

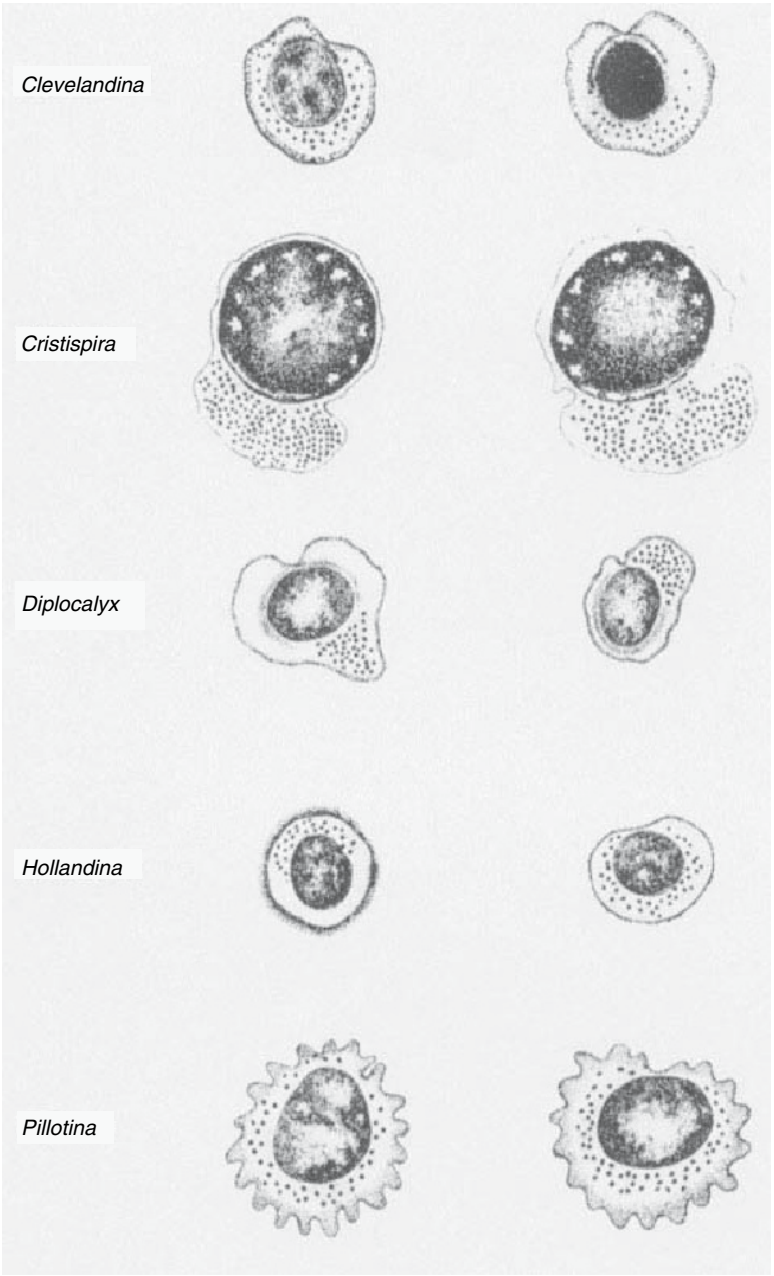


Fig. 4. Idealized drawings of transverse electron microscopic sections showing the range of morphotypes in the five genera of large, symbiotic spirochetes. Not drawn to scale. (Drawings by C. Lyons.)

tispira a figure-eight shape in cross-section, with the flagellar bundle often equal in size to the protoplasmic cylinder (Figs. 1 and 4). In highly motile *Cristispira*, the crest, which is merely the flagellar bundle, is difficult to discern. As the spirochetes lose vitality and speed, the crest swells and becomes more obvious. The periplasmic space in *Cristispira* tends to be minimal, but expands to much greater diameter where subtended by the periplasmic flagella.

Rosettes line the periphery of *Cristispira* along its length except where flagella insert (Fig. 5). Although the electron-luminous spheres making up the rosettes generally measure about 100 nm in diameter, the number of component spheres varies from 2 to as many as 9, such that the ultrastructure of the rosettes is not constant. "Chambers" or ovoid inclusions in light micrographs correspond to these rosettes; they have no known function.

The polar organelle, a structure first described in spirilla and subsequently found in other types of flagellated bacteria, is clearly present in *Cristispira* and lies conspicuously beneath the flagellar bundle (Figs. 2 and 5a). The proximity of the polar organelle to flagella insertions suggests a role in motility generation. Nothing is known directly about the reaction of *Cristispira* to gaseous oxygen, though if cytochrome oxidase were present at the polar organelle in *Cristispira* (as it is in

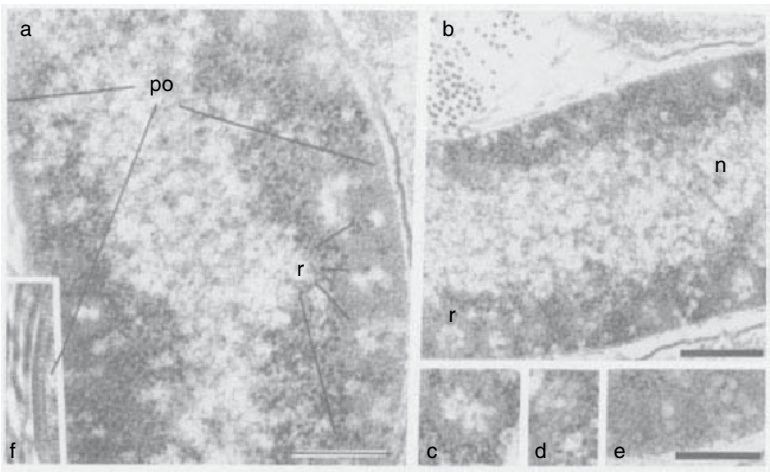


Fig. 5. *Cristispira pectinis*. (a) Relationship of rosettes (r) to polar organelles (po); four rosettes and two polar organelles in longitudinal electron microscopic sections. (Bar = 0.2 μm.) (b) Peripheral location of rosettes, one with seven-fold symmetry. (Bar = 0.2 μm.) (c to e) Rosettes with two to nine spherical components. (Bar = 0.1 μm.) (f) Detail of a polar organelle showing quadrilaminar structure. Abbreviations: n, nucleoid. See Margulis et al. (1991) for details.

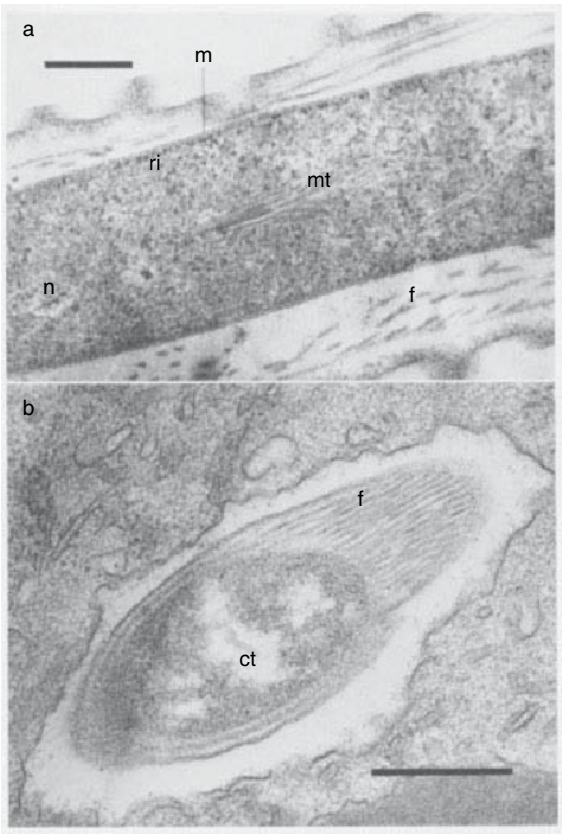


Fig. 6. (a) Cytoplasmic tubules (mt), 24 nm in diameter, in a longitudinal section of the termite *Pillotina calotermitidis* from Madeira. (Bar = 0.5 μm.) (b) Cytoplasmic tubules (ct), 21 nm in diameter, and flagella (f), in *Diplocalyx* from *Incisitermes minor* from Newbury Park, California. Abbreviations: m, inner membrane; n, nucleoid; ri, ribosomes. (Bar = 0.5 μm.) (Micrograph by D. G. Chase.)

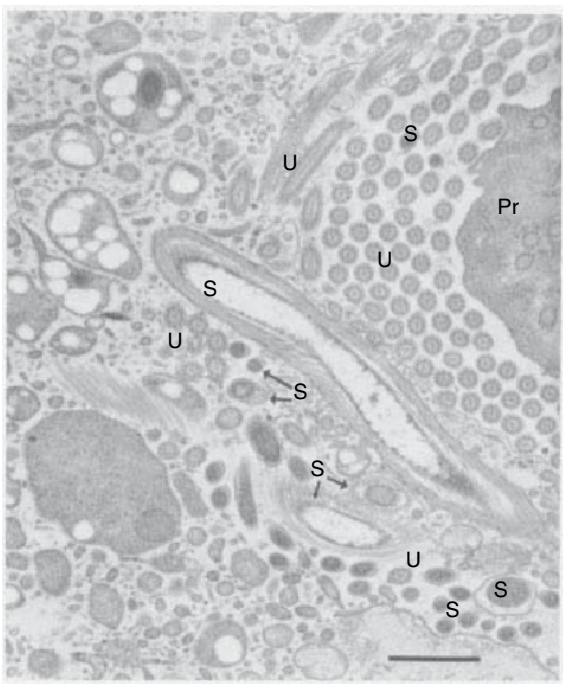


Fig. 7. Densely populated microbial community from the hindgut of the subterranean termite *Reticulitermes hesperus.* An unidentified protist (Pr), undulipodia (U), and unidentified and large spirochetes (S), including *Pillotina* and *Clevelandina,* are seen in this transmission electron micrograph. Bar = 0.5 μm.

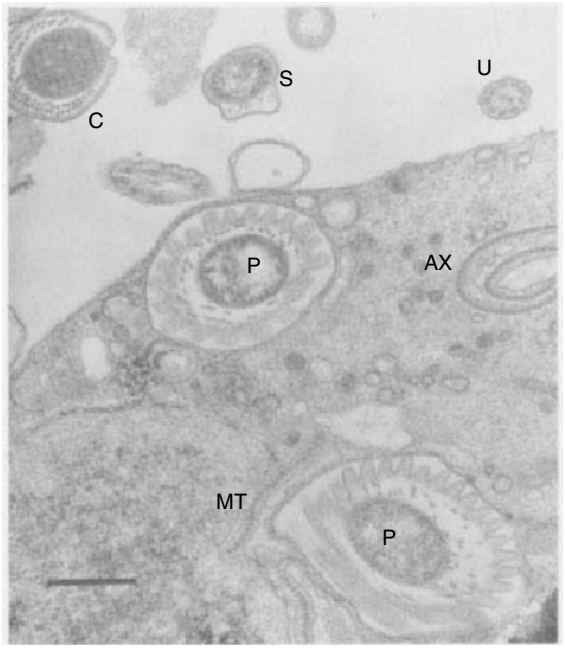


Fig. 8. Two *Pillotina* spirochetes (P) inside an unidentified hindgut protist, associated with host axostyle (AX) and microtubules (MT). Note the *Clevelandina* cell (C) and an unidentified small spirochete (S) at the upper left; U, undulipodium. (Bar = 0.5 μm.)

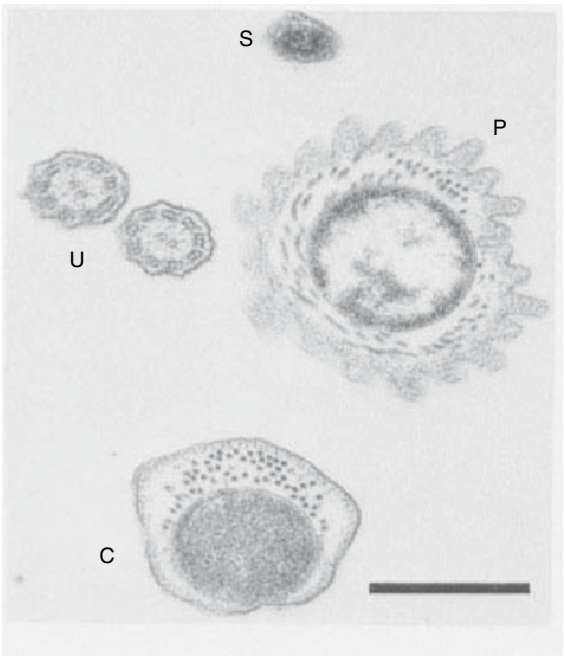


Fig. 9. *Clevelandina* (C), *Pillotina* (P), and an unidentified spirochete (S) coexisting with undulipodia (U) in the hindgut of *Reticulitermes hesperus.* Bar = 0.5 μm.

Sphaerotilus natans; Tauschel, 1985), this would suggest that the metabolism of this spirochete is aerobic or microaerophilic. The type species for the genus is *Cristispira pectinis* (Gross, 1910).

Clevelandina

Clevelandina spirochetes are symbiotic in the paunch of subterranean termites and therefore may be anaerobes. The cells are generally oblong in cross-section, are 0.4 to 0.8 μm in diameter and have 30 to 50 periplasmic flagella (Fig. 1, 4, and 9). The angle subtended by the flagella tends to be greater than 180°, and the inner coat (calyx) is correspondingly reduced. The cells are characterized by a thick inner coat (layer) of the outer membrane which tends to be chambered and an outer coat of the inner membrane which is present except where the circumference is covered by flagella. A sillon, the contact between the inner and outer membrane that extends longitudinally, is present and crenulations are lacking. The type species is *Clevelandina reticulitermitidis* from the California termite *Reticulitermes tibialis* (Bermudes et al., 1988).

Diplocalyx

The *Diplocalyx* spirochetes are symbionts in hindguts of dry wood-eating termites; they are probably either anaerobes or microaerophiles. The cells are helical and 0.7 to 0.9 μm in diameter. In *Diplocalyx* the 40 to 60 periplasmic flagella are tightly bundled between the inner and outer membranes (Figs. 1 and 4). A prominent sillon varies in position in different morphotypes relative to the flagellar bundle. A thick outer coat of the inner membrane, forming a calyx (cup), is present except under the flagellar bundle and sillon. Crenulations of the outer membrane are absent. Cytoplasmic tubules, about 21 nm in diameter (Fig. 6b), and polar organelles have been seen in some sections. The type species is *Diplocalyx calotermitidis* from the Mediterranean termite *Incisitermes flavicollis* (Gharagozlou, 1968).

Hollandina

Hollandina spirochetes, found in wood-eating cockroaches and termites, are probably anaerobes or microaerophiles. They are smooth, rounded to oblong, and helical when observed in transverse section; they are 0.4 to 1.0 μm in diameter (Figs. 1 and 4). *Hollandina* spirochetes generally have a thick coating on the outer surface of the outer membrane. Crenulations of the outer membrane are lacking. Cells have from 15 to 70 flagella dispersed in the periplasmic space. A sillon is absent or relatively inconspicuous. Polar organelles and cytoplasmic tubules have been observed in some sections. The type species

is *Hollandina pterotermitides* from the Sonoran desert termite *Pterotermes occidentis* (Bermudes et al. 1988).

Pillotina

Pillotina spirochetes are symbionts in the guts of wood-eating cockroaches and termites and are probably anaerobes or microaerophiles. They have helical cells 0.6 to 1.5 μm in diameter with prominent crenulations in the outer membrane (Figs. 1, 4, 6a, 7, 8, and 9). A stellate profile with approximately 30 to 70 flagella distributed throughout the periplasmic space is distinctive for the genus. The inner coat of the outer membrane is consistently prominent; an outer coat of the outer membrane is present in some populations. A deep and narrow crenulation forms the sillon. The type species is *Pillotina calotermitidis* from the Madeiran termite *Incisitermes praecox* (Hollande and Gharagozlou, 1967).

Cytoplasmic Tubules

Hollow tubules, called microtubules by Gharagozlou (1968; Fig. 6a) although they are somewhat smaller in diameter than the standard 25-nm microtubules of eukaryotes (Margulis et al., 1978, 1981; To, 1978), have been seen in the protoplasmic cylinders of many termite spirochete genera, including those listed in Table 1. In large spirochetes of the dry-wood-eating termites *Incisitermes schwarzi* and *Pterotermes occidentis,* the presence of a tubulin-like protein was detected by antitubulin immunofluorescence and by co-migration with authentic brain tubulin in acrylamide gel electrophoresis (Bermudes et al., 1987; Margulis et al., 1978). Tubulin proteins, which comprise eukaryotic cilia, flagella (undulipodia), and the mitotic apparatus, are ubiquitous in eukaryotes. This is the only known evidence of tubulin-like proteins in prokaryotes. An evolutionary relationship between the cytoplasmic tubules of these spirochetes and eukaryotic microtubules has been suggested (Margulis, 1981). Cytoplasmic tubules have not been reported in any *Cristispira* strains.

Distribution of Morphotypes

The geographical distribution of *Cristispira* (Table 2) and of large spirochetes from insect hindguts (Table 3) was summarized earlier. In any given termite, distinctive spirochete morphotypes are consistently and reliably found. Many undescribed spirochete morphotypes, especially smaller ones, are present in termite and cockroach hindguts, often in impressive numbers (Fig. 7). More than 25 species of kalotermitids and rhinotermitids have been examined, and they nearly all harbor some type of pillotina spirochete. Since more than 350 species of dry-wood termites (Krishna, 1961) and 158 species of subterranean termites are known (Wilson, 1971) and fewer than 30 termite species have been examined with the electron microscope, many more complex spirochetes likely await discovery.

Isolation

Collection and Examination of Symbiotic Spirochetes

Large spirochetes are easily observed live in wet mounts. Styles can be readily dissected from fresh mollusks and crushed with a coverslip for direct observations of *Cristispira*. Intestines are removed with forceps from termites or wood-eating cockroaches. The microorganisms are very densely packed and so are usually diluted with Trager's solution (1934) before observation. They may be maintained for observation for up to 2 hours (*Cristispira*) or 24 hours (some hindgut spirochetes), using petrolatum- or paraffin-sealed microscope slides. The style or gut, its microcosm intact, is prepared for electron microscopy by fixation with glutaraldehyde (Margulis et al., 1991; To, 1978) or observed directly.

Collection of *Cristispira*

To observe live *Cristispira*, the host mollusk must be freshly harvested since styles degenerate within a few hours after removal from their natural estuarine habitat. Oysters maintained in the laboratory quickly lose their spirochetes. *Cristispira* tend to be either plentiful or absent in any given style and when present tend to align longitudinally in the style matrix. The environmental conditions indicative of the presence and abundance of *Cristispira* in a mollusk are poorly understood. Mollusks of the same species sharing the same environment will not all contain *Cristispira*. The aeration of the general environment seems to have no correlation with the presence of *Cristispira*. Mollusks in well-aerated surf and mollusks buried deep in black, anaerobic muds both contain *Cristispira*. *Cristispira* can be collected the year round.

Maintenance of Motile *Pillotina* Cells

In spite of many efforts, no medium has yet been devised that permits the in vitro growth of any of these large, symbiotic spirochetes. The following medium, however, did maintain motile ter-

mite spirochetes in mixed culture for at least 72 and up to 120 hours:

> Sweet E broth (Holdeman and Moore, 1972) is adjusted to a pH of 6.8, rendered anaerobic by gassing with 80% Ar, 10% CO_2, and 10% H_2, and anaerobically sterilized in Hungate anaerobic tubes containing 1.5% agar. By the syringe method, fetal bovine serum and cocarboxylase are added to a final concentration of 5.0% and 0.05%, respectively. Hindgut contents from a surface-sterilized termite are inoculated into the medium.

The obvious sensitivity of the spirochetes to air bubbles on the slides and in the medium suggests that these organisms are microaerophiles or anaerobes.

Identification

Large symbiotic spirochetes may be identified by light microscopy to at least the family Pillotinaceae and in some cases to genus given identification of their animal hosts. For classification to species, ultrastructural identification is required. The use of molecular sequence analysis, in particular 16S ribosomal RNA sequence comparison, has not been applied to any large symbiotic spirochetes so the phylogenetic relation of these spirochetes with other spirochetes or any bacteria is unknown.

Symbiotic Relationships with Other Organisms

Cristispira

The crystalline styles of oysters digest cellulose in the absence of *Cristispira* symbionts. With respect to cellulose digestion in oysters, styles with spirochetes do not differ from styles without spirochetes (Margulis et al., 1991). Therefore, the role, if any, the spirochete plays in oyster digestion is unknown.

Termite Spirochetes

Termite spirochetes are always found in association with dense populations of hypermastigote and polymastigote protists, as well as with other bacteria. They are present in the hindguts of healthy, dry-wood, and subterranean termite hosts of all insect stages and castes: larvae, pseudergates, alates, and soldiers. The termite communities include some 10^5–10^6 protists per ml of fluid gut: lophomonads, trichonymphids, devescovinids, monocercomonads, and oxymonads. They also regularly contain 10^9–10^{11} bacteria per ml of hindgut fluid, including *Arthromitus* (Margulis et al., 1990b) and other Gram-negative rods of many kinds (To and Margulis, 1978; To et al., 1980; Figs. 7, 8, and 9). Live spirochetes are observed in many sorts of relationships with each other and with members of the community: 1) free in the lumen of the intestine (Grimstone, 1967; Margulis et al., 1979; Fig. 3, 7, and 9); 2) attached casually to the surfaces of protists, especially devescovinids and dinenymphids (Bloodgood and Fitzharris, 1976); 3) attached to other bacteria (Margulis and Schaadt, 1976); 4) attached to each other and translating in moving bundles (Margulis and Schaadt, 1976; To et al., 1978); 5) attached to debris and beating in synchrony without translation (Margulis and Schaadt, 1976); 6) attached in regular tufts to the surfaces of mastigotes (these populations of spirochetes may beat in synchrony and thus move their mastigote hosts, thereby forming motility symbioses; Cleveland and Grimstone, 1964; To et al., 1978); or 7) entirely engulfed within the cytoplasm of protists (Margulis et al., 1979, 1981; Fig. 8). Apparently, these spirochetes have sticky surfaces that facilitate attachment to each other and to other microbes. The hindgut spirochetes, along with other members of the hindgut microbial community, are actively transferred to newly hatched larvae and from insect to insect by proctodeal (mouth-to-anus or anus-to-anus) contact. If unprotected or exposed to oxygen, the spirochetes die in a few minutes.

The spirochetes are probably not essential for cellulose digestion because the community and the host can survive their absence, at least for some weeks. The spirochetes appear to depend on the protists in the hindgut community; the spirochetes do not survive more than a few days after the protists are removed by chemical or heat treatment of the termite hosts (Grosovsky and Margulis, 1982). None of the large symbiotic spirochetes seem to have any practical importance.

Literature Cited

Bermudes, D., Chase, D., Margulis, L. 1988. Morphology as the basis of taxonomy in large spirochetes symbiotic in termites. Int. J. Syst. Bacteriol. 38:291–302.

Bermudes, D., Fracek, S., Laursen, R., Margulis, L., Obar, R., Tzerzinis, G. 1987. Tubulin-like protein from *Spirochaeta bajacaliforniensis*. Ann. N.Y. Acad. Sci. 503:515–527.

Bloodgood, R. A., Fitzharris, T. P. 1976. Specific association of prokaryotes with symbiotic flagellate protozoa from the hindgut of *Reticulitermes* and the wood-eating cockroach *Cryptocercus*. Cytobios 17:103–122.

Breznak, J. A. 1984a. Genus II. *Cristispira*, (Gross 1910). 46–49. Krieg, N. R., and Holt, J. G. (ed.) Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co. Baltimore.

Breznak, J. A. 1984b. Hindgut spirochetes of termites and *Cryptocercus punctulatus*. 67–70. Krieg, N. R., and Holt, J. G. (ed.) Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co. Baltimore.

Certes, A. 1882. Les parasites et les commensaux de l'huitre. Soc. Zoologique de France Bulletin 7:347–353.

Cleveland, L. R., Grimstone, A. V. 1964. The fine structure of the flagellate *Mixotricha paradoxa* and its associated microorganisms. Proc. R. Soc. Lond. B. Biol. Sci. 157:668–683.

Czolij, R., Slaytor, M., O'Brien, R. W. 1985. Bacterial flora of the mixed segment and the hindgut of the higher termite *Nasutitermes exitiosus* Hill (Termitidae, Nasutitermitinae). Appl. Environ. Microbiol. 49:1226–1236.

Damon, F. 1925. A note on the spirochetes of termites. J. Bacteriol. 11:31–36.

Dobell, C. 1912. Researches on the spirochetes and related organisms. Archiv fur Protistenkunde 26:119–230.

Duboscq, O., Grassé, P.-P. 1929. Sur quelques protistes d'un *Calotermes* des; afiles Loyalty. Protistologica (Archives de Zoologie Experimentale et Generale) 14(68):8–15.

Gharagozlou, I. D. 1968. Aspect infrastructural de *Diplocalyx calotermitidis* nov. gen., nov. sp., spirochaetale de l'intestin de *Calotermes flavicollis*. Compte. Rendu. Acad. Sci. Ser. D. 226:494–496.

Grimstone, A. 1967. A note on the fine structure of a spirochete. Q. J. Microscopical Sci. 104:145–153.

Grosovsky, B. D. D., Margulis, L. 1982. Termite microbial communities. 519–532. Burns, R. G. and Slater, J. H. (ed.) Experimental microbial ecology, Ch. 30. Blackwell Scientific Publications. Oxford.

Gross, J. 1910. *Cristispira* nov. gen. Ein Beitrag zur Spirochaetenfrage. Mittelungen aus der Zoologischen Station zu Neapel 20:41–93.

Holdeman, L., Moore, W. 1972. Anaerobe laboratory manual. Virginia Polytechnic Institute Anaerobe Laboratory. Blacksburg, Virginia.

Hollande, A. 1922. Les spirochetes des termites; processus de division: formation du schizoplaste. Arch. Zool. Expt. Gen. Notes Rev. 61:23–28.

Hollande, A., Gharagozlou, I. 1967. Morphologie infrastructurale de *Pillotina calotermitidis* nov. gen., nov. sp., spirochaetale de l'intestin de *Calotermes praecox*. Compte Rendu, Acad. Sci. Ser. D. 265:1309.

Karimi, Y., Hovind-Hougen, K., Birch-Andersen, A., Asmar, M. 1979. *Borrelia persica* and *B. baltazardi* sp. nov.: experimental pathogenicity for some animals and comparison of the ultrastructure. Ann. Inst. Pasteur (Paris) 130 B:157–168.

Kirby, H., Jr. 1941. Organisms living on and in protozoa. 1009–1013. Calkins, G. N. and Summers, F. M. (ed.) Protozoa in biological research, Columbia University Press. New York.

Krishna, K. 1961. A generic revision and phylogenetic study of the family Kalotermitidae (Isoptera). Bulletin of the American Museum of Natural History 122:303–408.

Kuhn, D. A. 1981. The genus *Cristispira*. Stolp, M. P., Trueper, H. G., Balows, A., and Schlegel, H. G. (ed.) The prokaryotes: a handbook on habitats, isolation, and identification of bacteria. Springer-Verlag. Berlin.

Leidy, J. 1850. On the existence of entophyta in healthy animals, as a natural condition. Proc. Acad. Natur. Sci. Philadelphia 4:225–229.

Leidy, J. 1881. The parasites of the termites. Journal of the Academy of Natural Sciences, New Series 8:425–447.

Margulis, L. 1981. Symbiosis in cell evolution. W. H. Freeman Publishing Co. San Francisco, xxii:419.

Margulis, L., Chase, D., To, L. 1979. Possible evolutionary significance of spirochaetes. Proceedings of the Royal Society of London, Series B 204:189–198.

Margulis, L., Hinkle, G., Stolz, J. F., Craft, F., Esteve, I., Guerrero, R. 1990a. *Mobilifilum chasei:* Morphology and ecology of a spirochete from an intertidal stratified microbial mat community. Arch. Microbiolog. 153:422–427.

Margulis, L., Nault, L., Sieburth, J. 1991. *Cristispira* from oyster styles: Complex morphology of large symbiotic spirochetes. Symbiosis 11:1–19.

Margulis, L., Olendzenski, L., Afzelius, B. A. 1990b. Endosporeforming filamentous bacteria symbiotic in termites: ultrastructure and growth in culture of *Arthromitus*. Symbiosis 8:95–116.

Margulis, L., Schaadt, J. 1976. Spirochetes (16 mm film). Boston University. Boston, Massachusetts.

Margulis, L., To, L. P., Chase, D. 1978. Microtubules in prokaryotes. Science 200:1118–1124.

Margulis, L., To, L. P., Chase, D. 1981. Microtubules, undulipodia and *Pillotina* spirochetes. Ann. N.Y. Acad. Sci. 361:356–368.

Morton, J. 1952. A preliminary study of the land operculate *Murdochia pallidum* (Cyclophoridae, Mesogastropoda). Trans. Proc. R. Soc. New Zealand 80:69–79.

Noguchi, H. 1921. *Cristispira* in North American shellfish. A note on a spirillum found in oysters. J. Exp. Med. 34:295–315.

Orton, J. 1922. Occurrence of a crystalline style in the American slipper limpet (*Crepidula fornicata*) and its allies. Nature 110:149.

Pastor, B., Canale-Parola, E. 1985. *Treponema saccharophilum* sp. nov., a large pectinolytic spirochete from the bovine rumen. Appl. Environ. Microbiol. 50:212–219.

Ryter, M. A., Pillot, J. 1965. Structure des spirochètes. II. Étude du genre *Cristispira* au microscope optique et au microscope électronique. Ann. Inst. Pasteur 109:552–562.

Tauschel, H. 1985. ATPase and cytochrome oxidase activities at the polar organelle in swarm cells of *Sphaerotilus natans*: an ultrastructural study. Arch. fur Mikrobiol. 141:303–308.

Terasaki, Y. 1960. Studies on *Cristispira* in the crystalline style of a fresh water snail, *Semisulcospira bensoni* (Philippi). II. On a cyst-like cell. Bulletin of the Suzugamine Women's College 7:1–5.

To, L. 1978. The large pillotina spirochetes of dry wood termites and their microtubules. Ph. D. Thesis, Boston University.

To, L., Margulis, L. 1978. Ancient locomotion: prokaryotic motility systems. International Reviews of Cytology 54:267–293.

To, L., Margulis, L., Chase, D., Nutting, W. L. 1980. The symbiotic microbial community of the Sonoran Desert termite: *Pterotermes occidentis*. BioSystems 13:109–137.

To, L., Margulis, L., Cheung, A. T. W. 1978. *Pillotinas* and *Hollandinas:* Distribution and behavior of large spirochetes symbiotic in termites. Microbios 22:103–133.

Trager, W. 1934. The cultivation of a cellulose-digesting flagellate, *Trichomonas termopsidis*, and of certain other termite protozoa. Biological Bulletin (Woods Hole) 66:182–190.

Wilson, E. 1971. The insect societies. 548. Belknap Press of Harvard University. Cambridge, MA.

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CHAPTER 14.5

Streptobacillus moniliformis

JAMES R. GREENWOOD AND SYDNEY M. HARVEY

Introduction

The genus *Streptobacillus* is presently represented by one species, *Streptobacillus moniliformis*, the etiologic agent of rat-bite fever and Haverill fever. *S. moniliformis* has also been known as *Haverhillia multiformis, Actinomyces muris*, *Actinobacillus muris*, and *Haverhillia moniliformis.* Although the genus *Streptobacillus* was described in the 1920s, taxonomically it continues to be a genus of uncertain affiliation. Previously this genus was placed in the families Parvobacteriaceae (Tribe Haemophileae) and Bacteroidaceae. Since the 8th edition of *Bergy's Manual, Streptobacillus* has been described as a genus of "uncertain affiliation." It was recently suggested (Savage, 1989) that *S. moniliformis* is more similar to some of the Mycoplasmatales. This suggestion is based on the low GC content of the DNA (24–26 mol%), serum or blood requirements for growth, cholesterol incorporation into the cell membrane, animal parasitism, and production of L-phase organisms. Further clarification of the taxonomic position of this genus awaits genetic studies, such as DNA-RNA hybridizations.

Habitats

S. moniliformis is found in the nasopharynx of laboratory and wild rats, with some studies reporting as many as 50–100% of wild rats carrying it as a commensal organism and excreting it in their urine (McHugh et al., 1985). It is reported to have caused tendon sheath infections in turkeys, cervical abscesses in guinea pigs, epizootics in laboratory mice, and lesions in laboratory rats with bronchopneumonia. Humans become involved in the transmission cycle through rat bites or from the bites of squirrels, weasels, and such rat-eating carnivores as dogs, cats, and pigs. Rat bite fever has also been reported following the handling of dead rats. Haverhill fever may result from the ingestion of milk to which rats have had access.

Selective Enrichment

S. moniliformis is both fastidious and slow growing; consequently it is frequently overgrown by normal flora. Because it is an uncommon human pathogen, little work has been done to develop a selective culture medium. Recently, however, colistin nalidixic acid agar (CNA) was used in conjunction with other media to isolate Gram-negative *S. moniliformis* from a 2-month old with a fatal infection following a bite by a wild rat (Sens et al., 1989). CNA is inhibitory for many other Gram-negative bacteria and the use of this medium or a modification combining colistin, nalidixic acid and serum might provide a new approach to isolation of *S. moniliformis* from nonsterile body sites.

Isolation

S. moniliformis growth media should be supplemented with blood, serum, or ascitic fluid. Because L-phase variants might be present in clinical samples, media formulations must also take the specialized growth requirements of these forms into account. The bacterial phase has been isolated on media with either a meat infusion or tryptose base enriched with 20% horse serum or 15% sterile defibrinated rabbit blood. L-phase variants are more easily observed in culture when grown on the clear, serum-containing agar. Agar plates should be incubated in a humid environment with increased CO_2, such as that obtained in a candle jar, or in a humidified CO_2 incubator. Plates should be incubated at temperatures of 35–37°C. *S. moniliformis* colonies are greyish, are round, and have a butyrous consistency. They generally have a discrete edge and reach approximately 1–2 mm after incubation for 3 days. The L-phase

This chapter was taken unchanged from the second edition.

colonies exhibit a typical mycoplasma-type "fried egg" appearance and are considerably smaller than bacterial colonies.

When blood cultures are required to diagnose *S. moniliformis* endocarditis, thioglycolate broth appears adequate to support growth. However, it appears important not to use broth that contains sodium polyanethol sulformate, as this has been reported to be inhibiting at levels as low as 0.0125% (Lambe et al., 1973).

Identification

Table 1 lists the salient features of *S. moniliformis*. Because of the fastidious nature of these organisms, the literature frequently reports numerous discrepant biochemical features probably resulting from the use of different basal media. The most comprehensive studies on biochemical reactions of this genus are presently based on the studies of Aluotto et al. (1970) and Cohen et al. (1968).

Perhaps the most reliable and accessible method of testing carbohydrate fermentation is to inoculate a 24-h broth culture of *S. moniliformis* into a cystine trypticase agar (CTA) base that contains one drop of rabbit serum and 1% carbohydrate in final concentration. These tubes are incubated at 35°C and reactions read at 1, 2, 7, and 14 days. Inoculated tubes should be compared to control tubes consisting of CTA with added carbohydrate and serum. For detection of other biochemical features, the work of Cohen et al. (1968) should be consulted. Fatty acid profiles have also been used to rapidly identify *S. moniliformis* during an outbreak of Haverhill fever (Rowenbotham, 1983). In this work, strains were grown in serum broth for 24 h, and the cultures were used to prepare fatty acid methyl esters. *S. moniliformis* strains had consistent peaks of palmitic, linoleic, oleic, and stearic acid.

Table 1. Salient features of *Streptobacillus moniliformis*.

Test	Result
Catalase	–
Oxidase	–
Indole	–
Nitrate to nitrate	–
H_2S production	+
Arginine dihydrolase	+
Serum, blood, or ascitic fluid (required for growth)	+
Phenylalanine deaminase	–
Methyl red	–
Voges-Proskauer	–
Esculin in hydrolysis	d[a]
Gas produced from carbohydrate	–
Acid produced from:	
Fructose	+
Glucose	+
Maltose	+
Starch	+
Arabinose	–
Dulcitol	–
Sorbitol	–
Sucrose	–

[a]d = Different biotypes.

The microscopic morphology of *S. moniliformis* may vary depending on the media used, cultural conditions, and age of culture. Generally, cells appear as elongated Gram-negative rods, frequently in chains and filaments with occasional thickenings along the filaments giving rise to a necklace appearance ("moniliformis" means necklace-shaped). *S. moniliformis* is nonencapsulated, nonmotile, and facultatively anaerobic.

Preservation of Cultures

Cultures of *S. moniliformis* can be lyophilized for effective storage. They can also be subcultured in broth, but transfer to fresh broth medium is required as frequently as every 24 h to maintain viability. Refrigerated agar plate cultures can survive up to 15 days.

Applications

Animal research personnel are at risk from developing rat bite fever when working with laboratory rats. From 1958 to 1983, of 13 cases of rat bite fever reported in the United States, six of the cases were associated with bites of laboratory rats (Anderson et al., 1983). This is in keeping with the high rate of colonization by *S. moniliformis* in these animals. Studies on the mechanism of pathogenicity, virulence, and host range have not been reported.

Literature Cited

Aluotto, B. B., R. G. Wittler, C. D. Williams, J. E. Faber. 1970. Standardized bacteriologic techniques for characterization of *Mycoplasma* species. Int. J. Syst. Bacteriol. 20:35–58.

Anderson, L. C., S. L. Leary, P. J. Manning. 1983. Rat bite fever in animal research laboratory personnel. Lab. Animal Sci. 33:292–294.

Cohen, R. L., R. G. Wittler, J. E. Faber. 1968. Modified biochemical tests for characterization of L-phase variants of bacteria. Appl. Microbiol. 16:1655–1662.

Lambe, D. W., A. M. McPhedran, J. A. Mertz, P. Stewart. 1973. *Streptobacillus moniliformis* isolated from a case

of haverhill fever: biochemical characterization and inhibitory effect of sodium polyanethol sulfonate. Am. J. Clin. Pathol. 60:854–860.

McHugh, T. P., R. L. Bartlett, J. I. Raymond. 1985. Rat bite fever: Report of a fatal case. Ann. Emerg. Med. 14(2):1116–1118.

Rowenbotham, T. J. 1983. Rapid identification of *Streptobacillus moniliformis*. Lancet ii:567.

Savage, N. 1984. *Genus Streptobacillus* Levaditi, Nicolau and Poincloux 1925, 1188. 598–600. N. R. Krieg and J. G. Holt (ed.) Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins. Baltimore.

Sens, M. A., E. W. Brown, L. R. Wilson, T. P. Crocker. 1989. Fatal *Streptobacillus moniliformis* infection in a two-month old infant. Am. J. Clin. J. Clin. Pathol. 91:612–616.

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CHAPTER 14.6

The Genus *Toxothrix*

PETER HIRSCH

The first description of a bacterium that produced twisted bundles of thin filaments which contained oxidized iron was given by Cholodny (1924). He called this organism *Leptothrix trichogenes*, the filament-producing *Leptothrix*. The flexible bacterium was observed in a water basin next to the river Dnjepr near Kiev, Russia. Its cells (diameter approximately 0.5 μm) were connected to form a trichome of up to 400 μm in length. A peculiar movement was observed: the U-shaped trichome glided with its rounded part forward; both ends of the trichome left bundles of twisted filaments of polymer as parallel "railroad tracks." Occasionally, parts of the bundles appeared to be drawn out in a fan-shaped fashion (Figs. 1a, 2).

Although this first description was very accurate, as we know now, later scientists failed to notice the presence and importance of the flexible, gliding bacterium (trichome); they only observed the often rigid, brittle, iron-encrusted bundles of filaments. Consequently, these filaments were interpreted to be alive, to comprise the organism. It appears strange that the producer of these structures did not attract greater interest.

The explanation for the failure to recognize the true nature of *Toxothrix* came from Krul, Hirsch, and Staley (1970), who employed a partially immersed, phase-contrast microscope to study the formation of such filament bundles in an iron spring in Michigan. The bacterial trichome was observed to consist of up to 40 rods, each of 0.5–0.75 × 3–6 μm, with a total length of up to 240 μm. The forward movement of the often U-shaped trichome consisted of symmetrical rotation of both trichome ends, which thereby left a track on their attachment surface that consisted of twisted polymer fibers. Occasionally, the torque on the rounded center part of the U was released by an upward twist followed by a downward "printing" of polymer onto the surface of the glass slide. Later these polymer fibers became encrusted with iron oxide. Attempts to study the flexible filaments in the laboratory resulted in an explosive lysis that occurred within a few minutes after the living preparation had been made (Fig. 1b). Thus the absence of trichomes in samples of many earlier observers could be explained (Krul, Hirsch, and Staley, 1970).

The fact that the bundle of twisted fibers was different from the "true" sheaths produced by other *Leptothrix* spp. caused Molisch (1925) to name this organism *Toxothrix ferruginea* (*toxon,* Greek noun, a bow). Later, Beger and Bringmann (1953) changed the name partially back into *Toxothrix trichogenes*, a more proper name (Hirsch and Zavarzin, 1974).

This chapter was taken unchanged from the second edition.

Habitats

The organism is not at all common in nature, although in specific habitats it has been found with a worldwide distribution. Most observers have seen *Toxothrix trichogenes* in fairly cold iron springs under conditions of reduced oxygen tension and slightly acidic pH (Table 1). The highest water temperature at which *Toxothrix* has ever been reported to grow is 15.5°C; thus this bacterium is obviously a psychrotroph. Concentrations of ferric iron were, when measured, low: 1–2.7 mg/liter Fe^{2+}. Many authors do not mention, in their reports, the occurence of the gliding *Toxothrix* trichomes, and thus the organism itself could have been absent when the more persistent, iron-encrusted filament bundles were seen and the parameters measured.

If one studies the variety of other bacteria present with *Toxothrix trichogenes*, one discovers that *Gallionella ferruginea* usually accompanies it. *Leptothrix* spp., *Siderocapsa* spp. or *Sphaerotilus*, *Naumanniella* spp., and *Ochrobium tectum* also occur, but if present they appear in smaller numbers. The natural habitat of *Gallionella* seems to be very similar to that of *Toxothrix*. But while the former grows best in nutrient-poor environments, the *Toxothrix* trichomes seem to prefer a higher concentration of organic matter. Also, *Toxothrix* grows best under microaerophilic conditions (Table 1).

Occurrence of *Toxothrix* in lakes or ponds has rarely been reported. Gorlenko, Dubinina, and Kusnezov (1977) found this organism in a 7- to 8-m depth in a Karelian mesotrophic lake and state that, if meromictic lakes were studied, *Toxothrix* could be found in one out of three such lakes.

Enrichment Suggestions

Toxothrix trichogenes has not been obtained in pure culture to date. However, natural *Toxothrix* samples—especially from cold iron springs with low pH—can be kept in the laboratory for sev-

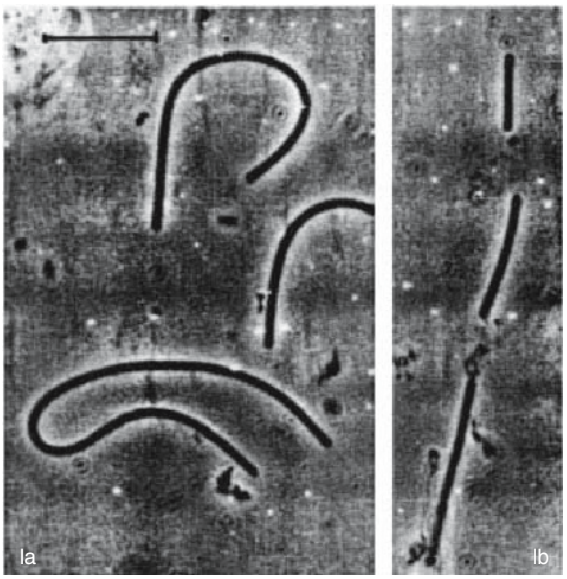

Fig. 1. *Toxothrix trichogenes* growing on a slide submerged in an iron-spring basin. The flexible trichomes with dense granules. (a) Typical U-shape of gliding cells. (b) A trichome has fragmented. Bar = 10 µm.

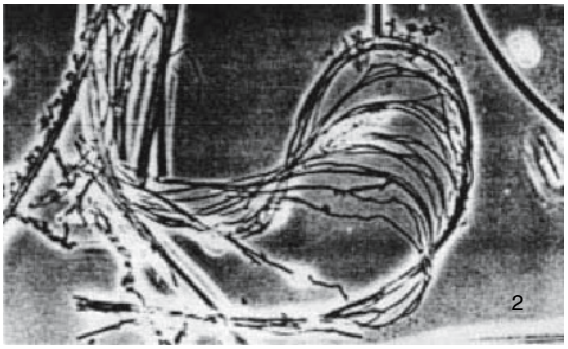

Fig. 2. *Toxothrix* polymer filaments (twisted bundle, fanshape) with iron encrustations. There are also sheaths of *Leptothrix* sp. and *Gallionella* bands. Preparation from the same site as in Fig. 1.

eral months, provided they contain some sediment with organic matter and are kept cold (5°C) and dark.

Microscopic examination of such stored samples often results in rapid lysis of the trichomes, a behavior also observed in some other gliding bacteria. The reason for the rapid lysis is not entirely clear. It could not be caused by a temperature that is too high, since lysis will occur below temperatures of some natural habitats. Light intensity and lack of oxygen could also be ruled out for the same reasons. *Toxothrix* trichomes may be extremely sensitive to pressure. Therefore, the cover slip of a preparation for microscopy should be carefully supported by small fragments of cover slip glass. It may also be quite harmful to these organisms to be streaked out, for the same reasons.

Future enrichments should be made with natural samples that contain many actively gliding trichomes; the number of twisted rope structures is quite irrelevant. The liquid medium should probably have a pH of 5.5–6.5, a constant iron supply of not more than 1–2 mg/liter Fe^{2+}, and an oxygen concentration of about 1 mg/liter. The incubation should be performed in the dark and at 5–10°C.

Identification

In the absence of iron-encrusted, excreted filament bundles, *Toxothrix trichogenes* could be mistaken for other gliding bacteria such as *Herpetosiphon* spp. The genus *Haliscomenobacter* (= *Streptothrix*), described from polluted environments (see The Genus *Haliscomenobacter* in this Volume), also shows some morphological similarities to *Toxothrix*. It is quite possible that gliding bacteria without such filamentous sheaths that have been observed in various freshwater, marine, or polluted habitats could have been *Toxothrix* spp. The absence, in *Toxothrix* trichomes, of constrictions at the cross-wall sites is quite characteristic. Their mode of gliding (in the

Table 1. Occurrence and habitats of *Toxothrix trichogenes*.[a]

Location and depth (m)	Time (months)[b]	Temp (°C)	pH	O_2 (mg/liter)	Redox potential (Eh, mV)	Fe^{2+} (mg/liter)	Trichomes	Cell density; other bacteria	References
Water basin, Dnjepr, Russia	7–8	.	.	.	.	.	+	Ga, Le	Cholodny, 1924
Iron spring, Braunschweig, Germany (<1)	9	9–11.5	5.5	<1	rH; 26–27	1.8–2.7	?	Ga, Le	Charlet and Schwartz, 1954
Iron spring, Reselithberg, Holstein (0.2)	7	13	6.6	.	.	+	++	Ga, Le, Si	P. Hirsch, unpublished observation
Iron spring, Kokkino Neró, Greece	.	15.5	6.1	0.4–1.2	+210 to +280	.	?	.	R. Schweisfurth, personal communication, 1976
Iron spring basin, Michigan (0–0.4)	1–4	4–9	6.8–6.9	.	.	+	+++	Ga, Le, Sp, Na, etc.	Krul, Hirsch, and Staley, 1970
	8–12	12–4		.	.	+	+++	Ga, Le, Sp	Krul, Hirsch, and Staley, 1970
Iron brook (before swamp), Karelia	.	.	6.25	.	+600	1.0	?	Na, Oc, Ga, Le; 1.3×10^3/mol	Dubinina and Derjugina, 1972
Iron brook (behind swamp), Karelia	.	.	5.12	.	+500	1.2	?	Ga, Le; 12.4×10^3/ml	Dubinina and Derjugina, 1972
Forest Pond, Michigan (0.6)	2–3	0.5–1.5	7.7	.	.	+	++	Sp, Si	Krul, Hirsch, and Staley, 1970
Lake Putis-Järvi, Karelia (7–8)	7	1	.	<3	.	.	?	5×10^3/ml	Gorlenko, Dubinina, and Kusnezov, 1977

[a]Other bacteria: GA = *Gallionella;* Le = *Leptothrix*; Si = *Siderocapsa*; Sp = *Sphaerotilus*; Oc = *Ochrobium;* Na = *Naumanniella.*
[b]Months in which *Toxothrix* has been observed; 7–8 stands for July–August, etc.

form of a U) separated them from the equally colorless, filamentous, and gliding *Achroonema* spp., which normally remain fairly straight.

Fragmentation of *Toxothrix* trichomes into short rods is more common in some habitats than in others and indicates the possibility of there being more than one *Toxothrix* species. Even before fragmentation, one recognizes many dense (electron-dense) granules in the *Toxothrix* trichomes. These may be polyphosphate granules (volutin). After fragmentation, each rod-shaped cell usually has two such granules, one at either end.

Literature Cited

Beger, H., Bringmann, G. 1953. Die Scheidenstruktur des Abwasserbakteriums *Sphaerotilus* und des Eisenbakteriums *Leptothrix* im elektronenmikroskopischen Bilde und ihre Bedeutung für die Systematik dieser Gattungen. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten, und Hygiene, Abt. 2 107:318–334.

Charlet, E., Schwartz, W. 1954. Untersuchungen über die Lebensweise von *Leptothrix ochracea* und einigen begleitenden Eisenmikroben. Schweizerische Zeitschrift für Hydrologie 16:318–341.

Cholodny, N. 1924. Über neue Eisenbakterienarten aus der Gattung *Leptothrix* Kütz. Zentralblatt für Bakteriologie, Parasitenkunde, and Infektionskrankheiten, Abt. 2 61:292–298.

Dubinina, G., Derjugina, Z. 1972. Vergleichende elektronmikroskopische Untersuchungen der Mikroflora von Moorerz und Seewasser. Archiv für Hydrobiologie 71:90–102.

Gorlenko, V. M., Dubinina, G., Kusnezov, S. I. 1977. Ecology of water microorganisms. "In Russian." Moscow, Izdatel'stvo Akademii Nauka.

Hirsch, P., Zavarzin, G. A. 1974. Genus *Toxothrix* Molisch 1925. 120. Buchanan, R. E., and Gibbons, N. E. (ed.) Bergey's manual of determinative bacteriology, 8th ed. Baltimore, Williams & Wilkins.

Krul, J. M., Hirsch, P., Staley, J. T. 1970. *Toxothrix trichogenes* (Chol.) Beger et Bringmann: The organism and its biology. Antonie van Leeuwenhoek Journal of Microbiology and Serology 36:409–420.

Molisch, H. 1925. Botanische Beobachtungen in Japan. 8. Mitteilung. Die Eisenorganismen in Japan. Tohoku Imperial University Science Reports. 4:135–168.

Prokaryotes (2006) 7:990–995
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CHAPTER 14.7

The Genus *Gallionella*

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The bacteria of the genus *Gallionella* belong to the so-called "iron bacteria," which achieved their theoretical importance after Winogradsky (1888, 1922) postulated his conception of chemolithotrophy for these organisms. These bacteria also have practical significance since they clog drains, water pipes, and wells with deposits of iron oxide compounds. In connection with freshwater supply problems and the rapid growth of agriculture, hydrotechnicians and land-reclamation (underground drainage) specialists have become increasingly interested in bacterial iron oxidation as a common source of interference with wells and drainage systems (Ford, 1978; Hanert, 1974b; Khrutskaya, 1970; Martin et al., 1978). Iron bacteria are also interesting for ecological and biogeochemical reasons. Like algae, iron bacteria may develop in their natural habitats in such masses that the idea of their participation in the sedimentary formation of iron ore is plausible, as was recognized in the first description of the iron bacterium *Gallionella ferruginea* (Ehrenberg, 1836).

Iron bacteria are reputed to be difficult to work with. The general opinion of microbiologists is that it is difficult to cultivate these organisms in the laboratory and that there are difficulties in isolating and identifying them. Therefore, it is one of the main purposes of this contribution to show that this poor reputation is unjustified. It will be demonstrated that cultivation and handling of the treated bacteria are in no way more difficult than for other microorganisms, if attention is paid to only a few fundamental peculiarities, such as "sessility" and "gradient growth." Disregard of these factors may be one of the most common practical reasons for failure to locate these organisms in natural habitats, for failure to cultivate them successfully, for misinterpretation of presumed developmental stages, and for discouragement which can often be observed when students are starting in this field. Therefore, the following text is narrowly restricted to methods and practical advice—without any theoretical discussion on the controversial life cycle, taxonomic position, and physiology, which are described in the literature cited.

This chapter was taken unchanged from the second edition.

Habitats

Chemistry of the Habitats

Gallionella ferruginea characteristically occurs most abundantly in very pure, iron-bearing waters that contain only traces of organic material. Habitats with the best prospects for a successful search are ferruginous mineral springs, water works, wells, and drainages, especially underground drainages in regions near the groundwater table. Swamp ditches and lakes are unusual habitats that contain *Gallionella* in large quantities only at places where pure, ferrous waters infiltrate. Occurrence is not restricted to fresh waters or to low-temperature habitats, as commonly assumed; *Gallionella* has been convincingly found in salt water, marine bays, and thermal springs with temperatures up to 47°C (Hanert, 1973b, 1981a; Sharpley, 1961; Volkova, 1939; Vouk, 1960).

Common to all of the various *Gallionella* localities investigated are sharply limited physicochemical conditions characterized by a low redox potential in an Eh range of +200 to +320 mV (rH_2 values generally vary from 19 to 21) and a slightly acidic environment caused by the solution of considerable amounts of CO_2. Adding the data determined by Volkova (1939) in slightly alkaline mineral waters, the pH environmental limits range from 6.0 to 7.6, which excludes growth in acid habitats. Occurrence of *Gallionella* in acid mine waters as described by Walsh and Mitchell (1969, 1970) may be a result of confusing *Gallionella* with *Metallogenium* and/or nonbiological structures.

These Eh and pH limits characterize *Gallionella* as a very good example of a gradient organism that develops under neither strongly reducing conditions nor in a highly oxidizing zone, but in a level between the two extremes with redox conditions about 200–300 mV lower than typical surface waters. This region in which *Gallionella* grows characterizes the lowest zone of Eh-pH environmental limits of iron bacteria

established by Baas-Becking et al. 1956, in which there are usually only low concentrations of oxygen.

Comparison with geochemical-stability field data for ferrous iron, published by Garrels and Christ (1965) and Hem (1972), shows that the *Gallionella* Eh-pH milieu, in all the cases that have been measured, lies significantly within the zone in which ferrous ions are stable. This stability appears to be the essential factor in the environmental conditions for the existence of *Gallionella,* much more important than the factors of temperature and oxygen content, to which *Gallionella* manifests greater adaptability than generally thought. The marked psychrophily and the microaerophily generally ascribed to *Gallionella* seem to be only secondary effects that depend on the fact that bivalent iron is most stable under these conditions (Hanert, 1975). Thus, it is easy to understand why *Gallionella* also occurs in O_2-saturated waters, for example, in aerated, iron-removal treatment plants in water works or in thermal springs, when the only essential condition, stability of bivalent iron in the presence of oxygen, is fulfilled. These observations do not invalidate the most common values of physicochemical factors measured in *Gallionella* habitats, which are 0.1–1 mg/liter O_2, 8–16°C, 5–25 mg/liter Fe(II), around 20 or more mg/liter CO_2, and a very slight content of organic material, not above 12 mg/liter $KMnO_4$ (Hanert, 1975).

Growth Measurement and Analysis of Iron-Oxidation Structures by In Situ Exposure

The red-brown deposits at *Gallionella* sites consist primarily of the typical spiral bands of dead matter excreted from the terminal *Gallionella* cells. These deposits may be extremely pure. However, their *Gallionella*-nature may be very difficult to recognize when the deposits have been altered by additional chemical iron-oxidation processes. False conclusions may then be drawn about the nature of the iron-oxide precipitation. A direct measurement of *Gallionella* growth is indispensable for analyzing such structures, as well as for determining whether or not *Gallionella* is actually growing on the site.

There are three proven methods of in situ exposure for light microscopy and for transmission and scanning electron microscopy: (1) The exposure of cover slips or slides for several hours or days, singly or in multiple vertical alignment, in plastic clamp fittings. This method, a modification of the on-growth method of Naumann (1919) and Cholodny (1924), allows the localization of the zones in which *Gallionella* develops in still waters, the densitometric and photometric registration of the total iron oxidation (Hanert, 1981a) and, by determining stalk production, the quantification of the momentary *Gallionella* development (Hanert, 1973a). (2) The exposure of Formvar-coated, platinum or gold, electron-microscope grids (copper grids are inappropriate due to the frequent presence of H_2S). With these procedures, the fine structure of the bands, as well as of the terminal *Gallionella* cells on the sessilely excreted intact stalks, may be observed. The latter is not included when suspension preparations of the stalk fragments are used (Hanert, 1970; Hirsch and Pankratz, 1970). (3) Exposure of round glass slides for scanning electron microscopy and electron probe microanalysis (FeKα). This procedure is especially important because it allows tiny globular particles (0.04–0.3 μm in diameter) to be chemically and physically analyzed (Hanert, 1981a). Until now, they were thought to be mycoplasma-like developmental stages and buds of *Gallionella,* but they have been shown to be pure Fe(III)-oxide particles.

The advantages of these three procedures, which all make use of the sessile way of life, are obvious when one considers that the failure to find the characteristic apical cells in natural habitats (van Iterson, 1958) was the starting point for the studies that aimed at proving the mycoplasma-like and *Metallogenium*-like viable nature of *Gallionella* stalks (Balashova, 1967b, 1969, 1974; van Iterson, 1958; Zavarzin, 1961). The three procedures described above may also be applied to pure cultures of *Gallionella.*

Isolation

Physiological Basis for Isolation

Although chemolithotrophy has not as yet been generally recognized for *Gallionella,* it must be emphasized that all of the successful cultures were able to develop only in mineral media with iron(II) as energy source. This is true for Lieske's first cultures (1911) containing metallic iron as well as for the excellent culturing procedures of Kucera and Wolfe (1957) that use ferrous sulfide as a source of reduced iron. Culture procedures that used organic media containing serum, as suggested by Balashova (1969), have remained unsuccessful. Classic *Gallionella* organisms with stalks and apical cells as well as developmental stages producing such forms have not been obtained. It thus seems evident that *Gallionella* is truly a chemolithotrophic bacterium and that ferrous-containing mineral media are best for cultivation and isolation.

Isolation Principles and Procedures

Two isolation procedures have proven useful. Both use mineral Wolfe's FeS medium (see below) as a selective isolation medium for *Gallionella,* in which neither sheathed nor capsulated iron bacteria will grow.

One procedure is a test-tube dilution method in which a final concentration of one colony per culture is reached. The ability to stick to the vessel walls is used to separate *Gallionella* from the contaminants (Engel and Hanert, 1967; Hanert, 1968; Hanert 1981b).

Isolation of *Gallionella* by Dilution and Serial Transfer (Engel and Hanert, 1967; Hanert, 1968)

> One Gallionella colony is withdrawn with a capillary pipette from the wall of a test tube containing an enrichment culture, suspended homogeneously in 10 ml of Wolfe's medium without FeS, diluted to 10^{-5} (maximally 10^{-6}, one colony of 1 mm in diameter consists of about 100,000 apical cells), and then inoculated into test tubes with Wolfe's FeS medium (30–50 parallel cultures starting with a 10^{-4} dilution).
>
> After 1 week of culture, a one-colony culture is treated, in essentially the same way as above, to obtain a pure culture. The colony, which is attached to the wall of the test tube, is carefully rinsed using a pipette with approximately 200 ml of fresh, sterile medium without FeS, after first disposing of the FeS sediment (1 ml) and the mineral medium (9 ml). Then the colony is transferred five times into test tubes containing 10 ml of sterile medium (without FeS) and washed with gentle shaking to rid the colony of clinging contaminations. The washed colony is then suspended into sterile medium (10 ml) and again inoculated into test tubes containing Wolfe's FeS medium at dilutions of maximally 10^{-6} (again 30–50 parallel cultures). Five to ten serial transfers are necessary to achieve pure culture in this manner. Although this procedure requires up to 10 weeks, it is a very certain method of continually reducing the number of contaminants and obtaining a pure culture (Hanert, 1975).

The second isolation technique requires much less time and leads, according to the authors, to a pure culture in 7 out of 10 cases. It is based on the observation that some strains of *Gallionella,* in contrast to most contaminants, are resistant to 0.5% formalin.

Isolation of *Gallionella* by Formalin Resistance (Nunley and Krieg, 1968)

> Five-tenths milliliter of formalin (40% formaldehyde solution) was added to a dilution bottle of Wolfe's medium containing 10 ml of ferrous sulfide agar and 100 ml of fluid medium. Samples of *Gallionella* from its natural source were centrifuged at $3000 \times g$ for 3 minutes. One to five milliliters of sediment was transferred to the Wolfe's medium with formalin. The medium was incubated at 25°C for 1–2 days. One-milliliter aliquots of the culture were transferred to fresh Wolfe's medium without formalin. Cultures were incubated at 25°C for 2–3 weeks. Tests for culture purity were performed using the third serial transfer after the formalin treatment.

Purity is checked by use of a variety of heterotrophic and autotrophic test media, for example, yeast extract bouillon, nutrient agar, *Nitrosomonas* medium, *Ferrobacillus* medium, and *Thiobacillus* agar. The length of observation is up to 4 weeks.

Micro- and Macrocultures and Axenic Maintenance

There are no principal difficulties in cultivating *Gallionella* in containers ranging from small chambers to 12-liter fermentors. The difficulties of culture that have been reported in almost all publications on *Gallionella* generally are due to overlooking interactions in the medium among the following factors: phosphate, ferrous and ferric iron, oxygen, pH, and redox potential. The most important growth-inhibiting chemical processes in the medium are: (1) iron phosphate precipitation caused by too high a concentration of phosphate; (2) chemical autoxidation of ferrous iron caused by too high a concentration of oxygen or by the redox potential being too high; and (3) the pH becoming more acidic when elevated O_2 concentration causes hydrolysis of ferric iron. All three processes eventually reduce the Fe(II) content of the mineral medium of the FeS cultures below the lower limit of 5 ppm. In order to retain the required Fe(II) concentration, it is absolutely necessary to have the following initial conditions in the medium: oxygen content 1 ± 0.2% (0.42 ppm), pH 6.0 (values from CO_2 bubbling, border values between 5.5 and 6.3), Eh +330 mV to +350 mV (Eh border values from –55 to +395 mV), phosphate (as K_2HPO_4) 0.05 g/liter. These conditions were established in optimum growth zones of 3-liter Erlenmeyer flasks and confirmed in test tube cultures (Hanert, 1975). They lead, following interactions between mineral medium and FeS-sediment, to an iron(II) concentration of 10–20 ppm which guarantees good growth of *Gallionella.* The conditions of gassing must be varied according to the volume in each vessel, so that common standardized instructions may not be given for all sizes of flasks. Identical for all cultures, though, is the production and storage of ferrous sulfide as well as the production of modified mineral Wolfe's medium. During the first process, essential mistakes may be made that inhibit the growth of *Gallionella.*

Preparation and Storage of Ferrous Sulfide

> For preparation of ferrous sulfide, two precipitation reactions with equal molar quantities are possible. FeS can be produced by reacting either 78 g ferrous ammonium sulfate "$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$" with 44 g sodium sulfide ($Na_2S \cdot 9H_2O$) or 140 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$) with 120 g sodium sulfide in deionized water (50°C; ferrous salts are added in solid form while stirring). The resulting FeS precipitate must be washed extensively using deionized water (decanting of the supernatant and its replacement with deionized water at 50°C), removing Na^+, NH_4^+, and, above all, S^{2-} ions until the precipitate reacts neutrally (pH measurement in FeS, not in the supernatant).

Washing of FeS while continually checking pH is indispensable, since hydrolysis of any residual sulfide ions ($S^{2-} + H_2O \rightarrow HS^- + OH^-$) raises the pH to 7.7 directly over the FeS sediment. The elevated pH prevents bivalent iron from dissolving and *Gallionella* development does not begin (Gebauer, 1978). Because of this indirect effect of sulfide on the development of *Gallionella,* sodium sulfide, used to manufacture FeS, is added in slightly less than the equimolar amount. The separation of the adsorbed sulfide ions from the FeS precipitate during the washing procedure is a slow process and takes approximately 5 days (5–9 washings at intervals of at least 4 h). Delayed FeS sedimentation caused by formation of FeS-hydrosol can easily be eliminated by including a few drops of a saturated $FeCl_3$ solution or by once washing with tap water. After this cautious washing, FeS precipitate can be stored without oxidation in glass-stoppered bottles that are completely filled.

Modified Mineral Wolfe's Medium

> The modified mineral medium contains per liter distilled water: 1 g NH_4Cl, 0.2 g $MgSO_4 \cdot H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, and only 0.05 g $K_2HPO_4 \cdot 3H_2O$ (one-tenth of the original concentration).

With the strongly reduced concentration of phosphate, it is no longer necessary to separately autoclave the individual components (no earth alkali phosphates are formed during autoclaving). Low phosphate concentration also prevents iron phosphate precipitate in the culture medium. Such precipitation would result in a total growth inhibition of *Gallionella* due to lack of dissolved iron(II).

Microculture Slide Chambers for In Vivo Studies on Cell Division, Cell Rotation, and Stalk Twisting

> In constructing a chamber, one cover glass (20 × 50 mm) is mounted on each slide (26 × 76 mm). The cover glass is maintained at 0.05 or 0.1 mm above the slide by initially inserting a 0.05- or 0.1-mm flat bar and then sealing the two sides with sealing wax. The bar is removed, the third side (bottom of the chamber) is then sealed, and the slide is held erect for filling. First, a tiny drop of FeS is inserted (by capillary pipette) and then mineral medium is added so that the chamber is two-thirds full, whereby the final ratio of FeS:medium is 1:10. The chamber is then inoculated and the fourth side is sealed. Microaerobic conditions are produced by replacing the air with CO_2 or with a mixture of 94% N_2–5% CO_2–1% O_2. *Gallionella* growth (stalk production) starts immediately after the chamber is closed (Hanert, 1974a).

Procedure for Maintenance of Axenic Cultures of *Gallionella*

> The following procedure has proven useful in maintaining pure cultures of *Gallionella*.
>
> Thirty-four test tube cultures are set up with the dilutions 10^{1-} (4 cultures; inoculum 150,000–300,000 cells), 10^{2-} (5 cultures), 10^{3-} (15 cultures), and 10^{4-} (10 cultures; inoculum 150–300 cells). For this purpose, the cotton-plugged test tubes are aseptically filled with 9 ml autoclaved mineral medium. Then, 22.8 cm^3 of cotton-filtered CO_2 is bubbled through each tube using a 1-ml pipette (length of bubbling approximately 5 s; the pH of the medium is now 4.5–4.8). One milliliter of sterile FeS precipitate is then slowly added with a pipette.
>
> Six hours after the addition of FeS, the inoculation follows subsequent to dissolving 5–10 ppm bicarbonatic Fe(II) in the medium; meanwhile, the tubes are stored in jars at 17°C under an atmosphere of 94% N_2–5% CO_2–1% O_2. In preparing the initial suspension for inoculation (10^0 dilution), two colonies with a diameter of 2 mm (or one colony with a diameter of 4 mm) are suspended in 10 ml of sterile medium using a vortex mixer. Dilution series and inoculation are repeated as described above. The cultures are then incubated in preserving jars at 17°C in an atmosphere of 94% N_2–5% CO_2–1% O_2. This gas mixture can also be used for bubbling through the cultures (135 cm^3 for each test tube). The best *Gallionella* growth is usually at a dilution of 10^{3-} and is macroscopically visible after 3–5 days. Enrichment cultures may be obtained in the same manner, using a drop of natural *Gallionella* sediment for inoculation. Cultures are transferred every 4 weeks.

Preservation of *Gallionella* culture material in viable form for at least 13 weeks has been reported by Nunley and Krieg (1968) using the following procedure.

Culture Preservation by Freezing (Nunley and Krieg, 1968)

> The organisms were centrifuged at 3000 × *g* for 3 minutes, resuspended in the fluid portion of fresh Wolfe's medium containing 15% glycerol, and stored in 1-ml quantities in a low-temperature cabinet at –80°C. Survival was tested by transfer to Wolfe's medium at weekly intervals; in every case, the organisms were viable, forming a distinct mat of growth on the submerged ferrous sulfide agar after 7–10 days.

Batch Cultures in Erlenmeyer Flasks for Physicochemical Studies in the Optimal *Gallionella* Growth Zone

> In order to obtain the described physicochemical growth conditions, FeS (100 ml of FeS precipitate or 100 ml of 1.5% FeS agar) is added to the sterile culture medium (2 liters) in 2-liter Erlenmeyer flasks (silicon stoppers with glass tubes for bubbling) and then bubbled with 94% N_2–5% CO_2–1% O_2 up to pH 6 (gassing takes approximately 90 min). Then, pH, O_2, and Eh values are checked electrometrically and the medium is additionally gassed with N_2 and CO_2 to adjust to the required conditions (see above). The inoculation is with a suspension containing 10–15 *Gallionella* colonies (2 mm in diameter) into 10 ml of mineral medium. *Gallionella* growth becomes macroscopically visible after a lag phase of 4–6 days, during which the redox potential of the culture medium is lowered 300–400 mV by the bacteria (Hanert, 1975).

Fermentor Cultures in Mineral Ferrous Bicarbonate Medium

> A clear $Fe(HCO_3)_2$ medium is made by adding 200 ml of FeS precipitate to 10 liters of modified Wolfe's medium, and CO_2 is bubbled through for approximately 24 h until a Fe(II) concentration of 50 ppm (pH 4.6) is reached. After FeS sedimentation, 4.5 liters of the supernatant, combined with 4.5 liters of modified Wolfe's medium

"without Fe(II)," is filtered into the autoclaved fermentor through a membrane-filter apparatus containing coarse prefilter (10-µm pore size) followed by a Sartorius membrane filter (0.2-µm pore size) using a gas mixture of 94% N_2–5% CO_2–1% O_2 under 2 atm pressure. This filtration results in 9 liters of culture volume with 25 ppm ferrous iron. For measurement, control, and eventual readjustment of the physicochemical conditions with N_2 or CO_2 gas, the solution is left in the fermentor vessel for 24 h. The conditions are so reductive that no autoxidation of the ferrous iron occurs (culture temperature, 17°C). Inoculation is with 10–15 colonies (4 mm in diameter), suspended in 10 ml of medium. In contrast to normal fermentation cultures, the medium should be only moderately stirred (25 rpm) and gassed (1 bubble/2 s).

Gallionella growth becomes macroscopically visible after 2 days (white flakes on the walls of the vessel; after 5 days, the entire Fe(II) in the culture medium has been oxidized. *Gallionella* grows on all the inner surfaces of the culture vessel without gradient formation. This procedure seems suitable for the development of *Gallionella* fermentor cultures with a continuous flow of culture solution, during which it is particularly important to prevent contamination of the culture (changing of redox potential during *Gallionella* growth; Hanert, 1975).

Identification

The characteristic, spirally twisted, stalk structure formed by rotation of the apical cell makes *Gallionella* very easy to identify. It is uncertain, though, whether the genus consists of one or more species. This question has repeatedly been discussed at length (Beger and Bringmann, 1953; Pringsheim, 1949; van Iterson, 1958; among others). These authors have pointed out—and rightly so—that in almost all cases the differentiation into species has been based only on differences visible under light microscopy in the bands of *Gallionella* collected in nature.

Studies on quantification of the iron content of the bands of pure cultures of *Gallionella ferruginea* (Hanert, 1975) have shown that, due to additional chemical-physical iron oxidation on the surface of the bands, these surfaces may vary greatly in morphology. Six stalk types have been differentiated. Thus, the differentiation into species on the basis of light-microscopic band differences is definitely unreliable.

The genus is presently differentiated into two species, *Gallionella ferruginea* (stalk consists of 40 filaments or more) and *Gallionella filamenta* (stalk consists of only 3–8 filaments), based on the electron-microscopic band differentiation of Balashova (1967a, 1968). But this differentiation is only a morphological definition in another dimension; it does not exclude the possibility that "filamenta" is only an underfed "ferruginea." It seems conceivable that the presumption of Pringsheim (1949), which we favor, is accurate—the genus may consist of only a single species *(Gallionella ferruginea)* and all the other described species are only ecological growth forms of this one. The proof of this presumption by the results of additional physiological investigations would show that this bacterium—unique in its physiology and stalk excretion—is not nearly as complex as *Gallionella* literature may give the impression.

Literature Cited

Baas-Becking, L. G. M., Wood, F. E. J., Kaplan, J. R. 1956. Biological processes in the estuarine environment. VIII. Iron bacteria as gradient organisms. Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen, Series B 59:398–407.

Balashova, V. V. 1967a. Enrichment culture of Gallionella filamenta n. sp. "In Russian, with English summary." Mikrobiologiya 36:646–650.

Balashova, V. V. 1967b. Structure of "stem" fibers of laboratory *Gallionella filamenta* culture "In Russian, with English summary." Mikrobiologiya 36:1050–1053.

Balashova, V. V. 1968. Taxonomy of the genus *Gallionella*. Microbiology "English translation of Mikrobiologiya" 37:590–598.

Balashova, V. V. 1969. The relationship of *Gallionella* to *Mycoplasma*. "In Russian." Doklady Akademii Nauk SSSR 184:1429–1432.

Balashova, V. V. 1974. Mikoplazmy i zhelezobakterii. 1–65. Akademiia Nauk SSSR, Insitut Mikrobiologii, Moscow.

Berger, H., Bringmann, G. 1953. Bisherige Anschauung über die Morphologie von *Gallionella* und neuere elektronenmikroskopische Befunde. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten, und Hygiene, Abt. 2 107:305–318.

Cholodny, N. 1924. Zur Morphologie der Eisenbakterien *Gallionella* und *Spirophyllum*.. Berichte der Deutschen Botanischen Gesellschaft 42:35–44.

Ehrenberg, C. G. 1836. Vorläufige Mitteilungen über das wirkliche Vorkommen fossiler Infusorien und ihre große Verbreitung. Poggendorf's Annalen der Physik und Chemie 38:213–227.

Engel, H., Hanert, H. H. 1967. Isolierung von *Gallionella ferruginea* Ehrenberg. Naturwissenschaften 54:147–148.

Ford, H. W. 1978. Fundamental characteristics of slime and ochre clogging in drainage and irrigation systems. Chicago, Winter Meeting American Society of Agricultural Engineers. 78:2538.

Garrels, R. M., Christ, C. L. 1965. Solutions, minerals, and equilibria. New York. Harper & Row. Tokyo, John Weatherhill.

Gebauer, A. 1978. Biochemische und physiologische Untersuchungen zur Autotrophie des Eisenbakteriums *Gallionella ferruginea* Ehrenberg. 1–62. Botanisches Institut der Technischen Universität Braunschweig. (Federal Republic of Germany).

Hanert, H. H. 1968. Untersuchungen zur Isolierung, Stoffwechselphysiologie, und Morphologie von *Gallionella*

ferruginea Ehrenberg. Archiv für Mikrobiologie 60:348–376.

Hanert, H. H. 1970. Struktur und Wachstum von *Gallionella ferruginea* Ehrenberg am natürlichen Standort in den ersten 6 Std der Entwicklung. Archiv für Mikrobiologie 75:10–24.

Hanert, H. H. 1973a. Quantifizierung der Massenentwicklung des Eisenbakteriums *Gallionella ferruginea* unter natürlichen Bedingungen. Archiv für Mikrobiologie 88:225–243.

Hanert, H. H. 1973b. Rezente marine Eisenerze auf Santorin, Griechenland. II. Bakterogenese von Eisenhdroxidsedimenten. Geologische Rundschau 62:786–812.

Hanert, H. H. 1974a. Untersuchungen zur individuellen Entwicklungskinetik von *Gallionella ferruginea* in statischer Mikrokultur. Archiv für Mikrobiologie 96:59–74.

Hanert, H. H. 1974b. In situ-Untersuchungen zur Analyse und Intensität der Eisen(III)-Fällung in Dränagen. Zeitschrift für Kulturtechnik und Flurbereinigung 15: 80–90.

Hanert, H. H. 1975. Entwicklung, Physiologie und Ökologie des Eisenbacteriums *Gallionella ferruginea* Ehrenberg —Beiträge zu einer Monographie. 1–104. Habilitationsschrift, Naturwissenschaftliche Fakultät der Technischen Universität Braunschweig.

Hanert, H. H. 1981a. Bakterielle und chemische Eisen(II)-Oxidation auf Palaea Kameni—Stereoscan, Elektronenstrahl-Mikroanalyse (FeKα) und Photometrie von in situ-Experimenten. Puchelt, H., Schroll, E. (ed.) Genesis of marine iron sediments from Santorini, Greece. Berlin, Springer-Verlag.

Hanert, H. H. 1981b. The genus *Gallionella*. 509–515. Starr, M. P., Stolp, H., Trüper, H. G., Balows, A., and Schlegel, H. G (ed.) The prokaryotes. Berlin, Springer-Verlag.

Hem, J. D. 1972. Chemical factors that influence the availability of iron and manganese in aqueous systems. Geological Society of America Bulletin 83:443–450.

Hirsch, P., Pankratz, J. T. 1970. Study of bacterial population in natural environments by the use of submerged electron microscope grids. Zeitschrift für Allgemeine Mikrobiologie 10:599–605.

Khrutskaya, Z. Ta. 1970. Clogging of drains with iron compounds. Moscow, M. "Kolos."

Kucera, S., Wolfe, R. S. 1957. A selective enrichment method for *Gallionella ferruginea*. Journal of Bacteriology 74:344–349.

Lieske, R. 1911. Beiträge zur Kenntnis der Physiologie von *Spirophyllum ferrugineum* Ellis, einem typischen Eisenbakterium. Jahrbücher für Wissenschaftliche Botanik 49:91–127.

Martin, J. P., Dumke, D. S., Meek, B., Ervin, J. V., Grass, L. 1978. Microbiological observations and chemical analyses of tile line drainage waters and deposits in Imperial Valley, California. 423–426. Loutit, M. W., Miles, J. A. R. (ed.) Microbial ecology. Berlin, Springer-Verlag.

Naumann, E. 1919. Eine einfache Methode zum Nachweis bzw. Einsammeln der Eisenbakterien. Berichte der Deutschen Botanischen Gesellschaft 37:76–78.

Nunley, J. W., Krieg, N. R. 1968. Isolation of *Gallionella ferruginea* by use of formalin. Canadian Journal of Microbiology 14:385–389.

Poindexter, J. S., Lewis, R. F. 1966. Recommendations for revision of the taxonomic treatment of stalked bacteria. International Journal of Systematic Bacteriology 16: 377–382.

Pringsheim, E. G. 1949. Iron bacteria. Biological Reviews of the Cambridge Philosophical Society 24:200–245.

Sharpley, J. M. 1961. The occurrence of *Gallionella* in salt water. Applied Microbiology 9:380–382.

Suessenguth, K. 1927. Zur Kenntnis der Eisenbakterien der *Gallionella*-Gruppe. Zentralblatt für Bakteriologie, Parasitenkunde, und Infektionskrankheiten, Abt. 2 69:327–339.

van Iterson, W. 1958. *Gallionella ferruginea* Ehrenberg in a different light. Verhandelingen der Koninklijke Nederlandse Akademie van Wetenschappen, Afdeling Natuurkunde, Series 2 52:1–185.

Volkova, O. J. 1939. The ferrobacteria of the mineral waters of Caucasian mineral waters district and their role in the formation of ferrous sediments. "In Russian." Mikrobiologiya 8:863–887.

Vouk, V. 1960. Ein neues Eisenbakterium aus der Gattung *Gallionella* in den Thermalquellen von Bad Gastein. Archiv für Mikrobiologie 36:95–97.

Walsh, F. M., Mitchell, R. 1969. Acid production by *Gallionella ferruginea* from coal-associated iron sulfides. Bacteriological Proceedings 69:25.

Walsh, F. M., Mitchell, R. 1970. Filterable iron autotroph. Bacteriological Proceedings 70:31–32.

Winogradsky, S. J. 1888. Über Eisenbakterien. Botanische Zeitung 46:262–270.

Winogradsky, S. 1922. Eisenbakterien als Anorgoxydanten. Zentralblatt für Bakteriologie, Parasitenkunde, und Infektionskrankheiten, Abt. 2 57:1–21.

Wolfe, R. S. 1958. Cultivation, morphology, and classification of the iron bacteria. Journal of the American Water Works Association 50:1241–1249.

Zavarzin, G. A. 1961. Budding bacteria. "In Russian." Mikrobiologiya 30:952–975.

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CHAPTER 14.8

The Genera *Caulococcus* and *Kusnezovia*

JEAN M. SCHMIDT AND GEORGI A. ZAVARZIN

Perfil'ev and Gabe (1965) observed bacteria of the genera *Caulococcus* and *Kusnezovia*, along with *Metallogenium* and *Siderococcus,* as manganese- and, to a lesser extent, iron-depositing or -oxidizing organisms of mud and sandy de-posits. The use of capillary devices that permit microscopic observation (peloscopes) has permitted some observations of structure and colonial morphologies. Their morphologies suggest that these organisms might be related to *Acholeplasma, Metallogenium,* or *Siderococcus,* but lack of axenic cultures has prevented thorough study of their physiology and composition; understanding of the nature of these rather rare bacteria is quite meager.

Habitats

Caulococcus has been found in samples of mud and sandy deposits as observed with microcapillary techniques (Kutuzova et al., 1972; Perfil'ev and Gabe, 1961; Perfil'ev et al., 1965). It was originally found in Lake Khepo-Yarvi (Karelian Isthmus), "especially in ore deposits," and was "frequently observed in the upper layers of bottom mud deposits, above the reducing horizon," or in the bottom water (periphyton) over the mud (fine mineral or sandy) surface (Perfil'ev et al., 1965).

Kusnezovia was observed, using the peloscope technique, only "in mud samples from Lake Ukshezero, Karelian ASSR" (Perfil'ev et al., 1965), and in secondary profiles in zones of manganese oxidation of mud samples stored in the laboratory.

As described by Perfil'ev and Gabe (1965), in capillary peloscope samples from many ore-bearing lakes, for all those "with a black-orange microzone, the blackish-brown horizon of the microzone in the peloscope canals showed a mass-development of *Metallogenium,* rarely *Caulococcus*, and sometimes *Kusnezovia*, the biogenic deposits of which consist mostly of manganese oxides."

Isolation

Neither *Caulococcus* nor *Kusnezovia* has been isolated in pure culture. Distinctive morphologies, particularly those of microcolonies using microcapillary techniques, have been used to define these two groups (Perfil'ev et al., 1965).

Organisms could be maintained in the isolated samples of mud (Perfil'ev et al., 1965): a sample of water (approximately $^1/_3$) and mud ($^2/_3$) from the natural habitat was mixed thoroughly and incubated in a 200-ml beaker at room temperature for months. Peloscopes were placed across the upper 3 cm of the mud. Development of manganese- and iron-depositing organisms was observed to occur in sharp, horizontal microzones (a few millimeters in width) in the upper layers of mud following the reduction of metal oxides in the bottom layers.

Identification

In the absence of axenic cultures and information on cultural traits, colonial morphology is used, along with the characteristic manganese deposition, for tentative recognition of these kinds of bacteria. To observe cell morphology, Perfil'ev and Gabe (1965) dissolved manganese deposits with 0.2–1.0% oxalic acid, washed them with distilled water, and stained them with a carbol solution of gentian violet. All steps were monitored with the light microscope. The bacteria appeared as minute bodies without sharp contours and appeared to reproduce by budding. They were embedded in a matrix or connected with thin filaments. For identification, the original illustrations of Perfil'ev et al. (1965) should be consulted. Some other characteristics are given in Table 1.

Colonial morphology is variable. Three sorts of microcolony morphologies have been described for *Caulococcus manganifer*. The most commonly observed microcolonies occur as dense, irregular clusters, the surfaces of which appear ribbed. The central portion of the microcolony is more heavily mineralized than the periphery, due to manganese deposition. The mineralized colonies are blackish brown in color.

This chapter was taken unchanged from the second edition.

Table 1. Characteristics of *Caulococcus* and *Kusnezovia* spp.

Trait	*Caulococcus manganifer*	*Kusnezovia polymorpha*
Coccoid cell diameter (μm)	0.5	0.5–1.5
Connecting filament diameter (μm)	0.1	0.1–0.2
Mode of reproduction	Budding (?)	Budding (?)
Motility	Present	Not observed
Gram reaction	Not reported	Not reported
Zoogleal accumulations	Present	Not observed
Oxygen relationship	Microaerophilic	Microaerophilic (presumed)
Occurrence	Rare, in iron-manganese ore from several lakes (Karelian Isthmus, ASSR)	Extremely rare, found in only one lake (Ukshezero, Karelia)

Occasionally, colonies have radial arms (termed "radial lobate" colonies); the rarest colonial form in *Caulococcus* microaccretions is the trichospherical colony with very fine, radiating processes.

Kusnezovia polymorpha microcolonies (polymorphous cenobia) also occur in a variety of forms: filamentous sprouts, open lily-of-the-valley leaves, or goblets with a toothed margin, with the wider portion of the microcolony pointing downward in the peloscope canals. Candelabra-like and clavate, filamentous cenobia structures attached to the peloscope wall, with an appearance analogous to *Cladonia* (a bryophyte), have also been attributed to *Kusnezovia*. Removal of manganese from these deposits revealed the cocci, connected by thread (Perfil'ev et al., 1965).

These organisms will require further attention in order to distinguish them from other quite similar manganese depositers, such as *Metallogenium.* In fact, we strongly suspect that these genera, particularly *Caulococcus*, are merely growth forms of *Metallogenium* in a certain habitat. The description of *Siderococcus* (see The Genus *Siderocapsa* (and Other Iron- and Maganese-Oxidizing Eubacteria in this Volume) should be consulted, since these iron-depositing organisms are very probably members of the same group, although they are not presently classified as such.

Literature Cited

Kutuzova, R. S., Gabe, D. R., Kravkina, I. M. 1972. Electron microscope study of overgrowths of iron-manganese microorganisms from ooze. Microbiology "English translation of Mikrobiologiya" 41:973–977.

Perfil'ev, B. V., Gabe, D. R. 1961. Capillary methods of investigating microorganisms. "In Russian." Moscow: Izdatel'stvo Akademii Nauk SSSR. Shewan, J. M., translator, 1969. Toronto, University of Toronto Press.

Perfil'ev, B. V., Gabe, D. R., Gal'perina, A. M., Rabinovich, V. A., Sapotnitskii, A. A., Sherman, É. É., Trosanov, É. P. 1965. Applied capillary microscopy: The role of microorganisms in the formation of iron-maganese deposits. Translated from the Russian by F. L. Sinclair. New York, Consultants Bureau.

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CHAPTER 14.9

The Genus *Brachyarcus*

PETER HIRSCH

Light-microscopic investigation of the microorganisms in the hypolimnion of many lakes shows the presence of a highly diverse population of bacteria. Here, most of the recognizable forms have never been cultured and many of them have not even been described morphologically. Among these bacteria are rod-shaped organisms that are bent in an arc by asymmetric, polar cell growth and that frequently assume the shape of a pretzel. Cell division in the center and separation result in two arcs that face each other. Additional cell divisions and the presence of masses of polymer lead to even numbers of arcs facing each other in a symmetric, mirror-image way (Fig. 1).

Phase-contrast light microscopy, using slides coated with water agar to improve resolution, reveals the presence of gas vesicles in most of these organisms. The wide distribution and often the high cell numbers of such arc-shaped bacteria warrant attempts to cultivate and study them, but such attempts have been unsuccessful. Skuja (1964) found similar bacteria in a Lappish lake, and he described them as *Brachyarcus thiophilus*. The species name was used to suggest a possible sulfur metabolism, since the cells occasionally possessed intracellular structures resembling sulfur globules.

Since *Brachyarcus* spp. have never been cultivated, not even in enrichments, their possible identification presents some problems. In fact, a rodlike, arc-shaped cell shape is quite common among other bacteria. But the cell arrangement after division, the presence of gas vesicles, the absence of photosynthetic pigments, and the frequent observation of these bacteria among other "thiophilic" organisms may indeed allow a recognition sufficient for a descriptive treatment here, as has been done in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Hirsch, 1974).

This chapter was taken unchanged from the second edition.

Habitats

The original observations were made by Skuja (1964) on a hypolimnion water sample collected April, 1953, at a depth of 12–13.5 m in Lake Vuolep Njakajaure, Swedish Lappland (408 m above sea level). The water sample had a temperature of 2°C; the lake was ice-covered. Together with *Brachyarcus thiophilus*, large numbers of other "thiobiontic" bacteria were found, many of which contained sulfur storage globules but none of which has ever been cultured (for nomenclature, see Skuja, 1964), e.g., *Macromonas bipunctata*, *M. mobilis*, *Gigantomonas cucullata, G. capitata, Thiovulumspora, Hyalobotrys hypolimnicus.* Also present in this sample were some photosynthetic bacteria *(Thiocystis violacea, Pelochromatium roseum consortium)* and sulfate reducers *(Microspira desulfuricans).*

Similar crescent-shaped organisms have been found by other authors (Table 1) in various lakes or ponds. Bacteria of such cell arrangement and/or shape were also found in soil (Nikitin, 1971, 1973; *"Renobacter vacuolatum"*) or even in reindeer rumen (Tarakanov, 1972). The latter bacteria were $0.4–0.5 \times 1.1–1.2$ μm and occurred in the above-described cell-plate arrangements. Their shape, however, was more that of a vibrio than that of an arc.

Isolation

Although isolation has not yet been successful, the data on occurrence in Table 1 allow us to speculate on possible enrichment techniques. In Wintergreen Lake, temperatures in the *Brachyarcus* layers during the time of observation varied between 11° and 20°C, with the maximum cell density of *Brachyarcus* spp. at 13°C (depth, 5 m). In the other sites—as far as data are available—temperatures ranged from 5° to 12.5°C. These observations indicate that *Brachyarcus* is psychrophilic.

In all cases observed, the organism occurred under anaerobic conditions. A pH of 7.5 and a phosphate concentration below 2.56 mg/liter would be additional parameters. No information is available on the carbon nutrition, although one could guess that *Brachyarcus* (being nonphotosynthetic) would have a chemoorganotrophic metabolism. Their presence in strata that contain methane (Wintergreen Lake) or reducible sulfate (Lake Vuolep Njakajaure) points to preference for a low redox potential in the environment. Storage of intracellular sulfur globules indicates participation in the sulfate reduction or sulfide oxidation processes.

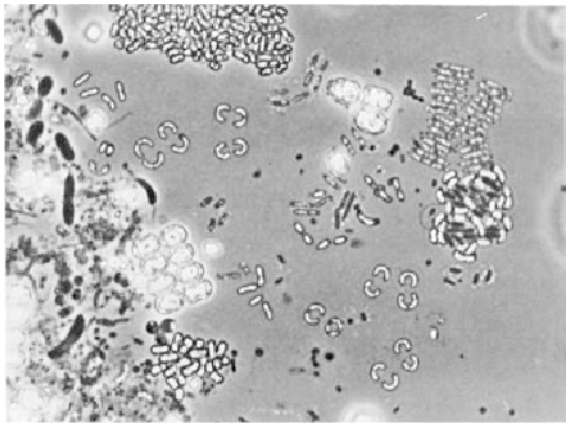

Fig. 1. Arc-shaped cell families of a *Brachyarcus* sp. from Wintergreen Lake (Michigan), collected from 4 m. Magnification × 1,280.

Identification

Brachyarcus Skuja 1964, 19, is a genus of colorless rod-shaped bacteria of approximately 1.0 × 1.5–2.5 µm that usually contain few to many gas vesicles (Fig. 1). Occasionally, globules resembling sulfur were observed by Skuja (1964), but these have not been reported by other authors nor have they been seen by this author. The cells are bent like a bow or arc, almost to a circle or even a pretzel shape. Upon division, a 3-shape is initially assumed until two semi-circles lie opposite each other, embedded in a thick polymer capsule with diffuse edges (Fig. 1). Families of two to 16 synchronously dividing cells are common; they often form cell plates, which may be free floating or grow attached to a surface. Three-dimensional, free-floating families of polymer-embedded cells of up to 100-µm family size or larger have been observed. The type species is *Brachyarcus thiophilus* Skuja 1964, 20, so named for the "thiophilic" environment in which it has been found.

Table 1. Occurrence of *Brachyarcus*-like bacteria.

Location	Date	Depth (m)	Temperature (°C)	Remarks	Reference
Lake Vuolep, Njakajaure (Lappland)	April 1953	12–13.5	2		Skuja, 1964
Burke Lake (Michigan)	23 Aug. 1972	9	6	Anaerobic; sulfide +	Caldwell and Tiedje, 1975
Cassidy Lake (Michigan)	1 Aug. 1968	7	8.5	Anaerobic; sulfide +	P. Hirsch, unpubl.
	11 Aug. 1970	7	7.6	Anaerobic; sulfide +; pH, 7.12	P. Hirsch, unpubl.
Knaack Lake (Wisconsin)	14 May 1975	17.5	5	Anaerobic	P. Hirsch, unpubl.
Wintergreen Lake (Michigan)	23 July 1970	4	16.5	O_2, 0.22 mg/liter; pH, 7.74	P. Hirsch, unpubl.
	22 July 1975	3.5–6.0	20–11	Anaerobic; pH, 7.8–7.3; DOC, 9–7 mg/liter; POC, 2–5 mg/liter; methane, 60–320 µM/liter × 10^{-3}; PO_4^{3-}, 0.23–2.56 mg/liter; max. distribution at 5 m (= 13°C)	P. Hirsch, unpubl. (M. Klug, R. Wetzel, unpubl.)
Lake Blunkersee (Holstein)	16 Sept. 1975	8	12.5	Anaerobic; sulfide +	P. Hirsch, unpubl.
Lake Plußsee (Holstein)	17 June 1967	27	5.5	Anaerobic	P. Hirsch, unpubl.
Forest Pond, Augusta (Michigan)	15 Oct. 1968	0.25–0.51	15.5	Anaerobic; sulfide (+)	P. Hirsch, unpubl.
	17 Nov. 1968	0.55–0.65	6	Anaerobic; sulfide +	P. Hirsch, unpubl.
Arco Lake (Minnesota)	Sept. 1972	6.5–9		Anaerobic ?	Walsby, 1974
	May, June 1973	6.5–9			Walsby, 1974

Identification is still largely on the basis of morphology. Cells or cell aggregates of similar shape are formed by the purple bacterium, *Rhodocyclus* (Rhodospirillaceae), or by the green bacteria, *Chlorobium vibrioforme* and *Pelodictyon luteolum* (Gorlenko, Dubinina, and Kuznetsov, 1977; Pfennig, 1978). The former two bacteria lack gas vesicles while *P. luteolum* does contain them. Since *Brachyarcus* has been described (Skuja, 1964) and has subsequently been found to be colorless (see Table 1), differentiation from these photosynthetic bacteria should be possible.

Morphological similarity with a colorless, gas-vacuolated soil bacterium called *Renobacter vacuolatum* (Nikitin, 1971, 448; 1973) also holds true for the size (0.7–1.0 × 1.6–1.8 μm). However, this bacterium appears to grow out, before cell division, to an S-shape rather than a 3-shape or pretzel. Similarity also exists with *Microcyclus* spp. Electron micrographs of the nonvacuolated *Microcyclus marinus* (Raj, 1977) or the gas-vacuolated strains of *M. aquaticus* (Van Ert and Staley, 1971) show cells that appear to be more vibrioid; in pure culture, the characteristic orderly arrangement in symmetric cell plates, as seen in *Brachyarcus* (in nature), is not seen in the genus *Microcyclus.* Furthermore, both of the *M. aquaticus* strains that were studied in greater detail had temperature optima of 37° and 30°C, respectively, and neither strain grew anaerobically. *Brachyarcus thiophilus* can be expected to be an anaerobic psychrophile.

Acknowledgments. Information on *Brachyarcus* kindly supplied by H. Skuja in 1968 is gratefully acknowledged. M. Klug and R. Wetzel (Kellogg Biological Station, Michigan State University) supplied data on Wintergreen Lake for 22 July 1975.

Literature Cited

Caldwell, D. E., Tiedje, J. M. 1975. A morphological study of anaerobic bacteria from the hypolimnia of two Michigan lakes. Canadian Journal of Microbiology 21:362–376.

Gorlenko, V. M., Dubinina, G. A., Kuznetsov, S. I. 1977. Ecology of water microorganisms. [In Russian.]. Moscow, Isdatel'stvo Nauka, Akademiya Nauk SSSR.

Hirsch, P. 1974. Genus *Brachyarcus* Skuja 1964. 216. Buchanan, R. E., Gibbons, N. E. (ed.) Bergey's manual of determinative bacteriology, 8th ed. Baltimore, Williams & Wilkins.

Nikitin, D.I. 1971. A new soil organism—*Renobacter vacuolatum* gen. et sp. n. (In Russian, with English summary.). Doklady Akademiia Nauk SSSR. 198:447–450.

Nikitin, D. I. 1973. Direct electron microscopic techniques for the observation of microorganisms in soil. 85–92. Rosswall, Th. (ed.) Modern methods in the study of microbial ecology. Bulletin of the Ecological Research Committee no. 17.

Pfennig, N. 1978. *Rhodocyclus purpureus* gen. nov. and sp. nov., a ring-shaped, vitamin B_{12}-requiring member of the family Rhodospirillaceae. International Journal of Systematic Bacteriology 28:283–288.

Raj, H. D. 1977. *Microcyclus* and related ring-forming bacteria. CRC Critical Reviews in Microbiology 5:243–270.

Skuja, H. 1964. Grundzüge der Algenflora und Algenvegetation der Fjeldgegenden um Abisko in Schwedisch-Lappland. Nova Acta Regiae Societatis Scientiarium Upsaliensis, Seria IV, 18(3).

Tarakanov, B. V. 1972. Microflora of reindeer rumen studied with the electron microscope. Microbiology [English translation of Mikrobiologiya.] 41:769–772.

Van Ert, M., Staley, J. T. 1971. Gas-vacuolated strains of *Microcyclus aquaticus*. Journal of Bacteriology 108:236–240.

Walsby, A. E. 1974. The identification of gas vacuoles and their abundance in the hypolimnetic bacteria of Arco Lake, Minnesota. Microbial Ecology 1:51–61.

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CHAPTER 14.10

The Genus *Pelosigma*

PETER HIRSCH

The Danish scientist E. Warming observed, during a walk along Kallebo Beach in 1874, a reddish discoloration of mud and decaying algae. His microscopic investigation of a sample started the now-famous study of "bacteria living on Denmark's coasts" (Warming, 1875). One of the organisms he found in "stinking mud at the height of decay" at several locations was a highly unusual, flat and striated, motile "bacterium" (which he called *Spiromonas cohnii*) that resembled a flagellate *Spiromonas* previously described from Switzerland. Although Warming himself was not quite sure of what he saw, his drawings are remarkably accurate illustrations of bacteria that can still be found in hypolimnia and on anaerobic sediment surfaces.

About 40 years later, these organisms were observed again and described as the bacterial genus *Pelosigma* (Lauterborn, 1913, 1915). Lauterborn clearly stated that a bacterium could not belong to a flagellate genus, but otherwise his observations confirmed the findings of Warming. Another 40 years later, a study on Swedish lakes revealed *Pelosigma* organisms in many different locations (Skuja, 1956). But now the old theory that this organism was a flagellate—at least in part—was revived. Skuja was convinced that he had found another example of a flagellate/bacterium symbiosis, and he produced detailed drawings of various stages of such consortia. Nevertheless, the genus name *Pelosigma* was retained for the consortium for both the flagellate and the bacterium organisms.

Detailed microscopic investigations of aquatic microorganisms and the study of several lakes and ponds in the United States and northern Germany revealed the presence of *Pelosigma* spp. in many locations, often in large numbers (P. Hirsch, unpublished observation). These studies allowed a preliminary description and interpretation for the eight edition of *Bergey's Manual of Determinative Bacteriology* (Hirsch, 1974). Since then, many additional samples have been found to contain *Pelosigma* spp., and evidence is mounting that these are, indeed, bacteria living in sigmoid, bundle-shaped aggregates just as originally drawn by Warming (1875) and described by Lauterborn (1913, 1915). Unfortunately, all attempts to cultivate these organisms so far have failed. Suggestions for enrichment cultures can be made, however, on the basis of the observed occurrence.

Habitats

The original description (Warming, 1875) does not contain many details on the natural habitat. It is not even clear whether *Spiromonas* (= *Pelosigma*) *cohnii* was found in mud of salt water, brackish water, or fresh water. However, the location "Limfjord" suggests brackish water, and the purpose of the article by Warming was to describe bacteria living at the coasts. Warming listed four locations for *S. cohnii* and stressed its occurrence in mud that was most active in the decaying process. He stated also that *S. cohnii,* like other bacteria discussed later in his paper, did not contain granules such as those of the organism now known as *Chromatium okenii*. His description of these granules makes it quite likely that he meant sulfur globules. Although obviously an anaerobe, *S. cohnii* thus did live in a sulfide-rich environment but did not store elementary sulfur.

The habitat of *Pelosigma* (= *Spiromonas*) *palustre* (Lauterborn, 1913) was likewise poorly described: fresh water; in the decaying sediment especially in ponds with characean algae.

The observations of Skuja (1956) could be added to the habitat description, if one accepts the interpretation that his *Pelosigma* organisms indeed were identical with those seen by Warming and Lauterborn. Skuja found *Pelosigma* spp. in Swedish lakes (Hönsan, Lushavet, Munkbosjön, Tjärnatjärn), usually at depths ranging from 10 to 14 m. Although he did not describe these environments chemically or physically, some information can be obtained from the list of accompanying bacteria. Skuja mentioned *Peloploca* spp. *(P. pulchra, P. fibrata, P. taeniata), Macromonas mobilis, Achroonema* sp.,

This chapter was taken unchanged from the second edition.

Table 1. Occurrence and morphological characteristics of *Pelosigma* spp. and some data on their environment.

Site	Time	Depth (m)	Length of aggregate (μm)	Width of aggregate (μm)	No. cells per aggregate	Width of cells (μm)	Flagella (no.)	Remarks
Lake Plußsee (Holstein)	June 1967	27	20.3	ND[a]	4 + 4	ND	1	T = 5.5°C; O_2(–)
		29	19.0	ND	4 + 3	0.31	0	T = 5°C; O_2(–)
		30	19.0	ND	12;6;8;4	ND	0	T = 5°C; O_2(–); very numerous
		31	19.0	ND	4 + 2	ND	0	T = 5°C; O_2(–)
Little Lake (Wisconsin)	May 1975	12	19.5	ND	3 + 4	ND	1	T = 4°C; O_2(–); actively motile
Wintergreen Lake Michigan	Aug. 1970	4.5	29.7	10.2	Numerous	0.23	0	O_2(–)
		4.5	19.5	5.1	4 + 4 (+4)	ND	0	O_2(–)
	July 1975	4.0	18.7	ND	4 + 4	ND	0	T = 19°C; O_2(–); pH = 7.65; S^{2-}+; CH_4+
		5.5	19.3	ND	4 + 4 + 4	ND	0	T = 10.5°C; O_2(–); pH = 7.25; S^{2-}+; CH_4+
		6.0	19.5	ND	4 + 4	ND	0	T = 10°C; O_2(–); S^{2-}+; CH_4+
Forest Pond (Michigan)	July 1968	0.7	19.6	10.1	3 + 8	ND	0	T = 17.5°C; O_2(–); S^{2-}(+)

[a]Not determined.
Data from P. Hirsch, unpublished observations.

Pelonema tenue, Achromatium oxaliferum, *Gigantomonas cucullata, Ochrobium tectum, Beggiatoa arachnoidea*, *Leukobium maior, Pelochromatium roseum,* and *Thiocystis violace* (nomenclature sensu Skuja, 1956). These bacteria are generally known to be anaerobic or microaerophilic, hypolimnetic organisms, many of which are found in sulfide-rich environments. Thus, we can expect the presence of low sulfide concentrations in an otherwise mostly anaerobic and dark environment.

More information comes from recent observations on *Pelosigma* habitats (Table 1; P. Hirsch, unpublished). The presence of *Pelosigma* spp. appears to be correlated with anaerobic conditions and, generally, temperatures below 11 °C. Like *Brachyarcus* spp., often found in the same location, *Pelosigma* spp. can be expected to be psychrophilic. It should be pointed out, however, that *Pelosigma* spp. have been found occasionally at higher temperatures.

The presence, in Wintergreen Lake (Michigan), of sulfide together with measurable concentrations of dissolved methane during *Pelosigma* occurrence points to a need of these organisms for a low redox potential.

Isolation

From the data given by Warming (1875), by Lauterborn (1915), by Skuja (1956), and in Table 1, an enrichment procedure could be conceived. It would consist of inoculating an anaerobic medium of pH 7.5 that contains organic matter (such as organic acids, amino acids, etc.), a mixed vitamin solution and, perhaps, a low concentration of sulfide (10^{-4} M or less). The sample for inoculation should be drawn anaerobically, and it should be kept at temperatures below 10°C. Incubation would be in the dark at 5–10°C.

Identification

All *Pelosigma* spp. observed were more or less flat, band-shaped aggregates of slender, long, and S-shaped bacterial rods. The aggregates appear to be formed preferentially by four cells or by multiples of four. At least one end of the aggregates appears to be more pointed. If a flagellar structure is present, it is located at the more pointed end. The overall length of the aggregates (9–25′30′ μm) is not the length of the individual, component rod, since uneven elongation and cross-division occur. The maximum width of aggregates is 5–11 μm and depends on the number of rods in the aggregate (Fig. 1).

At present, there are two species recognized (Hirsch, 1974): *Pelosigma cohnii* (Warming) Lauterborn 1913, 100 (synonym: *Spiromonas cohnii* Warming 1875, 370) and *P. palustre* Lauterborn 1915, 418. Both species show great morphological similarity but differ in size. Aggregates of *P. cohnii* are 1.2–4 μm wide and 9–20 μm long, while those of *P. palustre* are 8–10 μm wide and 20–25 μm long. The aggregates of *P. cohnii* are usually pointed at both ends

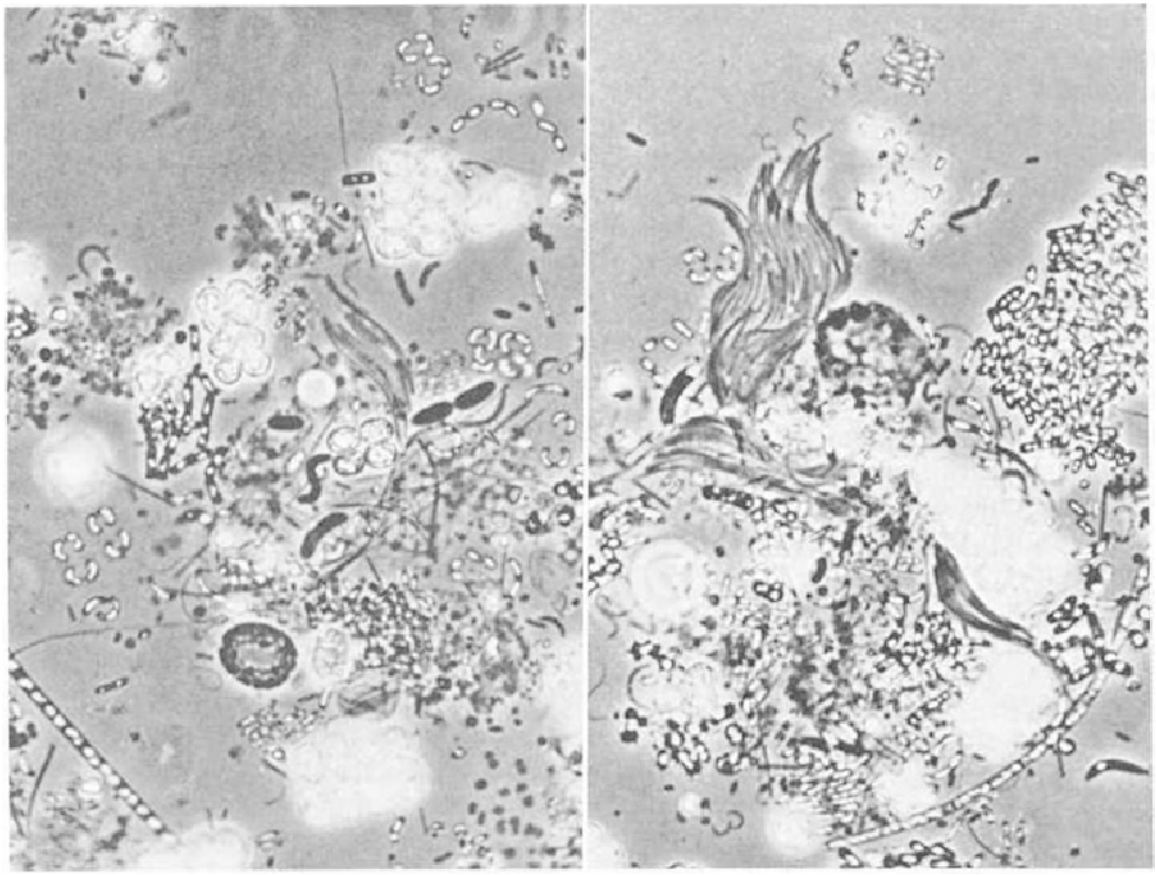

Fig. 1. A mixed bacterial flora with *Pelosigma* palustre cell aggregates from Wintergreen Lake (Michigan), collected from 4.5 m on 5 August 1970. × 1,280.

(and often flagellated on both ends), they are actively motile, and the individual cells measure approximately 0.4 µm in diameter (from the drawings of Warming, 1875). The aggregates of *P. palustre* normally show only one pointed end; they are much less actively motile, and flagella have not been seen. They occur in freshwater habitats.

The *Pelosigma* aggregates found by Skuja (1956) were thought to surround a colorless flagellate which multiplied by longitudinal fission. One to eight (usually four) colorless, S-shaped rods were kept by the flagellate "subpellicularly" with the ends sometimes sticking out. The aggregate (consortium) of bacteria and flagellate was described as "*Pelosigma cohnii*"; the overall sizes were 1–7 × 11–27 µm. The bacterial component was 0.3–0.4 × 7–15 (20) µm in size, colorless, without any granules, and sometimes yellowish pigmented. Cross-division was not synchronous with the longitudinal fission of the monad. The consortium did not have two flagella; the aggregates seen by Warming were likewise not supposed to have front and rear flagella—despite the fact that Warming (1875) mentions these and has actually drawn three double-flagellated aggregates (Table VII, Fig. 4a, 4c by Warming). The consortium was thought to be "oligothiophilic" and possibly involved in the decomposition of cellulose and/or hemicelluloses.

The aggregates described in Table 1 (Fig. 1) were, in general, 19.5 µm long and either 5–6 or 10–11 µm wide; thus, they resembled *P. palustre*. One sample (Wintergreen Lake, 4.5 m, August 1970) had much larger aggregates with thinner cells. None of the numerous aggregates seen contained anything that could have resembled a flagellate. Only two cases of flagellation and motility were seen; in both cases, the flagellum had the appearance of bacterial flagella, with a permanent, constant wavelength. Therefore, the author believes that such visible flagella constitute tufts of several prokaryotic flagella, attached polarly to each individual bacterium and combined to form the visible structure. Another argument against a eukaryotic flagellum would be the absence of two flagella from the "dividing" cell pole (as would be formed during longitudinal fission of a eukaryotic flagellate).

Instead, the *one* "flagellum" (tuft) is located on the nondividing cell pole. Occasional bipolar flagellation (as has been observed by Warming) would be expected to occur in polarly flagellated bacteria. The transparent area in between the aggregate's bacteria is probably filled with an extracellular, common polymer. It is also possible that there exist, in nature, *Pelosigma*-like consortia of S-shaped bacteria with colorless flagellates. Only observation of laboratory cultures and directly embedded and sectioned natural material could solve this problem. For the time being, it may be advisable to restrict the name *Pelosigma* to the bacteria that are agreed upon by all observers.

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Literature Cited

Hirsch, P. 1974. Genus *Pelosigma* Lauterborn, 1913. 215–216. Buchanan, R. E., and Gibbons, N. E. (ed.) Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins Baltimore.

Lauterborn, R. 1913. Zur Kenntnis einiger sapropelischer Schizomyceten. Allgemeine Botanische Zeitschrift 19: 97–100.

Lauterborn, R. 1915. Die sapropelische Lebewelt. Ein Beitrag zur Biologie des Faulschlammes natürlicher Gewässer. Verhandlungen des Naturkundlich-Medizinischen Vereins zu Heidelberg 13:395–481.

Skuja, H. 1956. Taxonomische und biologische Studien über das Phytoplankton schwedischer Binnengewässer. Nova Acta Regia Societatis Scientiarum Upsaliense, Seria IV 16 (3).

Warming, E. 1875. Om nogle ved Danmarks kyster levende Bakterier. Videnskaplige Meddelelser fra den naturhistoriske Forening i Kjöbenhavn 30:Nr. 20–28, 307–420.

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CHAPTER 14.11

The Genus *Siderocapsa* (and Other Iron- and Manganese-Oxidizing Eubacteria)

HANS H. HANERT

This chapter discusses those species of *Siderocapsa* and members of the family Siderocapsaceae (Pribram, 1929) whose morphology, ecology, and, to some extent, culture physiology have been so exactly described that, in spite of disagreement concerning their taxonomic position, there can be no doubt of their existence and great ecological significance. The discussion concentrates on such existing and ecologically significant representatives of this family in order to preserve the practical orientation of this Handbook. Information on many genera and species whose position is uncertain has been drawn together in *Bergey's Manual of Determinative Bacteriology,* eighth edition (Buchanan and Gibbons, 1974; contributor G. A. Zavarzin) and seventh edition (Breed et al., 1957; contributor R. S. Breed in cooperation with H. Beger) and by Dorff (1934). A comparison of the genera and species treated in these references provides a bird's-eye view of this entire group of organisms.

The family Siderocapsaceae is defined as unicellular, nonfilamentous or nonstalkforming, iron and/or manganese bacteria that are able to deposit these metal oxides under natural conditions, on or in capsules or on extracellular mucoid material excreted by these bacteria. This definition is taxonomically rather imperfect, but for practical purposes it is quite adequate. That the validity of this family, in particular of the species of the genus *Siderocapsa*, has often been questioned is to be ascribed exclusively to the present taxonomic inadequacy of the definition. Such questions have been raised most recently by Dubinina and Zhdanov (1975); but a final clarification of the taxonomic question will not be possible until the complete developmental cycle of these bacteria in their natural habitat has been clearly described. Culture experiments are doubtless very helpful here, but they cannot provide a final clarification of the taxonomic question as long as the natural development of the organisms remains unknown.

Merely because there have been controversial questions of taxonomy it does not follow that these bacteria do not exist. Their existence and great ecological significance have become firmly established in the last decades through the discovery of their mass development in the hypolimnion of many lakes.

Habitats

Occurrence and Natural Mass Development

Siderocapsaceae are widely distributed in nature (see Table 1). They are found in all habitats which contain iron and manganese. They were first found in meadow and swamp ditches and mesosaprobic flowing streams, as well as in stagnant waters (Dorff, 1934; Drake, 1965; Galinsky and Hanert, 1979a; Hardman and Henrici, 1939; Molisch, 1910; Naumann, 1921). The organisms occur in these habitats as epiphytes on submerged water plants or on growths of exposed slides, or they are free-living and neustonic in metallic, glossy, iron oxide films on the water. A second important source of these organisms is deep wells, pipes, and waterworks, as described by Beger (1949) and by Hässelbarth and Lüdemann (1967). Greatest development of Siderocapsaceae has been found in the hypolimnion of lakes, often near growths of *Metallogenium.* At present, five species of the genus *Siderocapsa* (see Table 1) have been shown to be planktonic, not sessile, and so are not susceptible to slide growth (Kalbe, 1965; Redinger, 1931; Skuja, 1948, 1956; Wawrik, 1956).

The occurrence of Siderocapsaceae is not limited to typical freshwater habitats. As the investigations by Ten (1967, 1968, 1969) have shown, the family Siderocapsaceae *(Naumanniella polymorpha)* occurs in brown forest soils and iron-manganese crusts on soil-forming rocks as well. A marine *Siderocapsa* has been found in the iron bay of Palaea Kameni in Santorini, Greece (Hanert, 1981).

In all habitats, the most characteristic feature of *Siderocapsa* is its capacity to form

This chapter was taken unchanged from the second edition.

Table 1. Spectrum of Siderocapsaceae habitats and environmental parameters.

Habitats	Organisms	Environment	Physicochemical basic conditions inferred from natural occurrence or culture experiments
Iron-rich meadow and swamp ditches, flat pools (often stagnant) and mesosaprobic flowing streams	*Siderocapsa*		
	S. treubii	Typically epiphytic (attached to submerged roots and leaves of water plants; also on growth on slides; mesosaprobic waters)	Aerobic, organotrophic, pH 6.2–8.7
	S. major	Neustonic and epiphytic	As above
	S. monoica	Epiphytic	Aerobic tending to microaerobic, organotrophic
	Siderocystis		
	S. vulgaris	Ongrowth in the upper water layer	Aerobic, organotrophic, pH 6–7
	S. confervarum	Forming large iron aggregates on algae	Aerobic, organotrophic, pH 6–7
Wells, pipes, and waterworks	*Siderocapsa*		
	S. botryoides		
	Naumanniella		
	N. pygmaea	All found in cool waters of pipes and deep wells by Beger (1949)	Microaerobic, psychrophilic, organotrophic
	N. elliptica		
	N. minor		
	N. catenata		
	Siderobacter		
	S. gracilis		
	S. brevis		
	S. latus		
Lakes (planktonic forms)	*Siderocapsa*		
	S. anulata	Epilimnetic (water surface up to 6 m)	Aerobic, pH around 8.0, organotrophic
	S. coronata	Generally neustic, mass development at time of vernal thaw, also found at depths of 17–20 m	Aerobic to microaerobic, organotrophic
	S. arlbergensis	Epilimnetic in alpine pools with mass development at time of thaw	Aerobic, organotrophic, ferous iron up to 4 ppm, pH 4.5–8.5
	S. eusphaera	Generally hypolimnetic in larger meso and eutrophic lakes, at depths of 6–30 m, mass development	Microaerobic to aerobic, organotrophic
	S. geminata	Generally hypolimnetic, epilimnetic occurrence during vernal and autumnal circulation, at depths of 0–26 m, mass development in lakes	Microaerobic to aerobic, organotrophic
	Metallogenium		
	M. personatum	Hypolimnetic with mass development in the chemocline zone, where O_2 disappears, at an iron and manganese content of 1–5 ppm	Microaerobic, organotrophic
Soils, bottom deposits of water bodies, mud, iron manganese crusts on soil-forming rocks	*Siderocapsa* sp. (Ten, 1967)	Different soil types	Aerobic to microaerobic, organotrophic
	Naumanniella		
	N. polymorpha	Iron-manganese crusts and brown forest soils	Aerobic to microaerobic, possibly mixotrophic by manganese oxidation
	*Sidero*coccus		
	S. limoniticus	Mud horizons and bottom deposits of fresh waters	Microaerobic, neutral pH

large masses of iron and/or manganese oxide. This mass development of Siderocapsaceae, like that of the classical stalk- and threadforming iron bacteria, is often visible to the naked eye. Such readily visible deposits usually develop during vernal and autumnal circulation or during vernal thaw in alpine lakes and pools; deposits have been reported from all planktonic lake Siderocapsaceae, as well as from some epiphytic *Siderocapsa species* (Sokolova, 1959). A yearly cycle of Siderocapsaceae development with vernal and autumnal maxima in swamp ditches has been demonstrated and quantified for *Siderocystis vulgaris* (Galinsky and Hanert, 1979b). Detailed measurements which give some impression of Siderocapsaceae mass development in nature were taken by Dubinina, Gorlenko, and Suleimanov (1973) in the meromictic lake Gek-Gel', using the membrane-filter method (filters stained with erythrosine and treated with potassium ferricyanide and hydrochloric acid). These determinations counted up to 60,000 cells/ml at a depth of 27–29.5 m in the zone of massive development of *Siderocapsa eusphaera* (distinct narrow zone below *Metallogenium* growth zone) and also demonstrated the gradient growth of this organism (see also Sorokin, 1968).

Aspects of Siderocapsaceae Physiology Deduced from Environmental Variables

The old idea that Siderocapsaceae are aerobic iron bacteria arose from their initial discovery in epiphytic, neustonic, and surface film habitats. More recent discoveries of Siderocapsaceae in deep wells, and especially in the hypolimnion of lakes, have forced the revision of this idea. *Siderocapsa treubii*, *Siderocapsa major*, and *Siderocapsa monoica* (which are probably only growth forms of one species—Drake, 1965; Hardman and Henrici, 1939) as well as *Siderocystis vulgaris, Siderocystis confervarum, Naumanniella neustonica,* and possibly *Siderocapsa anulata* (see Table 1) appear to be the only markedly aerobic species. All the other Siderocapsaceae (*Siderococcus limoniticus, Naumanniella polymorpha,* and the lake *Siderocapsa* species) tend to be microaerophilic. The oxygen content of the mass growth zone in lakes is at the level where oxygen disappears (narrow water layer at the chemocline: $O_2 < 1$ ppm, $rH_2 < 19$, Fe^{2+} 1–2 ppm, Mn^{2+} 1–5 ppm; Dubinina, Gorlenko, and Suleimanov, 1973). These natural mass growth conditions established for *Siderocapsa eusphaera* are very similar to those for *Siderocapsa geminata* (Schmidt, 1976), which also grows under extremely microaerobic conditions (see later, Fig. 2a–f). Generalizing, and including the macroscopic observations concerning the onset of a mass development of lake *Siderocapsa* described by the first observers (see Table 1), it seems that optimal Siderocapsaceae growth takes place at the beginning of a change from extremely reduced to oxidized conditions in a neutral-to-light alkaline environment.

A more essential difference between classical stalk- or threadforming bacteria and capsular iron bacteria appears to be the latter's obvious growth dependence on organic material. It appears to be no accident that Siderocapsaceae have not yet been found in iron springs or mineral water very poor in organic substances (with the exception of certain results concerning very unsure forms obtained by Beger, 1949). From this standpoint, classifying Siderocapsaceae as a chemolithotrophic bacterial group (Buchanan and Gibbons, 1974) appears less justified and a physiologically indeterminate classification would correspond better to the facts and would avoid invalid inferences. Until now, only *Naumanniella olymorpha* cultivated by Ten (1968, 1969) in Beijerinck's $MnCO_3$ medium appeared to have a certain probable capacity for mixotrophic growth by manganese oxidation.

For the other capsular iron or manganese bacteria, the natural conditions of the habitat suggest that the highest probability is organotrophic nutrition, possibly of the sort first postulated by Aschan (1907) (utilizing the organic portion of iron humates or other iron organic substances and precipitating ferric iron in or on the capsules).

Isolation

Enrichment

Table 2 lists suitable enrichment procedures for Siderocapsaceae. In particular, the procedures with natural water are extraordinarily simple, straightforward, and characteristic for the speedy enrichment of iron bacteria in general. In habitat-water samples maintained in cold storage or at room temperature without any stirring, a rapid growth of iron bacteria begins within a few days (often overnight). The growth can be recognized mostly by the formation of yellow to black-brown (iron and/or manganese oxides) flocs which sediment slowly. The Siderocapsaceae in general are no exception; nor, for example, is *Siderocapsa geminata*, observed in the hypolimnion of the "Pluβsee" (Plön, Germany) and investigated by Schmidt (1976), among others. Our own

Table 2. Procedures for Siderocapsaceae enrichment in natural waters and synthetic media.

A. Procedures using natural waters:	Procedure suitability and incubation time:
1. Maintenance of sample flasks (totally or $^2/_3$ filled) at 4°C and room temperature without any stirring. Flask size: up to 1 liter. Modifications: addition of 1 mg $MnCO_3$/liter or 1 mg $FeCO_3$/liter.	These procedures with natural waters seem suitable for the majority of all known Siderocapsaceae
2. Test tubes filled with sample water underlayed by 1 ml natural sediment. Modification as above.	
3. 30- to 40-liter aquarium cultures adding 0.07 g yeast extract/liter and 1 mg $FeCO_3$/liter or 1 mg $MnCO_3$/liter (divided in 5 portions added in intervals of 2 days).	Incubation time: up to 3 weeks
B. Procedures with synthetic liquid media (frequently used with 0.1% agar):	
1. Yeast extract-$MnSO_4$ medium; Pringsheim (1949)—medium in modification of Tyler and Marshall (1967) and application according to Dubinina and Zhdanov (1975). Composition (%): yeast extract, 0.005; $MnSO_4$, 0.002; distilled water, 1 liter—in place of manganese, to the bottom of the test tubes iron oxalate or ferrous sulfide were added.	
2. Peptone-$MnCO_3$ medium; manganese-peptone media have been frequently used, see Schweisfurth, 1972. Composition according to Schmidt (1976) with addition of glucose (%): peptone, 0.00002; $MnCO_3$, 0.00002; glucose, 0.00002; distilled water, 1 liter.	Growth usually within 2–3 days
3. Yeast and beef extract-manganese-iron medium (according to Mulder and van Veen, 1963). Composition (%): $MnCO_3$, 0.2; $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 0.015; Difco yeast extract, 0.0075; beef extract, 0.1; cyanocobalamin, 0.005 mg/liter; Na-citrate-2-hydrate, 0.015; distilled water, 1 liter.	
4. Starch-$MnCO_3$ medium (Zavarzin, 1964—medium modified according to Dubinina, 1970). Composition (%): starch, 0.1; $MnCO_3$, 0.5 (freshly prepared); distilled water, 1 liter.	
C. Procedures with solid media for isolation: Media as B 1–4 with 1–1.5% agar in petri dishes.	Growth within 2 days; iron and/or manganese oxidation within 7 days

samples from the same site behaved in the same way and led within a week to an intensive development of *Siderocapsa geminata* in the sample flasks.

A second suitable enrichment method involves the use of 30- to 40-liter aquaria cultures with habitat water after the addition of 0.007% yeast extract and 0.0001% $FeCO_3$ or $MnCO_3$. At first, within 2 days after the culture start and before the addition of yeast extract and $FeCO_3$, thread-forming bacteria develop. Then, 7 days after the addition of $FeCO_3$ and yeast extract and the start of desulfurication, an intensive development of Siderocapsaceae takes place at the end of the desulfurication phase. The Siderocapsaceae form an ochreous layer, 2–3 mm thick, on the water surface. This method makes enrichment possible by more closely simulating natural conditions, particularly with *Siderocapsa treubii* and *Naumanniella neustonica,* which both form dense nests of bacterial cells. The first enrichment of a *Siderocapsa* strain, as carried out by the discoverer of this organism group, is fundamentally similar; *Siderocapsa treubii* was cultivated as an epiphytic growth on water plants in aquaria with the addition of 0.1% $MnCl_2$ (Molisch, 1910). Test tubes with sample water underlayed with natural sample mud were successfully used for the enrichment of *Siderococcus limoniticus* and showed growth 3–4 days after the beginning of the culture (Dorff, 1934). The enrichment of *Siderocystis confervarum,* an organism which grows in direct contact with filamentous algae, also uses natural sample water.

In Table 2, section B, four synthetic media are presented which have proven to be particularly suitable for cultivating Siderocapsaceae. Yeast extract is particularly suitable as an organic carbon and nitrogen substrate, as is pep-tone and beef extract in weak concentration; iron and manganese are best given as carbonate or sulfate salts. On the basis of the presently available facts, in particular the culture experiments by Dubinina and Zhdanov (1975) and Schmidt (1976), as well as indications from the older literature (summarized by Schweisfurth, 1972), Siderocapsaceae can be regarded as organisms that grow well in weak concentrations of organic media, when cultivated in a neutral environment under aerobic to microaerobic conditions. Growth without oxygen appears to be impossible. Table 3 provides a bird's-eye

Table 3. Enriched or presumably isolated Siderocapsaceae.

Organism	Enrichment or isolation procedure[a]
Siderocapsa	
S. treubii	Enriched: method A3 (Moldau-water resp. tap water + 0.1% $MnCl_2$ as epiphytic growth; Molisch, 1910). Enriched: method A3 (swamp-ditch water + 0.007% yeast extract + 0.0001% $FeCO_3$; growing as neustic form in the water surface layer).
S. eusphaera	Enriched: method B1; presumably isolated: on medium B1 with 1% agar by Dubinina and Zhdanov (1975).
S. geminata	Enriched: method A1; presumably isolated: on media B1-4 by Schmidt (1976).
Naumanniella	
N. neustonica	Enriched: method A3 (swamp-ditch water + 0.007% yeast extract + 0.0001% $FeCO_2$; growing as neustic form in the water surface layer).
N. polymorpha	Presumably isolated: on Beijerinck's medium (K_2HPO_4, 0.05%; NH_4Cl, 0.05%; $MnCO_3$ or $Mn(HCO_3)_2$, 1%; agar, 2%); further on Aristovskaya's (1965) medium (ulminofulvate complex) and on iron ammonium salt of citric acid by Ten (1968, 1969).
Ferribacterium	
F. duplex	Enriched: modified method A2 underlayed with peat and iron bars, Brussoff (1916); presumably isolated: in iron ammonium citrate medium, Brussoff (1916).
Siderocystis	
S. vulgaris	Enriched: method A1 in 3-liter Erlenmeyer flasks $^3/_4$ filled with swamp-ditch water; growing as rust-spot ongrowth on the flask walls at room temperature, H. H. Hanert (unpublished observations).
S. confervarum	Enriched: method A1 in 100-ml flasks filled to the top with swamp-ditch water and some algae threads, cultivated at room temperature and near a window (daylight).
Siderococcus	
S. limoniticus	Enriched: method A2, coupled with slide and capillary ongrowth, Dorff, 1934; Zavarzin, 1972; Kutuzova, 1974.
S. communis	Enriched: in ferrous ammonium citrate, Dorff, 1934.

[a]See Table 2 for methods.

view of all Siderocapsaceae that have been enriched so far.

Isolation

The isolation of Siderocapsaceae is much more a problem of identification than the application of particular isolation techniques. As Dubinina and her co-workers indicated for *Siderocapsa eusphaera* (1973, 1975), Schmidt for *Siderocapsa geminata* (1976), and Ten for *Naumanniella polymorpha* (1968, 1969), pure cultures of these organisms could be conventionally produced without difficulty in liquid and solid media, using serial solution or plating techniques, respectively (Table 3).

The main problem in isolating Siderocapsaceae is the question of relating the isolated organism with the Siderocapsaceae organism of the inoculated material from the natural environment. The isolated strains of *Naumanniella polymorpha, Siderocapsa eusphaera*, and *Siderocapsa geminata* show—as described by the authors—an extreme polymorphism and a quite complex cycle of development, whereas it has not yet been possible to demonstrate corresponding changes directly in the natural environment immediately from an individual object. This is one of the reasons why the task of identifying Siderocapsaceae has such crucial importance at the moment and why isolation work cannot lead to really secure results so long as the individual developmental cycle remains unexplained.

Identification

Initial Identification

Siderocapsaceae are defined purely morphologically, without physiological features, and the morphological definition provided by the people who first described them refers to their morphology in their natural environment or in habitat water cultures. This fact is the starting point from which all identification work has to proceed. The second important point to make is that the first morphological descriptions of the organisms corresponded to the then present state of microscopic technology; the descriptions used only light-microscopic photographs or drawings and the natural ultramorphology and ultrastructure remained unknown. *Siderocapsa anulata* is the only Siderocapsaceae which, at the time of its discovery, was described by means of an electron microscope (Kalbe et al., 1965) so that a clear picture of its morphology was produced which was completely adequate for purposes of identification.

The description which defines the morphology of the Siderocapsaceae contains three components: the bacterial cell, the capsule or excreted gelatinous material, and iron and/or manganese compounds deposited in or on the excreted

material. The capacity to form capsule-like material has been recently shown for *Siderococcus limoniticus* (Kutuzova, 1974), which had previously been regarded as a non-capsule-forming Siderocapsaceae, so that this component appears to be common to all Siderocapsaceae. For the identification of Siderocapsaceae, the analysis of natural ultramorphology of these three components is of crucial importance, and thus, in virtue of new ideas concerning the pleomorphic development of these organisms, the analysis of the individual natural development must be added as a further new component.

Identification by Analysis of the Natural Ultramorphology and Development

The methods that are indispensable for a clear identification, which must be carried out with all Siderocapsaceae whose existence has been made certain by repeated finds, and ecological descriptions are summarized in Table 4. They relate to the visualization of the bacterial cell, the differentiation of cell and capsule or slime material, the ultramorphology of the capsular material, the specific evidence for the presence of iron and manganese, the ultramorphology of the iron deposits, and the analysis of the individual development under natural conditions.

Light Microscopy, Staining, and Differentiation of the Bacterial Cell. In phase contrast microscopy, the central cells can only be distinguished from the surrounding capsule which contains metal oxide by their differing light reflection. This appearance can also be created by the shape of the capsule, and cell and capsule staining are indispensable. Carbolfuchsin and fluorescence staining have been found suitable for cell staining. Erythrosine staining has been applied just as successfully by other authors. In combination with the Prussian blue

Table 4. Procedures necessary for identification of Siderocapsaceae.

Procedure	Methods for realization
1. Exact light-microscopic visualization of the bacterial cell and surrounding iron capsule	a. Phase-contrast observation: Yellowish iron capsule with light-refrangible central bacterial cell. b. Direct microscopic control of capsular ferric iron dissolution by adding 5% HCl to the edge of the cover glass (suspension preparations), immediately followed by carbolfuchsin staining of the central cell under microscope. c. Carbolfuchsin-Prussian blue double-staining (air-dried preparations): Prussian blue solution (70 ml 0.1% HCl + 30 drops 2% $K_4[Fe(CN)_6]\cdot 3H_2O$); 10 min (cuvette); washing with water (cuvette); 10 min into nonheated Ziehl-Neelsen carbolfuchsin (cuvette). d. Benzidine staining for manganese reaction: Material on slides or filters is first wetted with benzene, and 2 or 3 drops of 0.5% benzidine hydrochloride in 50% acetic acid are applied to the wet surface. After 1–2 s, the benzidine is removed with filter paper (Dubinina, Gorlenko, and Suleimanov, 1973). e. Flourescence staining of the bacterial cell: air-dried preparations covered with a filtered FITC solution for 5 min (0.1 N Na_2CO_3 + 0.1 $NaHCO_3$ in 3:2 proportion + 3.5 mg FITC per 10 ml)—washing with the same buffer. Very suitable for visualization of bacterial cells in rust-spot-forming bacteria.
2. Scanning electron microscopy and electron probe microanalysis	a. Stereoscan: Necessary for detection of the slimy nature of the capsule as well as the globular ferrihydrite particles in rust spots. Preparation in the usual way, placing a drop of material directly onto the specimen stub or using ongrowth method (specimens metal-coated). Figs. 1 (without 1h), 2a–d, and 2h–k. b. Electron-probe microanalysis for proof concerning iron or manganese incorporation of the capsules using X-ray analysis (area- or point-scanning). Figs. 1h, 2e, f, and l.
3. Transmission electron microscopy	Realization in the usual way using formvar-coated grids (ongrowth method for sessile Siderocapsaceae; see also Kutuzova, 1974, 1975; Kutuzova, Gabe, and Kravkina, 1972); cell observation is better when capsular iron or manganese is removed by dissolving.
4. Direct microscopic observation of developmental cell cycle in micro-chambers or reverse light microscope	The possibility of pleomorphic development of Siderocapsaceae members makes it necessary to compare cell development in chambers filled with habitat water or culture media for identifying growing cells with the natural originals. This should be possible by directly proving stalk excretion from the apical cell in the way done for *Gallionella* (Hanert, 1974) or by using the reverse microscope (Schmidt, 1976, cell cycle of *Siderocapsa geminata*; see also Dubinina and Zhdanov, 1975, cell cycle of *Arthrobacter siderocapsulatus*).

reaction for iron and the benzidine hydrochloride reaction for manganese, a differentiating double staining of the cell and metal-containing capsule is possible. Fluorescein isothiocyanate (FITC) fluorescence staining is especially suitable for the visualization and differentiation of the bacterial cells from iron/manganese oxides in rust-spot-forming bacteria, since the inorganic deposits are not stained (Hanert, 1981). A specific staining of the capsular polysaccharides has not yet been carried out, so that the capsule until now has only been made recognizable by staining the iron or manganese which it contains.

Ultramorphology of the Capsule and Excreted Gelatinous Material. In all the Siderocapsaceae material which has been sampled in the natural environment or in habitat water cultures, the capsules showed a very characteristic slime-thread ultramorphology and structure, which can be recognized best with scanning electron microscopy. The capsules are slimy but, nevertheless, form casings which the bacteria vacate, leaving a noticeable hole when they depart. The bacteria produce this gelatinous thread slime, cover themselves with it—at this stage the bacteria cannot be recognized with a scanning electron microscope—and break through the capsule after a certain time. Even though this process cannot be observed in vivo, the electron microscope pictures allow scarcely any other inference. (See Fig. 1a–c, *Siderocapsa treubii*; Fig. 1d–f, *Siderocapsa* sp.; Fig. 2a–d, *Siderocapsa geminata* from flocs.) On the basis of this characteristic ultramorphology, it seems reasonable to think that the slime capsule might be a very characteristic feature of genuine Siderocapsaceae. It should be noted that the gelatinous slime of the Siderocapsaceae also resembles ultramorphologically those thread slimes which we have observed without the formation of capsules in *Metallogenium* habitat water cultures (Fig. 2g–k, *Metallogenium* flocs).

Table 5. Siderocapsaceae which have clearly been shown to exist by repeated finds.

Organisms	Morphological features and characteristic ecology
Siderocystis	
S. confervarum	Large ovoid iron precipitations on *Conferva*-algae; iron-containing swamp ditches.
S. vulgaris	Forming rust spots up to 1 mm in diameter on exposed glass slides (Fig. 1j); iron-containing swamp ditches.
Naumanniella	Slim rods with a thin, sharply limited iron capsule in the form of a torus (marginal thickening); single and in short chains.
N. neustonica	Rod-shaped cells, including the torus, 1.8–3.3 by 4.9–10 μm; surface of iron-bearing well and swamp water.
N. polymorpha	Ellipsoid rods of 0.7–1.0 × 1.0–2.0 μm that form coccoid cells by budding and fission; thin capsules; manganese- not ferrous iron-oxidizing; colony-forming in manganese carbonate or manganese acetate agar; brown forest soils and rock crusts.
Siderococcus limoniticus	Cocci (0.2–0.5 μm in diameter) without capsules (recently, capsule-like formations in which iron-oxide deposition takes place have been found by Kutuzova, 1974); in mud horizons and zone growth in culture.
Siderocapsa	Coccus resp. ovoid or short rods, single or in groups in a very thick, slimy iron capsule.
S. treubii	(Including the intermediary form of *S. major* and *S. monoica* found by Hardman and Henrici, 1939 and Drake, 1965.) Coccoid cells of 0.6–1.6 μm in diameter and small rods 0.4–1.0 × 2 μm; single or in groups; most frequently of 6–8 up to 30 cells and more in mucoid capsules averaging about 2 μm by 4.5 μm with round, elliptical, or irregular outer edge surrounded by thick, rust-brown deposits of iron (light microscopic photographs by Hardman and Henrici, 1939).
S. anulata	Single coccus (0.2–0.5 μm in diameter) surrounded by sharply limited ferric iron ring (1.2–1.9 μm), excellent electron microscopic photographs by Kalbe, Keil, and Theile, 1965; planktonic and epilimnetic.
S. coronata	Two to eight cocci (maximal 1.2 μm) in one capsule (up to 24 μm in diameter); aggregating to coenobia of up to 90 capsules and a length of 400 μm (described and pictured by drawings; Redinger, 1931); in alpine lakes.
S. arlbergensis	Cocci (0.4–1.0 μm) normally in pairs in capsules (diameter 6–15 μm); coenobia similar to *S. coronata*, but single capsules not fusing; light-microscopic photographs by Wawrik, 1956; planktonic and neustic in alpine pools.
S. eusphaera	Coccoid to ovoid (1–2 μm) cells up to 60 in large capsules (regular spheres up to 50 μm in diameter); capsules occur singly (drawings by Skuja, 1948); planktonic and hypolimnetic in large lakes.
S. geminata	Ovoid cells (0.5 × 0.8 μm) usually in pairs in round capsules (7–11 μm in diameter); capsules occur singly (drawings by Skuja, 1956); planktonic and hypolimnetic in lakes.

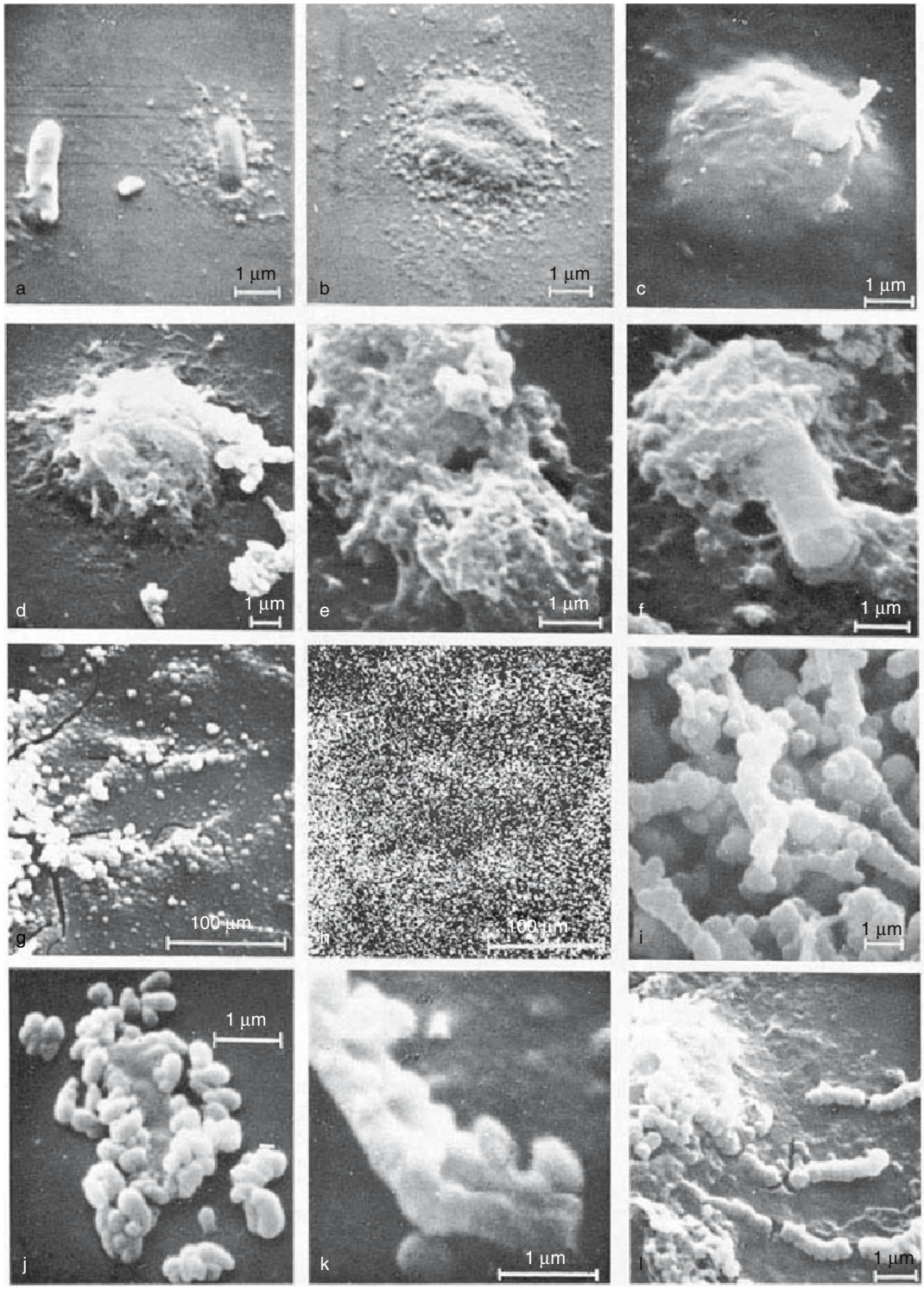

Fig. 1. Identification and differentiation of Siderocapsaceae cells, capsules, and ferrihydrite particles. (a–c) *Siderocapsa treubii*, in situ growth on slide in aquarium-natural water culture: (a) single cells at the initial phase of capsule formation; (b) two cells coated with slime and iron particles; (c) slime capsule completely coating the cell. (d–f) *Siderocapsa* sp. from the Siderocapsaceae zone in the marine iron bay of Palaea Kameni, in situ growth on slide, Hanert, 1981: (d) thread-slime capsule; (e) capsule with two holes; (f) cell half-coated with thread-slime capsular material. (g–i) Globular, chain-forming ferrihydrite particles on the slime surface of the rust-spot bacteria (formed in the marine bay): (g and i) ferrihydrite particles; (h) FeKα picture to (g). (j) Single cell of *Siderocystis vulgaris* surrounded by ferrihydrite particles. (k) Single cell of the marine rust-spot-former surrounded by ferrihydrite particles. (l) Slime capsule of the marine *Siderocapsa* sp. coated with ferrihydrite particles.

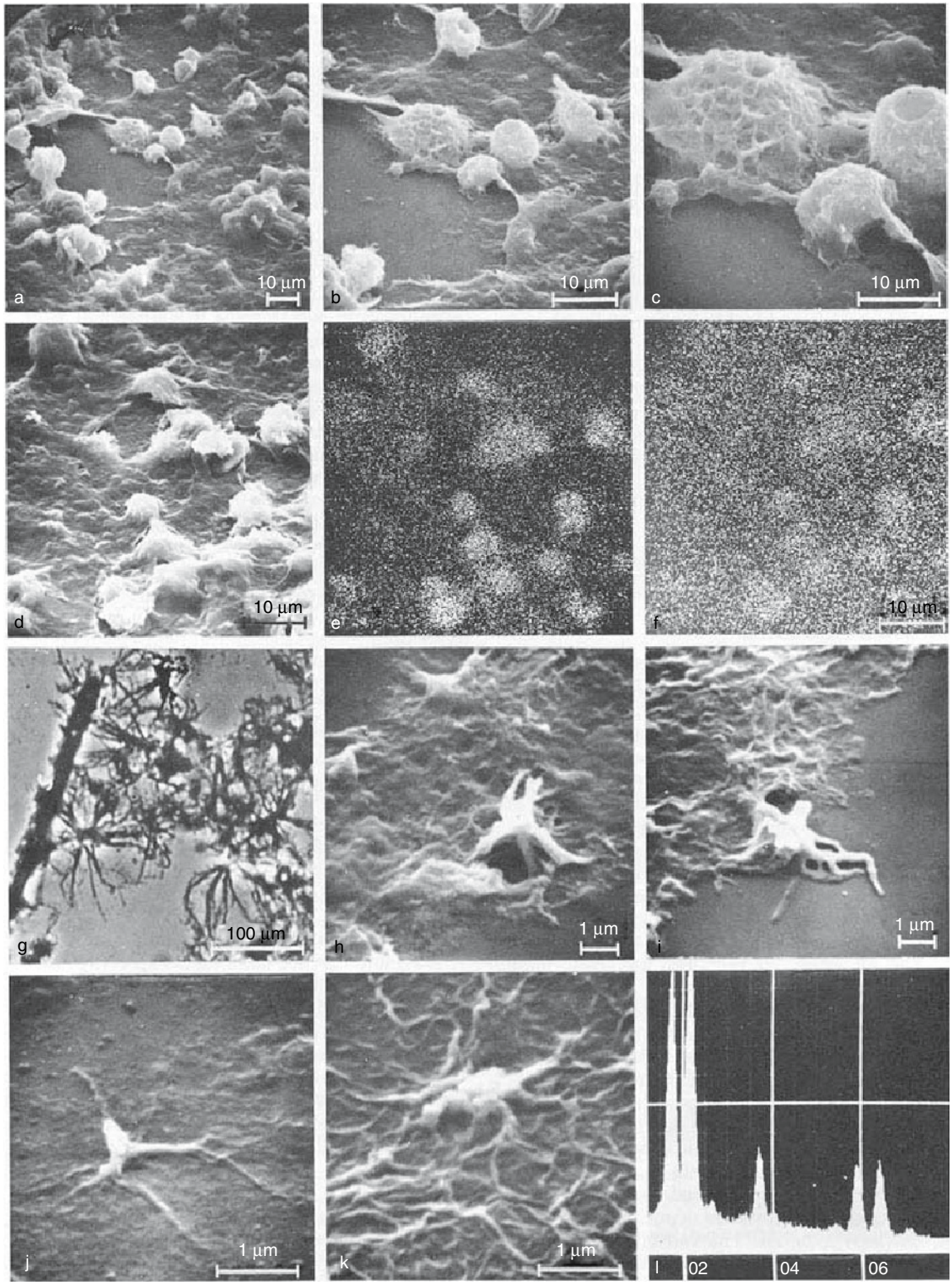

Fig. 2. Iron/manganese capsules and slimes of *Siderocapsa geminata* and *Metallogenium personatum,* (a–f) *Siderocapsa geminata*, flocs grown in Pluβsee sample flasks, three-quarters filled at a depth of 26 m in the natural habitat and maintained in cold storage at 4°C, where the *S. geminata* flocs formed in clear, natural habitat water: (a–d) capsules closed or with a hole; (e) MnKα (f) FeKα picture to (d), proving simultaneous presence of manganese and iron, the latter at a much lower level. (g–l) *Metallogenium personatum,* flocs grown in habitat water-sample flasks from 4°C at a depth of 27 m, Pluβsee: (g) light microscopic; (h–k) young *Metallogenium* organisms and thread-slime structure; (l) simultaneous manganese (MnKα, 5894keV) and iron (FeKα, 6398keV) evidence in the center of *Metallogenium* (k).

Ultramorphology of Deposited Iron Particles and Element-Specific, X-Ray Determination of the Presence of Iron/Manganese in the Capsules in Combination with Postulated Developmental Stages. There has been intensive discussion concerning the existence of tiny mycoplasma-like developmental and budding stages in certain iron and manganese bacteria, for example, in *Gallionella* (Balashova, 1969), in *Metallogenium* (Dubinina, 1970), and also in Siderocapsaceae (*Sidero*c*occus limoniticus,* Kutuzova, 1974; Kutuzova, et al., 1974; *Naumanniella polymorpha,* Ten, 1968, 1969; *Siderocapsa geminata*, Schmidt, 1976). Evidence which has previously been presented in favor of this hypothesis has largely been pictures obtained by transmission electron microscopy which, however, show only tiny dense particles.

Relevant here are observations on rust-spot-forming bacteria (rust spots in the Palaea Kameni Bay, Hanert, 1981; *Siderocystis vulgaris* rust spots, Galinsky and Hanert, 1979a). The iron oxides in these rust spots consist of tiny, spherical ferric iron particles which are often chainlike in form; these particles correspond to the iron bodies which have been analyzed as ferrihydrite by Tschukrov (1974). These particles consist exclusively of iron, which can easily be demonstrated by X-ray analysis (point-scanning) and dissolving in acids.

Another useful aid in structural analysis in this field is area-scanning electron microscopy in combination with element-specific scanning. The existence of both iron and manganese incorporation in the capsules can be indicated simultaneously (see Fig. 2d–f for *Siderocapsa geminata* and Fig. 2l for *Metallogenium*).

The methodological possibilities for analyzing natural individual development in vivo are represented in Table 4.

In summary, it should be emphasized that an adequate number of identification and culture methods are available for the investigation of Siderocapsaceae, which can be applied to those genera and species (Table 5) whose existence has been made certain. One of these forms is *Siderocystis confervarum,* which forms large, round, iron oxide aggregates on algae and can be found in profusion in iron-bearing swamp ditches in the vicinity of Braunschweig. The application of the methods described here to this bacterium has resulted in the surprising discovery that this organism lives not only on the surface of the algae but also within it. This bacterium might have a lytic function for this algae, similar to those lytic bacteria which colonize on the hyphae of fungi in natural habitats (e.g., *Phytophthora cinnamomi;* Nesbitt, Malajczuk, and Glenn, 1978).

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Literature Cited

Aristovskaya, T. V. 1965. Microbiology of podzolic soils. "In Russian." Moscow-Leningrad, Nauka.

Aschan, O. 1907. Die Bedeutung der wasserlöslichen Humusstoffe (Humussole) für die Bildung der See- und Sumpferze. Zeitschrift für Praktische Geologie 15:56.

Balashova, V. V. 1969. Ob otnoshenii *Gallionella* k mikoplazmam. Doklady Akademii Nauk SSSR 184:1429–1432.

Beger, H. 1949. Über *Zoogloea filipendula*-Formen und einige neue Eisenbakterien. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten, und Hygiene, Abt. 1 Orig. 154:61–68.

Breed, R. S., Murray, E. G. D., Smith, N. R. (ed.). 1957. Bergey's manual of determinative bacteriology 7th ed. 217–228. Baltimore, Williams & Wilkins.

Brussoff, A. 1916. *Ferribacterium duplex,* eine stäbchenförmige Eisenbakterie. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten, und Hygiene, Abt. 2 45:547–554.

Buchanan, R. E., Gibbons, N. E. (ed.). 1974. Bergey's manual of determinative bacteriology 8th ed. Baltimore, MD. Williams & Wilkins. 464–468.

Dorff, P. 1934. Die Eisenorganismen. Pflanzenforschung. Jena, Gustav Fischer Verlag.

Drake, C. H. 1965. Occurrence of Siderocapsa treubii in certain waters of the Niederrhein. Gewässer und Abwässer 39/40:41–63.

Dubinina, G. A. 1970. Untersuchungen über die Morphologie von *Metallogenium* und die Beziehungen zu *Mycoplasma*. Zeitschrift für Allgemeine Mikrobiologie 10: 309–320.

Dubinina, G. A., Gorlenko, V. M., Suleimanov, Ya. I. 1973. A study of microorganisms involved in the circulation of manganese, iron, and sulfur in meromictic lake Gek-Gel'. Mikrobiologiya 42:918–924.

Dubinina, G., Zhdanov, A. V. 1975. Recognition of the iron bacteria "*Siderocapsa*" as arthrobacters and description of *Arthrobacter siderocapsulatus* sp. nov. International Journal of Systematic Bacteriology 25:340–350.

Galinsky, G., Hanert, H. H. 1979a. Bakterogenese des Ferrihydrit durch *Siderocystis vulgaris*. Mitteilungen der Deutschen Bodenkundlichen Gesellschaft 29:485–492.

Galinsky, G., Hanert, H. H. 1979b. Jahresrhythmik der Eisenoxidation in Hochmoor-Standorten. Mitteilungen der Deutschen Bodenkundlichen Gesellschaft 29:479–484.

Hanert, H. H. 1974. Untersuchungen zur individuellen Entwicklungskinetik von *Gallionella ferruginea* in statischer Mikro kultur. Archiv für Mikrobiologie 96:59–74.

Hanert, H. H. 1981. Bakterielle und chemische Eisen(II)-Oxidation auf Palaea Kameni—Stereoscan, Elektronenstrahl-Mikroanalyse (FeKα) und Photometrie von in situ-Experimenten. Puchelt, H., and Schroll, E. (ed.) Genesis of marine iron sediments from San-

torini, Greece. Berlin, Heidelberg, New York. Springer-Verlag.

Hardman, Y., Henrici, A. 1939. Studies of freshwater bacteria. V. The distribution of Siderocapsa treubii in some lakes and streams. Journal of Bacteriology 37:97–104.

Hässelbarth, U., Lüdemann, D. 1967. Die biologische Verockerung von Brunnen durch Massenentwicklung von Eisen- und Manganbakterien. Bohrtechnik-Brunnenbau-Rohrleitungsbau, H. 10 and 11:363–368 and 401–406.

Kalbe, L., Keil, R., Theile, M. 1965. Licht- und elektronenmikroskopische Studien an Arten von *Leptothrix*, *Siderocapsa* and *Planctomyces*. Archiv für Protistenkunde 108:29–40.

Kutuzova, R. S. 1974. Electron microscopic study of ooze overgrowths of an iron-oxidizing coccus related to *Siderococcus limoniticus* Dorff. Mikrobiologiya 43:285–288.

Kutuzova, R. S. 1975. Study of iron-manganese microorganisms by the overgrowth method. Mikrobiologiya 44:156–159.

Kutuzova, R. S., Gabe, D. R., Kravkina, I. M. 1972. Electron microscope study of overgrowths of iron-manganese microorganisms from ooze. Mikrobiologiya 41:1099–1102.

Molisch, H. 1910. *Siderocapsa Treubii* Molisch, eine neue, weit verbreitete Eisenbakterie. Annales de Jardin Botanique de Buitenzorg. Suppl. III(1):29–34.

Mulder, E. G., van Veen, W. L. 1963. Investigations on the Sphaerotilus-Leptothrix group. Antonie van Leeuwenhoek Journal of Microbiology and Serology 29:121–153.

Naumann, E. 1921. Untersuchungen über die Eisenorganismen Schwedens I. Kungl. Svenska Vetenskapsakademiens Handlingar 62:1–68.

Nesbitt, H. J., Malajczuk, N., Glenn, A. R. 1978. Bacterial colonization of *Phytophthora cinnamomi* Rands. 371–375. Loutit, M. W., and Miles, J. A. R. (ed.) Microbial ecology. Berlin, Springer-Verlag.

Pribram, E. 1929. A contribution to the classification of microorganisms. Journal of Bacteriology 18:361–394.

Pringsheim, E. G. 1949. Iron bacteria. Biological Reviews of the Cambridge Philosophical Society 24:200–245.

Redinger, K. 1931. *Siderocapsa coronata* Redinger, eine neue Eisenbakterie aus dem Lunzer Obersee. Archiv für Hydrobiologie 22:410–414.

Schmidt, W.-D. 1976. Zur Biologie der Eisenbakterien—*Siderocapsa geminata-Leptothrix echinata*-des Plußsees. Diplomarbeit, Mathematisch-Naturwissenschaftliche Fakultät der Universität Kiel. Federal Republic of Germany.

Schweisfurth, R. 1972. Manganoxidierende Bakterien. Literaturübersicht (Bestandsaufnahme beschriebener Arten). Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten, und Hygiene, Abt. 1 233:257–270.

Skuja, H. 1948. Taxonomie des Phytoplanktons einiger Seen in Uppland, Schweden. Symbolae Botanicae Upsalienses IX(3):12–13.

Skuja, H. 1956. Taxonomische und biologische Studien über das Phytoplankton schwedischer Binnengewässer. Nova Acta Regiae Societatis Scientiarum Upsaliensis, Ser. IV XVI(3):19–20.

Sokolova, G. A. 1959. Iron bacteria of Lake Glubokoe. Mikrobiologiya 28:246–250.

Sorokin, Yu. I. 1968. Primary production and microbiological processes in lake Gek-Gel'. Mikrobiologiya 37:345–354.

Ten, H.-M. 1967. Iron- and manganese-oxidizing microorganisms in soils of South Sakhalin. Mikrobiologiya 36:337–344.

Ten, H.-M. 1968. The biological nature of iron-manganese crusts of soil-forming rocks in Sakhalin Mountain soils. Mikrobiologiya 37:749–753.

Ten, H.-M. 1969. A new manganese-oxidizing soil microorganism. Doklady Akademii Nauk SSSR 188:697–699.

Tschukrov, F. W., Ermilova, L. P., Gorschkov, A. I., Zviagin, B. B., Shukchlistov, A. P., Siderenko, O. W., Balaschova, W. W. 1974. Über die Natur der Eisenoxide in geologisch jungen Bildungen. Chemie der Erde 33:109–124.

Tyler, P. A., Marshall, K. C. 1967. Pleomorphy in stalked bacteria. Journal of Bacteriology 93:1132–1136.

Wawrik, F. 1956. *Siderocapsa arlbergensis* nova spec., eine Eisenbakterie aus den Hochgebirgs-Kleingewässern des Arlberggebietes. Oesterreichische Botanische Zeitschrift 103:19–23.

Zavarzin, G. A. 1964. *Metallogenium symbioticum*. Zeitschrift für Allgemeine Mikrobiologie 4:390–395.

Zavarzin, G. A. 1972. Lithotrophic microorganisms "In Russian." Moscow, Nauka.

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CHAPTER 14.12

The Genus *Fusobacterium*

TOR HOFSTAD

The genus *Fusobacterium* includes several species of obligately anaerobic, nonsporeforming, motile or nonmotile, Gram-negative rods. Some are slender, spindle-shaped bacilli, while others are pleomorphic rods with parallel sides and rounded ends. Their habitat is the mucous membranes of humans and animals.

During the last decade of the nineteenth century, several authors, among them Miller (1898), Plaut (1894), and Vincent (1896, 1899, 1904), observed spindle-shaped, or fusiform, bacilli in material from both the diseased and the healthy human mouth. Veillon and Zuber (1998), Lewkowicz (1901), and Ellermann (1904) were the first to cultivate fusiform bacilli. Loeffler, in 1884, observed pleomorphic rods in diphtheritic lesions of calves and doves. The same organism, identifiable with the *F. necrophorum* of today, was cultured by Bang (1890–1891) from necrotic lesions of a number of domestic animals and by Schmorl (1891) from an epizootic in rabbits.

The more pleomorphic fusobacteria without tapering ends have been described under different generic names. Examples are *Bacteroides*, *Sphaerophorus, Bacterium, Necrobacterium, Pseudobacterium, Bacillus*, *Actinomyces*, *Corynebacterium*, *Ristella,* and *Zuberella.*

The family name Bacteroidaceae was first used by Pribram (1929) for strictly anaerobic rods. Ten years earlier, Castellani and Chalmers (1919) proposed that the genus *Bacteroides* should only contain obligately anaerobic bacilli that did not form spores. Eggerth and Gagnon (1933) and Weiss and Rettger (1937) excluded the Gram-positive rods from the genus. The generic name *Fusobacterium* was proposed by Knorr (1923) for obligately Gram-negative bacilli that were fusiform. Prévot (1938), who argued that the generic name *Fusobacterium* (and also the names Bacteroidaceae and *Bacteroides*) was invalid, used the term *Sphaerophorus* for the nonmotile, pleomorphic fusobacteria, and the term *Fusiformis* for the fusobacteria that had tapered ends. The seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed et al., 1957) divided the family Bacteroidaceae into three genera: *Bacteroides*, defined as rods with rounded ends; *Fusobacterium*, defined as rods with tapering ends; and *Sphaerophorus,* defined as rods with rounded ends that showed a marked pleomorphism and where filaments were common.

Cell morphology, therefore, had so far been the main criterion for the classification of the nonsporeforming, anaerobic rods. Physiological studies (Beerens et al., 1962; Werner, 1972a; Werner et al., 1971) and studies of DNA base ratios (Sebald, 1962) showed that there was insufficient evidence to separate the genera *Fusobacterium* and *Sphaerophorus.* In the eight edition of *Bergey's Manual* (Moore and Holdeman, 1974a) the genus *Fusobacterium* was restricted to anaerobic, nonsporeforming, Gram-negative rods which form butyric acid as a major endproduct from peptone or glucose (without isobutyric and isovaleric acids). Further, *Leptotrichia* (see The Genus *Leptotrichia* in the second edition) was reestablished as a genus for the saccharolytic fusiform bacilli producing lactic acid as the only major fermentation product.

Up to now, 12 human species of *Fusobacterium* have been adequately described (Table 1). The GC content of these species ranges from 26 to 34 mol%. In addition, *F. prausnitzii*, which has a GC content of 52–57 mol%, has been included in the genus. The species isolated most frequently from humans and animals are *F. nucleatum* and *F. necrophorum*, respectively. *F. simiae* is a new species which has been isolated from the mouth of the stumptailed macaque (*Macaca arctoides*) (Slots and Potts, 1982).

Habitats

As Normal Microbiota of Humans and Animals

All *Fusobacterium* species are parasites of humans and animals. Anaerobic Gram-negative rods with the same morphological and biochemical properties as *Fusobacterium* species have also been isolated from the hindgut of the

This chapter was taken unchanged from the second edition.

Table 1. The main human sources of *Fusobacterium* isolates.

Species	Normal flora: Mouth	Normal flora: Gastrointestinal tract	Clinical specimens
F. nucleatum	+		+
F. necrophorum		+	+
F. alocis	+		
F. gonidiaformans		+	+
F. mortiferum		+	+
F. naviforme	+		+
F. necrogenes		+	
F. periodonticum	+		
F. prausnitzii		+	
F. russii		+	
F. sulci	+		
F. ulcerans			+
F. varium		+	+

Adapted from Moore et al. (1984), Slots et al. (1983), Cato et al. (1985), and Adriaans and Shah (1988).

cockroach *Eublaberus posticus* (Foglesong et al., 1984) and the gastrointestinal tract of the grass carp *Ctenopharyngodon idella* (Trust et al., 1979).

The main human habitats of the different *Fusobacterium* species are listed in Table 1.

F. nucleatum is a constant member of the oral microflora of adults, but has also been isolated from the oral cavity of predentate children (Hurst, 1957; McCarthy et al., 1965). The principal habitat of *F. alocis* and *F. sulci* is the human gingival sulcus. The incidence of *F. periodonticum* in the human oral cavity is unknown. Occasionally, *F. naviforme* has been found in the mouth or the upper respiratory tract (Holdeman et al., 1977). The isolation of *F. necrophorum* from pleuropulmonary infections suggests that this organism is also able to live as a parasite on the mucous membranes of the oral cavity and upper respiratory tract of humans. The number of fusobacteria per milliliter of saliva has been estimated to be 5.6×10^4 (Richardson and Jones, 1958). In different surveys, fusobacteria have been found to make up from 0.4 to 7% of the cultivable dental plaque flora (Hardie and Bowden, 1974). There are, however, great individual variations. In patients with different forms of periodontitis, *F. nucleatum* is a predominant member of the subgingival plaque flora (Williams et al., 1976; Moore et al., 1982, 1983). Hadi and Russel (1969) reported a mean viable count of *F. nucleatum* per gram wet weight of gingival plaque material from patients with advanced chronic periodontal disease and acute ulcerative gingivitis of 3.3×10^7 and 9.3×10^7, respectively. In subjects with healthy gingivae, the corresponding figure was 5.7×10^6.

Fusobacterium makes up a small part of the fecal microflora of man, with individual variation ranging from about 7% to less than 1% of the cultivable fecalflora (Finegold et al., 1974; Finegold et al., 1975; Holdeman et al., 1976; Moore and Holdeman, 1974b; van Houte and Gibbons, 1966). The most prevalent species seem to be *F. prausnitzii*, *F. russii*, and *F. mortiferum*. Both the number of fusobacteria in feces and the relative frequency of the different species are influenced by the diet (Finegold et al., 1974; Maier et al., 1974; Peach et al., 1974). Thus, Japanese on a traditional diet rich in carbohydrate have a relatively high number of *F. necrophorum* in feces (Ohtani, 1970a; Ueno et al., 1974).

The occurrence of fusobacteria on the mucous membranes of the genitourinary tract is virtually unknown. Fusobacteria were not present in the normal microflora of the cervix of 30 healthy females examined by Gorbach et al. (1973), and Hite et al. (1947) found no fusobacteria in the healthy vaginas of pregnant women. Fusobacteria, particularly *F. necrophorum*, were, however, present in the vagina of pregnant women with trichomoniasis and in the postpartum uterus of such women. Spaulding and Rettger (1937) found *F. nucleatum* in the normal vagina but not in the vagina of pregnant women. Davis and Pilot (1922) and Brams et al. (1923) isolated fusiform bacilli (and spirochetes) from the clitoris region in females and from preputial secretions of 50 out of 100 men.

The habitat of *F. ulcerans* is unknown. The organism has been isolated from tropical ulcers (Adriaans and Drasar, 1987) and from a few specimens of mud (Adriaans and Shah, 1988). Tropical ulcer is a form of skin ulceration predominantly affecting children and is most commonly in the tropics (Robinson and Hay, 1985).

F. necrophorum is a normal inhabitant of the alimentary tract of cattle, horses, sheep, and pigs. Fuller and Lev (1964), in a study of the Gram-negative bacteria of the pig alimentary tract, found *F. necrophorum* to be present from the age of 43 days. Aalbæk (1972) isolated the organism from the colon of pigs in numbers up to 10^3 per gram of wet material, but in considerably higher number in the ileus, cecum, and colon of pigs with experimental enteritis. *F. necrophorum* has also been found in infections and in feces of other animals, such as mules (Nolechek, 1918), goats (Jensen, 1913), reindeer (Horne, 1898–1899), antelope (Mettam and Carmichael, 1933), macropods (Bang, 1890–1891; Samuel, 1983), wildebeest (Grainer, 1983), dogs (Jensen, 1913), rabbits (Cameron and Williams, 1926; Schmorl, 1891), rats (Lewis and Rettger, 1940), chickens (Jensen, 1913), and apes (Dack et al., 1935; Dack et al., 1937). It has also been reported in buffaloes, cats, guinea pigs, mice, snakes, tortoises, and fowl (Simon and Stovell, 1969; Weinberg et al., 1937).

Less is known about the presence in animals of the other *Fusobacterium* species. Fusiform bacilli, probably *F. nucleatum*, have been isolated from the alimentary tract of pigs (Aalbæk, 1977) and mice (Syed, 1972), and from the oral cavity and the throat of monkeys (Krygier et al., 1973; Pratt, 1927; Slanetz and Rettger, 1933), dogs (Slanetz and Rettger, 1933), cats (Prèvot et al., 1951), rabbits (Pratt, 1927; Slanetz and Rettger, 1933), and guinea pigs (Pratt, 1927; Spaulding and Rettger, 1937). Terada et al. (1976) isolated *F. necrogenes* and *F. mortiferum* from pig feces, and *F. perfoetens* has been found in piglet feces (van Assche and Wilssens, 1977). *F. necrogenes* is a member of the cecal flora of poultry (Holdeman et al., 1977). *F. russii* is a member of the normal oral flora of cats (Love et al., 1987) and has been mentioned as being part of the normal microflora of mice and pigs, as well as of the rumen flora of cattle (Smith, 1975).

As Pathogens of Humans and Animals

Next to members of the "*Bacteroides fragilis* group" and the black-pigmented bacteroides, *F. nucleatum* is the Gram-negative anaerobic organism most often encountered in human infections. Also, *F. necrophorum* is clearly pathogenic in humans. Before the advent of antibiotics and other antimicrobial drugs, this organism was frequently isolated from suppurative infections of the oral cavity and the upper respiratory tract and from pleuropulmonary infections. Reviewing the literature concerning anaerobic pleuropulmonary infections, Finegold (1977) found that *F. necrophorum* accounted for 24% of all anaerobic bacteria isolated from 358 cases. Today the organism is less commonly isolated from human infections. *F. nucleatum* is usually found associated with other anaerobic and/or facultative organisms. On a percentage basis, *F. nucleatum* and *F. necrophorum* have been isolated in pure culture from pyogenic infections more frequently than have other anaerobic bacteria (Beerens and Tahon-Castel, 1965; Werner and Pulverer, 1971; Bartlett et al., 1974). However, when in mixture with other organisms the recovery of fastidious strains of *F. nucleatum* and *F. necrophorum* may fail. Other species of *Fusobacterium* are occasionally isolated from clinical specimens, and nearly always in mixed culture.

Pathogenic fusobacteria are in particular isolated from inflammatory processes accompanied by necrosis and ulceration. They are most frequently found in head and neck infections, pleuropulmonary infections, abscesses of the brain and the liver, and in infections following human and animal bites. *Fusobacterium* species are also recovered from subcutaneous and soft-tissue abscesses, obstetrical and gynecological infections, and abdominal abscesses. Because of variations in nomenclature and anaerobic culture technique, the incidence of *Fusobacterium* infections is difficult to determine from the literature. In a series of 15,844 clinical specimens submitted over 12 years (1973–1985) to the microbiological laboratories in two military hospitals, *Fusobacterium* species accounted for 4% of all anaerobic isolates (Brook, 1988). *F. nucleatum* was the most common Fusobacterium species (47% of all *Fusobacterium* species). The incidence of fusobacteria was somewhat higher in a 10-year series of anaerobic isolates from hospital patients (Sutter et al., 1985). *F. nucleatum* and *F. necrophorum* may also be encountered in blood cultures, particularly when the upper respiratory and the female genital tract are the portals of entry (Felner and Dowell, 1971; Henry et al., 1983). *Fusobacterium* species may be a cause of chorioamnionitis (Altshuler and Hyde, 1985). *F. nucleatum* is invariably present in Plaut-Vincent's angina (fusospirochetal angina) and in acute necrotizing ulcerative gingivitis.

F. necrophorum is the cause of human necrobacillosis. This is a rare, but life-threatening septicemia which predominantly affects healthy young adults. The infection is characterized by sore throat, followed by rigor and the formation of abscesses, usually involving the lung (Lemierre, 1936; Moore-Gillon et al., 1984).

F. necrophorum is an animal pathogen that is frequently isolated from necrotic and gangrenous lesions in cattle, sheep, and pigs and less frequently from other animals. Carnivorous animals appear to be resistant. The most common manifestations of diseases associated with *F. necrophorum* are liver abscess (hepatic necrobacillosis) and footrot.

Liver abscesses are especially encountered in heavily fattened cattle. Ninety % or more of such abscesses contain *F. necrophorum* as the only organism or in combination with other organisms (Hussein and Shigidi, 1974; Kanoe et al., 1976; Newsom, 1938; Simon and Stovell, 1971; Berg and Scanlan, 1982). The disease is associated with inflammation of the forestomach, presumably caused by irritating substances produced by fermentation of the high-caloric feed or by foreign bodies (Jensen and Mackay, 1965). *F. necrophorum* present in the stomach contents is thought to gain entry to the vascular system through the injured mucosa. Liver abscesses have thus been produced experimentally in cattle and sheep by intraportal injections of viable cells of a bovine isolate of *F. necrophorum* (Jensen et al., 1954). Hepatic necrobacillosis in feedlot cattle may present an economic problem in meat-producing countries (Langworth, 1977; Panel report, 1973). Footrot is frequently encountered in sheep and cattle. The disease

affects the epidermal tissues of the interdigital skin and hoof and leads to separation of the hoof from the soft tissues. Lameness and morbidity from infection lead to a reduction in productivity and economic losses. Footrot in sheep is caused by *Bacteroides nodosus* in combination with *Fusobacterium necrophorum*. *B. nodosus* is the principal causative agent transmitting the disease from one animal to another (Beveridge, 1941), whereas *F. necrophorum* is essential for the later inflammatory destruction of tissue (Egerton et al., 1969; Roberts and Egerton, 1969). Injury to the foot and damp soil are predisposing factors (Graham and Egerton, 1968). Effective protection against ovine footrot is achieved through immunization with killed, fimbriated *B. nodosus* cells (Egerton and Roberts, 1971; Stewart et al., 1982).

The primary cause of epizootic footrot in cattle has not been found. *F. necrophorum* is present in the lesions as a concurrent pathogen or a secondary invader. Typical lesions have been produced in cattle by the intradigital or intradermal inoculation of *F. necrophorum* alone or in combination with black-pigmented bacteroides (Berg and Loan, 1975; Clark et al., 1985).

Another disease associated with *F. necrophorum* is calf "diphtheria," which is necrotic laryngitis that occurs in calves up to 2 years of age. *F. necrophorum* is also involved in several other suppurative or gangrenous processes in domestic animals, such as interdigital dermatitis and heel abscess in sheep (Parsonson et al., 1967; Roberts et al., 1968), neonatal bacteremias in calves and lambs, necrotic enteritis of pigs, necrotic rhinitis of growing pigs, and oral infections in several animals.

Necrobacillosis has long been known as a serious cause of death of macropods, mainly in zoological collections, but also in wild habitats. The body sites most commonly affected are the face, the stomach wall, and the hind limbs. There is good evidence that *F. necrophorum* is the main etiological agent of the disease, but other organisms, especially *Bacteroides* species, are often also present in the lesions in high numbers (Oliphant et al., 1984; Samuel, 1983). *Fusobacterium* species are the predominant species of the mixed flora in soft tissue infections of cats caused by contamination from the oral flora (Love et al., 1980).

Virulence Determinants

The natural infections have verified the infectivity and invasiveness in animals of *F. necrophorum* and in man of *F. nucleatum* and *F. necrophorum*. Experimental investigations in animals have shown that synergistic mechanisms may be of importance in the pathogenesis of mixed infections involving fusobacteria (Brook and Walker, 1986; Hamp and Mergenhagen, 1963; Hill et al., 1974; Kaufman et al., 1972; Onderdonk et al., 1976; Roberts, 1967a, b).

Our current knowledge of virulence determinants is cursory. Fimbriation has been seen in *F. necrophorum* (Shinjo and Kiyoyma, 1986), but not in *F. nucleatum* or other *Fusobacterium* species. Encapsulation has been observed in *F. nucleatum* and *F. necrophorum* (Brook, 1986; Emery, 1988).

F. necrophorum isolates from cattle and sheep have been assigned to biovars A and B, and to an intermediate type, AB, on the basis of cultural characteristics (Fievez, 1963). A and AB biovars are frequently isolated from lesions, are highly virulent in mice, are hemolytic, and produce a hemagglutinin. B biovars are of low virulence in mice and nonhemolytic. The agglutinin is associated with the cell vall, has a subunit molecular weight of 19,000, and is heat labile (Nagai et al., 1984). Certain strains of *F. necrophorum* produce a leukocidin which is also destructive for erythrocytes and a variety of cultivated cells (Coyle-Dennis and Lauerman, 1978; Fales et al., 1977; Garcia et al., 1975a; Ishii et al., 1988; Roberts, 1967a, b; Scanlan et al., 1982). The toxin is possibly a phospholipase (Abe et al., 1979). High amounts of leukocidin are produced by A and AB biovars of *F. necrophorum*, indicating a correlation between toxin production and virulence. (Coyle-Dennis and Lauerman, 1979; Emery and Vaughan, 1986; Scanlan et al., 1982).

A hemagglutinin is also produced by *F. nucleatum* (Dehazya and Coles, 1980; Falkler and Hawley, 1977). *F. nucleatum* and *F. necrophorum*, as well as other *Fusobacterium* species, posses a cell wall lipopolysaccharide with the characteristics of an endotoxin (Garcia et al., 1975; Hofstad and Kristoffersen, 1971; Sveen et al., 1977; Warner et al., 1975).

Physiology

Fusobacterium species are not particularly demanding with regard to a low oxidation-reduction potential. The maximum E_h value permitting growth varies, depending on the species, the size of inoculum, and the medium. Stock strains of *F. nucleatum* were able to grow in an oxygen tension of up to 6% (Loesche, 1969). Fusobacteria are, however, fairly readily killed by exposure to air. This is possibly due to their susceptibility to hydrogen peroxide and is especially noticeable when thioglycolate or cysteine HC1 is incorporated into media that are exposed to air before inoculation and incubation.

Fusobacteria grow readily on ordinary solid media, such as Brucella blood agar and brain heart infusion agar, and in fluid media with a

base of peptone and yeast extract. Proteose peptone, tryptone, and trypticase have better growth-promoting effects than casamino acids (Gharbia and Shah, 1988a).

Energy is obtained from peptides and amino acids, which are fermented to give a mixture of butyric and acetic acids. Peptides represent the most important energy source (Gharbia et al., 1989). Lysine is likely catabolized by the 3-keto, 5-aminohexanoate pathway (Barker et al., 1982). Glutamate may be degraded through different pathways (Gharbia, 1987). *F. necrophorum* has an absolute need for protein-containing polypeptides (Wahren and Holme, 1973). Most *Fusobacterium* species convert threonine to proprionate. Lactate is converted to proprionate by *F. necrophorum*.

All the *Fusobacterium* species are either nonfermentative or only weakly fermentative. All species examined are able to utilize glucose, which is incorporated into cellular components (Robrish et al., 1987; Gharbia and Shah, 1988a). The accumulation of glucose is dependent upon energy supplied by fermentation of amino acids (Robrish et al., 1987). A low terminal pH (seldom lower than pH 5.5) in glucose-containing media indicates that, in at least a few species, some glucose is fermented.

Glutamate dehydrogenase is produced by all *Fusobacterium* species. The activity at different pH values and the electrophoretic mobility of the enzyme differ between species or groups of species (Gharbia and Shah, 1988b). Possibly all fusobacteria produce deoxyribonuclease (Porschen and Sonntag, 1974). *F. necrophorum* is able to deconjugate bile salts (Shimada et al., 1969).

The fusobacteria are susceptible to many of the commonly used antibiotics, including penicillins and cephalosporins. They are, however, resistant or relatively resistant to vancomycin, neomycin, and erythromycin. *F. nucleatum* may produce beta-lactamase. *F. varium* and *F. mortiferum* are resistant to rifampicin (rifampin). The growth of these two organisms is not inhibited by bile, to which other species of *Fusobacterium* are susceptible. Along with several other Gram-negative bacteria, the fusobacteria will grow in the presence of low concentrations of various dyes.

Isolation

Sampling

Fusobacterium nucleatum is best isolated from saliva or centrifuged salivary deposits or from the crevice or pocket that exists between the gingiva and the tooth surface. Sampling from the crevice area is performed by the use of sterile filter paper points (absorbent dental points), which are gently inserted into the crevice. Saliva or salivary deposits may be inoculated into the medium either directly or after being resuspended in a reducing diluent, such as the serum-containing diluent of Bowden and Hardie (1971), or in prereduced anaerobically sterilized (PRAS) one-fourth-strength Ringer solution (Sutter et al., 1985). The inoculated tapering end of the paper point is streaked on a small area of the solid medium, and further spreading of the deposited material is carried out by a wire loop. Because of their presence in small numbers, isolation of other *Fusobacterium* species from their natural habitat in man can be difficult. Detailed directions for collection, transport, and processing of fecal specimens have been given by Sutter et al. (1985). It is essential that the specimens are thoroughly homogenized and adequately diluted in a reducing diluent before inoculation. This applies also to the isolation of *F. necrophorum* from the intestinal tract of animals.

Isolation Under Nonselective Conditions

When present in clinical specimens, *Fusobacterium nucleatum* and *F. necrophorum* and the less commonly isolated *Fusobacterium* species are usually recovered on solid nonselective media. If the colonies are carefully inspected, the Gram stain is properly used and subculturing is performed promptly, isolation is usually straight forward.

Nonselective isolation of fusobacteria from their natural habitats on the mucous membranes in man and animals is laborious and time-consuming. Such isolation attempts should be avoided in those instances where isolation on selective media is possible. However, isolation under nonselective conditions seems to be the most reliable method for examination and quantitation of viable cells in normal flora specimens. For this purpose the roll tube method (Holdeman et al., 1977; Moore, 1966) or the use of a glove box (Aranki et al., 1969) is to be recommended. By inoculating roll tubes with 1 ml each of 10^8, 10^9, and 10^{10} dilutions of homogenized feces, bacterial species—including fusobacteria—present in numbers as low as 3×10^{10} per gram of fecal dry matter (0.06% of the fecal bacterial population) were counted (Moore and Holdeman, 1974).

Selective Isolation

Media formulations have been developed and evaluated for selective isolation of fusobacteria

from human (Baird-Parker, 1957; Morgenstein et al., 1981; Ohtani, 1970b; Omata and Disraely, 1956; Sutter et al., 1971; Walker et al., 1979) and animal (Fales and Teresa, 1972a) sources.

The following medium of Morgenstein et al., (1981) is recommended for the isolation of *Fusobacterium* species from their natural habitats and from clinical specimens.

Fusobacterium Egg Yolk Agar (FEA) Medium (Morgenstein et al., 1981; Sutter et al., 1985)

Brucella agar base	37 g
Disodium phosphate	5 g
Monopotassium phosphate	1 g
Magnesium sulfate	0.1 g
Hemin solution (5 mg/ml)	1 ml
Polysorbate 80	1 ml
Neomycin solution (100 mg/ml)	1 ml
Distilled water	1,000 ml
Vancomycin solution (7.5 mg/ml)	0.67 ml
Josamycin (Yamanouchi Pharmaceuticals)	3 mg
Egg yolk emulsion (Difco)	50 ml
(or Egg yolk emulsion) (Oxoid)	74 ml

The vancomycin, the josamycin and the egg yolk emulsion are added to the medium after adjustment of pH to 7.6, dissolving by boiling, autoclaving at 121°C for 15 min, and cooling to 50°C.

The FEA medium is selective because of its content of antibiotics. Addition of egg yolk makes it differential for *F. necrophorum*, which is lipase positive. All species of *Fusobacterium* grow on the medium with only minimal inhibition. The typical colonial morphology is translucent to white, convex, round, and entire colonies. *Leptotrichia* grows with white, raised, and granular colonies, and *Veillonella* forms small and translucent colonies. The growth of a majority of facultative Gram-negative organisms is inhibited. Gram-positive organisms do not grow on the medium.

The CVE medium of Walker et al. (1979) may be used in addition to the FEA medium for isolation of *F. nucleatum* from the oral cavity.

Crystal Violet Erythromycin Agar (CVE) Medium (Walker et al., 1979)

Trypticase	10 g
Yeast extract	5 g
Sodium chloride	5 g
Glucose	2 g
Tryptophane	0.2 g
Agar	15 g
Crystal violet	5 mg
Distilled water	1,000 ml
Sterile defibrinated sheep blood	50 ml
Erythromycin (dissolved in small volume of 95% ethanol)	4 mg

The blood and the erythromycin are added to the medium after adjustment of the pH to 7.0–7.2, autoclaving at 121°C for 20 min, and cooling to 50°C.

The selectivity of the CVE medium depends on its content of erythromycin and crystal violet. Strains of *F. nucleatum* exhibit one of two different colony types on CVE. Some strains form transparent, smooth, round, blue colonies having an entire edge with a darker blue center. Other strains form transparent, rounded or irregular, blue colonies with a speckled internal appearance. Several species of Gram-positive and Gram-negative organisms grow on the medium, but with a colonial morphology distinguishable from those of *F. nucleatum*.

The medium of Sutter et al. (1971) is selective for *F. varium* and *F. mortiferum* and can be used for isolation of these species from feces or from other sources. The selectivity of this medium depends on the addition of rifampin to standard blood agar.

Rifampin Blood Agar (Sutter et al., 1971; Sutter et al., 1985)

A total of 50 µg/ml of rifampin is added to Brucella blood agar just before the plates are poured. Rifampin stock solution: 0.1 g of rifampin is dissolved in 20 ml of absolute ethyl alcohol, and 80 ml of sterile distilled water is added. This gives a final concentration of 1,000 µg/ml. The solution can be stored at 4°C for up to 2 months.

F. varium and *F. mortiferum* grow freely on this medium, while the growth of *Bacteroides* and most other organisms present in human feces in high numbers is inhibited.

A medium selective for the isolation of *F. necrophorum* from bovine liver abscesses was reported by Fales and Teresa (1972a). The medium is based on the trypticase and egg yolk medium of McClung and Toabe (1947) and contains crystal violet and phenethyl alcohol as selective agents.

Isolation Medium for Fusobacterium necrophorum (Fales and Teresa, 1972a)

To 415.0 ml of distilled water add the following:

Trypticase	16.0 g
Biosate	4.0 g
Thiotone	2.0 g
Glucose	0.5 g
$MgSO_4$ (5% solution)	0.1 ml
Na_2HPO_4	2.5 g
Agar	8.3 g

Adjust to pH 7.3.

After autoclaving, the basal medium is cooled to 50°C, and 1.35 ml (0.27% vol/vol) of phenethyl alcohol is added. One egg yolk mixed with an equal volume of a 0.9% sterile saline solution (total volume, approximately 45.0 ml) and blended with the basal medium and then 11.5 mg of crystal violet dissolved in 25.0 ml of sterile distilled water are added. Finally, the volume is adjusted to 500 ml with sterile distilled water.

Small colonies of *Proteus* species appearing on the medium are easily distinguished from the

Table 2. Differential characteristics of *Fusobacterium* species encountered in clinical specimens.

Characteristic	*F. nucleatum*	*F. necrophorum*	*F. gonidiaformans*	*F. naviforme*	*F. mortiferum*	*F. russi*	*F. varium*
Growth in 20% bile	–	–	–	–	+	–	+
Production of							
Gas from PYG[a]	–	+	+	–	+	+	+
Indole	+	+	+	+	–	–	+
Lipase	–	+	–	–	–	–	–
Hydrolysis of esculin	–	–	–	–	+	–	–
Propionate from							
Lactate	–	+	–	–	–	–	–
Threonine	+	+	+	–	+	–	+
Fatty acids from PYG	Acetic, propionic, butyric; sometimes formic, lactic, succinic	Acetic, propionic, butyric; sometimes lactic, succinic	Acetic, propionic, butyric; sometimes formic, lactic, succinic	Acetic, butyric, lactic; sometimes formic, propionic, succinic	Acetic, propionic, butyric; sometimes formic, lactic, valeric, succinic	Acetic, butyric, lactic; sometimes formic	Acetic, propionic, butyric, lactic; sometimes succinic

[a]PYG, peptone-yeast extract-glucose broth.
+, positive reaction for majority of strains; –, negative reaction for majority of strains.

larger colonies (1.5–1.7 mm in diameter after 48 h of incubation) of *F. necrophorum*.

As previously mentioned, the various selective media designed for isolation of *Fusobacterium* species allow other organisms to grow to a varying extent. In order to gain experience with these media it is important, therefore, to check the different colony types by Gram-staining.

Axenic Cultivation and Maintenance

Fusobacterium strains can be maintained by weekly serial subcultures on blood agar. Viable cells can be stored at –70°C in Greave's solution. Stock cultures can also be prepared in skim milk (Sutter et al., 1985).

Greave's Solution

Bovine serum albumin	50 g
Sodium glutamate	50 g
Glycerol	100 g
Distilled water	1,000 ml

Batch cultivation is best performed in a nutrient broth with a tryptone base, which is supplemented with yeast extract (0.3%), glucose (0.25%), and cysteine HC1 (0.1%), or in the selective media bases. If narrow-necked, well-filled containers are used, PRAS media are usually not necessary.

F. necrophorum has been grown in continuous culture with glucose as the growth-limiting factor (Wahren et al., 1971). Maximal cell yields (3.5 mg/ml dry weight) were achieved at dilution rates between 0.19 h^{-1} and 0.40 h^{-1}, at a pH of 6.8, and at temperatures of 33–36°C.

Identification

All *Fusobacterium* species are susceptible to colistin and kanamycin, are resistant to vancomycin, and produce butyric acid without isobutyric or isovaleric acids. In the clinical diagnostic laboratory identification is based on both cell and colony morphology and on biochemical properties. Chemotaxonomic methods, such as DNA-DNA hybridization, lipid analysis, electrophoretic mobility of glutamate dehydrogenase, outer membrane protein patterns, and peptidoglycan composition, are useful for taxonomical purposes.

Morphology

Fusobacterium nucleatum has a characteristic cell morphology that makes presumptive identification relatively easy. The cells are Gram-negative, slender, spindle-shaped bacilli with sharply pointed ends, often appearing in pairs and end-to-end. Most cells are 5–10 μm long, but both shorter and longer rod forms may be seen. *F. periodonticum* has a similar cell morphology. This organism has, however, not been isolated from clinical specimens. The fusiform cells of *Capnocytophaga* species are generally smaller than those of *F. nucleatum*. Those of *Leptotrichia buccalis* are thicker and usually larger (distinguishing characters are given in Table 1 of The Genus *Leptotrichia* in the second edition). The cells of *F. necrophorum* are pleomorphic, are often curved, and may have spherical enlargements. Free coccoid bodies and, especially, filaments are common. *F. naviforme* strains may have boat-shaped cells. Gonidial forms may be seen in old cultures of *F. gonidiaformans*. The other *Fusobacterium* species have no distinctive cellular morphology.

Colonies of *F. nucleatum* on blood agar are low convex, glistening, and slightly irregular in form. Those of *F. necrophorum* are circular, rough, and often β-hemolytic. The other *Fusobacterium* species form smooth, small-to-punctiform colonies on blood agar after incubation for two days.

Biochemical Properties

Differential characteristics of the most common *Fusobacterium* species are shown in Table 2. In addition, *F. periodonticum* hydrolyzes hippurate; nitrate is reduced by *F. ulcerans*; and the pH may be slightly lowered in cultures of *Fusobacterium* species grown in carbohydrate-containing media, but also in cultures grown without added carbohydrate. This makes the interpretation of fermentation reactions difficult.

Commercial multitest systems for identification of bacteria are generally associated with a percentage of misidentifications, which may be either system or laboratory dependent. Experience is limited with respect to identifications of *Fusobacterium* species.

DNA probes for identification of *Fusobacterium* species are not available. The fluorescent antibody technique has been used for identification of fusobacteria in clinical specimens from man (Griffin, 1970; Stauffer et al., 1975) and for *F. necrophorum* in bovine liver abscesses (Fales and Teresa, 1972b). Simon (1975) has described a hemagglutination inhibition test for rapid identification of *F. necrophorum*.

Literature Cited

Aalbaek, B. 1972. Gram-negative anaerobes in the intestinal flora of pigs. Acta Vet. Scand. 13:228–237.

Abe, P. M., C. J. Kendall, L. R. Stauffer, J. W. Holland. 1979. Hemolytic activity of *Fusobacterium necrophorum* culture supernatants due to presence of phospholipase A and lysophospholipase. Am. J. Vet. Res. 40:92–96.

Adriaans, B., B. S. Drasar. 1987. The isolation of fusobacteria from tropical ulcers. Epidem. Infect. 99:361–372.

Adriaans, B., H. Shah. 1988. *Fusobacterium ulcerans* sp. nov. from tropical ulcers. Int. J. Syst. Bacteriol. 38:447–448.

Altshuler, A., S. Hyde. 1985. Fusobacteria. An important cause of chorioamnionitis. Arch. Pathol. Lab. Med. 109:739–743.

Aranki, A., S. A. Syed, E. B. Kenney, R. Freter. 1969. Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. Appl. Microbiol. 17:568–576.

Baird-Parker, A. C. 1957. Isolation of *Leptotrichia buccalis* and *Fusobacterium* species from oral material. Nature 180:1056–1057.

Bang, B. 1890–1891. Om aarsagen til lokal nekrose. Maanedskrift for Dyrlaeger. 2:235–259.

Barker, H. A., J. M. Kahn, L. Hedrick. 1982. Pathway of lysine degradation in *Fusobacterium nucleatum*. J. Bacteriol. 152:201–207.

Bartlett, J. G., V. L. Sutter, S. M. Finegold. 1974. Anaerobic pleuropulmonary disease: Clinical observations and bacteriology in 100 cases. 327–344. A. Balows, R. M. DeHaan, V. R. Dowell, Jr., and L. B. Guze (ed.) Anaerobic bacteria: role in disease. Charles C. Thomas. Springfield, IL.

Beerens, H., M. M. Castel, L. Fievez. 1962. Classification des Bacteroidaceae. 120. Abstr VIII Int. Congress Microbiol. Montreal.

Beerens, H., M. M. Tahon-Castel. 1965. Infectiones humaines à bactèries anaérobies non toxigénes. Presses Académiques Européennes. Brussels.

Berg, J. N., R. W. Loan. 1975. *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* as etiologic agents of footrot in cattle. Am. J. Vet. Res. 36:1115–1122.

Berg, J. N., C. M. Scanlan. 1982. Studies of *Fusobacterium necrophorum* from bovine hepatic abscesses: Biotypes, quantitation, virulence, and antibiotic susceptibility. Am. J. Vet. Res. 43:1580–1586.

Beveridge, W. I. B. 1941. Foot rot in sheep: A transmissible disease due to infection with *Fusiformis nodosus* (n.sp.). Council for Scientific and Industrial Research, Commonwealth of Australia, Bulletin 140, Melbourne.

Bowden, G. H., J. M. Hardie. 1971. Anaerobic organisms from the human mouth. 177–205. D. A. Shapton and R. G. Board (ed.) Isolation of anaerobes. Society for Applied Bacteriology Technical Series No. 5. Academic Press, London.

Brams, J., I. Pilot, D. J. Davis. 1923. Studies of fusiform bacilli and spirochetes. II. Their occurrence in normal preputial secretions and in erosive and gangrenous balanitis. J. Infect. Dis. 32:159–166.

Breed, R. S., E. G. D. Murray, N. R. Smith (ed.). 1957. Bergey's manual of determinative bacteriology, 7th ed. Williams & Wilkins, Baltimore.

Brook, I. 1986. Encapsulated anaerobic bacteria in synergistic infections. Microbiol. Rev. 50:452–457.

Brook, I. 1988. Recovery of anaerobic bacteria from clinical specimens in 12 years at two military hospitals. J. Clin. Microbiol. 26:1181–1188.

Brook, I., R. I.Walker. 1986. The relationship between *Fusobacterium* species and other flora in mixed infection. J. Med. Microbiol. 21:93–100.

Cameron, G. R., F. E. Williams. 1926. An epidemic affecting stock rabbits. J. Pathol. Bacteriol. 29:185–188.

Castellani, A., A. J. Chalmers. 1919. Manual of tropical medicine. William Wood & Company, Baltimore.

Cato, E. P., L. V. H. Moore, W. E. C. Moore. 1985. *Fusobacterium alocis* sp. nov. and *Fusobacterium sulci* sp. nov. from the human gingival sulcus. Int. J. Syst. Bacteriol. 35:475–477.

Clark, B. L., D. J. Stewart, D. L. Emery. 1985. The role of *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* in the aetiology of interdigital necrobacillosis in cattle. Aust. Vet. J. 62:47–49.

Coyle-Dennis, J. E., L. H. Lauerman. 1978. Biological and biochemical characteristics of *Fusobacterium necrophorum* leukocidin. Am. J. Vet. Res. 39:1790–1793.

Coyle-Dennis, J. E., L. H. Lauerman. 1979. Correlations between leukocidin production and virulence of isolates of *Fusobacterium necrophorum*. Am. J. Vet. Res. 40:274–276.

Dack, G. M., L. R. Dragstedt, T. E. Heinz. 1937. Further studies on *Bacterium necrophorum* isolated from cases of chronic ulcerative colitis. J. Infect. Dis. 60: 335–355.

Dack, G. M., T. E. Heinz, L. R. Dragstedt. 1935. Ulcerative colitis. Study of bacteria in the isolated colons of three patients by cultures and by inoculation of monkeys. Arch. Surg. 31:225–240.

Davis, D. J., I. Pilot. 1922. Studies of *Bacillus fusiformis* and Vincent's spirochete. I. Habitat and distribution of these organisms in relation to putrid and gangrenous processes. J. Amer. Med. Ass. 79:944–951.

Dehazya, P., R. J. Coles, Jr. 1980. Agglutination of human erythrocytes by *Fusobacterium nucleatum*: factors influencing hemagglutination and some characteristics of the agglutinin. J. Bacteriol. 143:205–211.

Egerton, J. R., D. S. Roberts. 1971. Vaccination against ovine foot-rot. J. Comp. Pathol. 81:179–185.

Egerton, J. R., D. S. Roberts, I. M. Parsonson. 1969. The aetiology and pathogenesis of ovine foot-rot. I. A histological study of the bacterial invasion. J. Comp. Pathol. 79:207–216.

Eggerth, A. H., B. H. Gagnon. 1933. The bacteroides of human feces. J. Bacteriol. 25:389–413.

Ellermann, V. 1904. Über die Kultur der fusiformen Bacillen. Cbl Bakteriol. Parasiten. Infektkr. Abt. I Orig. 37:729–730.

Emery, D. L. 1988. Approaches to identify and neutralize virulence determinants of *Fusobacterium* and *Bacteroides* spp. 343–362. J. A. Roth (ed.) Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington D.C.

Emery, D. L., J. A. Vaughan. 1986. Generation of immunity against *Fusobacterium necrophorum* in mice inoculated with extracts containing leucocidin. Vet. Microbiol. 12:255–268.

Fales, W. H., G. W. Teresa. 1972a. A selective medium for the isolation of *Sphaerophorus necrophorus.*. Am. J. Vet. Res. 33:2317–2321.

Fales, W. H., G. W. Teresa. 1972b. Fluorescent antibody technique for identifying isolates of *Sphaerophorus necrophorus* of bovine hepatic abscess origin. Am. J. Vet. Res. 33:2323–2329.

Fales, W. H., J. F. Warner, G. W.Teresa. 1977. Effects of *Fusobacterium necrophorum* leukotoxin on rabbit peritoneal macrophages in vitro. Am. J. Vet. Res. 38:491–495.

Falkler, W. H., Jr., C. E. Hawley. 1977. Hemagglutinating activity of *Fusobacterium nucleatum*. Infect. Immun. 15:230–238.

Felner, J. M., V. R. Dowell, Jr. 1971. "Bacteroides" bacteremia. Am. J. Med. 50:787–796.

Fievez, L. 1963. Etude comparée des souches de *Sphaerophorus necrophorus* isolées de L'homme et chez L'animal. Monographie, Presses Académiques Européennes. Brüssel.

Finegold, S. 1977. Anaerobic bacteria in human disease. Academic Press, New York.

Finegold, S. M., H. R. Attebery, V. L. Sutter. 1974. Effect of diet on human fecal flora: Comparison of Japanese and American diets. Am. J. Clin. Nutr. 27:1456–1469.

Finegold, S. M., D. J. Flora, H. R. Attebery, V. L. Sutter. 1975. Fecal bacteriology of colonic polyp patients and control patients. Cancer Res. 35:3407–3417.

Foglesong, M. A., D. L. Cruden, A. J. Markovetz. 1984. Pleomorphism of fusobacteria isolated from the cockroach hindgut. J. Bacteriol. 158:474–480.

Fuller, R., M. Lev. 1964. Quantitative studies on some of the Gram-negative anaerobic bacteria in the pig alimentary tract. J. Appl. Bacteriol. 27:434–438.

Gainer, R. S. 1983. Necrobacillosis in wildebeest calves. J. Wildlife Dis. 19:155–156.

Garcia, M. M., D. C. Alexander, K. A. McKay. 1975a. Biological characterization of *Fusobacterium necrophorum* cell fractions in preparation for toxin and immunization studies. Infect. Immun. 11:609–616.

Garcia, M. M., K. M. Charlton, K. A. McKay. 1975b. Characterization of endotoxin from *Fusobacterium necrophorum*. Infect. Immun. 11:371–379.

Gharbia, S. E. 1987. A biochemical approach to the study of the genus *Fusobacterium*. The University of Kuwait.

Gharbia, S. E., H. N. Shah. 1988a. Glucose utilization and growth response to protein hydrolysates by *Fusobacterium* species. Curr. Microbiol. 17:229–234.

Gharbia, S. E., H. N. Shah. 1988b. Characteristics of glutamate dehydrogenase, a new diagnostic marker of the genus *Fusobacterium*. J. Gen. Microbiol. 134:327–332.

Gharbia, S. E., H. N. Shah, S. G. Welch. 1989. The influence of peptides on the uptake of amino acids in *Fusobacterium*; predicted interactions with *Porphyromonas gingivalis*. Curr. Microbiol. 19:231–235.

Gorbach, S. L., K. B. Menda, H. Thadepalli, L. Keith. 1973. Anaerobic microflora of the cervix in healthy women. Am. J. Obst. Gyn. 117:1053–1055.

Graham, N. P. H., J. R. Egerton. 1968. Pathogenesis of ovine foot-rot: The role of some environmental factors. Aust. Vet. J. 44:235–240.

Griffin, M. H. 1970. Fluorescent antibody techniques in the identification of Gram-negative nonsporeforming anaerobes. Hlth Lab. Sci. 7:78–83.

Hadi, A. W., C. Russel. 1969. Fusiforms in gingival material. Quantitative estimations from normal individuals and cases of periodontal disease. Brit. Dent. J. 126:83–84.

Hamp, E. G., S. E. Mergenhagen. 1963. Experimental intracutaneous fusobacterial and fusospirochetal infections. J. Infect. Dis. 112:84–99.

Hardie, J. M., G. H. Bowden. 1974. The normal flora of the mouth. 47–83. F. A. Skinner and J. G. Carr (ed.) The normal microbial flora of man. Society for Applied Bacteriology Symposium Series No. 3. Academic Press, London.

Henry, S., A. DeMaria, Jr., W. R. McCabe. 1983. Bacteremia due to *Fusobacterium* species. Am. J. Med. 75:225–231.

Hill, G. B., S. Osterhout, P. C. Pratt. 1974. Liver abscess production by nonsporeforming anaerobic bacteria in a mouse model. Infect. Immun. 9:599–603.

Hite, K. E., H. C. Hesseltine, L. Goldstein. 1947. A study of the bacterial flora of the normal and pathological vagina and uterus. Am. J. Obst. Gyn. 53:233–240.

Hofstad, T., T. Kristoffersen. 1971. Preparation and chemical characteristics of endotoxic lipopolysaccharide from three strains of *Sphaerophorus necrophorus*. Acta Pathol. Microbiol. Scand., Sect B 79:383–390.

Holdeman, L. V., E. P. Cato, W. E. C. Moore. 1977. Anaerobe laboratory manual. 4th ed., Blacksburg. VPI Anaerobe Laboratory, Virginia Polytechnic Institute and State University, VA.

Holdeman, L. V., I. J. Good, W. E. C. Moore. 1976. Human fecal flora: Variation in bacterial composition within individuals and a possible effect of emotional stress. Appl. Environ. Microbiol 31:359–376.

Horne, H. 1898–1899. Renens klovsyge. Nor. Vet. tidsskr. 10–11:97–110.

Hurst, V. 1957. *Fusiformis* in the infant mouth. J. Dent. Res. 36:513–515.

Hussein, H. E., M. T. A. Shigidi. 1974. Isolation of *Sphaerophorus necrophorus* from bovine liver abscess in the Sudan. Trop. An. Hlth. 6:253–254.

Ishii, T., M. Kanoe, T. Inoue, K. Kai, H. Blobel. 1988. Cytotoxic effects of a leukocidin from *Fusobacterium necrophorum* on bovine hepatic cells. Med. Microbiol. Immunol. 177:27–32.

Jensen, C. O. 1913. Die vom Nekrosebacillus (Bacillus necroseos) hervorgerutenen Krankheiten. 234–250. W. Kolle and A. von Wassermann (ed.) Handbuch der pathogenen Mikroorganismen, vol. 6. Gustav Fischer Verlag, Jena.

Jensen, R., J. Flint, L. A. Griner. 1954. Experimental hepatic necrobacillosis in beef cattle. Am. J. Vet. Res. 15:5–14.

Jensen, R., D. R. MacKay. 1965. Diseases of feedlot cattle. Lea & Febiger. Philadelphia,

Kanoe, M., H. Imagawa, M.Toda, A.Sato, M.Inoue, Y.Yoshimoto. 1976. Bacteriology of bovine hepatic abscesses. Jpn. J. Vet. Sci. 38:263–268.

Kaufman, E. J., P. A. Mashimo, E. Hausmann, C. T. Hanks, S. A. Ellison. 1972. Fusobacterial infection. Enhancement by cell free extracts of *Bacteroides melaninogenicus* possessing collagenolytic activity. Arch. Oral Biol. 17:577–580.

Knorr, M. 1923. Uber die fusospirilläre Symbiose, die Gattung *Fusobacterium* (K. B. Lehmann) und *Spirillum sputigenum*. II. Mitteilung. Die Gattung Fusobacterium. Zbl. Bakteriol. Parasitenk. Infektkr. Abt. 1 Orig. 89:4–22.

Krygier, G., R. J. Genco, P. A. Mashimo, E. Hausmann. 1973. Experimental gingivitis in *Macaca speciosa* monkeys: Clinical, bacteriological and histological similarities to human gingivitis. J. Periodontol. 44:454–463.

Langworth, B. F. 1977. *Fusobacterium necrophorum*: Its characteristics and role as animal pathogen. Bacteriol. Rev. 44:373–390.

Lemierre, A. 1936. On certain septicaemias due to anaerobic organisms. Lancet ii:701–703.

Lewis, K. H., L. F. Rettger. 1940. Non-sporulating anaerobic bacteria of the intestinal tract. J. Bacteriol. 40:287–307.

Lewkowicz, X. 1901. Recherches sur la flore microbienne de la bouche des nourrissons. Arch. Med. Exp. 13:633–660.

Loeffler, F. 1884. Bacillus der Kälberdiphterie. Mittheilungen aus dem Kaiserlichen Gesundheitsamte 2:493–499.

Loesche, W. J. 1969. Oxygen sensitivity of various anaerobic bacteria. Appl. Microbiol. 18:723–727.

Love, D. N., E. P. Cato, J. L. Johnson, R. F. Jones, M. Bailey. 1987. Deoxyribonucleic acid hybridization among

strains of fusobacteria isolated from soft tissue infections of cats: comparison with human and animal type strains from oral and other sites. Int. J. Syst. Bacteriol. 37:23–26.

Love, D. N., R. F. Jones, M. Bailey. 1980. Characterization of *Fusobacterium* species isolated from soft tissue infections in cats. J. Appl. Bacteriol. 48:325–331.

McCarthy, C., M. L. Snyder, R. B. Parker. 1965. The indigenous oral flora of man. I. The newborn to the 1-year-old infant. Arch. Oral Biol. 10:61–70.

McClung, L. J., R. Toabe. 1947. The egg yolk plate reaction for the presumptive diagnosis of *Clostridium sporogenes* and certain species of the gangrene and botulinum groups. J. Bacteriol. 53:139–147.

Maier, B. R., M. A. Flynn, G. C. Burton, R. K. Tsukakawa, D. J. Hentges. 1974. Effects of a high-beef diet on bowel flora: A preliminary report. Am. J. Clin. Nutr. 27:1470–1474.

Mettam, R. W. M., J. Carmichael. 1933. Necrobacillosis in recently captured antelope in Uganda. J. Comp. Pathol. 46:16–24.

Miller, W. D. 1889. Die Mikroorganismen der Mundhöhle. Die örtlichen and allgemeinen Erkrankungen welche durch dieselben hervorgerufen werden. Georg Thieme Verlag, Leipzig.

Moore, W. E. C. 1966. Techniques for routine culture of fastidious anaerobes. Int. J. Syst. Bacteriol. 16:173–190.

Moore, W. E. C., L. V. Holdeman. 1974a. Genus II *Fusobacterium*. Knorr 1922. 404–416.R. E. Buchanan, and N. E. Gibbons (ed.) Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Company, Baltimore.

Moore, W. E. C., L. V. Holdeman. 1974b. Human fecal flora: The normal flora of 20 Japanese-Hawaiians. Appl. Environ. Microbiol. 27:961–979.

Moore, W. E. C., L. V. Holdeman, E. P. Cato, M. Smibert, J. A. Burmeister, R. R. Ranney. 1983. Bacteriology of moderate (chronic) periodontitis in mature adult humans. Infect. Immun. 42:510–515.

Moore, W. E. C., L. V. Holdeman, R. M. Smibert, D. E. Hash, J. A. Burmeister, R. R. Ranney. 1982. Bacteriology of seven periodontitis in young adults human. Infect. Immun. 38:1137–1148.

Moore-Gillon, J., T. M. Lee, S. J. Eykyn, I. Philips. 1984. Necrobacillosis: a forgotten disease. Brit. Med. J. 288:1526–1527.

Morgenstein, A. A., D. M. Citron, S. M. Finegold. 1981. New medium selective for *Fusobacterium* species and differential for *Fusobacterium necrophorum*. J. Clin. Microbiol. 13:666–669.

Nagai, S., M. Kanoe, M. Toda. 1984. Purification and partial characterization of *Fusobacterium necrophorum* hemagglutins. Zbl. Bakteriol. A258:232–241.

Newsom, I. E. 1938. A bacteriologic study of liver absceses in cattle. J. Infect. Dis. 63:232–233.

Nolechek, W. F. 1918. Necrobacillosis in horses and mules. J. Am. Vet. Med. Ass. 54:150–155.

Ohtani, F. 1970a. Selective media for the isolation of Gram-negative anaerobic rods. Jpn. J. Bacteriol. 25:222–232.

Ohtani, F. 1970b. Selective media for the isolation of Gram-negative anaerobic rods. Part II. Distribution of Gram-negative anaerobic rods in feces of normal human beings. Jpn. J. Bacteriol. 25:292–299.

Oliphant, J. C., R. Parsons, G. R.Smith. 1984. Aetiological agents of necrobacillosis in captive wallabies. Res. Vet. Sci. 36:382–384.

Omata, R. R., M. N. Disraely. 1956. A selective medium for oral fusobacteria. J. Bacteriol. 72:677–680.

Onderdonk, A. B., J. G. Bartlett, T. Louie, N. Sullivan-Seigler, S. L. Gorback. 1976. Microbial synergy in experimental intra-abdominal abscess. Infect. Immun. 13:22–26.

Panel, Report. 1973. Foot rot among cattle. Panel Report. Modern Vet. Practice 54:63–65.

Parsonson, I. M., J. R. Egerton, D. S. Roberts. 1967. Ovine interdigital dermatitis. J. Comp. Pathol. 77:309–313.

Peach, S., F. Fernandez, K. Johnson, B. S. Drasar. 1974. The nonsporing anaerobic bacteria in human faeces. J. Med. Microbiol. 7:213–221.

Plaut, H. C. 1894. Studien zur Bakteriellen Diagnostik der Diphterie und der Anginen. Dtsch Med. Wschr. 20:920–923.

Porschen, R. K., S. Sonntag. 1974. Extracellular deoxyribonuclease production by anaerobic bacteria. Appl. Microbiol. 27:1031–1033.

Pratt, J. S. 1927. On the biology of *B. fusiformis*. J. Infect. Dis. 41:461–466.

Prévot, A. R. 1938. Études de systématique bactérienne. III. Invalidité du genre Bacteroides. Castellani et Chalmers. Demembrement et reclassification. Ann. Inst. Pasteur 60:285–307.

Prévot, A. R., P. Goret, L. Joubert, P. Tardieux, N. Aladame. 1951. Recherches bactériologiques sur une infection purulente d'allure actinomycosique chez le chat. Ann. Inst. Pasteur 81:85–88.

Pribram, E. 1929. A contribution to the classification of microorganisms. J. Bacteriol. 18:361–394.

Richardson, R. L., M. Jones. 1958. A bacteriolytic census of human saliva. J. Dent. Res. 37:697–709.

Roberts, D. S. 1967a. The pathogenic synergy of *Fusiformis necrophorus* and *Corynebacterium pyogenesngenuspec 6*. I. Influence of the leucocidal exotoxin of F. necrophorus. Brit. J. Exp. Pathol. 48:665–673.

Roberts, D. S. 1967b. The pathogenic synergy of *Fusiformis necrophorus* and *Corynebacterium pyogenes*. II. The response of *F. necrophorus* to a filterable product of *C. pyogenes*.. Brit. J. Exp. Pathol. 48:674–679.

Roberts, D. S., J. R. Egerton. 1969. The aetiology and pathogenesis of ovine foot-rot. II. The pathogenic association of *Fusiformis nodosus* and *F. necrophorus*. J. Comp. Pathol. 79:217–227.

Roberts, D. S., N. P. H. Graham, J. R. Egerton, I. M. Parsonson. 1968. Infective bulbous necrose (heel abscess) of sheep, a mixed infection with *Fusiformis necrophorus* and *Corynebacterium pyogenes*. J. Comp. Pathol. 78:9–17.

Robinson, D. A., R. J. Hay. 1985. Tropical ulcer in Zambia. Trans. Roy. Soc. Trop. Med. Hyg. 80:132–137.

Robrish, S. A., C. Oliver, J. Thomson. 1987. Amino acid-dependent transport of sugar by *Fusobacterium nucleatum* ATCC 10953. J. Bacteriol. 169:3891–3897.

Samuel, J. L. 1983. Jaw disease in macropod marsupials: Bacterial flora isolated from lesions and from the mouths of affected animals. Vet. Microbiol. 8:373–387.

Scanlan, C. M., J. N. Berg, W. H. Fales. 1982. Comparative in vitro leukotoxin production of three bovine strains of *Fusobacterium necrophorum*. Am. J. Vet. Res. 43:1329–1333.

Schmorl, G. 1891. Über ein pyogenes Fadenbacterium (*Streptothrix cuniculi*). Dsch. Z. Tiermed. Pathol. 17:375–408.

Sebald, M. 1962. Étude sur les bactéries anaérobies gramnégatives asporulées. University of Paris.

Shimada, K., K. S. Bricknell, S. M. Finegold. 1969. Deconjugation of bile acids by intestinal bacteria: Review of

literature and additional studies. J. Infect. Dis. 119:273–281.

Shinjo, T., H. Kiyoyma. 1986. Pathogenicity of a non-haemagglutinating mutant strain of *Fusobacterium necrophorum* biovar A in mice. Jpn J. Vet. Sci. 48:523–527.

Simon, P. C. 1975. A simple method for rapid identification of *Sphaerophorus necrophorus* isolates. Can. J. Comp. Med. Vet. Sci. 39:349–353.

Simon, P. C., P. L. Stovell. 1969. Diseases of animals associated with *Sphaerophorus necrophorus:* characteristics of the organism. Vet. Bull. 39:311–315.

Simon, P. C., P. L. Stovell. 1971. Isolation of Sphaero phorus necrophorus from bovine hepatic abscesses in British Columbia. Can. J. Comp. Med. Vet. Sci. 35:103–106.

Slanetz, L. W., L. F. Rettger. 1933. A systematic study of the fusiform bacteria. J. Bacteriol. 26:599–617.

Slots, J., T. V. Potts. 1982. *Fusobacterium simiae*, a new species from monkey dental plaque. Int. J. Syst. Bacteriol. 32:191–194.

Slots, J., T. V. Potts, P. A. Mashimo. 1983. *Fusobacterium periodonticum*, a new species from the human oral cavity. J. Den. Res. 62:960–963.

Smith, L. D. 1975. The pathogenic anaerobic bacteria 2nd ed. 62–63. A. Balows (ed.) American lectures in clinical microbiology. Charles C. Thomas, Springfield, IL.

Spaulding, E. H., L. F. Rettger. 1937. The *Fusobacterium* genus. I. Biochemical and serological clasification. J. Bacteriol. 34:535–548.

Stauffer, L. R., E. O. Hill, J. W. Holland, W. A. Altemeier. 1975. Indirect fluorescent antibody procedure for the detection and identification of *Bacteroides* and *Fusobacterium* in clinical specimens. J. Clin. Microbiol. 2:337–344.

Stewart, D. J., B. L. Clark, J. E. Peterson, D. A. Griffiths, E. F. Smith. 1982. Importance of pilus-associated antigen in *Bacteroides nodosus* vaccines. Res. Vet. Sci. 32:140–147.

Sutter, V. L., D. M. Citron, M. A. C. Edelstein, S. M. Finegold. 1985. Wadsworth anaerobic bacteriology manual, 4th. ed. Star Publishing Company, Belmont, CA.

Sutter, V. L., P. T. Sugihara, S. M. Finegold. 1971. Rifampin-blood-agar as a selective medium for the isolation of certain anaerobic bacteria. Appl. Microbiol. 22:777–780.

Sveen, K., T. Hofstad, K. C. Milner. 1977. Lethality for mice and chick embryos, pyrogenicity in rabbits and ability to gelate lysates from amoebocytes of *Limulus polyphemus* by lipopolysaccharides from *Bacteroides*, *Fusobacterium* and *Veillonella*. Acta Pathol. Microbiol. Scand. Sect. B 85:388–396.

Syed, S. A. 1972. Biochemical characteristics of *Fusobacterium* and *Bacteroides* species from mouse cecum. Can. J. Microbiol. 18:169–174.

Terada, A., K. Uchida, T. Mitsuoka. 1976. Die Bacteroidaceenflora in den Faeces von Schweinen. Zbl. Bakteriol. Parasitenk. Infektkr. Hyg. Orig. A 234:362–370.

Tissier, H. 1905. Répartition des microbes dans l'intestin du nourrison. Ann. Inst. Pasteur 19:109–123.

Trust, T. J., L. M. Bull, B. R. Currie, J. T. Buckley. 1979. Obligate anaerobic bacteria in the gastrointestinal microflora of the grass carp (*Ctenopharyngodon idella*), goldfish (*Carassius auratus*), and rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 36:1174–1179.

Ueno, K., P. T. Sugihara, K. S. Brichnell, H. R. Attebery, V. L. Sutter, S. M. Finegold. 1974. Comparison of characteristics of Gram-negative anaerobic bacilli isolated from feces of individuals in Japan and the United States. 135–148. A. Balows, R. M. DeHaan, V. R. Dowell Jr., and L. B. Guze (ed.) Anaerobic bacteria: Role in disease. Charles C Thomas, Springfield, IL.

van Assche, F. F., A. T. Wilssens. 1977. *Fusobacterium perfoetens* (Tissier) Moore and Holdeman 1973: Description and proposed neotype strains. Int. J. Syst. Bacteriol. 27:1–5.

van Houte, J., R. J. Gibbons. 1966. Studies of the cultivable flora of normal human feces. Antonie van Leeuwenhoek J. 32:212–222.

Veillon, A., A. Zuber. 1898. Recherches sur quelques microbes strictement anaérobies et leur rôle en pathologie. Arch. Med. Exp. 10:517–545.

Vincent, H. 1896. Sur l'étiologie et sur les lesions anatomopathologique de la pourriture d'Hôpital. Ann. Inst. Pasteur 10:488–510.

Vincent, H. 1899. Recherches bactériologiques sur l'angine a *Bacillus fusiformis*. Ann. Inst. Pasteur 13:609–620.

Vincent, H. 1904. Étiologie de la stomatite ulcéro-membraneuse primitive. Compt. Rend. Soc. Biol. (Paris) 56:311–313.

Wahren, A., K. Bernholm, T. Holme. 1971. Formation of proteolytic activity in continuous culture of *Sphaerophorus necrophorus.*. Acta Pathol. Microbiol. Scand. Sect. B 79:391–398.

Wahren, A., T. Holme. 1973. Amino acid and peptide requirement of *Fusiformis necrophorus*. Bacteriol. 116:279–284.

Walker, C. B., D. Ratlitt, D. Muller, R. Mandell, S. S. Socransky. 1979. Medium for selective isolation of *Fusobacterium nucleatum* from human periodontal pockets. J. Clin. Microbiol. 10:844–849.

Warner, F., W. H. Fales, M. C. Sutherland, G. W. Teresa. 1975. Endotoxin from *Fusobacterium necrophorum* of bovine hepatic abscess origin. Am. J. Vet. Res. 36:1015–1019.

Weinberg, M., R. Nativelle, A. R. Prévot. 1937. Les microbes anaérobies. Masson et Cie, Paris.

Weiss, J. E., L. F. Rettger. 1937. The Gram-negative bacteroides of the intestine. J. Bacteriol. 33:423–434.

Werner, H. 1972a. A comparative study of 55 *Sphaerophorus* strains. Differentiation of 3 species: *Sphaerophorus necrophorus, Sph. varius* and *Sph. freundii*. Med. Microbiol. Immunol. 157:299–314.

Werner, H. 1972b. Anaerobierdifferenzierung durch gaschromatographische Stoffwechselanalysen. Zbl Bakteriol. Parasitenk. Infektkr. Hyg. Orig. A 220:446–451.

Werner, H., F. Neuhaus, H. Hussels. 1971. A biochemical study of fusiform anaerobes. Med. Microbiol. Immunol. 157:10–16.

Werner, H., G. Pulverer. 1971. Haufigkeit und medizinische Bedeutung der eiterregenden *Bacteroides* and *Sphaerophorus*-arten. Dsch. Med. Wschr. 96:1325–1329.

Williams, B. L., R. M. Pantalone, J. C. Sherris. 1976. Subgingival microflora and periodontitis. J. Periodont. Res. 11:1–18.

Prokaryotes (2006) 7:1028–1037
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CHAPTER 14.13

Prokaryotic Symbionts of Amoebae and Flagellates

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Amoebae and flagellates have long been known to be associated with both extracellular and intracellular symbionts (Hall, 1969; Kirby, 1941a; Lee et al., 1985). The presence of prokaryotic symbionts on and in flagellates and in some amoebae, as observed by light microscopy, was reported by several authors during the late 1800s and the early part of this century, as was comprehensively reviewed by Kirby (1941a). Symbiont-bearing flagellates were chiefly found in termite guts, and only a few free-living flagellates were found to have adhering symbionts. Hall (1969) extensively reviewed the literature on symbionts of protozoa published since 1941. Both in flagellates and amoebae, the suspected presence of some of the small bacterial symbionts had to be confirmed later by more sophisticated methods such as electron microscopy and specific staining.

Amoebae and flagellates represent two very diverse groups of protozoa and it is not possible to cover all known prokaryotic symbionts in depth. In this chapter, we shall simply list known symbionts described in the above two reviews in a tabular form (Tables 1 and 2) and then consider newly found symbionts or results of recent studies on earlier symbionts in some detail. The significance of symbiotic relationships remains obscure in most cases and in only a few instances has the host-symbiont relationship been studied in detail. Some disagreements remain about whether the term "symbiosis" should be limited to associations where definite benefits have been proven to exist or not. In this chapter, we shall use the broader definition of symbiosis to include parasitism, commensalism, and mutualism, as did Kirby (1941a) and Hall (1969). Thus, the list of prokaryotic symbionts will include those whose relationships to their hosts are not known or may not be mutually beneficial.

The heightened interest in cellular symbiosis in recent years has been stimulated, in part, by the notion that eukaryotic cell organelles such as mitochondria, chloroplasts, and microtubules may have originated from endosymbionts, i.e., the Serial Endosymbiosis Theory (Sagan, 1967; Margulis, 1970, 1981; Taylor, 1974; papers in Lee and Fredrick, 1987). Some authors have felt that the role of endosymbiosis in the origin of eukaryotic cell organelles has not yet been clearly established (Gray and Doolittle, 1982), but the theory is gaining wider support in view of recent results on the close relationship between the ribosomal RNAs of prokaryotes and those of chloroplasts and mitochondria (e.g., Watson et al., 1987). It should be noted that an opposing view has existed, according to which such organelles evolved as a result of autogenous intracellular differentiation without involving symbionts (Cavalier-Smith, 1975; Raff and Mahler, 1972; Uzzell and Spolsky, 1974, 1981).

Meanwhile, it is interesting to note that *Pelomyxa palustris*, which does not have mitochondria (Daniels et al., 1965; Leiner and Wohlfeil, 1953) contains several types of intracellular symbionts (Daniels, 1973), and the suggestion has been made that such symbionts may carry out metabolic functions in place of mitochondria (Chapman-Andresen, 1971). Bacteria present in *P. palustris* have been found to be methanogenic (van Bruggen et al., 1983, 1985; see also The Methanogenic Bacteria) and may function as electron sinks related to energy production, comparable to mitochondrial function in aerobic eukaryotic cells.

In the case of amoeba-bacteria symbiosis, the D strain of *Amoeba proteus* became spontaneously infected with a large number (60,000–150,000 bacteria per amoeba) of rod-shaped Gram-negative bacteria (Jeon and Lorch, 1967). Initially, the bacteria were harmful and brought about damaging effects to their hosts, called xD amoebae, such as reduced cell size, slower cell growth, increased membrane fragility, sensitivity to starvation, and a poor clonability. When introduced into symbiont-free D amoebae, the bacteria multiplied and killed their new hosts within a few host cell generations. However, adverse effects of infection gradually diminished over a period of about 1 year, and the bacteria became

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Table 1.

Symbiont name or type	Host species (origin)	Reference
Methanobacterium	*Mastigella*	Goldschmidt, 1907
Methanobacterium	*Pelomyxa*	Gould-Veley, 1905
Schizomycetes	*Pelomyxa*	Penard, 1902
Bacilli	*Acanthamoeba* sp.	Hall and Voelz, 1985
Bacilli	*Acanthamoeba castellanii*	Drozanski, 1956
Bacilli	*Amoeba proteus* (Chicago)	Roth and Daniels, 1961
Bacilli (X-bacteria)	*A. proteus* (Scotland)	Jeon and Lorch, 1967
Cocci	*A. albida*	Nagler, 1910
Cocci	*A. proteus*	Cohen, 1957
Micrococci	*Entamoeba minchini*	Mackinnon, 1914
Micrococci	*E. muris*	Wenyon1907
Micrococci	*Sappinia*	Dangeard, 1896

Table 2.

Symbiont name or type	Host species (origin)	Reference
Ectosymbionts		
Green bacteria	*Mastigamoeba*	Lauterborn, 1916
Fusiformis	*Caduceia*	Kirby, 1936
Fusiformis	*Devescovina*	Duboscq and Grasse, 1926
Fusiformis	*Foaina signata*	Kirby, 1942b
Fusiformis	*Lophomonas*	Grasse, 1926
Fusiformis	*Mactrichomonas*	Kirby, 1942a
Fusiformis	*Polymastix*	Grasse, 1926
Treponema	*Devescovina*	Duboscq and Grasse, 1926
Bacilli	*Barbulanympha*	Cleveland et al., 1934
Bacilli	*Bullanympha*	Kirby, 1938b
Bacilli	*Chrysostephanosphaera*	Geitler, 1948
Bacilli	*Kalotermes*	Kirby, 1938a
Bacilli	*Lophomonas striata*	Beams et al., 1960
Bacilli	*Macrotrichomonas*	Kirby, 1938b
Bacilli	*Metapolystoma*	Skuja, 1958
Bacilli	*Oxymonas grandis*	Cross, 1946
Bacilli	*Rhynchonympha*	Cleveland et al., 1934
Bacilli	*Streblomastix strix*	Grimstone, 1961
Bacilli	*Trichonympa*	Kirby, 1932
Bacilli	*Urinympha*	Cleveland et al., 1934
Spirochetes	*Devescovina vestita*	Kirby, 1941b
Spirochetes	*Holomastigotoides*	Koidzumi, 1921
Spirochetes	*Hyperdevescovina*	Nurse, 1945
Spirochetes	*Mixotricha*	Cleveland and Grimstone, 1964
Spirochetes	*Rostronympha*	Duboscq et al., 1937
Spirochetes	*Spirotrichonympha*	Sutherland, 1933
Spirochetes	*Spirotrichonymphella*	Sutherland, 1933
Endosymbionts		
Carococcus	*Trichonympha*	Kirby, 1944
Pseudomonas	*Volvox aureaus*	Hamburger, 1958
Bacilli	*Costia pyriformis*	Davis, 1943
Bacilli	*Euglenoids*	Tschermak-Woess, 1950
Bacilli	*Gigantomonas*	Kirby, 1949
Bacilli	*Hyperdevescovina*	Kirby, 1946
Bacilli	*Macrotrichomonas*	Kirby, 1942a
Bacilli	*Volvox carteri*	Kochert and Olson, 1970
Bacilli (bipolar body)	*Strigomonas*	Newton and Horne, 1957
Bacilli (diplosome)	*Blastocrithidia*	Novey et al., 1907

less virulent, bacteria-bearing xD amoebae growing well with near-normal growth rates. Also, some of the newly infected D amoebae survived, indicating a reduced virulence as compared to earlier infection.

Within a few years, host amoebae became dependent on their endosymbionts (Jeon, 1972). Thus, xD amoebae lost viability when they were deprived of endosymbionts either by nuclear transplantation (Jeon and Jeon, 1976), by treatment with antibiotics (Jeon and Hah, 1977), or by raising the culture temperature (Jeon and Ahn, 1978). Aposymbiotic xD amoebae could be resuscitated only by reintroducing live X-bacterial symbionts (Lorch and Jeon, 1980). Newly infected amoebae became dependent on their symbionts after about 200 cell generations or 18 months. The reason for the hosts' dependence is not known, but preliminary evidence suggests that a symbiont-synthesized protein may be required for the survival of hosts. When xD amoebae are grown in the presence of chloramphenicol (100–700μg/ml) or rifampicin (125μg/ml), the synthesis of a unique 29-kDa polypeptide by endosymbionts is instantly suppressed and xD amoebae die much sooner than do symbiont-free D amoebae (Kim and Jeon, 1986, 1987a). The symbiont's gene coding for the xD-specific protein has been cloned (Park and Jeon, 1988) and its nucleotides sequenced (Park and Jeon, 1989). The symbiotic bacteria were found to accumulate host actin selectively (Kim and Jeon, 1987b), as studied using a monoclonal antibody against the amoeba actin. Thus, in this example, the transition of spontaneously infecting parasites to required cell components was observed while it occurred (Jeon, 1980, 1983, 1986, 1987). While the host's dependence on symbionts developed over 200 host cell generations, some physiological characters changed after a few host cell divisions (Lorch and Jeon, 1981, 1982).

In *Blastocrithidia culicis* and *Crithidia oncopelti,* symbiotic bacteria were found to supply their hosts with lysine (Gill and Vogel, 1962, 1963), hemin (Guttman and Eisenman, 1965; Chang and Trager, 1974; Newton, 1956, 1957), and other nutritional factors. An aposymbiotic host, produced by growing symbiont-bearing cells in the presence of chloramphenicol, required exogenous hemin for growth. The ectosymbiotic spirochetes on *Myxotricha* were found to help their host move by their coordinated undulation while the host's flagella functioned only to steer its movement (Cleveland and Grimstone, 1964). Flagella of prokaryotic symbionts attached to *Cryptotermes* were also found to help propel the host cell (Tamm, 1978b).

These are a few examples in which protozoan hosts and prokaryotic symbionts have developed an intimate relationship, and symbiont integration and host-symbiont interactions have been experimentally studied.

Habitats

General

The host-symbiont relationship appears to be somewhat specific for both ecto- and endosymbionts, since certain symbionts are almost always found associated with given hosts (Hall, 1969; Kirby, 1941a). However, the mechanism for specific recognition of the host by symbionts is not known. For ectosymbionts, their only requirement for continuing symbiotic association with their hosts would be to stay attached to their host-cell surfaces. Some symbionts cover the whole surface of their host, while others are limited to certain areas of the host's body (Kirby, 1941a). In some cases, specialized attachment sites such as brackets and underlying network of fibrous strands are present, as in *Myxotricha* (Fig. 1A; Cleveland and Grimstone, 1964). On the basis of extensive electron-microscopic observations, Cleveland and Grimstone reconstructed the attachment complexes as shown in Fig. 1B. Tamm (1978a) also found membrane specialization where two kinds of bacteria, rod-shaped and filamentous, were attached to *Crytptotermes cavifrons.* Thus, the attachment of ectosymbionts is not haphazard but appears to be helped by structural adaptations on the part of host cells.

For endosymbionts, their habitat in the cytoplasm of host cells can be considered to be an extreme environment comparable to hot springs and salt lakes (Moulder, 1979), although most symbionts live within symbiont-containing vesicles and the membranes protect them from direct exposure to the host cytoplasm. These symbiont-containing vesicles have been called "symbiosomes" (Fig. 2; Roth et al., 1988). It appears that endosymbionts have adapted in various ways to overcome adverse effects of the harsh environment, e.g., the possession of rigid cell walls to withstand digestive action of the hosts' hydrolytic enzymes (Drozanski and Chmielewski, 1979; Han and Jeon, 1980) or prevention of lysosomal fusion with symbiosome membranes, thus avoiding exposure to hosts' digestive enzymes (Ahn and Jeon, 1979; Armstrong and Hart, 1971; Jeon, 1983).

Amoebae

In amoebae, all known bacterial symbionts are intracellular. This is understandable since amoebae do not have a firm cortex and ectosymbionts

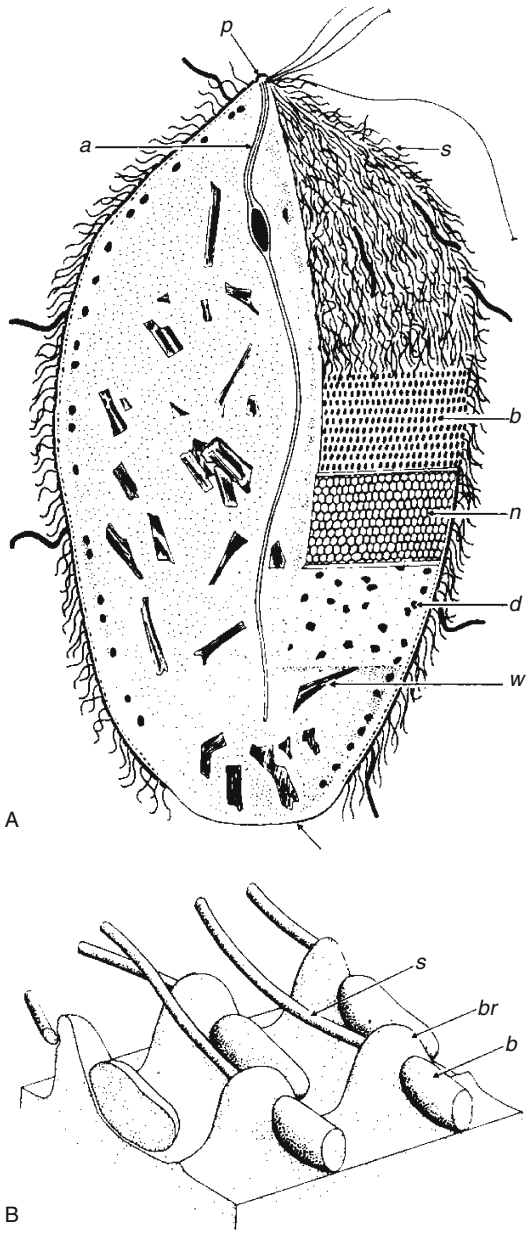

Fig. 1. A diagram of *Myxotricha paradoxa* showing the attachment of ectosymbionts on the cell surface (A) and a reconstruction of a small area of the cell surface showing the attachment complex (B). An optical section is shown on the left and surface structures are shown on the right. b, bacteria; br, brackets; n, fibrous network; s, spirochetes. (From Cleveland and Grimstone, 1964.)

cannot permanently attach to them as do flagellates. Several different bacterial types have been reported in the cytoplasm of amoebae, mostly enclosed in symbiosomes singly or in groups (Fig. 3). Roth and Daniels (1961) were among the first to confirm by electron microscopy the bacterial nature of previously reported bacteria-like particles (0.5×2μm) in vacuoles of *Amoeba proteus* (Cohen, 1957). These symbionts could not be eliminated by starvation or penicillin treatment. Chapman-Andresen and Hayward (1963) found rod-shaped bacteria (0.5 × 3–5μm) in a strain of *A. proteus* (about 4000 bacteria per cell), that could not be detected by light microscopy. Drozanski (1963a) reported Gram-negative rods (0.6–0.8 × 1.3–2.1μm) that caused a fatal infection in *Acanthamoeba castellanii.* These bacteria first multiplied in food vacuoles and later in the cytoplasm. Other reports followed that confirmed the presence of a large number of bacteria in various strains of amoebae (Jeon and Lorch, 1967; Wolstenholme and Plaut, 1964). In one strain of *Pelomyxa palustris*, Daniels et al. (1966; Daniels and Breyer, 1967) found rod-shaped bacteria (0.3 × 3μm) within individual vesicles characteristically surrounding the nuclei, while another type of bacteria of similar size was located in other parts of the cytoplasm. In *Acanthamoeba* (Drozanski, 1963a; Hall and Voelz, 1985; Proca-Ciobanu et al., 1975), symbionts are found throughout the cytoplasm. In the large, free-living *Amoeba,* all symbionts were enclosed in symbiosomes located in all parts of the cell (Jeon and Lorch, 1967; Wolstenholme and Plaut, 1964). So far, no endonuclear symbionts have been found in amoebae. Many unsuccessful attempts have been made to grow symbiotic bacteria in vitro (Drozanski, 1963b; Jeon and Lorch, 1967; K. Jeon, unpublished observations), and none of the reported bacterial symbionts of amoebae has been grown outside living cells.

The fact that symbionts of amoebae cannot be cultured outside amoebae indicates the symbionts' dependence on their hosts, but in many cases the dependence does not appear to be species-specific. Thus, bacteria isolated from

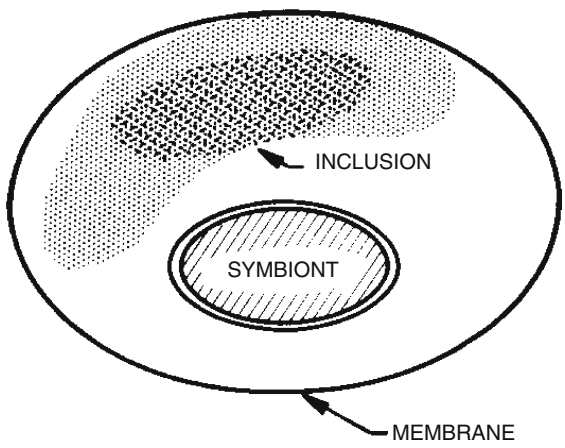

Fig. 2. A schematic diagram of a symbiosome to show its components. Each line represents a membrane and the intrasymbiosome space is shown to contain inclusions. (From Roth et al., 1988.)

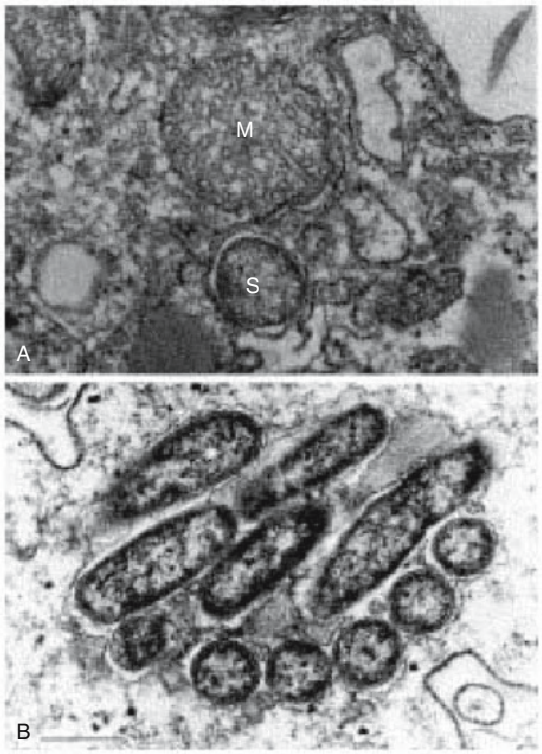

Fig. 3. Electron micrographs of symbiosomes in amoebae. (A) A small symbiosome with a single round symbiont (S) called a "DNA-containing body" and (B) a larger symbiosome with several rod-shaped symbionts. (A) A mitochondrion (M) with tubular cristae is shown for comparison. (B) The interbacterial space is filled with inclusions containing fibrous matter. Bar = 0.5 µm. (Fig. 3B from Jeon, 1987.)

infected *Acanthamoeba castellanii* were able to infect trophic forms of *Hartmannella rhysodes*, *Schzopyrenus resselli*, *Didasculus thorntoni*, and other unidentified amoebae of *Limax* type (Drozanski, 1963b). Jeon and Jeon (1982) found that X-bacteria isolated from xD amoebae *(Amoeba proteus)* cross-infected an unrelated species of giant amoeba, *Chaos carolinensis*.

Flagellates

Unlike amoebae, all of whose symbionts are intracellular, flagellates have symbionts in various parts of the cells, some as ectosymbionts and others in the nucleus, in the endoplasmic reticulum, in chloroplasts, or free in the cytoplasm. Most of the prokaryotic symbionts of flagellates were first found as ectosymbionts by light microscopy early in the 20th century (Table 2), and the hosts were mostly flagellates living in termite guts. Some suspected prokaryotic endosymbionts were reported in those flagellates, but confirmation of the bacterial nature of such endosymbionts had to await electron-microscopic observation and advanced biochemical tests. Thus, definitive reports of the presence of endosymbionts in flagellates started to appear in the late 1950s. For example, Roth (1959) found rod-like bacteria within the nucleus of *Peranema*, several hundred per nucleus, while others reported endocytoplasmic symbionts in *Volvox* (Kochert and Olson, 1970). Since then many other flagellates have been found to harbor endosymbiotic bacteria. Gromov (1977) described the presence of Gram-negative bacteria in the surface cortical region of *Trichonympha turkestanica* and suggested that the symbiotic bacteria play a role in the host flagella operation. Gerola (1978) reported inclusion bodies of several bacteria-like elements in *Euglena* cells from an alpine water pool rich in organic residues; the endosymbionts were free in the host cytoplasm, but there was no sign of cytoplasmic reaction around the symbionts detectable by electron microscopy. Endosymbiotic bacteria living inside the endoplasmic reticulum were reported in *Ochromonas monicis* isolated from a saline pool (Doddema and van der Veer, 1983), and these authors thought that the symbionts enabled their hosts to survive in vitamin-poor water. Sousa-Silva and Franca (1985) studied the ultrastructure of two species of dinoflagellates, *Gyrodinium instriatum* and *Glenodinium foliaceum*, harboring bacterial symbionts, some of which were in the nucleus while others were in the cytoplasm, and the third group appeared to live in both.

Chesnick and Cox (1986) presented a summary of results from 17 studies reporting the presence of endosymbiotic bacteria in algal species. In most cases, bacterial symbionts were usually found in small vesicles. However, in the marine alga *Penicillus*, bacteria were contained in one large central vacuole, with occasional presence found in the tip cytoplasm (Turner and Friedmann, 1974). Wilcox (1986) found small bacteria-like symbionts within chloroplasts of the dinoflagellate, *Woloszynskia pascheri*, and suggested that the bacteria may have been in a symbiotic relationship for some time, although no supporting evidence was presented. The author also consider it possible that the "symbionts" may represent regions where chloroplasts' nucleic acids are packaged in membranes. Different species of *Giardia* have been found to have mycoplasma-like organisms attached on the surface of trophozoites while harboring intracellular bacteria in trophozoites and cysts (Feely et al., 1988).

While the roles of many symbionts are not clear, the presence of symbionts has some effects on the structure and physiology of their hosts. Freymuller and Camargo (1981) compared the ultrastructure of symbiont-bearing and sym-

biont-free species of trypanosomatids and found some differences. For example, paraxial rods of flagella or intraflagellar structures were present only in symbiont-free species, while branching of mitochondria was found exclusively in symbiont-bearing species. Endonuclear symbionts of *Peranema trichophorum* appeared to cause structural changes in their hosts, such as fragmented karyosome and granulation of DNA and RNA within the nucleus (Radchenko, 1983). McLaughlin et al. 1983 found differences in lectin agglutinability between symbiont-bearing and aposymbiotic strains of *Crithidia* and *Blastocrithidia.* Later, it was found that aposymbiotic hosts failed to incorporate detectable amounts of fucose into a major surface glycopeptide (McLaughlin and Cain, 1985a). These authors attributed the differences in lectin agglutinability to different carbohydrate compositions of the flagellates' surfaces. In contrast, McLaughlin and Cain (1985b) failed to detect differences in the incorporation of labeled leucine and methionine between symbiont-bearing and aposymbiotic strains of these hemoflagellates. Krylov et al. 1985 detected many differences between symbiont-bearing (Sym^+) and symbiont-free (Sym^-) strains of *Crithidia oncopelti* and proposed that the two strains be named as different species; observed differences included cell size, flagellum length, colony shape and size, rate of movement, electrophoretic mobility of malate dehydrogenases, growth rate at 32°C, oxygen consumption, and sensitivity to antibiotics. In most other cases, however, host-symbiont relationships have not been clarified.

There have been some reports for the presence of blue-green algae as endosymbionts in various species of dinoflagellates (reviewed by Gaines and Elbrachter, 1987; Steidinger and Baden, 1984). Some of the symbiotic algae appeared to have nutritional importance to their hosts and others were thought to produce toxins.

Isolation

Since all the known endosymbiotic prokaryotes of flagellates and amoebae are obligatory symbionts and do not grow in vitro, the only sure source for their isolation would be symbiont-bearing hosts. Even for facultative ectosymbionts of flagellates that may live free from their hosts at one time or another, their natural host is the best source. *Amoeba proteus* cells are grown in dilute salt solution (Goldstein and Ko, 1976; Jeon and Jeon, 1975). Since these amoebae are strictly phagocytic, they have to be fed live prey organisms, commonly used food organisms for mass culture being *Tetrahymena* cultured axenically in a rich, complex medium containing various vitamins and mineral additives with proteose peptone and liver extract as the main ingredients (Goldstein and Ko, 1976). Amoebae are fed with washed *Tetrahymena* daily or every other day, depending on the desired growth rate. *Acanthamoeba* are cultured axenically in a medium containing proteose peptone, yeast extract, and glucose (Drozanski, 1984). Various media have been used to culture hemoflagellates and other flagellates as hosts of bacterial endosymbionts (e.g., Chang and Trager, 1974).

Bacterial endosymbionts have been isolated in mass from a few species of amoebae and flagellates. No reports are found for mass isolation of ectosymbionts from flagellates. For example, symbiotic bacteria have been isolated from xD amoebae *Amoeba proteus* (Ahn and Jeon, 1982; Han and Jeon, 1980) and *Acanthamoeb castellanii* (Drozanski et al., 1984). In the first example (Han and Jeon, 1980), symbionts were collected by Ficoll gradient centrifugation from lysed amoebae. Later, the method was modified to apply filtration through nylon screens to remove larger pieces of cell debris first (Ahn and Jeon, 1982). Pure bacteria were collected after centrifugation on a sucrose-step gradient. In either procedure, the recovery of symbionts could be greater than 90%. Symbiotic bacteria from *A. castellanii* were obtained by centrifugation from lysed amoebae (Drozanski et al., 1984).

Chang (1975) isolated endosymbionts of hemoflagellates by a sequential treatment of host flagellates by hypotonic shock, complement-dependent immune lysis, and needle passage. Host flagellate cells were incubated in Trager buffer (Trager, 1959) containing guinea-pig serum and rabbit antiserum against flagellates, followed by DNase digestion and differential centrifugation. The final fraction was free of contamination, and the recovery of symbionts was about 20%.

Identification

Since symbionts of amoebae and flagellates cannot be cultured in vitro, the traditional methods of identifying and classifying them (e.g., *Bergey's Manual*, see Buchanan and Gibbons, 1974) cannot be used. Thus, these symbionts have been simply identified as fusiform, rod-shaped, round, or filamentous on the basis of their external morphology based on light or electron-microscopic observations. Most of the bacterial endosymbionts have been reported to be Gram negative, either as a result of Gram staining or by electron microscopic examination for the presence of characteristic surface membranes. More specialized methods are available to

identify bacterial species, such as serological tests, bacteriophage typing, and DNA and rRNA sequencing. In particular, the use of specific oligodeoxynucleotide probes, which can be applied in identifying single microbial cells (Giovannoni et al., 1988), should be helpful in dealing with endosymbionts that cannot be grown in vitro. It is also desirable to culture isolated symbionts in vitro for their definitive identification based on their metabolic, physiological, and biochemical characteristics.

Concluding Remarks

This short survey reiterates that many prokaryotic organisms live on and in a wide variety of flagellates and amoebae as symbionts. Their presence is known to bring about various structural, physiological, and biochemical changes in the host. However, many questions still remain unanswered regarding the host-symbiont relationship. For example, it is not clear in many instances how the host and symbionts recognize each other and establish a specific association, how endosymbionts escape digestion by their host, how the symbiont population is controlled, and how the host and symbionts benefit or suffer from their association. Studies of prokaryotic symbionts in flagellates and amoebae have lagged behind those in ciliates (see Prokaryotic Symbionts of Ciliates), where identification and culture of symbionts in vitro and the clarification of the genetic interaction between the host's and symbionts' genomes have been well established. It is hoped that similar advancement will be achieved soon with symbionts in flagellates and amoebae.

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Literature Cited

Ahn T. I.; Jeon K. W. 1979. Growth and electron microscopic studies on an experimentally established bacterial endosymbiosis in amoebae. J. Cell. Physiol. 98:49–58.

Ahn T. I.; Jeon K. W. 1982. Structural and biochemical characteristics of the plasmalemma and vacuole membranes in amoebae. Exp. Cell Res. 137:253–268.

Armstrong J. A.; Hart P. D. 1971. Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. J. Exp. Med. 134:713–740.

Beams H. W.; King R. L.; Thamisian T. N.; Devine R. 1960. Electron microscope studies on Lophomonas striata with special reference to the nature and position of the striations. J. Protozool. 7:91–101.

Buchanan R. E.; Gibbons N. E. (ed.) 1974. Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins Baltimore.

Cavalier-Smith T. 1975. The origin of nuclei and of eukaryotic cells. Nature 256:463–468.

Chang K.-P. 1975. Reduced growth of Blastocrithidia culicis and Crithidia oncopelti freed of intracellular symbiotes by chloramphenicol. J. Protozool. 22:271–276.

Chang K.-P.; Trager W. 1974. Nutritional significance of symbiotic bacteria in two species of hemoflagellates. Science 183:532–533.

Chapman-Andresen C. 1971. Biology of the large amoebae. Annu. Rev. Microbiol. 25:27–48.

Chapman-Andresen C.; Hayward A. F. 1963. Bacterial complexes in Amoeba proteus. British-Dutch-Scandinavian Mtg. Soc. Exp. Biol. Oxford, England.

Chesnick J. M.; Cox E. R. 1986. Specialization of endoplasmic reticulum architecture in response to a bacterial symbiosis in Peridinium balticum (Pyrrhophyta). J. Phycol. 22:291–298.

Choi E. Y.; Jeon K. W. 1988. The presence of a spectrin-like protein on symbiosome membranes of symbiont-bearing Amoeba proteus as studied with monoclonal antibodies. Endocyt. Cell Res. 6:99–108.

Cleveland L. R.; Grimstone A. V. 1964. The fine structure of the flagellate Mixotricha paradoxa and its associated micro-organisms. Proc. Roy. Soc. B159:668–686.

Cleveland L. R.; Hall S. R.; Sanders E. P.; Collier J. 1934. The wood-feeding roach Cryptocercus, its Protozoa and the symbiosis between Protozoa and roach. Am. Acad. Arts Sci. Mem. 17:185–342.

Cohen A. I. 1957. Electron microscopic observations of Amoeba proteus in growth and inanition. J. Biophys. Biochem. Cytol. 3:859–866.

Cross J. B. 1946. The flagellate subfamily Oxymonadinae. Univ. Calif. Pub. Zool. 53:67–162.

Dangeard P. A. 1896. Contribution a l'etude des Acrasiees. Bot. 5:1–20.

Daniels E. W. 1973. Ultrastructure. 125–169. In: K. W. Jeon, (ed.). The Biology of amoeba. Academic Press. New York.

Daniels E. W.; Breyer E. P. 1967. Ultrastructure of the giant amoeba Pelomyxa palustris. J. Protozool. 14:167–179.

Daniels E. W.; Breyer E. P.; Kudo R. R. 1965. Fine structure of the giant, algae-eating amoeba Pelomyxa palustris. Am. Zool. 5:734–740.

Daniels E. W.; Breyer E. P.; Kudo R. R. 1966. Pelomyxa palustris Greeff II. Its Ultrastructure. Z. Zellforsch. 73:367–383.

Davis H. S. 1943. A new polymastigine flagellate, Costia pyriformis, parasitic on trout. J. Parasitol. 29:385–386.

Doddema H.; van der Veer J. 1983. Crypt. Algol. 4:89–98.

Drozanski W. 1963a. Studies of intracellular parasites of free-living amoebae. Acta Microbiol. Pol. 12:3–8.

Drozanski W. 1963b. Observations on intracellular infection of amoebae by bacteria. Acta Microbiol. Pol. 12:9–24.

Drozanski W.; Chemielewski T. 1979. Electron microscopic studies of Acanthamoeba castellanii. Acta Microbiol. Pol. 28:123–130.

Drozanski W.; Drozanska D.; Wicinska M. 1984. The cell wall of the obligate intracellular bacterial parasite of small free-living amoebae. I. Morphology and chemical composition of the rigid layer and peptidoglycan. Acta Microbiol. Pol. 33:195–206.

Duboscq O.; Grasse P.-P. 1926. Les Schizophytes de Devescovina billi *n. sp.*. Compt. Rend. Soc. Biol. Paris 94:33–34.

Duboscq O.; Grasse P.-P.; Rose M. 1937. Les Flagelles de l'Anacanthotermes ochraceus Sjost du Sud-Algerien. Compt. Rend. Acad. Sci. Paris 205:574–576.

Freymuller E.; Camargo E. P. 1981. Ultrastructural differences between species of trypanosomatids. J. Protozool. 28:175–182.

Gaines G.; Elbrachter M. 1987. Heterotrophic nutrition. 224–268. In: F. J. R. Taylor (ed.) The biology of dinoflagellates. Blackwell Publications Oxford.

Geitler L. 1948. Symbiosen zwischen Chrysomonaden und knospenden bakterien-artigen Organismen sowie Beobachtungen über Organisationseigentümlichkeiten der Chrosomonaden. Österreich. Bot. Zeitschr. 95:300–324.

Gerola F. M.; Bassi M. 1978. A case of parasitism in Euglena. J. Submicr. Cytol. 10:261–263.

Gill J. W.; Vogel H. J. 1962. Lysine synthesis and phylogeny: biochemical evidence for a bacterial-type endosymbiote in the protozoon Herpetomonas (Strigomonas) oncopelti. Biochim. Biophys. Acta 56:200–201.

Gill J. W.; Vogel H. J. 1963. A bacterial endosymbiote in Crithidia (Strigomonas) oncopelti: biochemical and morphological aspect. J. Protozool. 10:148–152.

Giovannoni S. J.; DeLong E. F.; Olsen G. J.; Pace N. R. 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. 170:720–726.

Goldschmidt R. 1907. Lebensgeschichte der Mastigamoben, Mastigella vitrea n. sp.. und Mastigina setosa n. sp. Arch. Protistenk 1 (Suppl.):83–168.

Goldstein L.; Ko C. 1976. A method for the mass culturing of large free-living amoebas. Methods Cell Biol. 13:239–246.

Gould-Veley L. J. 1905. A further contribution to the study of Pelomyxa palustris (Greeff). J. Linn. Soc. 29:374–395.

Grasse P. P. 1926. Sur la nature des cotes cuticulaires des Polymastix et Lophomonas striata. Compt. Rend. Soc. Biol. Paris 94:1014–1015.

Gray M. W.; Doolittle W. F. 1982. Has the endosymbiont hypothesis been proven?. Microbiol. Rev. 46:1–42.

Grimstone A. V. 1961. The fine structure of Streblomastix strix. Proc. I Int. Congr. Protozool. p. 121.

Gromov V.; Seravin L. N.; Gerasimova Z. P. 1977. Bacteriacorticae symbionts of Trichonympha turkestanica, a protozoan from the gut tract of termite Hodotermes murgabicus. Mikrobiol. (russ.) 46:971–973.

Guttman H. N.; Eisenman R. N. 1965. "Cure" of Crithidia (Strigomonas) oncopelti of its bacterial endosymbiote. Nature 206:113–114.

Hall G. H. 1969. Organisms living on and in protozoa. 566–718. In: T.-T. Chen (ed.) Research in protozoology. 3:Pergamon Press. London.

Hall J.; Voelz H. 1985. Bacterial endosymbionts of Acanthamoeba sp. J. Parasitol. 71:89–95.

Hamburger B. 1958. Bakteriensymbiose bei Volvox aureus Ehrenberg. Arch. Mikrobiol. 29:291–310.

Han J. H.; Jeon K. W. 1980. Isolation and partial characterization of two plasmid DNAs from endosymbiotic bacteria in Amoeba proteus. J. Bacteriol. 141:1466–1469.

Jeon K. W. 1972. Development of cellular dependence on infective organisms: Micrurgical studies in amoebas. Science 176:1122–1123.

Jeon K. W. 1980. Symbiosis of bacteria with Amoeba. 245–262. In: C. B. Cook, P. Pappas, and E. Rudolph (ed.) Cellular interactions in symbiotic and parasitic relationships. Ohio State University Press Columbus.

Jeon K. W. 1983. Integration of bacterial endosymbionts in amoebae. Int. Rev. Cytol. Suppl. 14:29–47.

Jeon K. W. 1986. Bacterial endosymbionts as extrachromosomal elements in Amoebas. 363–371. In: R. B. Wickner, A. Hinnebusch, A. Labowitz, I. C. Gunsalus, and A. Hollaender (ed.) Extrachromosomal elements in lower eukaryotes. Plenum Press. New York.

Jeon K. W. 1987. Change of cellular "pathogens" into required cell components. Ann. N. Y. Acad. Sci. 503:359–371.

Jeon K. W.; Ahn T. I. 1978. Temperature sensitivity: A cell character determined by obligate endosymbionts in amoebas. Science 202:635–637.

Jeon K. W.; Hah J. C. 1977. Effect of chloramphenicol on bacterial endosymbiotes in a strain of Amoeba proteus. J. Protozool. 24:289–293.

Jeon K. W.; Jeon M. S. 1975. Cytoplasmic filaments and cellular wound healing in Amoeba proteus. J. Cell Biol. 67:243–249.

Jeon K. W.; Jeon M. S. 1976. Endosymbiosis in amoebae: Recently established endosymbionts have become required cytoplasmic components. J. Cell. Physiol. 89:337–347.

Jeon K. W.; Jeon M. S. 1982. Experimental cross-infection of Chaos carolinensis with endosymbiotic bacteria from Amoeba proteus. J. Protozool. 29:493A.

Jeon K. W.; Lorch I. J. 1967. Unusual intra-cellular bacterial infection in large, free-living amoebae. Exp. Cell Res. 48:236–240.

Kim H. B.; Jeon K. W. 1986. Protein synthesis by bacterial endosymbionts in amoebae. Endocyt. Cell Res. 3:299:309.

Kim H. B.; Jeon K. W. 1987a. A monoclonal antibody against a symbiont-synthesized protein in the cytosol of symbiont-dependent amoebae. J. Protozool. 34:393–397.

Kim H. B.; Jeon K. W. 1987b. Actin-like protein accumulated within symbiont-containing vesicles of amoebae as studied using a monoclonal antibody. Endocyt. Cell Res. 4:151–166.

Kirby H. 1932. Protozoa in termites of genus Armitermes. Parasitol. 24:289–304.

Kirby H. 1936. Two polymastigote flagellates of the genera Pseudodevescovina and Cauduceia. Quart. J. Micros. Sci. 79:309–335.

Kirby H. 1938a. The devescovinid flagellates Caduceia theobromae Franca, Pseudodevescovina ramosa new species, and Macrotrichomanas pulchra Grassi. Univ. Calif. Pub. Zool. 43:1–40.

Kirby H. 1938b. Polymastigote flagellates of the genus Foaina Janicki, and two new genera Crucinympha and Bulanympha. Quarterly J. Micros. Sci. 81:1–25.

Kirby H. 1941a. Organisms living on and in protozoa. 1009–1013. In: G. N. Calkins and F. M. Summers, (ed). Protozoa in biological research. Columbia Univ. Press. New York.

Kirby H. 1941b. Devescovinid flagellates of termites I. The genus Devescovina. Univ. Calif. Pub. Zool. 45:1–92.

Kirby H. 1942a. Devescovinid flagellates of termites II. The genera *Caduceia* and *Macrotrichomonas*. Univ. Calif. Pub. Zool. 45:93–166.

Kirby H. 1942b. Devescovinid flagellates of termites. II. The genera *Foaina* and *Parajoenia*. Univ. Calif. Pub. Zool. 45:167–246.

Kirby H. 1944. The structural characteristics and nuclear parasites of some species of Trichonympha in termites. Univ. Calif. Pub. Zool. 49:185–282.

Kirby H. 1946. Gigantomonas herculea Dogiel, a polymastigote flagellate with flagellated and amoeboid phases of development. Univ. Calif. Pub. Zool. 53:163–226.

Kirby H. 1949. Devscovinid flagellates of termites V. The genus *Hyperdevescovina,* the genus *Bullanympha,* and undescribed or unrecorded species. Univ. Calif. Pub. Zool. 53:319–422.

Kochert G.; Olson L. W. 1970. Endosymbiotic bacteria in Volvox carteri. Trans. Am. Micros. Soc. 89:475–478.

Koidzumi M. 1921. Studies on the intestinal Protozoa found in the termites of Japan. Parasitol. 13:235–309.

Krylov M. V.; Podlipaev S. A.; Khaetskii A. S.; Belova L. M.; Frolov A. O.; Niyazbekova; B. Ya1985. Is only one species present in a culture of Crithidia oncopelti kinetoplastmonada trypanosomatidae?. Zool. Zh. 64:165–171.

Lauterborn R. 1916. Die sapropelische Lebewelt. Ein Beitrag zur Biologie des Faulschlammes naturlicher Gewasser. Verh. Naturwis. Ver. Heidelberg 13:395–481.

Lee J. J.; Fredrick J. F. (ed). 1987. Endocytobiology III. 530, Ann. N.Y. Acad. Sci. N.Y. Academy of Sciences. New York.

Lee J. J.; Soldo A. T.; Lee M. J.; Reisser W.; Jeon K. W.; Gortz H.-D. 1985. The extent of algal and bacterial endosymbiosis in protozoa. J. Protozool. 32:391–403.

Leiner M.; Wohlfeil W. 1953. Pelomyxa palustris Greeff und ihre symbiontischen Bakterien. Arch. Protistenk. 98:227–286.

Lorch I. J.; Jeon K. W. 1980. Resuscitation of amoebae deprived of essential symbiotes: Micrurgical studies. J. Protozool. 27:423–426.

Lorch I. J.; Jeon K. W. 1981. Rapid induction of cellular strain specificity by newly acquired cytoplasmic components in amoebas. Science 211:949–951.

Lorch I. J.; Jeon K. W. 1982. Nuclear lethal effect and nucleocytoplasmic incompatibility induced by endosymbionts in Amoeba proteus. J. Protozool. 29:468–470.

Margulis L. 1970. Origin of eukaryotic cells. Yale Univ. Press. New Haven.

Margulis L. 1981. Symbiosis and cell evolution. W. H. Freeman San Francisco.

Mackinnon D. L. 1914. Observations on amoebae from the intestine of the crane-fly larva Tipula sp. Arch. Protistenk. 32:267–277.

McLaughlin G. L.; Cain G. D. 1985a. Cell surface proteins of symbiotic and aposymbiotic strains of Crithidia oncopelti and Blastocrithidia culicis. Comp. Biochem. Physiol. 82B:469–477.

McLaughlin G. L.; Cain G. D. 1985b. Characterization of whole-cell and organelle protein synthesis in normal and aposymbiotic strains of Crithidia oncopelti and Blastocrithidia culicis. Comp. Biochem. Physiol. 82B:479–486.

McLaughlin G. L.; Wood D. L.; Cain G. D. 1983. Lipids and carbohydrates in symbiotic and aposymbiotic Crithidia oncopelti and Blastocrithidia culicis. Comp. Biochem. Physiol. 76B:143–152.

Moulder J. W. 1979. The cell as an extreme environment. Proc. Roy. Soc. Lond. B204:199–210.

Nagler K. 1910. Fakultative parasitische Micrococcen in Amoben. Arch. Protistenk. 19:246–254.

Newton B. A. 1956. A synthetic growth medium for the trypanosomid flagellate Strigomonas (Herpetomonas) oncopelti. Nature 177:279–280.

Newton B. A. 1957. Nutritional requirements and biosynthetic capabilities of the parasitic flagellate Strigomonas oncopelti. J. Gen. Microbiol. 17:708–717.

Newton B. A.; Horne R. W. 1957. Intracellular structures in Strigomonas oncopelti I. Cytoplasmic structures containing ribonucleoprotein. Exp. Cell Res. 13:563–574.

Novey F. G.; McNeal W. J.; Torrey H. N. 1907. The trypanosomes of mosquitoes and other insects. J. Infect. Dis. 4:223–276.

Nurse F. M. 1945. Protozoa from New Zealand termites. Trans. Roy. Soc. N.Z. 74:305–314.

Park M. S.; Jeon K. W. 1988. A symbiont gene coding for a protein required for the host amoeba: Cloning and expression in phage-transformed Ecoli. Endocyt. Cell Res. 5:215–224.

Park M. S.; Jeon K. W. 1989. Nucleotide sequence of a symbiont gene coding for a protein required for the host amoeba. Endocyt. Cell Res. 7:37–44.

Penard E. 1902. Faune rhizopodique du bassin du Leman. H. Køundig Geneva.

Proca-Ciobanu M.; Lupascu G. H.; Petrovic A. L.; Ionescu M. D. 1975. Electron microscopic study of a pathogenic Acanthamoeba castellanii strain. *The presence of bacterial endosymbionts*. Int. J. Parasitol. 5:49–54.

Radchenko A. I. 1983. Morphology and ultrastructure of the euglenoid flagellate Peranema trichophorum. Tsitol 25:141–147.

Raff R. A.; Mahler H. R. 1972. The non-symbiotic origin of mitochondria. Science 177:575–582.

Roth E.; Jeon K.; Stacey G. 1988. Homology in endosymbiotic systems: The term "Symbiosome,". 220–225. In: Molecular genetics of plant-microbe interactions. R. Palacios and D. P. S. Verma (ed.) APS Press St. Paul. MN.

Roth L. E. 1959. An electron-microscope study of the cytology of the protozoan Peranema trichophorum. J. Protozool. 6:107–116.

Roth L. E.; Daniels E. W. 1961. Infective organisms in the cytoplasm of Amoeba proteus. J. Biophys. Biochem. Cytol. 9:317–323.

Sagan L. 1967. On the origin of mitosing cells. J. Theoret. Biol. 14:225–275.

Skuja H. 1958. Eine neue vorwiegend sessil oder rhizopodial aufretende synbakteriotische Polytomee aus einem Schwefelgewasser. Sven. Bot. Tidkr. 52:379–390.

Sousa-Silva E.; Franca S. 1985. The association dinoflagellate-bacteria: Their ultrastructural relationship in two species of dinoflagellates. Protistol. 21:429–446.

Steidinger S. A.; Baden D. G. 1984. Toxic marine dinoflagellates. 201–262. In: D. L. Spector (ed.) Dinoflagellates. Academic Press. New York.

Sutherland J. L. 1933. Protozoa from Australian termites. Quart. J. Micros. Sci. 76:145–173.

Taylor F. J. R. 1974. Implications and extensions of the serial endosymbiosis theory of the origin of eukaryotes. Taxon 23:229–258.

Trager W. 1959. The enhanced folic and folinic acid contents of erythrocytes infected with malaria parasites. Exp. Parasitol. 8:265–273.

Tschermak-Woess E. 1950. Über eine Synbakteriose und ähnliche Symbiosen. Oesterreich. Bot. Zeitschr. 97:188–206.

Turner J. B.; Friedmann E. I. 1974. Fine structure of capitular filaments in the coenocytic green alga Penicillus. J. Phycol. 10:125–134.

Uzzell T.; Spolsky C. 1974. Mitochondria and plastids as endosymbionts: a revival of special creation? Am. Sci. 62:334–343.

Uzzell T.; Spolsky C. 1981. Two data sets: Alternative explanations and interpretations. Ann. N.Y. Acad. Sci. 361:481–499.

van Bruggen J. J. A.; Stumm C. K.; Vogels G. D. 1983. Symbiosis of methanogenic bacteria and sapropelic protozoa. Arch. Microbiol. 136:89–95.

van Bruggen J. J. A.; Stumm C. K.; Zwart K. B.; Vogels G. D. 1985. Endosymbiotic methanogenic bacteria of the sapropelic amoeba Mastigella. EEMS Microbiol. Ecol. 31:187–192.

Watson J. D.; Hopkins N. H.; Roberts J. W.; Steiz J. A.; Weiner A. M. 1987. Molecular biology of the gene, 4th ed. 1154–1155. Benjamin Cummins Menlo Park. CA.

Wenyon C. M. 1907. Observations on Protozoa in the intestine of mice. Arch. Protistenk. Suppl. 1:169–201.

Wilcox L. W. 1986. Prokaryotic endosymbionts in the chloroplast stroma of the dinoflagellate Woloszynskia pascheri. Protoplasma 135:71–79.

Wolstenholme D. R.; Plaut W. 1964. Cytoplasmic DNA synthesis in Amoeba proteus III. Further studies on the nature of the DNA-containing elements. J. Cell Biol. 22:505–513.

Index

A

Abdomen habitat, *Bacteroides*, 402
Abortion, septic
  Borrelia, 237, 279
  Campylobacter, 130–32
  Chlamydia, 743
  Helicobacter, 161, 169
  Leptospira, 305–6
  Treponema, 226
Acenaphthene degradation, *Sphingomonas*, 621
Acetaldehyde, *Pelobacter*, 6, 8–9
Acetate production
  Bacteroides, 387–88, 414
  Brachyspira, 330, 340–41
  Capnocytophaga, 578, 710
  Chloroflexus, 831
  Flavobacterium, 509
  Opitutus, 891
  Porphyromonas, 428
  Propionigenium, 957
  Spirochaeta, 204, 206–7
  termite gut spirochete, 323, 325–26
  Thermotogales, 899, 915
  Ultramicrobacteria, 891
  Victivallis, 894
Acetate utilization. *See* Organic acid utilization
Acetobacter, 965
Acetobacterium carbinolicum, 5
Acetoin utilization, *Pelobacter*, 5–9
Acetyl CoA utilization
  Bacteroides, 388
  Brachyspira, 340–41
  Chloroflexaceae, 830
  Pelobacter, 6, 9
  Spirochaeta, 206–7
  Zoogloea, 966
Acetylene utilization, *Pelobacter*, 5, 7–8
N-acetylgalactosamine, *Capnocytophaga*, 580
N-Acetylglucosamine (NAG)
  Borrelia, 243
  Brachyspira, 339
  Capnocytophaga, 580
  Gemmata, 782
  Pirellula, 780
  Planctomyces, 777
  Prosthecobacter, 888
  Verrucomicrobium, 887
Achromatium, 941–46
Achromatium gigas, 943. *See also Achromatium oxaliferum*
Achromatium mobile, 946. *See also Macromonas mobilis*
Achromatium oxaliferum, 942–46, 1001
Achromatium volutans, 942, 945–46
Achroonema, 989, 1001
Achroonema spiroideum, 591. *See also Saprospira albida*
Aconitase dehydrogenase, *Bacteroides*, 390
Actidione tolerance, *Leptospira*, 304
Actinobacillus actinomycetemcomitans, 704
Actinobacillus muris, 983. *See also Streptobacillus moniliformis*
Actinomyces, 1016
Actinomyces israelii, 580
Actinomyces muris, 983. *See also Streptobacillus moniliformis*
N-Acyl capnine, *Capnocytophaga*, 710
Aequorivita, 455, 460, 466–67, 471, 481, 678, 685
Aequorivita antarctica, 460, 678, 680, 697
Aequorivita crocea, 460, 678, 680
Aequorivita lipolytica, 460, 678, 680, 685
Aequorivita sublithincola, 458, 460, 678, 680
Aerobacter, 41
Aeromonas hydrophila, 664
Aeromonas salmonicida, 20–21
African swine fever virus, 251
Agar degradation
  Aequorivita, 467
  Arenibacter, 467
  Bergeyella, 467, 534, 535
  Capnocytophaga, 467
  Cellulophaga, 467, 683
  Chryseobacterium, 467
  Coenonia, 467
  Croceibacter, 467
  Cytophaga, 467, 539, 553
  Cytophagale, 557, 574
  Empedobacter, 467
  Flavobacteriaceae, 470
  Flavobacterium, 467, 494–95, 501–2, 520
  Fucobacter, 467
  Gelidibacter, 468, 469
  Mesonia, 468, 469
  Muricauda, 468, 469
  Myroides, 468, 469
  Ornithobacterium, 468, 469
  Polaribacter, 468, 469
  Psychroflexus, 468, 469
  Psychroserpens, 468, 469
  Riemerella, 468, 469
  Salegentibacter, 468, 469
  Tenacibaculum, 468, 469
  Ulvibacter, 468
  Vitellibacter, 468
  Weeksella, 469, 534, 535
  Zobellia, 469, 683
Agar shakes purification, *Pelobacter*, 6
Aglycone production, *Bacteroides*, 399–400
Agrobacterium radiobacter, 669
Agrobacterium tumefaciens, 14, 20, 167, 252
AIDS, Campylobacter, 132
Alanine production, Thermotogales, 915
Alanine utilization. *See* Amino acid utilization
Alcohol utilization
  Acetobacterium, 5
  Bergeyella, 649
  Borrelia, 243
  Chryseobacterium, 649, 651, 653–54
  Clostridium, 5
  Elizabethkingia, 649, 653–54
  Fervidobacterium, 905
  Flavobacterium, 543–44
  Geotoga, 905
  Haliscomenobacter, 604
  Herpetosiphon, 862
  Hymenobacter, 728
  Marinitoga, 905
  Meiothermus, 802
  Myxococcus, 35, 82
  Opitutus, 891
  Pelobacter, 5–9
  Petrotoga, 905
  Rhodothermus, 715
  Riemerella, 649, 700
  Salinibacter, 733–34
  Thermomicrobium, 850
  Thermosipho, 905
  Thermotoga, 905

Verrucomicrobium, 887
Victivallis, 894
Zoogloea, 965
Algae environment
Achromatium, 944
Cellulophaga, 678, 690
Cytophaga, 681
Flavobacteriaceae, 689–90
Formosa, 690
Psychroflexus, 679
Psychroserpens, 690
Alginate degradation
Cytophaga, 539
Cytophagale, 574
Flavobacterium, 494–95, 501, 520
Sphingomonads, 615–16
Aliphatic diols, *Flavobacterium*, 579
Alkaline phophatase hydrolysis, *Helicobacter*, 141, 143, 149–59, 161–65
Alkane utilization, *Thermoleophilum*, 844–45
Alteromonadales, 685
Alzheimer's disease, *Chlamydia*, 753
Amblyomma americanum, 241
Ambruticin, 91–92
Amino acid requirement
Bacteroides, 389–90
Chloroflexus, 831
Leptospira, 298
myxobacteria, 59
Saprospira, 599
Amino acid utilization
Fusobacterium, 1019–20
Leptospira, 298
Propionigenium, 957
Zoogloea, 965
Aminoglycoside resistance/sensitivity
Bacteroides, 406, 409, 411
Bergeyella, 534–35
Chryseobacterium, 667
Cytophagale, 575
Elizabethkingia, 667
Myroides, 633, 635
Rhodothermus, 721
Thermotogales, 908–9
Weeksella, 534–35
Ammonia production
Helicobacter, 167
myxobacteria, 54
Propionigenium, 957
Saprospira, 599
Ammonia utilization, *Verrucomicrobium*, 887
Ammonium requirement
Bacteroides, 383, 388–89
Chlorobiaceae, 369
Chloroflexus, 831
Cytophagas, 558
Haliscomenobacter, 604
Leptospira, 299
Sporocytophagas, 558
Thermoleophilum, 844
Amoebae habitat
Chryseobacterium, 644
Elizabethkingia, 643
Amoebinatus massiliae, 458
Amoxicillin resistance/sensitivity
Borrelia, 277
Helicobacter, 167
Ornithobacterium, 705
Riemerella, 704
Amphotericin B resistance/sensitivity
Borrelia, 241
Gemmata, 782
Ampicillin resistance/sensitivity
Bacteroides, 407
Chryseobacterium, 664
Flavobacterium, 545
Gemmata, 782
Helicobacter, 150
Ornithobacterium, 705
Planctomycetales, 766
Salinibacter, 733
Thermotoga, 908
Ultramicrobacteria, 891
Anaeroflexus maritimus, 713
Anaerolinea, 867, 870
Anaeromyxobacter, 85
Anaeromyxobacter dehalogenans, 38, 59
Ancalochloris, 360, 885
Ancalochloris perfilievii, 361–63
Ancalomicrobium, 766, 885
Anguillina coli. See Brachyspira pilosicoli
Animal environment. *See also specific* animals
Bacteroides, 381, 397, 400
Bdellovibrio, 15–16
Bergeyella, 532–33
Borrelia, 235–41
Brachyspira, 330, 332, 335, 344–45
Chryseobacterium, 638, 646
Elizabethkingia, 638, 646
Flavobacteriaceae, 473
Fusobacterium, 1016
Leptospira, 305–6, 308–11
Porphyromonas, 447–48
Treponema, 212–13
Weeksella, 532–33
Anisomycin resistance/sensitivity, *Salinibacter*, 733
Anoxic environment
Chlorobiaceae, 359, 362–63, 365, 370, 373
Chloronema, 822
Muricauda, 682
Opitutus, 889
Oscillochloris, 822
Planctomycetales, 764
Propionigenium, 956
Rhodothermus, 722
termite gut spirochete, 320, 323
Antenna complex
Chlorobiaceae, 359–61, 366–8
Chloroflexaceae, 834
Anthracene degradation, *Sphingomonas*, 621
Antibiotic purification procedure, myxobacteria, 39–42, 46–47
Antibiotic resistance. *See specific* antibodies
Antibiotic therapy
Brachyspira, 346
Chlamydia, 748
Chryseobacterium, 664, 667–68
Elizabethkingia, 667–68
Helicobacter, 166–67
Leptospira, 307
Sphingomonas, 621
Antigenic variation
Bacteroides, 403
Borrelia, 272–76
Antigens
Leptospira, 299–301
Porphyromonas, 442
Aphidicolin resistance, *Salinibacter*, 733
Apple tree, *Sphingomonas*, 610–11
Applications
Bdellovibrio, 26
Bergeyella, 535
Chlorobiaceae, 373–74
Chryseobacterium, 668–69
Cytophagale, 578–81
Elizabethkingia, 668–69
Empedobacter, 635
Flavobacteriaceae, 473–74, 690
Flavobacterium, 519–20, 545
Myroides, 635
myxobacteria, 91–94
Pelobacter, 10
Propionigenium, 958
Rhodothermus, 722
Saprospira, 600
Sphingomonads, 621
Streptobacillus, 984
Weeksella, 535
Wolinella, 188
Zoogloea, 967–68
Aquaspirillum gracile, 203
Aquaspirillum serpens, 17, 20–21, 205
Aquaspirillum sinuosum, 20–21
Aquifex, 916, 925–30, 932, 934
Aquifex aeolicus, 925–32, 934–35
Aquifex pyrophilus, 713, 902, 926–31, 934–35
Aquificaceae, 925–296
Aquificae, 925
Aquificales, 899, 925–35
α-L-Arabinofuranosidase production, *Rhodothermus*, 715, 717, 722
Arabinogalactan utilization, *Bacteroides*, 384–85, 398
Arabinose utilization. *See* Monosaccharide utilization
Archaeoglobus, 915
Archangium, 38, 42–43, 53, 56, 64, 71, 73, 82, 85–86, 88
Archangium gephyra, 74–75, 80–82, 88
Archangium serpens, 36
Archangium violaceum, 31–32, 54, 88
Arcobacter, 119–20, 124, 127, 142, 178
Arcobacter butzleri, 120, 125, 179
Arcobacter cryaerophilus, 120, 125, 179
Arcobacter nitrofigilis, 120
Arcobacter skirrowii, 120, 125

Arenibacter, 455, 460, 466–67, 471, 481, 678
Arenibacter latericius, 460, 678, 680, 690, 697
Arenibacter troitsensis, 458, 460, 678, 680
Arginine aminopeptidase
  Helicobacter, 153–55, 157, 162
  Wolinella, 180
Arginine dihydrolase, *Streptobacillus*, 984
L-Arginine arylamidase, *Helicobacter*, 157
Arginine utilization
  Leptospira, 298
  Meiothermus, 802
  Saprospira, 599
Arsenate reduction, Sphingomonads, 616
Arthritis
  Borrelia, 239, 262
  Campylobacter, 132
  Chlamydia, 749, 751
  Elizabethkingia, 667
  Helicobacter, 168
  Psychroflexus, 690
Arthrobacter, 605
Arthropod habitat
  Borrelia, 235, 247, 251, 257, 263–67, 269, 271
  spirochetes, symbiotic, 971
  vaccines, 278
Arylsulfatase, *Campylobacter*, 125–26
L-Asparaginase, *Wolinella*, 188
Asparagine utilization
  Flavobacteriaceae, 683
  Flavobacterium, 510
  Meiothermus, 802
Aspartate utilization
  Hymenobacter, 728
  Leptospira, 298
  Rhodothermus, 715
Asthma, *Chlamydia*, 751
Asticcacaulis, 766, 888
A system motility
  Flavobacterium, 499
  myxobacteria, 68–69
Atherosclerosis
  Chlamydia, 746, 751, 753
  Treponema, 211
ATP generation
  Bacteroides, 387
  Brachyspira, 340
  Capnocytophaga, 573–74
  Chlorobiaceae, 368
  Propionigenium, 955, 957–58
  Spirochaeta, 206–8
  Thermus, 804
  Treponema, 224
Autoimmune disease, *Borrelia*, 263
Avian borreliosis, *Borrelia*, 237
Azocasein, *Bacteroides*, 389
Azocasein hydrolysis assay, *Bacteroides*, 398
Azocoll, *Bacteroides*, 389
Azospirillum brasilense, 26
Azotobacter, 39, 93

B

Bacillus, 45–46, 412, 482, 556, 1016
Bacillus anthrasis, 252
Bacillus brevis, 630. *See also Empedobacter brevis*
Bacillus subtilis, 252, 869, 926
Bacitracin resistance/sensitivity
  Hymenobacter, 728
  Leptospira, 304
  Porphyromonas, 431
  Salinibacter, 733
  Sphingomonads, 618
  Ultramicrobacteria, 891
Bacteremia
  Bergeyella, 536
  Campylobacter, 132
  Chryseobacterium, 667–68
  Elizabethkingia, 666
  Fusobacterium, 1019
  Helicobacter, 147
  Myroides, 631, 634
  Sphingomonas, 621
Bacteriochlorophyll
  Chlorobiaceae, 359–61, 364, 366–67, 369–70, 372
  Chloroflexaceae, 816, 828
  Chloroflexus, 815, 818, 819, 828, 833–34
  Chloronema, 815, 818, 819, 828–29
  Heliothrix, 815, 818, 819, 829
  Oscillochloris, 815, 818, 819, 828–29, 833
  Roseiflexus, 818, 819, 829
Bacteriochlorophyll a, 366–67, 815–16, 818, 820, 822, 828–29, 833–34
Bacteriochlorophyll c, 359, 364, 366–67, 815–16, 818, 828–29, 833–34
Bacteriochlorophyll d, 359, 364, 366, 818, 826, 828–29
Bacteriochlorophyll e, 359, 364, 366
Bacteriocins
  Cytophaga, 572–73
  Cytophagale, 572–73
  Flavobacterium, 507–8
  myxobacteria, 39, 67–68
  Sporocytophaga, 572
Bacteriophages
  Bacteroides, 395, 397
  Bdellovibrio, 14
  Borrelia, 255–56
  Brachyspira, 344
  Leptospira, 302
  Myxococcus xanthus, 35, 67
  Planctomycetales, 765
Bacteroidaceae, 5, 381, 429, 431, 900
Bacteroides, 119, 359, 381–416, 428–29, 459, 561, 575, 761, 1016, 1019, 1021
Bacteroides acidifaciens, 382
Bacteroides caccae, 381, 395, 414
Bacteroides distasonis, 381–83, 385, 389–92, 395–97, 399, 401–2, 407, 413–15
Bacteroides eggerthii, 381–83, 385, 388, 391–92, 396, 414–15
Bacteroides forsythus, 382, 428, 432
Bacteroides fragilis, 381–408, 410–15, 713, 1018
Bacteroides heparinolyticus, 381–82
Bacteroides merdae, 381–82, 414
Bacteroides nodosus, 1018
Bacteroides ochraceus, 578
Bacteroides ovatus, 381–86, 389, 391–92, 395–402, 407–8, 411, 413–15
Bacteroides putredinis, 382
Bacteroides splanchnicus, 382
Bacteroides stercoris, 381–82, 414
Bacteroides thetaiotaomicron, 381–82, 384–89, 391–93, 395–402, 406–7, 410–11, 413–15, 473
Bacteroides uniformis, 381–83, 385, 389–92, 394–95, 397–98, 400–402, 407–8, 411, 413–15
Bacteroides ureolyticus, 120
Bacteroides vulgatus, 381–83, 385, 389–92, 394–402, 407–8, 411, 413–15
Bacteroides zoogleoformans, 381–82
Baiting technique, myxobacteria, 40–41
Bark environment. *See specific* tree species
Bass, *Chryseobacterium*, 644
Bdellocysts, 21
Bdellophages, 14
Bdellovibrio, 12–26
Bdellovibrio bacteriovorus, 13–14, 20, 22–24
Bdellovibrio starrii, 13–14
Bdellovibrio stolpii, 13–14, 20
Bdellovibrio strain W, 13–14
Beech tree, myxobacteria, 37
Beggiatoa, 208, 600, 822–23, 855, 941, 951
Beggiatoa arachnoidea, 1001
Bell's palsy, *Borrelia*, 263
Bergeyella, 455, 460, 466–67, 470, 473, 488, 532–36, 638–41, 655
Bergeyella zoohelcum, 458, 460, 471, 532–33, 535–36, 540–41, 543–44, 639, 647–51, 656–57, 664, 680, 697
Bifidobacterium, 397
Bile acid hydrolases, *Bacteroides*, 399
Bile esculin tolerance, *Campylobacter*, 121
Bile metabolism, *Bacteroides*, 382, 398, 414
Bile sensitivity, *Fusobacterium*, 1020
Bile tolerance, *Helicobacter*, 150, 152, 154, 156–57, 164, 168
Bilophila, 413
Biofilms
  Bdellovibrio, 16
  Chryseobacterium, 645
  Elizabethkingia, 645
  Treponema, 221, 226
Biotin production, Sphingomonads, 621

Biotin requirement
Leptospira, 299
myxobacteria, 56
Biphenyl catabolism, Sphingomonads, 619
Bird habitat
Borrelia, 235, 238–39, 279
Brachyspira, 331, 347–48
Campylobacter, 122
Chlamydia, 747
Chryseobacterium, 646, 701
Coenonia, 695, 701, 703
Elizabethkingia, 664
Flavobacteriaceae, 473
Helicobacter, 140
Ornithobacterium, 695, 701–2
Pasteurella, 701
Riemerella, 695, 701, 703
Blastocaulis spaerica, 760. *See also Planctomyces bekefii*
Blastomonas, 606
Blastomonas natatoria, 606–7. *See also Sphingomonas natatoria*
Blastomonas ursincola, 606–7
Blattabacterium, 465
Blood-brain barrier, *Borrelia*, 263
Blood habitat
Bacteroides, 402
Bergeyella, 533
Borrelia, 236, 238, 240–41, 256, 260, 263, 273–74, 279
Brachyspira, 330, 336, 348
Campylobacter, 121–24
Chryseobacterium, 460, 640, 663, 665
Elizabethkingia, 640, 664–65
Empedobacter, 631
Flavobacterium, 541
Helicobacter, 153
Leptospira, 309–10
Myroides, 631
Porphyromonas, 446
Riemerella, 462
Sphingomonads, 613
Sphingomonas, 610, 621
Weeksella, 533
Blue crab, *Bdellovibrio*, 15–16
Bobcat, *Helicobacter*, 161
Bog environment, *Saprospira*, 592
Bordetella avian, 704
Bordetella pertussis, 167
Borrelia, 195, 197, 213, 235–79, 340, 342
Borrelia afzelii, 239, 257, 263–65
Borrelia andersonii, 239
Borrelia anserina, 237, 249, 279
Borrelia bissettii, 239
Borrelia burgdorferi, 197, 214, 235, 237–40, 242–55, 260, 262–63, 265–69, 271–79, 326
Borrelia caucasica, 237
Borrelia coriaceae, 237, 239, 249, 270, 279
Borrelia crocidurae, 237, 249, 261
Borrelia dipodilli, 237
Borrelia duttonii, 236–38, 240, 251–52, 261–62
Borrelia garinii, 239, 263–65
Borrelia hermsii, 197, 214, 237, 240, 242, 249, 255, 257, 270, 272–74, 276
Borrelia hispanica, 237, 261
Borrelia japonica, 239
Borrelia latyschewii, 237
Borrelia lonestari, 237, 241
Borrelia lusitaniae, 239
Borrelia mazziottii, 237
Borrelia merionesi, 237
Borrelia microti, 237
Borrelia miyamotoi, 239, 274
Borrelia parkeri, 237, 249, 253, 279
Borrelia persica, 237, 971
Borrelia recurrentis, 236–37, 240, 261
Borrelia takunii, 239
Borrelia theileri, 237, 279
Borrelia turdi, 239
Borrelia turicatae, 237, 249, 257, 261, 270, 273–74
Borrelia valaisiana, 239
Borrelia venezuelensis, 237
Bovine borreliosis, *Borrelia*, 237
Bovine habitat
Bacteroides, 382, 395, 397
Bergeyella, 533
Borrelia, 237
Campylobacter, 122, 130, 132
Chryseobacterium, 460, 645
Fusobacterium, 1017–19
Helicobacter, 142, 160
Leptospira, 305–7, 309
Porphyromonas, 429
Treponema, 212, 215, 222
Weeksella, 533
Wolinella, 178
Brachyarcus, 998–1000, 1002
Brachyarcus thiophilus, 998–1000
Brachyspira, 195, 197, 212–13, 235, 255, 330–49
Brachyspira aalborgi, 214, 330–37, 344–45
Brachyspira alvinipulli, 330–34, 336–39, 344, 346, 348
Brachyspira canis, 337
Brachyspira christiani, 330
Brachyspira hyodysenteriae, 197, 212, 214, 326, 330–40, 342–49
Brachyspira innocens, 197, 212, 330–34, 336, 338–39, 344
Brachyspira intermedia, 330–34, 336–39, 346
Brachyspira murdochii, 330–34, 336, 338–39
Brachyspira pilosicoli, 330–39, 342–46, 348
Brachyspira pulli, 330
Brain heart infusion agar/broth
Brachyspira, 338, 412
Capnocytophaga, 709
Chryseobacterium, 646–47
Elizabethkingia, 647
Fusobacterium, 1019
Sphingomonads, 614
Thermomicrobium, 850
Brevibacterium, 555
Brevibacterium linens, 555
Brevinema, 195, 197, 213, 235
Brevinema andersonii, 195, 197, 214, 326
Bright field microscopy, Chlorobiaceae, 364
Brocadia, 759, 764, 773
Brocadia anammoxidans, 768–70
Budding
Caulococcus, 996–97
Gemmata, 781–84
Isosphaera, 764, 784–88
Kusnezovia, 996–97
Planctomyces, 774–77
Planctomycetales, 757, 766
Siderocapsaceae, 1011, 1014
Burkholderia cepacia, 120, 697
2,3-Butanediol utilization, *Pelobacter*, 5–9
Butanol utilization, *Pelobacter*, 7
Butyrate production
Brachyspira, 330, 340–41
Pelobacter, 8–9
Butyrivibrio, 388
Byssophaga, 59, 73, 85–86, 89–90
Byssophaga cruenta, 97

C

Cabbage habitat, *Bdellovibrio*, 14–16
Calcium requirement
Bacteroides, 383
Leptospira, 299
myxobacteria, 57, 65
Spirochaeta, 205
Calderobacterium, 925
Calderobacterium hydrogenophilum, 925. *See also Hydrogenobacter hydrogenophilus*
Caldilinea, 867, 870
Calvin-Bassham-Benson cycle, *Oscillochloris*, 830
Campylobacter, 119–33, 139–42, 162, 178, 180
Campylobacter cinaedi. See Helicobacter cinaedi
Campylobacter coli, 119–23, 125, 127–31
Campylobacter concisus, 119–24, 126, 128, 181
Campylobacter cryaerophila. See Arcobacter cryaerophilus
Campylobacter curvus, 119–22, 124, 126, 180–81
Campylobacter fetus, 119–25, 129–30, 132
Campylobacter gracilis, 119–22, 126, 181
Campylobacter helveticus, 119–22, 124, 126
Campylobacter hyoilei, 119–21, 125
Campylobacter hyointestinalis, 119–24, 126, 145
Campylobacter jejuni, 119–23, 125, 127–31, 133, 179
Campylobacter lanienae, 119–21
Campylobacter lari, 119–23, 125, 127–28

Campylobacter mucosalis, 119–24, 126
Campylobacter nitrofigilis. *See Arcobacter nitrofigilis*
Campylobacter pyloridis. *See Helicobacter pylori*
Campylobacter rectus, 119–22, 124, 126, 179–81
Campylobacter showae, 119–22, 126
Campylobacter sputorum, 119–24, 126
Campylobacter upsaliensis, 119–23, 125, 127–28
Campylobacter ureolyticus, 119, 124, 126. *See also Bacteroides ureolyticus*
Canine habitat
Bergeyella, 533, 536
Brachyspira, 331
Campylobacter, 122
Capnocytophaga, 460, 578, 581, 709
Chryseobacterium, 646
Elizabethkingia, 664
Empedobacter, 634
Fusobacterium, 1017
Helicobacter, 139–40, 142, 150–54, 156, 161
Leptospira, 305, 307
Porphyromonas, 428–29, 447
Streptobacillus, 983
Treponema, 212
Weeksella, 541
Capnocytophaga, 455, 460, 466–67, 470, 473, 509, 554, 561–62, 567, 569–71, 573–76, 578, 580–81, 662–63, 696, 709–10, 1023
Capnocytophaga canimorsus, 458, 460, 581, 697, 709
Capnocytophaga cynodegmi, 458, 460, 581, 697, 709
Capnocytophaga gingivalis, 458, 460, 580–81, 697, 709
Capnocytophaga granulosa, 458, 460, 697, 709
Capnocytophaga haemolytica, 458, 460, 697, 709
Capnocytophaga ochracea, 458, 460, 574, 580, 680, 697, 709
Capnocytophaga sputigena, 458, 460, 580–81, 697, 709
Capnoids, 570–71
Capsular polysaccharide complex, *Bacteroides*, 402–4
Carbapenem resistance/sensitivity
Bacteroides, 407–8
Elizabethkingia, 667
Myroides, 635
Carbofuran metabolism
Chryseobacterium, 669
Sphingomonads, 618
Carbohydrate fermentation. *See specific* carbohydrate groups
Carbon dioxide production
Brachyspira, 330, 340
Spirochaeta, 204, 206–7
Thermotogales, 899, 915
Wolinella, 180
Carbon dioxide requirement
Capnocytophaga, 473, 561, 573, 578, 709–10
Coenonia, 473, 699
Cytophagale, 562, 565
Flavobacterium, 509
Ornithobacterium, 473
Riemerella, 473, 699
Carbon dioxide utilization
Aquificales, 934
Bacteroides, 383–84, 387
Chlorobiaceae, 368–69, 372
Helicobacter, 148
Leptospira, 299
Streptobacillus, 983
termite gut spirochete, 323, 326–27
Carbon metabolism
Chlorobiaceae, 368–69, 372
Flavobacterium, 510
Carbon monoxide dehydrogenase, termite gut spirochete, 325
Carboxymethylcellulose degradation, *Flavobacterium*, 494–95, 500–502
Cardiolipin
Cytophagale, 571
myxobacter, 63
Cardiovascular disease
Chlamydia, 753
Porphyromonas, 446, 448
Cardiovascular environment, *Chlamydia*, 753
Carotenoids
Brevibacterium, 555
Chlorobiaceae, 361, 366
Chloroflexus, 819, 828, 833
Chloronema, 819, 828
Cytophagale, 571
Flavobacteriaceae, 466, 681
Flavobacterium, 471, 500
Flexibacter, 553
Haliscomenobacter, 604
Heliothrix, 819, 829
Herpetosiphon, 868
Hymenobacter, 725
Isosphaera, 786
myxobacteria, 63–64
Oscillochloris, 819, 828, 833–34
Rhodothermus, 720–21
Roseiflexus, 819, 829
Saprospira, 599
Thermomicrobium, 850–51
Thermonema, 723
Thermus, 806
Carp habitat
Elizabethkingia, 664
Flavobacterium, 516, 518
Fusobacterium, 1016
Casein hydrolysis
Bacteroides, 389
Bergeyella, 535
Chryseobacterium, 645
Flavobacterium, 493–95
Herpetosiphon, 869
Pirellula, 780
Thermonema, 724
Thermus, 799
Weeksella, 535
Catalase activity
Aequorivita, 467, 685
Arenibacter, 467, 685
Bacteroides, 384, 391, 414
Bergeyella, 467, 534–36
Campylobacter, 121, 123, 125–26
Capnocytophaga, 467
Cellulophaga, 467, 685
Chryseobacterium, 467, 651, 701
Coenonia, 467, 701
Croceibacter, 467, 685
Cytophaga, 467, 685
Empedobacter, 467, 633
Flavobacteriaceae, 683
Flavobacterium, 467, 493
Formosa, 685
Fucobacter, 467
Gelidibacter, 468, 469, 685
Gemmata, 781
Gillisia, 685
Helicobacter, 141, 143, 148–59, 161–66
Herpetosiphon, 868
Hymenobacter, 725
Leptospira, 298
Maribacter, 685
Meiothermus, 802
Mesonia, 468, 469, 685
Muricauda, 468, 469, 685
Myroides, 468, 469, 633
Novosphingobium, 609
Ornithobacterium, 468, 469, 701–2
Pasteurella, 701
Polaribacter, 468, 469, 685
Psychroflexus, 468, 469, 685
Psychroserpens, 468, 469, 685
Riemerella, 468, 469, 701
Robiginitalea, 685
Salegentibacter, 468, 469, 685
Salinibacter, 731
Sphingobium, 609
Sphingomonas, 609
Sphingopyrix, 609
Tenacibaculum, 468, 469, 685
Thermoleophilum, 846–47
Thermomicrobium, 852
Ulvibacter, 468, 685
Vitellibacter, 468, 685
Weeksella, 469, 534, 535
Wolinella, 180
Zobellia, 469, 685
Catfish habitat, *Flavobacterium*, 516–17
Cattle. *See* Bovine
Caulobacter, 766, 777, 888
Caulococcus, 996–97
Caulococcus manganifer, 996–97
Cecum
Bacteroides, 382, 395–96, 398, 400
Brachyspira, 332, 334, 336, 344
Campylobacter, 122
Helicobacter, 147
Cefoperazone tolerance
Campylobacter, 123
Helicobacter, 152, 156
Cefoxitin, *Bacteroides*, 407–8
Ceftizoxime, *Bacteroides*, 407
Cellobiose utilization. *See* Di- and trisaccharide utilization

Cellulitis
Campylobacter, 132
Elizabethkingia, 667
Cellulophaga, 455, 460, 466–67, 471, 481, 484, 678, 683, 690
Cellulophaga algicola, 458, 460, 678, 680, 690
Cellulophaga baltica, 458, 460, 678, 680
Cellulophaga fucicola, 458, 460, 678, 680
Cellulophaga lytica, 458, 460, 678, 680–81, 697
Cellulophaga uliginosa, 458, 460
Cellulose hydrolysis
Bacteroides, 384, 398
Cytophaga, 539, 553, 555, 558
Cytophagale, 554–55, 557–60, 574
Flavobacteriaceae, 470–71
Flavobacterium, 493, 500–501
Herpetosiphon, 869, 871
myxobacteria, 37–38, 40–43, 47, 59–60
Rhodothermus, 715, 722
Spirochaeta, 207
Sporocytophagas, 558
Tenacibaculum, 683
Cell wall
Fervidobacterium, 908
Flavobacterium, 503–4
Helicobacter, 145
Herpetosiphon, 865, 867
myxobacteria, 61–62
Thermomicrobium, 852
Cephalosporin resistance/sensitivity
Bacteroides, 407, 410
Elizabethkingia, 667
Fusobacterium, 1020
Myroides, 635
Ornithobacterium, 705
Cephalothin, *Helicobacter*, 141, 143, 149–59, 162–65
Cephamycin, *Bacteroides*, 408
Ceramides
Cystobacter, 63
Cytophagales, 571
Cerebrosides, *Cystobacter*, 63
Cerebrospinal fluid (CSF)
Bergeyella, 533
Borrelia, 240–41
Chryseobacterium, 460, 640
Elizabethkingia, 640
Flavobacterium, 541
Leptospira, 309–10
Sphingomonas, 621
Weeksella, 533
Cheetah habitat, *Helicobacter*, 140, 151, 168
Chemotaxis
Achromatium, 945
Bacteroides, 406
Bdellovibrio, 20
Brachyspira, 344, 348
Flavobacteriaceae, 470
Flavobacterium, 504, 520
myxobacteria, 69, 79–80
Spirochaeta, 200, 205–8
Thiospira, 947
Thiovulum, 942, 948, 950
Chemotherapy
Borrelia, 277–79
Flavobacterium, 541
Porphyromonas, 432
Chicken habitat
Bacteroides, 382
Bergeyella, 533
Borrelia, 279
Brachyspira, 330–31, 334, 345, 347–48
Campylobacter, 127, 129
Fusobacterium, 1017
Helicobacter, 140, 155
Ornithobacterium, 695–96, 702–3
Riemerella, 695, 703–4
Weeksella, 533
Chimpanzee habitat, *Treponema*, 212, 217
Chipmunk habitat, *Borrelia*, 237, 257
Chitin hydrolysis
Chryseobacterium, 653
Cytophaga, 539
Cytophagale, 553–54, 557, 579
Flavobacteriaceae, 470
Flavobacterium, 493–95, 501, 520
Herpetosiphon, 869
Chitinophaga, 482, 549, 568. *See also Flexibacter filiformis*
Chitobiose, *Borrelia*, 243
Chlamydia, 741–53, 760
Chlamydiae, 757–58, 881, 900
Chlamydia pecorum, 742–43
Chlamydia pneumoniae, 741–43, 746, 751–52
Chlamydia psittaci, 742–43, 746–47, 751, 759. *See also Chlamydophila psittaci*
Chlamydia trachomatis, 741–43, 746–51, 883
Chlamydophila, 742
Chlamydophila psittaci, 704
Chloramphenicol resistance/sensitivity
Bacteroides, 406–7
Bergeyella, 534–35
Borrelia, 277
Brachyspira, 344
Capnocytophaga, 581
Chryseobacterium, 664, 667–68
Cytophagale, 575, 581
Elizabethkingia, 667–68
Flavobacterium, 508
Helicobacter, 162
Hymenobacter, 728
Rhodothermus, 721
Riemerella, 704
Salinibacter, 733
Weeksella, 534–35
Wolinella, 188
Chlorobactene, Chlorobiaceae, 361, 366
Chlorobenzoic acid, *Flavobacterium*, 579
Chlorobiaceae, 359–74
Chlorobium, 360, 362–63, 368–69, 372
Chlorobium chlorovibrioides, 361
Chlorobium limicola, 360–63, 368–69, 371, 374, 713–14
Chlorobium phaeobacteroides, 361–63, 370
Chlorobium phaeovibrioides, 360–63, 367
Chlorobium tepidum, 362–63, 368–69, 713
Chlorobium vibrioforme, 360, 363, 368–69, 713, 999
Chlorochromatium aggregatum, 371–73
Chlorochromatium glebulum, 371–73
Chlorochromatium lunatum, 371, 373
Chloroflexaceae, 359, 815–37
Chloroflexus, 359–60, 818, 820–26, 828, 844, 849, 854–55, 858, 867, 873–74
Chloroflexus aggregans, 817–21, 823, 828–30, 832, 870
Chloroflexus aurantiacus, 798, 815–25, 828–36, 870
Chloroflexus aurantiacus mesophilus, 822
Chloroherpeton, 360, 823
Chloroherpeton thalassium, 360–64
Chlorohexidine, *Porphyromonas*, 433
Chloromycetin resistance/sensitivity, *Thermomicrobium*, 850
Chloronema, 371, 815, 818, 820, 822, 826, 828–29, 870
Chloronema giganteum, 816–17, 819–20, 822, 829, 870
Chloronema spiroideum, 819–20, 829
Chloronium mirabile, 371
Chlorosomes
Chlorobiaceae, 359–60, 366–67
Chloroflexaceae, 816
Chloroflexus, 815, 818–19, 828, 834
Chloronema, 815, 819, 828
Oscillochloris, 815, 818–20, 828
Cholestenols production, *Nannocystis*, 63
Cholesterol requirement, *Brachyspira*, 338, 340
Chondrococcus columnaris, 93. *See also Flavobacterium columnare*
Chondroitin sulfate utilization
Bacteroides, 384–86, 398, 405
Flavobacteriaceae, 470
Flavobacterium, 502
Riemerella, 700
Chondromyces, 31–32, 36, 39, 43–44, 51–52, 56, 58, 60, 71, 73, 75, 85–86, 90
Chondromyces apiculatus, 31–32, 36–37, 51, 76–77, 79–80, 90
Chondromyces aurantiacus. See Stigmatella aurantiaca
Chondromyces catenulatus, 90
Chondromyces crocatus, 32, 34, 39, 51, 60–61, 75, 78–80, 82, 90
Chondromyces lanuginosus, 51, 78–79, 90

Chondromyces pediculatus, 36, 51, 78–79, 90
Chondromyces robustus, 90
Choroiditis, *Borrelia*, 261
Chromatiaceae, Chlorobiaceae, 359, 362, 364, 366–72
Chromatium okenii, 1001
Chromatium vinosum, 371
Chryseobacterium, 455, 460, 466–67, 470, 472–73, 484, 488, 533–35, 632, 638–69, 677, 689
Chryseobacterium balustinum, 458, 460, 519, 540, 542–43, 638–39, 641, 644–53, 655, 657–63, 669
Chryseobacterium daecheongense, 642–43, 647, 652–53, 655, 657, 661–63
Chryseobacterium defluvii, 458, 460, 639, 642, 644, 647–53, 655, 657, 661, 663
Chryseobacterium formosense, 642–43, 647, 652–53, 655, 657, 663
Chryseobacterium gleum, 458, 460, 638–39, 642–62, 665, 667, 669, 697
Chryseobacterium indologenes, 458, 460, 472, 508, 638–39, 641–52, 654–62, 664–65, 667, 669, 680
Chryseobacterium indoltheticum, 458, 460, 540, 542–43, 638–39, 641, 643, 646–53, 655, 658–60
Chryseobacterium joostei, 458, 460, 639, 646–53, 655, 657–58, 661
Chryseobacterium massiliae, 458, 668
Chryseobacterium meningosepticum, 458, 460, 471, 508, 510, 519, 539–43, 545, 638–39, 646–52, 658–59, 700, 713. *See also Elizabethkingia meningoseptica*
Chryseobacterium miricola, 458, 639, 647–51. *See also Elizabethkingia miricola*
Chryseobacterium proteolyticum, 458, 460, 466, 639, 642–43, 647–53, 655, 657, 662–63, 669
Chryseobacterium scophthalmum, 458, 460, 472, 519, 639, 641, 646–55, 658–63
Chryseobacterium taichungense, 642–43, 647, 652–53, 655, 657, 663
Ciprofloxacin resistance/sensitivity
  Bacteroides, 406–12
  Capnocytophaga, 710
  Salinibacter, 733
Citrate synthase
  Rhodothermus, 716
  Thermoleophilum, 847
  Wolinella, 183
Citrate utilization
  Bergeyella, 535
  Roseiflexus, 831
  Weeksella, 535
Citric acid cycle
  Aquifex, 934
  Hydrogenobacter, 934
  Saprospira, 599
Cladonia, 997
Cladophora, 37–38, 83
Clams, *Cristispira*, 971
Clarithromycin, *Helicobacter*, 167
Clathrochloris, 360
Clathrochloris sulfarica, 361, 363
Clevelandina, 213, 971–81
Clevelandina reticulitermitidis, 971, 977
Clindamycin resistance/sensitivity
  Bacteroides, 407–8
  Brachyspira, 349
  Capnocytophaga, 710
  Chryseobacterium, 668
  Elizabethkingia, 668
  Wolinella, 180
Clinical environment
  Campylobacter, 181
  Chryseobacterium, 638, 642, 665
  Elizabethkingia, 638, 642–43, 665–67
  Empedobacter, 630–31
  Fusobacterium, 1017
  Myroides, 630–31
Clostridium, 397, 398
Clostridium aceticum, 5
Clostridium acetobutylicum, 899
Clostridium rectum, 957
Clostridium thermocellum, 207
Coagulase, *Staphylococcus*, 84
Coccobacillus, *Porphyromonas*, 428
Cochleobolus, 83
Cockroache habitat
  Blattabacterium, 465
  Chryseobacterium, 644
  Fusobacterium, 1016
  Hollandina, 975, 979
  Pillotina, 979
  spirochetes, symbiotic, 974–75
Coenonia, 455, 461, 466, 473, 509, 695–706
Coenonia anatina, 458, 461, 466–67, 471, 680, 695–706
Coliphage, *Myxococcus xanthus*, 35
Colistin resistance/sensitivity
  Bacteroides, 413
  Fusobacterium, 1023
  Hymenobacter, 728
  Porphyromonas, 431
  Riemerella, 705
  Salinibacter, 733
  Streptobacillus, 983
Colitis
  Brachyspira, 334, 348
  Campylobacter, 130
  Helicobacter, 153, 159, 163–64
Colon habitat
  Bacteroides, 381, 383, 387, 395–400, 404
  Brachyspira, 332, 334, 336, 344
  Fusobacterium, 1017
  Helicobacter, 147, 153, 156, 163
  Treponema, 213
Colonies. *See also* Swarms
  Bacteroides, 382–83
  Bergeyella, 534
  Brachyspira, 335–36
  Capnocytophaga, 561, 709, 710
  Caulococcus, 996
  Chlorobiaceae, 361
  Chryseobacterium, 647, 652
  Coenonia, 699
  Cytophaga, 559–60
  Cytophagale, 550, 552, 554, 559–60, 573
  Elizabethkingia, 647, 652
  Flavobacterium, 490–91, 493, 496–99, 503, 517
  Fusobacterium, 1020–21
  Gemmata, 781–82
  Helicobacter, 148–49, 156
  Herpetosiphon, 854, 865–67
  Hymenobacter, 725
  Kusnezovia, 997
  Leptospira, 297, 304
  Myroides, 632–33
  myxobacteria, 70–72
  Planctomyces, 774–75, 777
  Porphyromonas, 428, 431
  Proteus, 1021
  Riemerella, 699
  Roseiflexus, 827
  Salinibacter, 731
  Saprospira, 594, 598
  Spirochaeta, 204
  Sporocytophaga, 559–60
  Thermocrinis, 929
  Thermoleophilum, 845
  Thermomicrobium, 849
  Thiobacterium, 947
  Treponema, 221
  Weeksella, 534
  Wolinella, 179
  Zoogloea, 963–64
Colorless sulfur bacteria, 941–52
Columnaris disease
  Cytophagale, 554
  Flavobacterium, 515–18
Commercial fermentation, myxobacteria, 55–56
Complex organic substrate utilization
  Fervidobacterium, 905
  Geotoga, 905
  Marinitoga, 905
  Petrotoga, 905
  Thermosipho, 905
  Thermotoga, 905
Congenital syphilis, *Treponema*, 226
Congo red adsorption, *Flavobacterium*, 494–95, 499–500
Conjugation, *Bacteroides*, 394
Conjunctivitis, *Chlamydia*, 747–48, 751
Corallococcus, 38, 43, 48, 56, 59, 62–64, 71–73, 82, 85–87
Corallococcus coralloides, 33, 36–37, 60, 74–75
Corallococcus macrosporus, 56
Coral plaque, *Sphingomonas*, 620
Corynebacterium, 1016
Corynebacterium glutamicum, 865
Cotton-wool disease. *See* Columnaris disease
Coumermycin, *Borrelia*, 256

Crab, *Saprospira*, 592
Creosote
  Sphingomonads, 612–13
  Sphingomonas, 611–12
Cristispira, 195, 197, 213–14, 235, 591, 971–81
Cristispira pectinis, 326, 971, 978
Croceibacter, 455, 461, 466, 471, 481, 678
Croceibacter atlanticus, 458, 461, 467, 678, 680–81, 688
Croceibacter latercula, 678
Croceibacter marinoflava, 678
Crohns disease, *Bacteroides*, 400
CSF. *See* Cerebrospinal fluid
Cultivation methods
  Bdellovibrio, 22–23
  Chlorobiaceae, 365–66
  Chloroflexus, 825–26
  Chryseobacterium, 646–47
  Cytophagale, 562–65
  Elizabethkingia, 646–47
  Flavobacteriaceae, 688–89
  Flavobacterium, 489–92
  Helicobacter, 147–48
  Herpetosiphon, 861–62
  Isosphaera, 786
  Leptospira, 297–98, 303–5
  myxobacteria, 48–57
  Saprospira, 595–96
  Sphingomonads, 614
  Thermoleophilum, 844–45
  Thermomicrobium, 850
  Treponema, 217–18
  Wolinella, 181–82
Cyanide sensitivity, *Thermomicrobium*, 852
Cyanobacteria, 360, 364, 366, 370, 816, 820–24, 826–28, 830, 832–33, 835, 900
Cyanocobalamin requirement
  Cytophagale, 565
  Leptospira, 299
Cyclitis, *Borrelia*, 261
Cycloartenol production, myxobacteria, 63
Cycloheximide resistance/sensitivity
  Campylobacter, 123
  Gemmata, 782
  myxobacteria, 40–42, 47
  Pirellula, 779
  Planctomycetales, 766
Cycloserine resistance/sensitivity
  Thermonema, 724
  Thermotogales, 905
Cyclosporin
  Cytophaga, 580
  Porphyromonas, 432
Cylindrogloea bacterifera, 371
Cysteine metabolism
  Bacteroides, 383
  Chloroflexus, 831
Cysteine proteinases, *Porphyromonas*, 428–29, 434–37, 442, 444
Cystobacter, 36, 39, 42, 49, 53, 56, 60, 62–64, 71–77, 80, 82, 85–86, 88, 90, 94
Cystobacter badius, 97
Cystobacter disciformis, 89
Cystobacter ferrugineus, 32, 36, 54, 62, 74–75, 84, 88, 95
Cystobacter fuscus, 36–37, 43–44, 54, 63, 75, 82, 84, 88, 94
Cystobacter gracilis, 96
Cystobacter miniatus, 96
Cystobacter minus, 63, 76–77, 88
Cystobacter velatus, 32, 36, 74–75, 95
Cystobacter violaceus, 33, 36, 63–64, 71, 88, 95–96
Cytochrome
  Aquifex, 932
  Bacteroides, 384, 387
  Chlorobiaceae, 368–69
  Chloroflexus, 834–35
  Hydrogenobacter, 932
  Leptospira, 298
  myxobacteria, 59
  Pelobacter, 7
  Saprospira, 599
  Sphingomonads, 619
  Wolinella, 180
Cytochrome oxidase production
  Aequorivita, 685
  Arenibacter, 685
  Cellulophaga, 685
  Croceibacter, 685
  Cytophaga, 685
  Flavobacteriaceae, 683
  Flavobacterium, 494–96, 503
  Formosa, 685
  Gelidibacter, 685
  Gillisia, 685
  Hymenobacter, 725
  Maribacter, 685
  Mesonia, 685
  Muricauda, 685
  Polaribacter, 685
  Psychroflexus, 685
  Psychroserpens, 685
  Robiginitalea, 685
  Salegentibacter, 685
  Tenacibaculum, 685
  Thermus, 804
  Ulvibacter, 685
  Vitellibacter, 685
  Zobellia, 685
Cytolethal distending toxin, *Campylobacter*, 131
Cytophaga, 549, 555, 567, 576–77, 579–80, 761, 867
Cytophaga allerginae, 482, 486–87, 489, 492, 511, 554, 580
Cytophaga aquatilis, 549, 553. *See also Flavobacterium hydatis*
Cytophaga aurantiaca, 550, 569
Cytophaga columnaris, 552, 554, 561–62, 564, 569–70, 572–74, 577–78, 581
Cytophaga diffluens, 574
Cytophaga flevensis, 552–53, 557, 573
Cytophaga heparina, 549, 576, 578
Cytophaga hutchinsonii, 473, 510, 569–71, 574, 680, 713
Cytophaga johnsonae, 549, 553, 567, 570–74, 576, 579–80. *See also Flavobacterium johnsoniae*
Cytophaga krzemieniewskae, 571
Cytophaga latercula, 456, 458–59, 466–67, 680, 682–85, 687
Cytophagale, 359, 501, 511, 539–40, 549–81
Cytophaga lytica, 549–50, 571, 681. *See also Cellulophaga lytica*
Cytophaga marinoflava, 456, 458–59, 466–67, 681–85
Cytophaga maritima, 577–78, 581
Cytophaga psychrophila, 554, 561, 574, 577–78, 581. *See also Flavobacterium psychophilum*
Cytophaga saccharophila, 557
Cytophaga salmonicolor, 573–74
Cytophaga succinicans, 550, 562, 572–74. *See also Flavobacterium succinicans*
Cytophaga uliginosa, 573, 580, 681. *See also Zobellia uliginosa*
Cytophaga xantha. See Flavobacterium xanthum
Cytoplasmic filaments, *Treponema*, 217
Cytoplasmic membrane
  Borrelia, 244
  Treponema, 215–17
Cytoplasmic tubules, spirochetes, symbiotic, 980
Cytotoxin
  Campylobacter, 131
  Helicobacter, 139, 155

D

Dairy products. *See also* Milk
  Bergeyella, 533
  Chryseobacterium, 638, 640, 643, 645, 653
  Elizabethkingia, 638, 640, 643
  Flavobacterium, 488, 645–46
  Myroides, 631
  Weeksella, 533
Deer habitat, *Borrelia*, 237
Dehalococcoides ethanogenes, 817
Deinococcus, 797, 806, 844
Deinococcus geothermalis, 798
Deinococcus grandis, 798
Deinococcus indicus, 798
Deinococcus murrayi, 798
Deinococcus proteolyticus, 798
Deinococcus radiodurans, 798
Deinococcus radiophilus, 798
Deinococcus radiopugnans, 798
Dendritic cells, *Porphyromonas*, 443
Desiccation resistance
  Cytophagales, 555
  Herpetosiphon, 859, 863–64
  myxobacteria, 82
  Sporocytophaga, 567
Desulfovibrio desulfuricans, 14, 374
Desulfurobacterium, 925
Desulfurobacterium thermolithotrophum, 926
Desulfuromonas acetexigens, 5
Desulfuromonas acetoxidans, 5
Desulfuromonas succinoxidans, 5

Detergent sensitivity, *Bdellovibrio*, 16
Dextran utilization, *Bacteroides*, 385
Diabetes, *Porphyromonas*, 446–48
Diagnosis, *Leptospira*, 309–11
Di- and trisaccharide utilization
Bacteroides, 383–84, 414
Bergeyella, 467, 534, 649
Borrelia, 243
Brachyspira, 339, 414
Chryseobacterium, 467, 649, 651, 653
Cytophaga, 467, 574
Cytophagale, 563
Elizabethkingia, 649, 653
Empedobacter, 467, 633
Fervidobacterium, 905
Flavobacterium, 467, 543
Geotoga, 905
Haliscomenobacter, 604
Hymenobacter, 728
Marinithermus, 808
Marinitoga, 905
Meiothermus, 802
Myroides, 468–69, 633
Oceanitherums, 808
Opitutus, 891
Petrotoga, 905
Planctomyces, 777
Propionigenium, 957
Rhodothermus, 715, 717
Riemerella, 468–69, 649, 700
Salinibacter, 733
Sphingomonads, 615–16
Streptobacillus, 984
Thermomicrobium, 850
Thermosipho, 905
Thermotoga, 905
Ultramicrobacteria, 891
Verrucomicrobium, 887
Victivallis, 894
Vulcanitherums, 808
Weeksella, 469, 534
Diarrhea
Bacteroides, 397, 405, 415
Borrelia, 180
Brachyspira, 348–49
Campylobacter, 129–30, 132
Helicobacter, 153–54, 159, 162
Leptospira, 306
Dichelobacter nodosus, 704
Dichelobacter nodulans, 222
2-(2,4-Dichlorophenoxy)-propionic acid, *Sphingomonas*, 606, 620
Digital dermatitis
Fusobacterium, 1019
Treponema, 222, 320
Dimethyl sulfoxide (DMSO) reduction, *Wolinella*, 178, 182, 184
Dimethyl sulfoxide (DMSO) sensitivity, *Saprospira*, 597
Diphosphatidylglycerol, *Salinibacter*, 731
Diplocalyx, 213, 971–81
Diplocalyx calotermitidis, 971
Direct Fluorescent Antibody Test, *Treponema*, 219
Dithionite utilization, Chlorobiaceae, 362
DNA-DNA homology
Bacteroides, 382, 413, 415
Bdellovibrio, 13–14
Borrelia, 251
Brachyspira, 330–32
Campylobacter, 120, 128
Capnocytophaga, 709
Chryseobacterium, 656–61, 663
Cytophaga, 540
Cytophagale, 576–77
Elizabethkingia, 656–61, 663
Flavobacteriaceae, 473
Flavobacterium, 482, 504–6, 510–11, 539–40, 542–44
Fusobacterium, 1023
Helicobacter, 144–45, 151–52, 154
Herpetosiphon, 871–72
Leptospira, 294, 303
Pelobacter, 5
Pirellula, 780
Planctomycetales, 768
Rhodothermus, 712–13
Sphingomonads, 615
Thermomicrobium, 850
Thermus, 797–98
Treponema, 211–12
DNase activity
Aequorivita, 685
Arenibacter, 685
Cellulophaga, 685
Croceibacter, 685
Cytophaga, 685
Empedobacter, 633
Formosa, 685
Gelidibacter, 685
Gillisia, 685
Herpetosiphon, 869
Maribacter, 685
Mesonia, 685
Muricauda, 685
Myroides, 633
Polaribacter, 685
Psychroflexus, 685
Psychroserpens, 685
Robiginitalea, 685
Salegentibacter, 685
Tenacibaculum, 685
Thermonema, 724
Ulvibacter, 685
Vitellibacter, 685
Zobellia, 685
Dolphin habitat, *Helicobacter*, 142, 164
Doxycycline resistance/sensitivity
Borrelia, 278
Chlamydia, 747
Leptospira, 307
Porphyromonas, 432–33
Duck habitat
Borrelia, 279
Brachyspira, 330
Coenonia, 461, 703
Ornithobacterium, 703
Riemerella, 462, 695, 700, 703–5
Dysentery, *Brachyspira*, 334, 346–47
Dyspepsia, *Helicobacter*, 148
Dysuria, *Chlamydia*, 749–50

E

Ear habitat
Bergeyella, 533
Weeksella, 533
Ectothiorhodospiraceae, Chlorobiaceae, 364, 367
Eikenella corrodens, 180
Elastase activity, *Bacteroides*, 389
Elastase production, *Myroides*, 635
Elasticotaxis, myxobacteria, 69, 72
Elastin degradation
Flavobacterium, 513–14
Thermonema, 724
Thermus, 799
Electron paramagnetic resonance, *Rhodothermus*, 720
Electroporation
Bacteroides, 395
Borrelia, 256
ELISA. *See* Enzyme-linked immunosorbent assay
Elizabethkingia, 638–69
Elizabethkingia meningoseptica, 638–39, 641–44, 646–57, 660–68
Elizabethkingia miricola, 638, 642, 644, 646–53, 656–57, 661–63
Embden-Meyerhof-Parnas pathway (EMP)
Bacteroides, 387
Brachyspira, 340–41
Chloroflexus, 830
Flavobacteriaceae, 682
Thermotoga, 915
Thermus, 804
EMP. *See* Embden-Meyerhof-Parnas pathway
Empedobacter, 455, 461, 466, 470, 473, 484, 488, 534–35, 630–35, 638, 641, 645, 652, 655, 657, 667
Empedobacter brevis, 458, 461, 467, 533, 539–43, 545, 549, 630–32, 634, 639, 641, 656, 664–65, 680, 697, 713
Encephalitis, *Borrelia*, 277
Endocarditis
Campylobacter, 132
Porphyromonas, 448
Endocytosis, *Chlamydia*, 743
Energy metabolism
Bacteroides, 384–88
Bdellovibrio, 24
Borrelia, 242–43
Brachyspira, 330
Campylobacter, 120
Flavobacterium, 509
Fusobacterium, 1019
Helicobacter, 139, 149, 167
Leptospira, 298
myxobacteria, 59–60
Propionigenium, 957–58
Spirochaeta, 200, 206–7
Thermotoga, 916
Wolinella, 182–83
Enhygromyxa, 85
Enrofloxacin resistance/sensitivity, *Ornithobacterium*, 705

Enteritis
Campylobacter, 129–30
Fusobacterium, 1019
Ornithobacterium, 704
Enterobacteriaceae, 397
Enterococcus, 397, 398
Enterococcus faecalis, 302, 404, 411–12
Enterotoxin
Bacteroides, 401, 405, 415
Campylobacter, 131
Entner-Doudoroff pathway
Salinibacter, 733
Sphingomonads, 617
Thermotoga, 915
Environmental pollutant sensitivity. *See also specific* pollutants
Bdellovibrio, 16
Flavobacterium, 579
Herpetosiphon, 858
myxobacteria, 37–38
Sphingobium, 610, 612, 621
Sphingomonads, 612–13, 616, 619–20
Sphingomonas, 610
Enzyme immunoassay, *Leptospira*, 310
Enzyme-linked immunosorbent assay (ELISA)
Borrelia, 257, 279
Brachyspira, 346
Flavobacterium, 507, 514, 519
Leptospira, 311
Ornithobacterium, 702
Riemerella, 700, 705
Enzyme production, Flavobacteriaceae, 470–71
Epibiont, Chlorobiaceae, 371–74
Epixenosomes, 881, 883, 889, 891–92
Epothilone, 93
Erwinia amylovora, 17
Erwinia carotovora, 17, 26
Erwinia herbicola. See Pantoea ananatis
Erythema migrans, *Borrelia*, 239, 241, 262–63
Erythrobacter, 606
Erythromonas ursincola, 606. *See also Blastomonas ursincola*
Erythromycin resistance/sensitivity
Bergeyella, 534–35
Borrelia, 277
Brachyspira, 349
Chlamydia, 748
Chryseobacterium, 668
Cytophagale, 581
Elizabethkingia, 668
Flavobacterium, 514, 545
Fusobacterium, 1020
Helicobacter, 150, 162
Hymenobacter, 728
Leptospira, 307
Ornithobacterium, 705
Rhodothermus, 721
Thermomicrobium, 850
Thermonema, 724
Weeksella, 534–35
Escherichia coli, 326, 703–4, 713, 715, 717–19, 722, 784, 835, 846–47, 857, 859–61, 869
Bacteroides, 386, 390, 393–95, 404–6, 410
Bdellovibrio, 16, 20–24
Borrelia, 249–50, 256
Brachyspira, 342, 344, 347
Campylobacter, 131, 133
Chlorobiaceae, 369–70
Coenonia, 699
Cytophagale, 556
Helicobacter, 167
Leptospira, 296, 298, 300, 302
mutagenesis with, 958, 966
myxobacteria, 33, 42, 44, 46–47, 51, 57, 65, 67, 69, 71, 93
phylogenetic tree, 14, 926
Porphyromonas, 438, 444
Sphingomonads, 618
Spirochaeta, 205
Treponema, 216–17
Wolinella, 184–85
Esculin hydrolysis
Aequorivita, 467, 685
Arenibacter, 467, 685
Bacteroides, 386, 412, 414
Bergeyella, 467, 534, 535
Capnocytophaga, 467
Cellulophaga, 467, 685
Chryseobacterium, 467, 651, 655, 701
Coenonia, 467, 701
Croceibacter, 467, 685
Cytophaga, 467, 685
Empedobacter, 467
Flavobacterium, 467, 494–95, 543
Formosa, 685
Fucobacter, 467
Fusobacterium, 1022
Gelidibacter, 468, 469, 685
Gillisia, 685
Herpetosiphon, 869
Maribacter, 685
Meiothermus, 802
Mesonia, 468, 469, 685
Muricauda, 468, 469, 685
Myroides, 468, 469
Opitutus, 891
Ornithobacterium, 468, 469, 701
Pasteurella, 701
Polaribacter, 468, 469, 685
Psychroflexus, 468, 469, 685
Psychroserpens, 468, 469, 685
Riemerella, 468, 469, 700, 701
Robiginitalea, 685
Salegentibacter, 468, 469, 685
Streptobacillus, 984
Tenacibaculum, 468, 469, 685
Thermus, 799
Ulvibacter, 468, 685
Verrucomicrobium, 887
Vitellibacter, 468, 685
Weeksella, 469, 534, 535
Zobellia, 469, 685
Estuarine environment
Bdellovibrio, 14–16
Chlorobiaceae, 359
Cytophagale, 553, 561
Flavobacteriaceae, 682
Pelobacter, 5–6
Sphingomonas, 610
Ethanol production
Brachyspira, 330, 340–41
Chloroflexus, 831
Propionigenium, 957
Spirochaeta, 204, 206–7
termite gut spirochete, 325
Thermotogales, 915
Victivallis, 894
Ethanol tolerance, *Sphingomonas*, 617
Ethanol utilization. *See* Alcohol utilization
Ethidium bromide, *Bacteroides*, 411
Ethylene glycol utilization, *Pelobacter*, 7–9
Euplotidium arenarium, 889
Exotoxin, *Campylobacter*, 131
Extrusomes, Epixenosomes, 881, 892
Exudative septicemia, *Riemerella*, 695
Eyes. *See* Ocular habitat

F

FAME composition. *See* Fatty acid methyl ester composition
Fatty acid β oxidation pathway, myxobacteria, 59
Fatty acid composition
Aquifex, 930–31
Bergeyella, 651, 656
Capnocytophaga, 709
Chloroflexus, 819, 828, 832
Chloronema, 819
Chryseobacterium, 651, 656
Coenonia, 699
Elizabethkingia, 651, 656
Flavobacteriaceae, 685–87
Fusobacterium, 1022
Heliothrix, 819
Herpetosiphon, 867–68, 871
Hydrogenobacter, 930–31
Hydrogenobaculum, 930–31
Hydrogenothermus, 930–31
Hymenobacter, 725, 728
Isosphaera, 784, 786
Marinithermus, 808, 809
Meiothermus, 801, 803, 806–7
Oceanitherums, 808, 809
Ornithobacterium, 699, 702
Oscillochloris, 819, 832
Planctomycetales, 761–62
Rhodothermus, 720–21
Riemerella, 651, 656, 695, 699
Roseiflexus, 819, 832
Sejongia, 656
Streptobacillus, 984
Thermocrinis, 930–31
Thermomicrobium, 851
Thermonema, 723
Thermotogales, 899, 908
Thermus, 797–98, 800, 806–7
Verrucomicrobium, 886
Vulcanitherums, 808, 809

Fatty acid methyl ester composition (FAME composition)
Chryseobacterium, 656
Coenonia, 696
Elizabethkingia, 656
Flavobacterium, 504
Helicobacter, 144
Ornithobacterium, 696
Riemerella, 696
Feces habitat
Archangium, 36
Bacteroides, 382–83, 390, 396–98, 400, 402
Brachyspira, 330, 334, 346–49
Campylobacter, 122–24, 127
Chondromyces, 36
Corallococcus, 36–37
Cystobacter, 36–37
Cytophaga, 579
Cytophagale, 553
Flavobacterium, 541
Fusobacterium, 1017, 1020–21
Helicobacter, 147–48, 153–55, 157, 159, 163–65
Herpetosiphon, 855
Hymenobacter, 724
Melittangium, 36
Myroides, 631, 634
myxobacteria, 36–37, 40
Myxococcus, 36–37
Nannocystis, 36
Polyangium, 36
Sporocytophaga, 579
Stigmatella, 36–37
Taxeobacter, 726
Victivallis, 882, 893
Feline habitat
Bergeyella, 533
Campylobacter, 122
Chryseobacterium, 646
Elizabethkingia, 664
Fusobacterium, 1017
Helicobacter, 139–40, 142, 148, 150, 153–54, 162, 165
Leptospira, 305
Porphyromonas, 428–29
Streptobacillus, 983
Ferredoxin
Chlorobiaceae, 367–68
Rhodothermus, 716
Spirochaeta, 207
Thermotoga, 899
Ferret habitat, *Helicobacter*, 140, 149
Ferribacterium, 1009
Ferribacterium duplex, 1009
Ferroglobus, 915
Fervidobacterium, 899, 902–3, 906, 908, 914
Fervidobacterium gondwanense, 901–5
Fervidobacterium islandicum, 901–8
Fervidobacterium nodosum, 901–5, 907–8, 926
Fervidobacterium pennivorans, 901, 905, 908
Fetal tissues
Campylobacter, 122
Helicobacter, 161, 169
Fetopathology, *Campylobacter*, 130
F. heparinum, 14
Fibrils
Cytophaga, 569
Cytophagale, 568
myxobacteria, 52, 70
Porphyromonas, 430
Fibrin hydrolysis, *Thermus*, 799
Fibrinogen
Bacteroides, 389, 405
Flavobacterium, 502
Porphyromonas, 434–35
Fibrobacter succinogenes, 713
Fibronectin
Borrelia, 269–70
Flavobacterium, 502
Porphyromonas, 434
Fimbriae
Gemmata, 781, 783
Pirellula, 779–80
Planctomyces, 777–78
Porphyromonas, 433–34, 442–43, 446
Verrucomicrobium, 882, 885–86
Fish habitat
Chryseobacterium, 460, 640, 643, 645, 646, 653, 657, 663–64
Cytophagale, 554, 561, 577–78, 581
Elizabethkingia, 640, 643, 664
Empedobacter, 631–32
Flavobacteriaceae, 473
Flavobacterium, 461, 472, 485–87, 489–91, 493, 499, 502, 511–15, 519, 540
Myroides, 631–32
Tenacibaculum, 462, 472, 679, 681, 687
Weeksella, 533
Flagella
Aquifex, 930
Bdellovibrio, 12–13, 21
Borrelia, 244–47
Brachyspira, 333, 336, 347
Campylobacter, 124, 128, 131
Clevelandina, 971–75, 977
Cristispira, 971–77
Diplocalyx, 971–75, 978–79
Helicobacter, 139–43, 148, 150–65
Hollandina, 971–75, 979
Hydrogenobacter, 930
Hydrogenobaculum, 930
Hydrogenothermus, 930
Leptospira, 295–96, 300–301
Macromonas, 946
Pelosigma, 1001–3
Pillotina, 971–75, 979
Pirellula, 779–80
Planctomyces, 775, 777
Rhodothermus, 714
Sphingomonads, 615
Spirochaeta, 195, 199–200, 203, 205, 207
Thermocrinis, 930
Thermotogales, 907
Thiospira, 947–48
Thiovulum, 948
Toxothrix, 986
Treponema, 215–16
Wolinella, 179, 181
Flavobacteriaceae, 455–74, 481, 483, 630, 638, 642, 645, 900. *See also* Marine clade of flavobacteriaceae
Flavobacterium, 359, 455, 461, 466–67, 470–71, 473, 481–521, 533, 539–45, 555, 575, 605, 630, 638, 641–42, 645, 656, 677, 689, 761
Flavobacterium acidificum, 482
Flavobacterium acidurans, 482
Flavobacterium antarcticum, 481, 484, 488, 496, 505, 508–9
Flavobacterium aquatile, 458, 461, 481–83, 485, 487–89, 493–94, 505, 539–40, 544–45, 549, 575, 639, 641, 680, 697, 713
Flavobacterium balustinum. See Chryseobacterium balustinum
Flavobacterium branchiophilum, 458, 461, 482–83, 485, 487, 489, 491, 493–94, 496–97, 502, 504–10, 512, 518–19, 544–45, 569, 581
Flavobacterium breve, 713. *See also Empedobacter brevis*
Flavobacterium capsulatum, 605
Flavobacterium columnare, 458, 461, 472, 482–83, 485, 487, 489–94, 497–510, 512–13, 515–18, 520
Flavobacterium degerlachei, 458, 461, 483–85, 487, 489, 494, 496, 507
Flavobacterium devorans, 605. *See also Sphingomonas paucimobilis*
Flavobacterium ferrugineum. See Chitinophaga
Flavobacterium flevense, 458, 461, 481–83, 485, 487, 489, 493–94, 505
Flavobacterium frigidarium, 458, 461, 481–85, 487–89, 493–94, 496–97, 500, 505, 508, 520
Flavobacterium frigoris, 458, 461, 483–85, 487, 489, 494, 496, 507
Flavobacterium fryxellicola, 484, 496, 505, 507
Flavobacterium gelidilacus, 458, 461, 482–85, 487, 489, 494, 496, 507
Flavobacterium genitale. See Bergeyella; Weeksella
Flavobacterium gillisiae, 458, 461, 481–82, 484–85, 487–90, 494, 496–97, 500, 505
Flavobacterium gleum, 540–42, 641
Flavobacterium gondwanense, 544
Flavobacterium grunuli, 481, 488, 496, 505, 509
Flavobacterium heparinum, 575, 579
Flavobacterium hibernum, 458, 461, 483–85, 487, 489, 494, 496–97, 503, 506
Flavobacterium hydatis, 458, 461, 481–83, 485, 487, 489, 491, 493, 495, 505, 509–11, 518, 520
Flavobacterium indologenes, 540–42, 641

Flavobacterium indoltheticum. See Chryseobacterium indoltheticum
Flavobacterium johnsoniae, 458, 461, 482–83, 485, 487, 489, 491, 493, 495–96, 501, 503–5, 507–11, 519–20, 540, 663, 667, 710
Flavobacterium limicola, 458, 461, 483–85, 487, 489, 493, 495–97, 503, 505
Flavobacterium meningosepticum. See Elizabethkingia meningoseptica
Flavobacterium micromati, 458, 461, 483–85, 487, 489, 495–96, 507, 510
Flavobacterium mizutaii, 482, 540, 543–45. *See also Sphingobacterium*
Flavobacterium multivorum, 540–41, 543–45
Flavobacterium oceanosedimentum, 482
Flavobacterium odoratum. See Myroides odoratus
Flavobacterium omnivorum, 458, 461, 481, 483–85, 487–90, 493, 495–97, 505, 510
Flavobacterium pectinovorum, 458, 461, 481–83, 486–87, 489, 493, 495–97, 499, 505, 510, 560, 575
Flavobacterium piscicida. See Pseudoalteromonas piscicida
Flavobacterium psychophilum, 458, 461, 466, 482–83, 485–87, 489–93, 495–97, 500–515, 518
Flavobacterium psychrolimnae, 484, 496
Flavobacterium ranacida. See Chryseobacterium meningosepticum
Flavobacterium resinovorum, 482
Flavobacterium saccharophilum, 461, 481–83, 486–87, 489, 493, 495, 505, 510
Flavobacterium salegens, 544
Flavobacterium scophthalmum. See Chryseobacterium scophthalmum
Flavobacterium spiritivorum, 540–41, 543–45
Flavobacterium succinicans, 458, 461, 482–83, 485–87, 489, 493, 495, 497, 504, 509–11, 520
Flavobacterium tegetincola, 458, 461, 483–84, 486–87, 489–90, 495–97, 509–10
Flavobacterium thalpophilum, 540–41, 543–45
Flavobacterium thermophilum, 482, 544
Flavobacterium uliginosum, 571, 575, 580–81
Flavobacterium xanthum, 458, 461, 481, 483–84, 486–87, 489, 495–97, 505
Flavobacterium xinjiangense, 458, 461, 481, 483–84, 486–88, 489–90, 493, 495–97, 505
Flavobacterium yabuuchiae. See Sphingobacterium spiritovorum
Flectobacillus, 459
Flexibacter, 511, 549, 553, 555, 567–68, 571–72, 576–77, 579–81, 869
Flexibacter auranticus, 483
Flexibacter auranticus copepodarum, 458
Flexibacter auranticus excathedrus, 458, 482–84, 486–87, 489, 492
Flexibacter canadensis, 510
Flexibacter echinicida, 458
Flexibacter elegans, 549, 564, 571–72, 577
Flexibacter filiformis, 549, 552, 564, 567–69, 571–72, 578
Flexibacter flexilis, 550, 577, 713
Flexibacter giganteus, 854. *See also Herpetosiphon*
Flexibacter maritimus, 554, 681
Flexibacter polymorphus, 568, 570, 685
Flexibacter tractuosus, 458
Flexirubin
  Bergeyella, 648
  Capnocytophaga, 710
  Chryseobacterium, 648, 652, 654
  Cytophaga, 555, 558, 682
  Cytophagale, 553, 558, 571–72
  Elizabethkingia, 648, 653–54
  Empedobacter, 633
  Flavobacteriaceae, 466, 471, 681
  Flavobacterium, 471, 494–95, 500–501, 545
  Flexibacter, 553, 555, 577
  Myroides, 633
  Riemerella, 648
  Sporocytophaga, 555, 558
Flexispira rappini. See Helicobacter flexispira
Flexithrix dorotheae, 567, 571, 576
Florfenicol resistance/sensitivity, *Flavobacterium*, 508, 515, 518
Fluoroquinolone resistance/sensitivity
  Bacteroides, 411
  Ornithobacterium, 705
5-Fluorouracil resistance/sensitivity
  Helicobacter, 150, 152–54
  Leptospira, 304
Fly habitat
  Empedobacter, 631
  Myroides, 631, 635
Footrot, *Fusobacterium*, 1018–19
Formalin treatment, *Flavobacterium*, 515, 518–19
Formate production
  Bacteroides, 387
  Chloroflexus, 831
  Propionigenium, 957
Formate utilization. *See* Organic acid utilization
Formosa, 481, 690
Formosa algae, 678, 680, 682–83
3-Formylindole, 92
Fowl. *See also specific* fowl
  Borrelia, 237, 279
  Brachyspira, 345
  Chryseobacterium, 645
  Flavobacterium, 488
  Fusobacterium, 1017
  Ornithobacterium, 695–96, 703
Fox habitat, *Helicobacter*, 153
Freshwater environment
  Acetobacterium, 5
  Achromatium, 943–44
  Bdellovibrio, 14–16
  Beggiatoa, 941
  Brachyarcus, 998–99
  Caulococcus, 996–97
  Chlorobiaceae, 359, 362–63, 370, 372
  Chloroflexaceae, 815, 820
  Chloroflexus, 818, 825
  Chloronema, 815, 816, 822, 826, 828–29
  Chryseobacterium, 638, 642–43, 645, 653, 663
  Clostridium, 5
  Cytophaga, 550, 552, 553, 555
  Cytophagale, 553–54, 560–61, 576
  Elizabethkingia, 638, 643
  Empedobacter, 631–32
  Flavobacteriaceae, 470
  Flavobacterium, 461, 482, 484–87, 489–90, 493–96, 511–15, 520, 540
  Flexibacter, 553, 555
  Gallionella, 990
  Gemmata, 781–82
  Herpetosiphon, 854–55, 858, 862, 872
  Kusnezovia, 997
  Leptospira, 305, 307
  Metallogenium, 1006
  myxobacteria, 37–38
  Oscillochloris, 815, 817, 822–23, 828, 830
  Pelobacter, 5–6
  Pelosigma, 1002
  Pirellula, 778
  Planctomyces, 762, 773
  Planctomycetales, 762–65
  Propionigenium, 955–56
  Prosthecobacter, 882, 887
  Salegentibacter, 462
  Saprospira, 591–99
  Siderocapsa, 1005, 1006
  Sphingomonas, 610
  Spirochaeta, 195, 198, 200, 205, 207
  Sporocytophaga, 555
  Thiospira, 947
  Toxothrix, 986–88
  Verrucomicrobia, 884
  Verrucomicrobium, 882, 885
Frog habitat
  Chryseobacterium, 646, 664
  Elizabethkingia, 664
Frozen environment
  Aequorivita, 678
  Cellulophaga, 678
  Flavobacteriaceae, 472

Flavobacterium, 461, 485–88, 490, 494–95, 497
Gelidibacter, 462, 678
Hymenobacter, 712
Maribacter, 679
Polaribacter, 462, 679
Prosthecobacter, 887
Psychroflexus, 462, 679
Sphingomonas, 612, 620
Verrucomicrobia, 881
Fructose utilization. *See* Monosaccharide utilization
Fruiting body
description of, 72–73
formation of, 77–80
Herpetosiphon, 857
myxobacteria, 31–35, 38–44, 48, 51, 57–59, 61, 70–91
Fucobacter, 455, 462
Fucobacter marina, 458, 462, 466–67
Fucoidan degradation
Flavobacteriaceae, 470
Fucophilus, 885
Fucophilus fucoidanolyticus, 881
Fucose utilization
Bacteroides, 400
Borrelia, 243
Sphingomonads, 615
α-Fucosidase
Bacteroides, 414
Chryseobacterium, 701
Coenonia, 701
Ornithobacterium, 701
Pasteurella, 701
Riemerella, 701
Fumarate reduction
Bacteroides, 387, 388, 390
Capnocytophaga, 710
Cytophagale, 565
Propionigenium, 957
Wolinella, 178–84, 184, 186
Furans
Chryseobacterium, 669
Flavobacterium, 515
Furazolidone, *Chryseobacterium*, 664
Fusidic acid sensitivity, *Hymenobacter*, 728
Fusobacteria, 957
Fusobacterium, 412, 957, 1016–23
Fusobacterium alocis, 1016–17
Fusobacterium gonidiaformans, 1017, 1022–23
Fusobacterium mortiferum, 413, 1017, 1020–22
Fusobacterium naviforme, 1016–17, 1022–23
Fusobacterium necrogenes, 1017
Fusobacterium necrophorum, 1016–23
Fusobacterium nucleatum, 428, 957, 1016–23
Fusobacterium periodonticum, 1016–17, 1023
Fusobacterium prausnitzii, 1016–17
Fusobacterium russii, 1017, 1022
Fusobacterium simiae, 1016
Fusobacterium sulci, 1016–17
Fusobacterium ulcerans, 1017, 1023
Fusobacterium varium, 1017, 1020–22

G

Galactosamine utilization, *Porphyromonas*, 439
Galactose utilization. *See* Monosaccharide utilization
α-Galactosidase production
Meiothermus, 802
Rhodothermus, 717–18
Thermus, 799
β-Galactosidase production
Aequorivita, 467
Arenibacter, 467
Bergeyella, 467, 535, 649
Capnocytophaga, 467
Cellulophaga, 467
Chryseobacterium, 467, 649, 654, 701
Coenonia, 467, 701
Croceibacter, 467
Cytophaga, 467
Elizabethkingia, 649, 654
Empedobacter, 467
Flavobacterium, 467, 543, 545
Fucobacter, 467
Gelidibacter, 468, 469
Mesonia, 468, 469
Muricauda, 468, 469
Myroides, 468, 469
Ornithobacterium, 468, 469, 701, 702
Pasteurella, 701
Polaribacter, 468, 469
Psychroflexus, 468, 469
Psychroserpens, 468, 469
Riemerella, 468, 469, 649, 700, 701
Salegentibacter, 468, 469
Tenacibaculum, 468, 469
Thermus, 799
Ulvibacter, 468
Vitellibacter, 468
Weeksella, 469, 535
Zobellia, 469
Gallic acid utilization, *Pelobacter*, 5, 7
Gallionella, 990–94, 1014
Gallionella ferruginea, 986, 990, 994
Gallionella filamenta, 994
Gas-liquid chromatography analysis, *Bacteroides*, 412–13
Gastric cancer, *Helicobacter*, 166
Gastric mucosa, *Helicobacter*, 139, 141, 143, 147–52, 159–60, 162, 164
Gastritis, *Helicobacter*, 166
Gastroenteritis
Helicobacter, 155, 163
Myroides, 634
Gastrointestinal tract. *See also* Intestinal tract
Bacteroides, 395–96, 400
Chlamydia, 749
Fusobacterium, 1016–17
Gas vesicles
Brachyarcus, 998–99
Chlorobiaceae, 360, 364, 370
Chloroflexus, 819
Chloronema, 818, 819
Heliothrix, 819
Isosphaera, 764, 787
Oscillochloris, 818, 819
Polaribacter, 681
Roseiflexus, 819
G+C content
Aequorivita, 467, 685
Aquifex, 929
Arenibacter, 467, 685
Bacteroides, 392
Bergeyella, 467, 534–35, 544, 650
Borrelia, 249
Brachyspira, 330, 333
Capnocytophaga, 467
Cellulophaga, 467, 685
Chlorobiaceae, 361, 364
Chloroflexus, 819, 828
Chloronema, 819
Chryseobacterium, 467, 534, 650, 653, 663
Coenonia, 467
Croceibacter, 467, 685
Cytophaga, 467, 540, 685
Cytophagale, 572, 575
Elizabethkingia, 650, 653, 663
Empedobacter, 467, 633
Fervidobacterium, 904
Flavobacteriaceae, 459, 473, 687
Flavobacterium, 467, 485–86, 493, 494–95, 501, 510, 539–40, 543–44
Formosa, 685
Fucobacter, 467
Fusobacterium, 1016
Gelidibacter, 468, 469, 685
Gemmata, 761, 781, 784
Geotoga, 904
Gillisia, 685
Haliscomenobacter, 604
Helicobacter, 141, 143, 149–56, 162
Heliothrix, 819
Herpetosiphon, 854–55, 868–69, 871–72, 874
Hydrogenobacter, 929
Hydrogenobaculum, 929
Hydrogenothermus, 929
Hymenobacter, 726, 731
Isosphaera, 761
Leptospira, 301
Lysobacter, 575
Maribacter, 685
Marinithermus, 808
Marinitoga, 904
Mesonia, 468, 469, 685
Muricauda, 468, 469, 685
Myroides, 468, 469, 534, 633
Novosphingobium, 609
Oceanitherums, 808
Opitutus, 882
Ornithobacterium, 468, 469
Oscillochloris, 819, 829
Petrotoga, 904
Planctomyces, 761, 777
Planctomycetales, 761, 766

Polaribacter, 468, 469, 685
Porphyromonas, 431
Prosthecobacter, 882
Psychroflexus, 468, 469, 685
Psychroserpens, 468, 469, 685
Rhodothermus, 712
Riemerella, 468, 469, 650
Robiginitalea, 685
Roseiflexus, 819, 829
Salegentibacter, 468, 469, 685
Saprospira, 593, 599–600
Sphingobacterium, 534, 543–44
Sphingobium, 609
Sphingomonads, 618
Sphingomonas, 609
Sphingopyrix, 609
Spirochaeta, 197, 204
Streptobacillus, 983
Streptomyces, 874
Taxeobacter, 575, 726
Tenacibaculum, 468, 469, 685
Thermocrinis, 929
Thermoleophilum, 845
Thermomicrobium, 849–50
Thermonema, 722–23
Thermosipho, 904
Thermotoga, 904
Thermotogales, 899
Treponema, 212, 223
Ulvibacter, 468, 685
Verrucomicrobium, 882, 886
Victivallis, 882, 894
Vitellibacter, 468, 685
Vulcanitherums, 808
Weeksella, 469, 534–35, 544
Wolinella, 188
Zobellia, 469, 685
Zoogloea, 966
Geese habitat
Borrelia, 279
Coenonia, 695, 703
Ornithobacterium, 703
Riemerella, 695, 703–4
Gelatin hydrolysis
Aequorivita, 467
Arenibacter, 467
Bacteroides, 383, 389, 399, 405
Bergeyella, 467, 534, 535
Capnocytophaga, 467
Cellulophaga, 467
Chryseobacterium, 467, 534, 645
Coenonia, 467
Croceibacter, 467
Cytophaga, 467
Empedobacter, 467
Flavobacterium, 467, 490, 493, 502
Fucobacter, 467
Gelidibacter, 468, 469
Herpetosiphon, 869
Mesonia, 468, 469
Muricauda, 468, 469
Myroides, 468, 469, 534
Ornithobacterium, 468, 469
Pirellula, 780
Polaribacter, 468, 469
Psychroflexus, 468, 469
Psychroserpens, 468, 469
Riemerella, 468, 469
Salegentibacter, 468, 469
Salinibacter, 733
Sphingobacterium, 534
Tenacibaculum, 468, 469
Thermonema, 724
Thermus, 799
Ulvibacter, 468
Vitellibacter, 468
Weeksella, 469, 534, 535
Zobellia, 469
Gelidibacter, 455, 462, 466–69, 471, 481, 488, 678, 689
Gelidibacter algens, 458, 462, 678, 680–81, 697
Gelidibacter mesophilus, 458, 462, 472, 678, 680
Gemmata, 760–72, 775, 781–84
Gemmata obscuriglobus, 759, 761, 763–64, 767, 769, 771–72, 781–84, 788
Genetics
Aquificales, 935
Bacteroides, 392–95
Borrelia, 249–56
Brachyspira, 342–44, 346
Campylobacter, 124, 128–29, 133
Chlorobiaceae, 369–70
Chloroflexus, 836
Chryseobacterium, 663
Elizabethkingia, 663
Flavobacteriaceae, 473
Flavobacterium, 510
Herpetosiphon, 873–74
Leptospira, 301–2
Planctomycetales, 772
Porphyromonas, 439–40
Sphingomonads, 617–18
Thermotogales, 915–17
Treponema, 223–24
Wolinella, 188
Zoogloea, 966–67
Genetic transformation
Borrelia, 255–56
Chlorobiaceae, 369
Leptospira, 302
Genitalia, *Treponema*, 212–13
Genome sequence
Aquifex, 935
Bacteroides, 392
Borrelia, 242, 247–50, 252–53
Chlorobiaceae, 369
Chloroflexus, 836
Gemmata, 772
Leptospira, 301
Pirellula, 772
Planctomycetales, 768–72, 772
Porphyromonas, 440
Sphingomonads, 618–19
Thermotoga, 916
Thermus, 804
Gentamycin resistance/sensitivity
Bacteroides, 411
Coenonia, 699
Hymenobacter, 728
Ornithobacterium, 700, 705
Rhodothermus, 721
Riemerella, 699, 705
Geosmin, 92, 94
Geotoga, 899
Geotoga petraea, 901–5
Geotoga subterranea, 901–5
Gigantomonas capitata, 998
Gigantomonas cucullata, 998, 1001
Gillisia, 481, 678
Gillisia limnaea, 678, 680
Gills
Chryseobacterium, 640, 643–45, 663–64
Cytophagale, 561
Elizabethkingia, 640, 643–44
Flavobacterium, 485, 487–88, 491, 502, 512–13, 516–20
Thermosipho, 903
Gingipains. *See* Cysteine proteinases
Gingival environment
Bergeyella, 533
Fusobacterium, 1018, 1020
Porphyromonas, 428–38, 440, 442, 446
Gingivitis
Fusobacterium, 1018
Porphyromonas, 428, 430–31
Treponema, 211, 213, 220
Gliding motility
Aequorivita, 682
Arenibacter, 682
Capnocytophaga, 709–10
Cellulophaga, 681, 682
Chloroflexus, 815, 824, 832
Chloronema, 815, 832
Chryseobacterium, 654
Croceibacter, 682
Cytophaga, 569, 681
Cytophagale, 553, 555, 570, 573, 575, 578
Elizabethkingia, 654
Flavobacteria, 575
Flavobacteriaceae, 681
Formosa, 682
Gelidibacter, 681, 682
Gillisia, 682
Heliothrix, 815, 817, 832
Herpetosiphon, 854
Hymenobacter, 725
Isosphaera, 784
Maribacter, 682
Mesonia, 682
Muricauda, 682
myxobacteria, 38, 68–70
Oscillochloris, 815, 832
Polaribacter, 682
Psychroflexus, 681, 682
Psychroserpens, 682
Robiginitalea, 682
Roseiflexus, 832
Salegentibacter, 682
Saprospira, 591, 594, 596–97
Tenacibaculum, 682
Thermonema, 722–23
Toxothrix, 986–87
Ulvibacter, 682
Vitellibacter, 682
Zobellia, 681, 682
Gluconate oxidation
Bergeyella, 535
Weeksella, 535
Gluconeogenesis, *Sorangium*, 60
Gluconobacter, 965
Gluconobacter oxydans, 965–66

Glucosamine utilization
Borrelia, 243
Brachyspira, 339
Haliscomenobacter, 604
Porphyromonas, 439
Glucosephosphate isomerase, *Leptospira*, 298
Glucose utilization. *See* Monosaccharide utilization
Glucosidase, *Bacteroides*, 399–400
Glutamate dehydrogenase
Bacteroides, 388–90
Fusobacterium, 1023
Salinibacter, 732
Glutamate production, *Bacteroides*, 388, 390
Glutamate utilization
Flavobacteriaceae, 683
Flavobacterium, 510
Fusobacterium, 1019
Haliscomenobacter, 604
Leptospira, 298
Rhodothermus, 715
Glutamine synthetase, *Bacteroides*, 388
Glutamine utilization, *Meiothermus*, 802
γ-Glutamyl transferase, *Helicobacter*, 141, 143, 161
γ-Glutamyl transpeptidase, *Helicobacter*, 149–59, 162, 164–66
Glycerol utilization. *See* Alcohol utilization
Glycine tolerance
Campylobacter, 121
Helicobacter, 141, 143, 149–59, 162–66
Glycine utilization
Leptospira, 298
Thermoleophilum, 845
Glycogen production, myxobacteria, 60
Glycogen utilization
Bacteroides, 414
Prosthecobacter, 888
Glycolipids
Marinithermus, 809
Vulcanitherums, 809
Glycoside production, myxobacteria, 63
Glycosphingolipids (GSL)
Erythrobacter, 606
Novosphingobium, 609
Porphyrobacter, 606
Sphingobium, 609
Sphingomonads, 615, 617, 621
Sphingomonas, 605–6, 609
Sphingopyrix, 609
Glyoxalate pathway, *Leptospira*, 298
Green alga, 854. *See also Cladophora*
Green sulfur bacteria, 815, 826, 833, 844, 849, 855, 900, 999. *See also Chlorobiaceae*
GSL. *See* Glycosphingolipids
Guar gum utilization, *Bacteroides*, 384–85, 398
Guillain-Barré syndrome (GBS), *Campylobacter*, 130
Guinea pig habitat
Brachyspira, 345
Fusobacterium, 1017
Helicobacter, 169
Leptospira, 307
Streptobacillus, 983
Treponema, 212

H

Habitats. *See specific* habitats
Haemophilus influenzae, 704
Halanaerobacter lacunarum, 714
Halanaerobium lacusrosei, 714
Haliangium, 36, 85–86, 91
Halibut habitat
Chryseobacterium, 644
Tenacibaculum, 687
Haliscomenobacter, 549, 602–4, 987
Haliscomenobacter hydrossis, 602–4
Haloanella gallinarum, 458, 639, 643, 668
Haloarcula marismortui, 731
Halobacterium salinarum, 731–32
Halomonas aquamarina, 714
Halorhodospira halophila, 714
Hamster habitat
Bacteroides, 404
Helicobacter, 140, 153, 156, 158
Leptospira, 300
Haploangium, 36–37, 39, 48, 85–86, 89
Haploangium minus, 89
Haploangium rugiseptum, 89
Hard tick, *Borrelia*, 235, 237–39
Haverhill fever, *Streptobacillus*, 983–84
Haverhillia moniliformis, 983. *See also Streptobacillus moniliformis*
Haverhillia multiformis, 983. *See also Streptobacillus moniliformis*
Hay, *Spirochaeta*, 203
Heart. *See* Cardiovascular environment
Heat purification, myxobacteria, 46, 82
Heat shock protein
Chlamydia, 746, 751
Leptospira, 302
Planctomycetales, 757
Rhodothermus, 715
Heat tolerant. *See also* Thermophile
Chloroflexus, 821
Herpetosiphon, 858
Heavy metals
Bdellovibrio, 16
Zoogloea, 967–68
Helicobacter, 119–20, 139–69
Helicobacter acinonychis, 140–41, 146, 151, 168
Helicobacter aurati, 139–42, 146, 153, 159
Helicobacter bilis, 139–42, 146, 154, 156–57, 159, 161, 168
Helicobacter Bird-B and Bird-C, 142, 162
Helicobacter bizzozeronii, 140–41, 144–46, 150–52, 161
Helicobacter bovis, 142, 146, 160
Helicobacter canadensis, 140–41, 146, 159, 164, 168
Helicobacter canis, 140–41, 144, 146, 153–54, 156, 159, 163–64, 168
Helicobacter cetorum, 142–43, 146, 164–65
Helicobacter cholecystus, 140–41, 146, 156–57, 164
Helicobacter cinaedi, 120, 124, 126, 140–41, 144–46, 153, 156, 162–63, 168
Helicobacter colifelis, 142, 162–63
Helicobacter Cottontop tamarin. *See Helicobacter tamarin*
Helicobacter felis, 140–41, 144–47, 150–52, 161, 168
Helicobacter fennelliae, 124, 126, 140–41, 144, 146, 153, 162, 164, 168
Helicobacter flexispira, 142, 145–46, 161–62, 164, 168
Helicobacter ganmani, 140–41, 146, 158–59, 164
Helicobacter heilmannii, 142–43, 145–46, 150, 161, 167
Helicobacter hepaticus, 140–41, 146, 155, 159, 163, 165, 169, 179
Helicobacter marmotae, 142–43, 146, 165
Helicobacter mesocricetorum, 140–41, 146, 158, 164
Helicobacter muricola, 142–43, 146, 165–66
Helicobacter muridarum, 139–41, 144–46, 153–54, 159, 166
Helicobacter mustelae, 139–41, 144–46, 149–50, 152, 162, 168
Helicobacter nemestrinae, 140–41, 144, 152, 169
Helicobacter pametensis, 140–41, 146, 155, 162
Helicobacter pullorum, 140–41, 146, 155–56, 159, 163–64, 168
Helicobacter pylori, 119–20, 139–41, 144–49, 151–52, 155, 164, 166, 168–69, 178
Helicobacter rappini, 124, 126, 142, 154, 156, 161–62, 169
Helicobacter rhesus, 142, 164–65
Helicobacter rodentium, 140–41, 146, 157–58, 164–65
Helicobacter salomonis, 140–41, 144–46, 150–52
Helicobacter strain CLO-3, 142, 162
Helicobacter suis, 142, 146, 160–61
Helicobacter suncus, 142–43, 146, 162–63
Helicobacter tamarin, 142, 163–64
Helicobacter trogontum, 140–42, 146, 156, 161
Helicobacter typhlonius, 140–41, 146, 159–60, 165
Helicobacter westmeadii. See Helicobacter cinaedi

Helicobacter winghamensis, 142–43, 146, 163–64, 168
Heliobactericeae, 360, 366–67, 815
Heliothrix, 818, 822–23, 826, 828–29, 870
Heliothrix oregonensis, 764, 815, 817, 819–20, 822–23, 826, 829–30, 832–33
Hemagglutinins
Chryseobacterium, 664
Flavobacterium, 513, 517
Fusobacterium, 1019
Porphyromonas, 434–39, 441
Hematoporphyrin utilization, *Bacteroides*, 384
Hemicellulose utilization, *Bacteroides*, 384, 386, 398
Hemin requirement
Bacteroides, 382–84, 387–88, 404
Porphyromonas, 437–38, 440–41
Hemoglobin utilization
Bacteroides, 384, 406
Leptospira, 299, 308
Porphyromonas, 434–35, 437
Hemopexin, *Bacteroides*, 404–5
Heparin sulfate utilization
Bacteroides, 385, 405
Cytophagale, 574
Hepatitis, *Helicobacter*, 156, 168–69
Herbicides
Sphingobium, 612
Sphingomonads, 612–13, 616
Herpetosiphon, 41, 815, 817–18, 844, 849, 854–74, 987
Herpetosiphon aurantiacus, 817, 854–59, 862–63, 866–73
Herpetosiphon cohaerens, 854. *See also Lewinella*
Herpetosiphon geysericola, 817, 854, 858, 861, 863, 868, 871–72
Herpetosiphon nigricans, 854. *See also Lewinella*
Herpetosiphon persicus, 854. *See also Lewinella*
Hexachlorocyclohexane degradation, *Sphingomonas*, 620–21
Hexose metabolism, myxobacteria, 60
Hillhousia mirabilis, 943. *See also Achromatium oxaliferum*
Hillhousia palustris, 943. *See also Achromatium oxaliferum*
Hippurate hydrolysis
Brachyspira, 333
Campylobacter, 121, 125–26
Fusobacterium, 1023
Helicobacter, 149–59, 162
Histidine aminopeptidase, *Helicobacter*, 150, 154
Histidine utilization, *Saprospira*, 599
HIV, *Porphyromonas*, 432, 448
Hollandina, 213, 971–81
Hollandina pterotermitidis, 971
Homospermidine
Flavobacteriaceae, 687
Hymenobacter, 725
Thermonema, 723
Homospermine, *Thermonema*, 723
Hooves, *Treponema*, 213
Horse habitat
Borrelia, 237
Fusobacterium, 1017
Treponema, 212
Hospital environment
Chryseobacterium, 646, 667
Elizabethkingia, 643, 646, 666
Sphingomonas, 621
Hot spring environment
Aquifex, 927, 928
Chloroflexaceae, 815, 818, 820
Chloroflexus, 820–21, 823–24, 828, 832
Fervidobacterium, 902–3
Gallionella, 990–91
Heliothrix, 815, 820, 822–23, 829
Herpetosiphon, 854, 871–72
Hydrogenobacter, 927
Hydrogenobaculum, 927
Hydrogenothermus, 928
Isosphaera, 764, 784–86
Marinitoga, 903
Meiothermus, 801–3
Pirellula, 778
Rhodothermus, 712, 714–15, 718–22
Roseiflexus, 820, 822–23, 829
Thermocrinis, 927–28
Thermoleophilum, 843–44
Thermomicrobium, 849–50
Thermonema, 722–24
Thermosipho, 903
Thermus, 798, 801–2
Verrucomicrobia, 881
Human habitat
Bacteroides, 381, 395–96
Bergeyella, 460, 532–33, 536
Borrelia, 235–39, 255, 257, 262, 269, 276
Brachyspira, 330–32, 335, 348
Campylobacter, 130, 132
Capnocytophaga, 460, 554, 561, 578, 581, 709
Chlamydia, 741, 746–53
Chryseobacterium, 460, 638, 640, 665–68, 701
Elizabethkingia, 638, 640, 665–68
Empedobacter, 461, 631
Flavobacteriaceae, 473
Flavobacterium, 488, 510–11, 541
Fusobacterium, 1016, 1018
Haloanella, 668
Helicobacter, 139–40, 142, 151, 153–54, 160–63, 166
Leptospira, 294, 305–6, 308–11
Myroides, 462, 631
Porphyromonas, 428–29, 438, 447
Sphingomonads, 613
Sphingomonas, 621
Streptobacillus, 983
Treponema, 211–13, 215, 217
Verrucomicrobia, 884
Weeksella, 463, 532–33, 541
Wolinella, 178
Human intestinal spirochetosis, *Brachyspira*, 348
Hyalangium, 85–86, 88–89
Hyalangium minutum, 97
Hyalobotrys hypolimnicus, 998
Hyaluronic acid utilization
Bacteroides, 385, 405
Flavobacteriaceae, 470
Flavobacterium, 502
Hydrogenase, *Wolinella*, 184
Hydrogenobacter, 925–30, 934
Hydrogenobacter acidophilus, 925. *See also Hydrogenobaculum acidophilum*
Hydrogenobacter halophilus, 927–30
Hydrogenobacter hydrogenophilus, 925–27, 929–30
Hydrogenobacter subterraneus, 926–27, 929–30
Hydrogenobacter thermophilus, 902, 926–32
Hydrogenobaculum, 925, 927–29
Hydrogenobaculum acidophilum, 925–27, 929–30, 934
Hydrogenothermaceae, 925–26
Hydrogenothermus, 929
Hydrogenothermus marinus, 925–30, 932, 934
Hydrogen peroxide sensitivity
Bacteroides, 391
Fusobacterium, 1019
Sphingomonas, 617
Hydrogen production
Brachyspira, 330, 340
Propionigenium, 957
Spirochaeta, 204, 206
termite gut spirochete, 325
Thermotogales, 899, 915
Victivallis, 894
Wolinella, 180
Hydrogen sulfide production
Aquifex, 934
Bergeyella, 535, 649
Campylobacter, 121, 125–26
Chryseobacterium, 645, 649
Elizabethkingia, 649
Flavobacterium, 494–95
Planctomycetales, 763
Riemerella, 649
Saprospira, 599
Spirochaeta, 204
Streptobacillus, 984
Thermocrinis, 934
Thermotogales, 915
Weeksella, 535
Wolinella, 180
Hydrogen sulfide reduction
Achromatium, 941–46
Aequorivita, 685
Arenibacter, 685
Cellulophaga, 685
Croceibacter, 685
Cytophaga, 685
Formosa, 685
Gelidibacter, 685
Gillisia, 685
Macromonas, 941–42
Maribacter, 685
Mesonia, 685
Muricauda, 685
Polaribacter, 685
Psychroflexus, 685
Psychroserpens, 685

Robiginitalea, 685
Salegentibacter, 685
Tenacibaculum, 685
Thiobacterium, 941–42, 947
Thiospira, 941–42, 947–48
Thiovulum, 941–42, 948–49, 952
Ulvibacter, 685
Vitellibacter, 685
Zobellia, 685
Hydrogen utilization
Aquifex, 934
Aquificales, 925, 934
Campylobacter, 121
Chlorobiaceae, 359, 368
Chloroflexus, 830
Helicobacter, 148, 153
Marinithermus, 808, 809
Oceanitherums, 808, 809
Oscillochloris, 830
termite gut spirochete, 323, 325–27
Thermotoga, 915
Vulcanitherums, 808, 809
3-Hydroxypropionate production, *Pelobacter*, 9
Hymenobacter, 724–29
Hymenobacter actinosclerus, 713, 725–26, 728–29
Hymenobacter aerophilus, 713, 725–26, 728–29
Hymenobacter roseosalivarius, 713, 725–28–729
Hyphomicrobium, 766

I

Ibuprofen, *Porphyromonas*, 432
Ice environment. *See* Frozen environment
Ilyobacter, 957
IM. *See* Inner membrane
Imipenem, *Bacteroides*, 407
Immune response, *Porphyromonas*, 445–47
Immunity
Chlamydia, 746
Leptospira, 308–9
Immunoelectron microscopy, *Borrelia*, 244–45
Immunoglobulin G (IgG), *Porphyromonas*, 445
Indole production
Aequorivita, 467
Arenibacter, 467
Bacteroides, 414
Bergeyella, 467, 534, 535, 649
Brachyspira, 333
Capnocytophaga, 467
Cellulophaga, 467
Chryseobacterium, 467, 534, 649, 701
Coenonia, 467, 701
Croceibacter, 467
Cytophaga, 467
Elizabethkingia, 649
Empedobacter, 467, 633
Flavobacterium, 467, 493, 539, 542, 543
Fucobacter, 467
Fusobacterium, 1022
Gelidibacter, 468, 469
Mesonia, 468, 469
Muricauda, 468, 469
Myroides, 468, 469, 534
Ornithobacterium, 468, 469, 701
Pasteurella, 701
Polaribacter, 468, 469
Psychroflexus, 468, 469
Psychroserpens, 468, 469
Riemerella, 468, 469, 649, 700, 701
Salegentibacter, 468, 469
Saprospira, 599
Sphingobacterium, 534
Tenacibaculum, 468, 469
Ulvibacter, 468
Vitellibacter, 468
Weeksella, 469, 534, 535, 543
Zobellia, 469
Indoxyl acetate hydrolysis
Campylobacter, 121, 125–26
Helicobacter, 141, 143, 149–59, 162–66
Inflammation, *Chlamydia*, 746
Innate immunity, *Borrelia*, 276–77
Inner membrane (IM)
Cytophaga, 570
Leptospira, 297
Treponema, 215
Insect habitat
Chryseobacterium, 644
spirochetes, symbiotic, 974
Treponema, 212
Interleukin, *Porphyromonas*, 434, 441–43, 447
Intestinal mucosa
Brachyspira, 332, 334, 344
Campylobacter, 122
Helicobacter, 139, 141, 143, 147–48, 152–59
Intestinal tract
Bacteroides, 381–82, 389, 397, 400–401
Brachyspira, 330, 336, 344–45
Chryseobacterium, 643–44, 663
Elizabethkingia, 643
Flavobacterium, 488
Fusobacterium, 1020
Myroides, 631
spirochetes, symbiotic, 974, 981
Treponema, 212–13
Iritis, *Borrelia*, 261
Iron requirement
Bacteroides, 391, 404–5
Borrelia, 243
Brachyspira, 340–41
Chloroflexus, 821
Leptospira, 299
Planctomyces, 763
Planctomycetales, 762–63
Porphyromonas, 440–41
Iron utilization
Caulococcus, 996–97
Gallionella, 990–92
Kusnezovia, 996–97
Siderocapsa, 1005
Siderocapsaceae, 1007
Thermotoga, 915
Toxothrix, 986–88
IS elements, *Leptospira*, 301–2
Isobutyrate production
Bacteroides, 414
Porphyromonas, 428
Spirochaeta, 206, 208
Isocitrate dehydrogenase
Bacteroides, 390
Thermoleophilum, 847
Isocitrate lyase, *Leptospira*, 298
Isocystis pallida, 760, 785. *See also Isosphaera pallida*
Isocystis salina, 785
Isoleucine fermentation
Saprospira, 599
Spirochaete, 206, 208
Isoleucine synthesis, *Bacteroides*, 390
Isorenieratene, Chlorobiaceae, 361, 366, 372
Isosphaera, 760–72, 784–88
Isosphaera pallida, 759, 760–61, 764, 767, 769–70, 772, 784–88, 826, 829
Isovalerate production
Bacteroides, 414
Porphyromonas, 428
Spirochaeta, 206, 208

J

Jahnia, 73, 85–86, 90
Jahnia thaxteri, 76–79, 97
Jarisch-Herxheimer reaction, *Borrelia*, 277–78
Jaundice, *Leptospira*, 294

K

Kaistella, 639–40
Kaistella koreensis, 458, 639, 647, 657
Kanamycin resistance/sensitivity
Bacteroides, 413
Cytophaga, 575
Flavobacterium, 508
Herpetosiphon, 860
Hymenobacter, 728
Rhodothermus, 721
Riemerella, 705
Salinibacter, 733
Sporocytophaga, 575
Thermomicrobium, 850
Thermonema, 724
Wolinella, 188
Keratin hydroloysis
Cytophaga, 539
Flavobacterium, 520
Keratitis, *Empedobacter*, 634
Kidney
Cytophagale, 561
Flavobacterium, 461, 512–13
Leptospira, 294, 300, 305–7, 309
Kofleria, 85–86, 91
Kofleria flava, 64, 91, 99
Kordia algicida, 458

Kouleothrix aurantiacus, 870
Krajian procedure, *Treponema*, 219
Krebs cycle
  Bacteroides, 390
  Helicobacter, 149
Kuenenia, 759, 764, 773
Kusnezovia, 996–97
Kusnezovia polymorpha, 997

L

β-Lactam resistance/sensitivity
  Bacteroides, 407–10
  Bergeyella, 534–35
  Capnocytophaga, 581, 710
  Chlamydia, 745
  Chryseobacterium, 667
  Elizabethkingia, 667
  Flavobacterium, 508
  Flexibacter, 580
  Fusobacterium, 1020
  Myroides, 633, 635
  Planctomycetales, 761
  Weeksella, 534–35
Lactate production
  Bacteroides, 387–88, 414
  Borrelia, 242
  Chloroflexus, 831
  Propionigenium, 957
  Spirochaeta, 204, 206
  Thermotogales, 915
Lactate utilization. *See* Organic acid utilization
Lactobacillus casei, 869
Lactobacillus plantarum, 846–47
Lactose utilization. *See* Di- and trisaccharide utilization
Lagoon environment. *See* Freshwater environment
Lake environment. *See* Freshwater environment
Laminarin utilization
  Bacteroides, 385
  Flavobacteriaceae, 470
  Flavobacterium, 501, 520
Lanosterol production, myxobacteria, 63
Laryngitis, *Fusobacterium*, 1019
Leptirospirosis, 294, 305–7
Leptonema, 195, 197, 214–15, 235, 255, 294
Leptonema illini, 197, 214, 294, 326, 713
Leptospira, 195, 197, 214–15, 235, 249, 255, 294–311, 340
Leptospira alexanderi, 295
Leptospira biflexa, 197, 294–99, 301–2, 326
Leptospira borgpetersenii, 295, 298–301, 308
Leptospira fainei, 295
Leptospira hardjo, 294
Leptospira icterohaemorrhagiae, 294
Leptospira illini. See Leptonema illini
Leptospira inadai, 295, 297–98
Leptospira interrogans, 197, 214, 294–95, 298–301, 308, 342
Leptospira kirschneri, 295–96, 308
Leptospira meyeri, 295, 298
Leptospira noguchii, 295, 298, 308, 321
Leptospira parva. See Turneria parva
Leptospira pomona, 294
Leptospira santarosai, 295, 298, 308
Leptospira weilii, 295, 298, 308
Leptospira wolbachii, 295
Leptothrichia, 957, 1021
Leptothrix, 604
Leptothrix trichogenes, 986. *See also Toxothrix trichogenes*
Leptotrichia buccalis, 957, 1023
Lettuce, *Chryseobacterium*, 643
Leucine aminopeptidase, *Helicobacter*, 150, 153
Leucine arylamidase, *Helicobacter*, 149
Leucine fermentation
  Leptospira, 298
  Saprospira, 599
  Spirochaete, 206, 208
Leucine synthesis, *Bacteroides*, 390
Leucothrix, 855
Leukobium maior, 1001
Levofloxacin, *Bacteroides*, 411
Lewinella, 854
Life cycle
  Bdellovibrio, 12–13, 20–21, 23, 25
  Chlamydia, 742–43, 750
  myxobacteria, 38
  Planctomyces, 777
  Planctomycetales, 769, 771
Limulus, 581
Lincomycin resistance/sensitivity, *Riemerella*, 704
Lincosamide resistance/sensitivity
  Bacteroides, 407, 409
  Ornithobacterium, 705
Lipase production
  Fusobacterium, 1022
  Leptospira, 298
Lipids
  Chloroflexus, 832
  Cytophaga, 539
  Cytophagale, 571
  Leptospira, 296–98
  myxobacteria, 62–63
  Pirellula, 761
  Planctomyces, 761
  Planctomycetales, 761–62
  Thermomicrobium, 851
  Thermotoga, 908
Lipooligosaccharide (LOS), *Brachyspira*, 346–47
Lipopolysaccharide (LPS)
  Bacteroides, 404
  Borrelia, 235
  Campylobacter, 128, 131–32
  Capnocytophaga, 570, 581
  Chlamydia, 746
  Chloroflexus, 832
  Chryseobacterium, 664
  Cytophagale, 568–69
  Flavobacterium, 503, 514–15
  Flexibacter, 570
  Fusobacterium, 1019
  Helicobacter, 139
  Herpetosiphon, 865, 867
  Leptospira, 294–97, 299–300, 302, 308, 310–11
  myxobacteria, 61–62
  Porphyromonas, 429, 438, 442–46
  Sphingomonads, 617, 621
  Thermonema, 723
  Treponema, 216, 224
  Wolinella, 180–81
Lipoproteins
  Borrelia, 244, 246–47, 253–54, 265–77
  Leptospira, 296–97, 300
Listeria monocytogenes, 631–32
Liver habitat
  Borrelia, 241
  Chlamydia, 747
  Fusobacterium, 1018
  Helicobacter, 147, 155–56, 165, 168
  Leptospira, 306–7, 309
Lizard, *Borrelia*, 238
LOS. *See* Lipooligosaccharide
Louse-borne relapsing fever, *Borrelia recurrentis*, 236, 240, 256–57, 260–62
Louse, *Borrelia*, 235–37
LPS. *See* Lipopolysaccharide
Lungs. *See* Pulmonary environment
Lyme disease (lyme borreliosis)
  Borrelia, 235, 237–40, 242–43, 245, 257–66, 269, 272, 275–78
  vaccine, 278–79
Lymphatic disease, *Chlamydia*, 751
Lymph, *Borrelia*, 260
Lymphocytic meningitis, *Borrelia*, 263
Lymphocytoma, *Borrelia*, 239
Lyophilization
  Brachyspira, 338
  Chryseobacterium, 662
  Elizabethkingia, 662
  Flavobacteriaceae, 687
  Hydrogenobacter, 934
  Hymenobacter, 728
  Planctomycetales, 788
  Rhodothermus, 721
  Salinibacter, 733
  Streptobacillus, 984
  Thermonema, 724
Lysine utilization, *Saprospira*, 599
Lysobacter, 555, 575–76, 579

M

Macaque habitat
  Fusobacterium, 1016
  Helicobacter, 140, 142, 152
Macrolide lincosamide streptogramin resistance/sensitivity, *Bacteroides*, 392–93, 408–9
Macrolide resistance, *Ornithobacterium*, 705
Macromonas, 941–42, 946–47
Macromonas bipunctata, 942, 946, 998

Macromonas mobilis, 942, 946, 998, 1001
Magnesium requirement
Bacteroides, 383
Leptospira, 299
myxobacteria, 57
Spirochaeta, 205
Thermoleophilum, 847
Major histocompatibility complex (MHC), *Chlamydia*, 745
Malate dehydrogenase
Salinibacter, 732
Thermoleophilum, 847
Malate production
Bacteroides, 387
Chloroflexus, 831
Malate synthase, *Leptospira*, 298
Malate utilization
Hymenobacter, 728
Meiothermus, 802
Malonate utilization
Bergeyella, 535, 649
Chryseobacterium, 649
Elizabethkingia, 649
Riemerella, 649
Weeksella, 535
Maltose production, *Myroides*, 635
Maltose utilization. *See* Di- and trisaccharide utilization
Maltotetraose production, *Myroides*, 635
Maltotriose production, *Myroides*, 635
Mammals. *See also specific* mammals
Bacteroides, 395–96
Borrelia, 238–39, 265–67, 269, 271–72, 276
Leptospira, 305
Pasteurella, 701
Manganese requirement
Bacteroides, 391
Haliscomenobacter, 604
Planctomyces, 763
Planctomycetales, 762–63
Thermoleophilum, 846–47
Manganese utilization
Caulococcus, 996–97
Kusnezovia, 996–97
Siderocapsa, 1005
Siderocapsaceae, 1007
Mannitol utilization. *See* Alcohol utilization
Mannose utilization. *See* Monosaccharide utilization
Mannosylglyceramide production, *Rhodothermus*, 718–19
Mannosylglycerate production, *Rhodothermus*, 718–19
Maribacter, 481, 678
Maribacter aquivivus, 678, 680
Maribacter orientalis, 678, 680
Maribacter sedimenticola, 679–80
Maribacter ulvicola, 679–80
Marine clade of flavobacteriaceae, 677–90
Marine environment
Achromatium, 943
Aequorivita, 460, 678
Aquifex, 927
Aquificales, 928
Arenibacter, 460, 678
Bdellovibrio, 14–16, 18, 20
Beggiatoa, 941
Cellulophaga, 460, 678
Chlorobiaceae, 359, 362
Chloroflexaceae, 815–16, 818, 820
Chloroflexus, 818
Chryseobacterium, 460, 638, 640, 643, 645, 663
Croceibacter, 461, 678
Cytophaga, 463, 553, 681
Cytophagale, 553–54, 576
Elizabethkingia, 638, 640, 643
Epixenosomes, 891–92
Flavobacteriaceae, 470, 472, 677–90
Flavobacterium, 461, 482, 484–88, 490, 494–96, 520, 540
Formosa, 678
Fucobacter, 462
Gallionella, 990
Gelidibacter, 462, 678
Gillisia, 678
Herpetosiphon, 854
Hydrogenobacter, 927
Hydrogenothermus, 928
Maribacter, 678
Marinithermus, 808
Marinitoga, 903
Mesonia, 462, 679
Muricauda, 462, 679
myxobacteria, 38
Novosphingobium, 610–11
Oceanitherums, 808
Pelobacter, 5–6
Pelosigma, 1001
Planctomyces, 773
Planctomycetales, 762, 764–65
Polaribacter, 462, 679
Propionigenium, 955
Psychroflexus, 462, 679
Psychroserpens, 462, 679
Robiginitalea, 679
Salegentibacter, 679
Salinibacter, 712
Saprospira, 591–99
Sphingomonas, 610–12, 617, 619–20
Spirochaeta, 198–99, 202, 204–5, 207
Tenacibaculum, 462, 679, 681
Thermosipho, 903
Thermotoga, 915–16
Thermotogales, 900, 902
Thermus, 802
Thiospira, 947–48
Thiovulum, 949
Ulvibacter, 463, 679
Verrucomicrobia, 884
Vitellibacter, 463, 679
Vulcanitherums, 808
Zobellia, 463, 679
Marinilabilia salmonicolor, 713
Marinithermus, 797, 807–9
Marinithermus hydrothermalis, 798, 808–9
Marinitoga, 899, 903
Marinitoga camini, 901–5
Marinitoga piezophila, 901–5, 915
Marsh environment
Gallionella, 990
Novosphingobium, 612
Spirochaeta, 195, 198, 208
Marsupials, *Leptospira*, 305
Meiothermus, 797–807, 809
Meiothermus cerbereus, 798, 801–3, 807
Meiothermus chliarophilus, 798, 801–3, 806–7
Meiothermus rosaceus, 801
Meiothermus ruber, 798, 801–3, 805, 807
Meiothermus silvanus, 798, 801–3, 807
Meiothermus taiwanensis, 798, 801–3, 805
Meiothermus timidus, 806
Melanoid pigment, myxobacteria, 64
Melezitose utilization, *Bacteroides*, 414
Melibiose utilization
Opitutus, 891
Sphingomonads, 615
Ultramicrobacteria, 891
Verrucomicrobium, 887
Melittangium, 36, 39, 43, 53, 71, 85–86, 89
Melittangium alboraceum, 89
Melittangium boletus, 75, 82, 89
Melittangium gracilipes, 89
Melittangium lichenicola, 76–77, 84, 89
Membrane-filter method, *Treponema*, 221
Membrane proteins, myxobacteria, 65–66
Menaquinone
Bergeyella, 534
Chloroflexus, 819, 828, 835
Chryseobacterium, 651
Cytophaga, 540
Cytophagale, 571, 575–76
Empedobacter, 633
Flavobacteriaceae, 470, 683
Flavobacterium, 493, 504, 540, 545, 575
Flexibacter, 577
Herpetosiphon, 867
Myroides, 633
myxobacteria, 65
Oscillochloris, 819, 835
Planctomycetales, 761
Rhodothermus, 719
Riemerella, 695
Roseiflexus, 819, 835
Saprospira, 599
Thermoleophilum, 843, 845–46
Thermus, 806
Verrucomicrobium, 886
Weeksella, 534
Wolinella, 184–87
Meningitis
Bergeyella, 536
Borrelia, 261, 263, 277
Campylobacter, 132
Chryseobacterium, 646, 667

Elizabethkingia, 664–68
Empedobacter, 634
Flavobacterium, 541
Leptospira, 306
Riemerella, 703
Sphingomonas, 621
Weeksella, 541
Meningoencephalitis, *Borrelia*, 263
Meropenem, *Bacteroides*, 407
Mesonia, 455, 462, 466, 471, 481
Mesonia algae, 458, 462, 467–69, 679–80, 688, 697
Mesosome, myxobacteria, 60
Metallogenium, 990, 996–97, 1006, 1014
Metallogenium personatum, 1006, 1013
Methanobacillus omelianskii, 6
Methanocaldococcus jannaschii, 926
Methanococcus, 915
Methanol production, *Opitutus*, 891
Methanopyrus, 915
Methanopyrus kandleri, 902
Methanospirillum hungatii, 594
Methionaquinone, *Hydrogenothermus*, 932
Methionine utilization
Bacteroides, 383, 390
Chloroflexus, 831
Saprospira, 599
Methoxyethanol utilization, *Pelobacter*, 6
2-Methylbutyrate production, *Spirochaeta*, 206, 208
Methylococcus capsulatus, 63
Methyl oleate utilization, myxobacteria, 59
Metronidazole
Bacteroides, 406–7, 410
Helicobacter, 149–65, 167
Porphyromonas, 432
Mice
Bacteroides, 398, 400
Borrelia, 238, 241, 245, 257, 260, 265–67, 275
Brachyspira, 347
Fusobacterium, 1017, 1019
Helicobacter, 140, 142, 153, 155–57, 159, 165–66, 168–69
Porphyromonas, 438–39, 441, 443, 447
Streptobacillus, 983
Treponema, 212
Verrucomicrobia, 884
Micrococcus, 580
Micrococcus luteus, 33, 41–42, 966
Microcoleus chthonoplastes, 822
Microcyclus, 1000
Microcyclus aquaticus, 1000
Microcyclus marinus, 1000
Microcystis aeruginosa, 593
Microimmunofluorescence, *Chlamydia*, 752
Micromonospora purpurea, 865
Micropredator, myxobacteria, 36, 59
Microscilla, 550, 567, 576–77
Microscopic agglutination test, *Leptospira*, 310–11
Microspira desulfuricans, 998
Microspira vacillans, 946. *See also Macromonas mobilis*
Milk habitat
Campylobacter, 127
Chryseobacterium, 645
Cytophagale, 554, 566
Mitomycin C, *Brachyspira*, 344
Mobilifilum chasei, 971
Mollusk habitat
Cristispira, 971–72, 974, 980
spirochetes, symbiotic, 971
Monas muelleri, 949. *See also Thiovulum majus*
Monkey habitat, *Helicobacter*, 164
Monosaccharide utilization
Bacteroides, 383, 386–87, 414
Bergeyella, 467, 534, 649
Borrelia, 242–43
Brachyspira, 330, 339–41, 344
Capnocytophaga, 467, 710
Chloroflexus, 830
Chryseobacterium, 467, 649, 654–56
Coenonia, 467
Cytophaga, 467, 574, 684
Cytophagale, 563, 574
Elizabethkingia, 649, 654
Empedobacter, 467, 633
Fervidobacterium, 905
Flavobacteriaceae, 468–69, 682–84
Flavobacterium, 467, 494–95, 509, 543–44
Fucobacter, 467
Fusobacterium, 1020, 1023
Gemmata, 781
Geotoga, 905
Haliscomenobacter, 604
Helicobacter, 149
Hymenobacter, 728
Marinithermus, 808
Marinitoga, 905
Meiothermus, 802
Myroides, 468–69, 633
Oceanitherums, 808
Opitutus, 891
Petrotoga, 905
Planctomyces, 777
Porphyromonas, 439
Propionigenium, 957
Prosthecobacter, 888
Rhodothermus, 715
Riemerella, 468–69, 649, 700
Roseiflexus, 831
Salinibacter, 733
Saprospira, 599
Sphingomonads, 615–16
Spirochaeta, 200, 204
Streptobacillus, 984
Thermosipho, 905
Thermotoga, 905
Thermotogales, 899
Ultramicrobacteria, 891
Verrucomicrobium, 887
Victivallis, 894
Vulcanitherums, 808
Weeksella, 469, 534
Zoogloea, 965
Moraxella anatipestifer, 695. *See also Riemerella anatipestifer*
Mosquitoe habitat
Chryseobacterium, 644
Flavobacterium, 485
Motility
Achromatium, 945–46
Aequorivita, 467
Aquifex, 930
Arenibacter, 467
Bergeyella, 467
Borrelia, 245–47
Brachyspira, 344, 347–48
Campylobacter, 122
Capnocytophaga, 467
Caulococcus, 997
Cellulophaga, 467
Chlorobiaceae, 360
Chryseobacterium, 467, 653, 701
Clevelandina, 971
Coenonia, 467, 701
Cristispira, 971
Croceibacter, 467
Cytophaga, 467, 569
Cytophagale, 549, 555
Diplocalyx, 971
Empedobacter, 467, 632
Flavobacteriaceae, 471–72
Flavobacterium, 467, 472, 494–99, 510, 517, 542, 545
Flexibacter, 568–69
Fucobacter, 467
Gelidibacter, 467–68, 472
Haliscomenobacter, 604
Helicobacter, 139, 149, 151–65
Hollandina, 971
Hydrogenobacter, 930
Hydrogenobaculum, 930
Hydrogenothermus, 930
Hymenobacter, 725
identification procedure, 493
Isosphaera, 784, 787
Macromonas, 946
Mesonia, 467–68
Muricauda, 467–68
Myroides, 467–68, 632
myxobacteria, 38, 68–70
Opitutus, 882
Ornithobacterium, 467–68, 701
Pasteurella, 701
Pelosigma, 1003
Pillotina, 971
Pirellula, 779
Planctomyces, 775
Polaribacter, 467–68
Porphyromonas, 428
Psychroflexus, 467–68
Psychroserpens, 467–68
Riemerella, 467–68, 701
Salegentibacter, 467–68
Saprospira, 591, 596–97
Sphingomonads, 615
Spirochaeta, 195, 200, 205–6
Tenacibaculum, 467–68, 472
Thermocrinis, 930
Thermonema, 723
Thermotogales, 907
Thiovulum, 948
Toxothrix, 986

Treponema, 211, 216–18
Ulvibacter, 467
Vitellibacter, 467
Weeksella, 468
Wolinella, 180–81
Zobellia, 468
Mouth fungus. *See* Columnaris disease
Mucosal surfaces
Bacteroides, 405
Bergeyella, 533
Campylobacter, 122
Chryseobacterium, 643, 664
Coenonia, 703
Elizabethkingia, 643
Fusobacterium, 1016–18, 1020
Helicobacter, 139, 141, 143, 147–60, 162–63, 166, 169
Sphingomonas, 621
Weeksella, 533
Mud environment
Achromatium, 943–44, 946
Caulococcus, 996
Cellulophaga, 460
Chlorobiaceae, 363
Chryseobacterium, 460, 640, 643
Elizabethkingia, 640
Flavobacteriaceae, 472
Flavobacterium, 461, 485–87, 490
Fusobacterium, 1017
Haliscomenobacter, 602
Kusnezovia, 996
Oscillochloris, 818
Pelosigma, 1001
Saprospira, 592
Sphingopyxis, 611–12
Spirochaeta, 195, 198–99, 207–8
Thermoleophilum, 843
Multiple sclerosis, *Chlamydia*, 753
Muramic acid, *Borrelia*, 245
Muricauda, 455, 462, 466, 471, 481
Muricauda ruestringensis, 458, 462, 466–69, 679–83, 697
Mussel habitat
Cristispira, 971
Spirochaeta, 198
Mutagenesis
Bacteroides, 381, 392–94, 399–400
Brachyspira, 342–44
Flavobacteriaceae, 473
Flavobacterium, 510
Planctomycetales, 772
Propionigenium, 958
Thermotoga, 916
Thermus, 806
Zoogloea, 966
Mutation
Bacteroides, 411
Borrelia, 272–73
myxobacteria, 57, 69–70
Myxococcus, 62
Porphyromonas, 433–34, 437
Sphingomonads, 617–18
Treponema, 217
Mycobacterium chelonae, 865
Mycoplasma pneumoniae, 752
Myocarditis
Borrelia, 263
Leptospira, 305
Myroides, 455, 462, 466–70, 473, 484, 488, 533–35, 630–35, 641, 645, 652, 655, 664–65, 667, 689
Myroides odoratimimus, 462, 631, 633–34
Myroides odoratus, 458, 462, 483, 539–42, 545, 630–35, 645, 680, 697
Myxalamid, 92
Myxaline, *Myxococcus*, 61, 93
Myxobacteria
applications, 91–94
characterization, 59–73
cultivation methods, 48–57
Cytophagale, 555–56
description of, 31–35
fruiting body, 73–91
genera key, 85–91
habitats, 35–40, 48
isolation procedure, 39–48
preservation methods, 57–59
as proteobacteria, 32–34
taxonomy, 84–85
Myxocin, myxobacteria, 67–68
Myxocoagulase, myxobacteria, 84
Myxococcus, 31–32, 35, 38, 42–44, 48, 52, 59, 62–64, 66–67, 71–73, 77, 79, 81–82, 85–87, 90
Myxococcus disciformis. *See Pyxicoccus*
Myxococcus filiformis. *See Flexibacter filiformis*
Myxococcus flavescens, 93
Myxococcus fulvus, 36–37, 50, 52, 56–57, 60–65, 67, 74–75, 83
Myxococcus stipitatus, 36, 42, 63, 65, 74–75
Myxococcus virescens, 31, 36–37, 39, 54, 56–57, 60–61, 64–65, 67, 83, 93
Myxococcus xanthus, 14, 31–33, 35–36, 38–39, 49–53, 55–57, 59–62, 64–72, 79–84, 93, 499, 713, 769
Myxophage, 67–68
Myxospores, 38–39, 46, 58–59, 65, 72–73, 76–77, 80–82
function of, 82–83
Myxothiazol, 92
Myxovirescin, 92–93

N

NADH dehydrogenase, *Thermus*, 804
NADH oxidase
Anaeromyxobacter, 59
Bacteroides, 387–89
Brachyspira, 330, 337, 340–43, 345, 347
Chloroflexus, 835
Flavobacterium, 509
Saprospira, 599
Thermoleophilum, 847
Treponema, 225
NADH reductase, *Thermoleophilum*, 847
NADPH oxidase
Bacteroides, 388–89, 410
Thermoleophilum, 847
NAG. *See* N-acetylglucosamine
Nalidixic acid resistance/sensitivity
Bergeyella, 534–35
Helicobacter, 141, 143, 149–59, 162–66
Leptospira, 304
Porphyromonas, 431
Thermonema, 724
Weeksella, 534–35
Nannocystis, 42–43, 52–53, 56, 60, 62–63, 69, 71, 80, 85–86, 91
Nannocystis agregans, 91
Nannocystis exedens, 33, 36, 39, 41, 62–63, 76–77, 91, 94
Nannocystis pusilla, 91
Naphthalene catabolism, Sphingomonads, 619, 621
Naproxen, *Porphyromonas*, 432
Naumanniella, 986, 1006, 1009, 1011
Naumanniella catenata, 1006
Naumanniella elliptica, 1006
Naumanniella minor, 1006
Naumanniella neustonica, 1007–9, 1011
Naumanniella polymorpha, 1006–7, 1009, 1011, 1014
Naumanniella pygmaea, 1006
Neisseria, 69, 412
Neisseria gonorrhoeae, 412, 704, 750
Neomycin resistance/sensitivity
Coenonia, 699
Cytophaga, 561
Flavobacterium, 490, 492
Fusobacterium, 1020
Leptospira, 304
Ornithobacterium, 705
Riemerella, 699, 705
Thermomicrobium, 850
Nephritis, *Leptospira*, 294
Neuraminidase, *Bacteroides*, 404
Neuroborreliosis, *Borrelia*, 239, 261, 264
Neurological disorders
Borrelia, 264
Chlamydia, 753
Neurosyphilis, *Treponema*, 226
Neutrophils
Bacteroides, 405–6
Capnocytophaga, 581
Porphyromonas, 442–43
Nifedipine, *Porphyromonas*, 432
Nitrate reduction
Aequorivita, 467, 685
Arenibacter, 467, 685
Bergeyella, 467, 534, 535, 649
Campylobacter, 121, 125–26
Capnocytophaga, 467
Cellulophaga, 467, 685
Chryseobacterium, 467, 534, 649, 701
Coenonia, 467, 701
Croceibacter, 467, 685
Cytophaga, 467, 685
Cytophagale, 565

Elizabethkingia, 649
Empedobacter, 467
Flavobacterium, 467, 494–95, 509, 543–44
Formosa, 685
Fucobacter, 467
Gelidibacter, 468, 469, 685
Gillisia, 685
Haliscomenobacter, 604
Helicobacter, 141, 143, 148, 150–59, 161–65
Herpetosiphon, 868, 871
Maribacter, 685
Mesonia, 468, 469, 685
Muricauda, 468, 469, 685
Myroides, 468, 469, 534
Novosphingobium, 607, 609
Opitutus, 891
Ornithobacterium, 468, 469, 701
Pasteurella, 701
Pirellula, 780
Polaribacter, 468, 469, 685
Psychroflexus, 468, 469, 685
Psychroserpens, 468, 469, 685
Riemerella, 468, 469, 649, 701
Robiginitalea, 685
Salegentibacter, 468, 469, 685
Sphingobacterium, 534
Sphingobium, 607, 609
Sphingomonas, 609
Sphingopyrix, 609
Tenacibaculum, 468, 469, 685
Thermus, 799, 805
Ulvibacter, 468, 685
Verrucomicrobium, 887
Vitellibacter, 468, 685
Weeksella, 469, 534, 535
Wolinella, 178–80, 183
Zobellia, 469, 685
Nitrite reductase, *Wolinella*, 185
Nitrite reduction
Bergeyella, 535, 649
Campylobacter, 121
Chryseobacterium, 649
Cytophaga, 574
Elizabethkingia, 649
Flavobacterium, 543–44
Myroides, 633
Riemerella, 649
Thermus, 805
Weeksella, 535
Wolinella, 178, 180, 184–85, 187
Nitrogen cycling, *Bacteroides*, 381, 398
Nitrogen fixation
Chlorobiaceae, 369
termite gut spirochete, 326–27
Nitrogen metabolism
Chloroflexus, 831
Flavobacterium, 510
Nitrogen requirement, termite gut spirochete, 325
5-Nitroimidazole, *Bacteroides*, 409–10
Nitrous oxide (N_2O) reduction, *Wolinella*, 178, 182
Norfloxacin, *Bacteroides*, 411
Nostocoida limicola, 870
Novosphingobium, 605–22
Novosphingobium aromaticivorans, 607, 610, 612, 618–19. *See also Sphingomonas aromaticivorans*
Novosphingobium capsulatum, 607, 611, 613, 618. *See also Sphingomonas capsulata*
Novosphingobium rosea, 607, 611–12. *See also Sphingomonas rosa*
Novosphingobium stygiae, 607, 611, 618. *See also Sphingomonas stygia*
Novosphingobium subarcticum, 607, 611–12. *See also Sphingomonas subarctica*
Novosphingobium subterraneum, 607, 611, 618. *See also Sphingomonas subterranea*
Nylon oligomers, *Flavobacterium*, 579

O

Ocean environment. *See* Marine environment
Oceanitherums, 797, 807–9
Oceanitherums desulfurans, 798, 808–9
Oceanitherums profundus, 798, 808–9
Ochrobium tectum, 986, 1001
Ocular disease
Borrelia, 261
Chlamydia, 741, 746–49
Ocular habitat, *Chlamydia*, 741, 746–49
Oil field environment
Flavobacterium, 520
Geotoga, 903
Petrotoga, 903
Pseudomonas, 536
Sphingomonas, 611–12
Spirochaeta, 198–99, 204
Thermosipho, 903
Thermotogales, 900, 902
Weeksella, 536
Opitutus, 881, 888–89
Opitutus terrae, 881, 883, 885, 889–90
Opossum habitat, *Treponema*, 212
Optic neuritis, *Borrelia*, 261
Opuntia cactus, myxobacteria, 37
Oral cavity
Bacteroides, 381
Campylobacter, 122
Capnocytophaga, 460, 554, 561, 578, 709–10
Flavobacterium, 516
Fusobacterium, 1016, 1018–19
Porphyromonas, 428–30, 439–40, 442
Weeksella, 541
Oral mucosa
Porphyromonas, 430
Treponema, 212–13, 215, 219–22, 226–27
Organic acid utilization
Achromatium, 945
Anaeromyxobacter, 38
Bacteroides, 388, 391
Borrelia, 242
Brachyspira, 339–41
Capnocytophaga, 710
Chlorobiaceae, 362, 369, 372
Chloroflexus, 830
Fervidobacterium, 905
Flavobacterium, 509
Fusobacterium, 1019, 1022
Gemmata, 781
Geotoga, 905
Hymenobacter, 728
Leptospira, 299
Marinithermus, 808
Marinitoga, 905
Meiothermus, 802
myxobacteria, 59
Oceanitherums, 808
Pelobacter, 6–7, 7, 8–9, 9
Petrotoga, 905
Propionigenium, 955, 957
Saprospira, 599
termite gut spirochete, 325
Thermoleophilum, 843, 847
Thermosipho, 905
Thermotoga, 905
Thermus, 805
Vulcanitherums, 808
Wolinella, 178–82, 184–85, 184–86
Zoogloea, 965
Ornithine
Borrelia, 245
Cytophagale, 571
Flexibacter, 569
Ornithine decarboxylase, *Helicobacter*, 156
Ornithobacterium, 455, 462, 466, 473, 509, 695–706
Ornithobacterium rhinotracheale, 458, 462, 466–69, 471, 639, 657, 680, 695–706
Oscillatoria, 822–23
Oscillochloris, 815, 817–18, 820, 822, 826, 828–29, 870
Oscillochloris chrysea, 818, 819–20, 822, 826, 829, 833
Oscillochloris trichoides, 816–17, 819–20, 822–23, 826, 828–30, 832–33, 870
Osmotic solutes, *Rhodothermus*, 718
Outer membrane
Borrelia, 244–46
Chlamydia, 743–45
Cytophaga, 570
Cytophagale, 568
Herpetosiphon, 865
Hollandina, 979
Leptospira, 296–97
Rhodothermus, 714
Thermotoga, 908
Treponema, 215–17
Outer membrane vesicles, *Bacteroides*, 404
Ovaries, *Borrelia*, 238
Ovine habitat
Bacteroides, 395, 397
Bergeyella, 533
Borrelia, 237

Campylobacter, 122, 130, 132
Chryseobacterium, 645
Fusobacterium, 1017–19
Helicobacter, 142, 161, 169
Porphyromonas, 447
Weeksella, 533
Owl habitat, *Borrelia*, 257
Oxaloacetate sensitivity, Chlorobiaceae, 368
Oxaloacetate utilization
Bacteroides, 387
Flavobacterium, 509
Oxidase activity
Aequorivita, 467
Arenibacter, 467
Bergeyella, 467, 534
Capnocytophaga, 467
Cellulophaga, 467
Chryseobacterium, 467, 534, 651, 701
Coenonia, 467, 701
Croceibacter, 467
Cytophaga, 467
Empedobacter, 467, 633
Flavobacterium, 467
Fucobacter, 467
Gelidibacter, 468, 469
Gemmata, 781
Helicobacter, 148–59, 162–64
Herpetosiphon, 868
Leptospira, 298
Mesonia, 468, 469
Muricauda, 468, 469
Myroides, 468, 469, 534, 633
Ornithobacterium, 468, 469, 701
Pasteurella, 701
Polaribacter, 468, 469
Psychroflexus, 468, 469
Psychroserpens, 468, 469
Riemerella, 468, 469, 701
Salegentibacter, 468, 469
Salinibacter, 731
Sphingobacterium, 534
Tenacibaculum, 468, 469
Ulvibacter, 468
Vitellibacter, 468
Weeksella, 469, 534
Wolinella, 180
Zobellia, 469
Oxidoreductase utilization, *Rhodothermus*, 719–20
Oxygen requirement
Achromatium, 945–46
Brachyspira, 330, 339, 345, 347
Chloroflexus, 819
Chloronema, 819
Heliothrix, 819
Oscillochloris, 819
Prosthecobacter, 882
Roseiflexus, 819
Siderocapsaceae, 1007
Thiobacterium, 947
Thiospira, 947
Thiovulum, 948–49
Verrucomicrobium, 882
Oxygen sensitivity/tolerance
Bacteroides, 390–92
Brachyspira, 335–36, 339, 345
Capnocytophaga, 561
Chloroflexus, 824–25
Fusobacterium, 1019
Gallionella, 990–92
Porphyromonas, 440
Propionigenium, 957
termite gut spirochete, 323, 325–26
Thiovulum, 952
Treponema, 219–20
Ultramicrobacteria, 891
Oxygen utilization, Aquificales, 925, 934
Oxytetracycline resistance/sensitivity
Chryseobacterium, 664
Flavobacterium, 515, 518
Ornithobacterium, 705
Oyster habitat
Bdellovibrio, 15–16
Cristispira, 971, 981
Cytophagale, 554
Saprospira, 591–93

P

Palmitate utilization, Chlorobiaceae, 360
Palmitoleate utilization, Chlorobiaceae, 360
Pantoea ananatis, 482
Paracoccus denitrificans, 926
Parathion, *Flavobacterium*, 579
Parrots, *Chlamydia*, 747
Pasteurella, 700
Pasteurella anatipestifer, 695. *See also Riemerella anatipestifer*
Pasteuria, 761
Pasteuria ramosa, 780. *See also Pirellula staleyi*
Pathogenesis
Bacteroides, 400–406
Chlamydia, 746
Flavobacteriaceae, 473
Flavobacterium, 510–20
Leptospirosis, 306–7
Pathogenicity
Bergeyella, 535
Borrelia, 260–77
Campylobacter, 130–33
Chryseobacterium, 663–68
Coenonia, 704
Elizabethkingia, 663–68
Empedobacter, 633–34
Flavobacteriaceae, 687
Myroides, 634–35
Ornithobacterium, 704
Riemerella, 704
Tenacibaculum, 687
Weeksella, 535
PCB. *See* Polychlorinated biphenyls
Peach tree, *Sphingomonas*, 610
Pectin degradation
Bacteroides, 384–86, 398
Cytophaga, 539, 560–61
Cytophagale, 574
Flavobacteriaceae, 470
Flavobacterium, 493–95, 501, 520
Herpetosiphon, 869
Opitutus, 891
Sporocytophaga, 560–61
Ultramicrobacteria, 891
Pedobacter, 484
Peduncle disease, *Flavobacterium*, 511–12
Pelobacter, 5–10
Pelobacter acetylenicus, 5–10
Pelobacter acidigallici, 5–10
Pelobacter carbinolicus, 5–10
Pelobacter propionicus, 5–10
Pelobacter venetianus, 5–10
Pelochromatium roseo-viride, 371, 373
Pelochromatium roseum, 371–74, 1001
Pelochromatium roseum consortium, 998
Pelochromatium selenoides, 371, 373
Pelodictyon, 360
Pelodictyon clathratiforme, 360–61, 363–64
Pelodictyon luteolum, 360–63, 999
Pelodictyon phaeoclathratiforme, 360–64, 368, 370
Pelodictyon phaeum, 361–62
Pelonema tenue, 1001
Peloploca, 1001
Pelosigma, 1001–3
Pelosigma cohnii, 1001–3
Pelosigma palustre, 1001–3
Pelvic inflammatory disease (PID), *Chlamydia*, 746–47, 750–51
Penguin, *Flavobacterium*, 488
Penicillin resistance/sensitivity
Aequorivita, 467
Arenibacter, 467
Bacteroides, 407–8, 410
Bergeyella, 467, 535
Borrelia, 277
Capnocytophaga, 467
Cellulophaga, 467
Chlamydia, 745
Chryseobacterium, 467
Coenonia, 467
Croceibacter, 467
Cytophaga, 467
Cytophagale, 575
Empedobacter, 467
Flavobacterium, 467, 508
Fucobacter, 467
Fusobacterium, 1020
Gelidibacter, 468, 469
Gemmata, 782
Hymenobacter, 728
Leptospira, 297, 307
Mesonia, 468, 469
Muricauda, 468, 469
Myroides, 468, 469, 635
Ornithobacterium, 468, 469, 705
Planctomyces, 774
Polaribacter, 468, 469
Psychroflexus, 468, 469
Psychroserpens, 468, 469
Rhodothermus, 721
Riemerella, 468, 469, 704
Salegentibacter, 468, 469
Salinibacter, 733
Tenacibaculum, 468, 469

Thermomicrobium, 850
Thermonema, 724
Thermotoga, 908
Ultramicrobacteria, 891
Ulvibacter, 468
Vitellibacter, 468
Weeksella, 469, 535, 539
Wolinella, 180–81
Zobellia, 469
Pentachlorophenol (PCP)
Chryseobacterium, 668–69
Flavobacterium, 579
Sphingobium, 610, 612
Sphingomonads, 612–13, 616
Pentose phosphate pathway
Bacteroides, 387
Chloroflexus, 830
Leptospira, 298
myxobacteria, 60
Oscillochloris, 816
Sphingomonads, 617
Peptic ulcers, *Helicobacter*, 166
Peptidoglycan
Borrelia, 245
Capnocytophaga, 581
Chlamydia, 745
Chloroflexus, 832
Cytophagale, 568–69
Deinococcus, 806
Fusobacterium, 1023
Herpetosiphon, 865–67
Leptospira, 295–97
Meiothermus, 806
myxobacteria, 72, 83
Oscillochloris, 833
Pirellula, 761
Planctomyces, 761
Planctomycetales, 757, 760–61
Sporocytophaga, 569
Thermus, 805–6
Peptone utilization
myxobacteria, 59–60
Thermomicrobium, 850
Perinatal, *Bacteroides*, 402
Periodontal disease
Capnocytophaga, 460, 554, 561, 581
Porphyromonas, 428–33, 435–36, 441–47
Treponema, 211, 226
Periplasmic flagella
Borrelia, 245–46
Helicobacter, 140–41, 143, 150, 153, 156–57, 161, 163
Leptospira, 295–97
Spirochaeta, 195, 197, 205–6
termite gut spirochete, 322
Treponema, 215–16
Peritonitis
Campylobacter, 132
Sphingomonas, 621
Weeksella, 536
Peroxidase activity, *Thermoleophilum*, 846
Peroxide sensitivity, *Campylobacter*, 123
Persicobacter diffluens, 713
Pesticide degradation, Sphingomonads, 616
Pesticide sensitivity, *Bdellovibrio*, 16
Petrotoga, 899, 903
Petrotoga miotherma, 901–5
Petrotoga mobilis, 901–5
Petrotoga olearia, 901–5, 915
Petrotoga sibirica, 901–5, 915
Phages
Bacteroides, 395, 397
Bdellovibrio, 14
Borrelia, 255
Brachyspira, 342
Cytophaga, 572
Cytophagale, 572–73
myxobacteria, 67–68
Porphyromonas, 442–43
Phage typing, *Campylobacter*, 128
Phagocytosis
Bacteroides, 403, 406
Campylobacter, 132
Chlamydia, 743
Porphyromonas, 435, 439
Phenanthrene degradation
Flavobacterium, 579
Sphingomonas, 621
Phenylacetate production
Bacteroides, 414
Porphyromonas, 428
Phenylalanine deamination
Bergeyella, 535, 649
Chryseobacterium, 649, 654–55
Elizabethkingia, 649, 654–55
Riemerella, 649
Weeksella, 535
Phenylalanine synthesis, *Bacteroides*, 390
Phenylalanine utilization
Leptospira, 298
Saprospira, 599
Phloroglucinolcarboxylate utilization, *Pelobacter*, 7
Phloroglucinol utilization, *Pelobacter*, 7
Phormidium geysericola, 871
Phormidium luridum, 83
Phosphatase production
Chryseobacterium, 651
Empedobacter, 633
Herpetosiphon, 869
Myroides, 633
Verrucomicrobium, 887
Phosphate production
Bergeyella, 534
Weeksella, 534
Phosphate requirement
Bacteroides, 383
Leptospira, 299
Phosphatidylcholine, *Salinibacter*, 731
Phosphatidylethanolamine (PE)
Cytophagale, 571
Hymenobacter, 725
myxobacteria, 69–70
Salinibacter, 731
Phosphatidylglycerol
Cytophagale, 571
Salinibacter, 731
Verrucomicrobium, 886
Phosphatidylinositol, Cytophagale, 571
Phosphatidylserine, Cytophagale, 571
Phosphogluconate, *Leptospira*, 298
Phospholipid requirement, *Brachyspira*, 340
Phospholipids
Isosphaera, 784
Marinithermus, 809
myxobacteria, 63, 65
Oceanitherums, 809
Pirellula, 761–62
Planctomyces, 761–62
Planctomycetales, 761–62
Rhodothermus, 720
Salinibacter, 731
Thermonema, 723
Thermus, 806
Verrucomicrobium, 886
Vulcanitherums, 809
Phosphomycin resistance
Borrelia, 241
Treponema, 221–22
Phosphotransferase system, *Borrelia*, 243
Photolithotrophs, Chlorobiaceae, 359, 370
Photooxidation
Chlorobiaceae, 367, 371
myxobacteria, 64
pH tolerance
Aquifex, 929
Aquificales, 928, 934
Bacteroides, 395–96, 398, 406
Borrelia, 271
Brachyarcus, 998
Brachyspira, 339
Chlorobiaceae, 634
Chloroflexus, 820
Cytophagale, 553, 563–64
Fervidobacterium, 904
Fusobacterium, 1023
Gallionella, 990, 992
Geotoga, 904
Haliscomenobacter, 604
Helicobacter, 167
Heliothrix, 822
Herpetosiphon, 869
Hydrogenobacter, 929
Hydrogenobaculum, 929
Hydrogenothermus, 929
Leptospira, 297
Marinitoga, 904
Meiothermus, 803
Opitutus, 891
Oscillochloris, 828–29
Pelosigma, 1002
Petrotoga, 904
Planctomycetales, 762
Porphyromonas, 440
Roseiflexus, 822, 829
Salinibacter, 733–34
Saprospira, 596
Sphingomonads, 616
Thermocrinis, 929
Thermoleophilum, 844
Thermomicrobium, 850
Thermosipho, 904
Thermotoga, 904
Thermus, 801

Toxothrix, 986–88
Ultramicrobacteria, 891
Victivallis, 894
Zoogloea, 962, 965
Phylogenetic tree
Aquificales, 926
Bacteroides, 382
Bdellovibrio, 14
Campylobacter, 120
Chlorobiaceae, 363
Chloroflexaceae, 816–17
Chryseobacterium, 639
Coenonia, 697
Elizabethkingia, 639
Flavobacteriaceae, 457–58
Helicobacter, 146
Hymenobacter, 713–14
Ornithobacterium, 697
Planctomycetales, 759
Porphyromonas, 429
Rhodothermus, 713–14
Riemerella, 697
Salinibacter, 713–14
Spirochaeta, 197
Thermoleophilum, 843–44
Thermonema, 713–14
Thermotogales, 900, 902
Verrucomicrobia, 881, 883
Wolinella, 179
Pig. *See* Swine
Pigeon habitat
Coenonia, 703
Ornithobacterium, 703
Riemerella, 462, 695, 701, 703
Pigments
Achromatium, 945
Aequorivita, 467, 682
Aquifex, 928
Arenibacter, 467, 682
Bergeyella, 467, 471, 534, 648
Brevibacterium, 555
Capnocytophaga, 467, 561, 710
Cellulophaga, 467, 682
Chlorobiaceae, 359–62, 364, 366–67, 370–71
Chloroflexaceae, 815–16, 818
Chloroflexus, 815, 824, 833
Chloronema, 828–29
Chryseobacterium, 467, 471, 534, 647, 648, 652, 654, 701
Coenonia, 467, 471, 701
Croceibacter, 467, 682
Cytophaga, 467, 555, 559, 561, 571–72, 682
Cytophagale, 549–50, 555, 561, 571–72, 575
Elizabethkingia, 647, 648, 652–54
Empedobacter, 467, 632
Flavobacteriaceae, 466, 471, 681–82
Flavobacterium, 467, 471, 490–91, 493, 497–98, 500–501, 539, 541–42, 545
Flexibacter, 555, 571
Flexithrix, 571
Formosa, 682
Fucobacter, 467
Gelidibacter, 467–68, 682
Gemmata, 781
Gillisia, 682
Haliscomenobacter, 604
Heliothrix, 822, 829
Herpetosiphon, 863, 868
Hydrogenobacter, 928
Hymenobacter, 712, 724–25
Isosphaera, 786, 788
Lysobacter, 555
Macromonas, 946
Maribacter, 682
Marinithermus, 807, 808
Meiothermus, 801–2, 806
Mesonia, 467–68, 682
Muricauda, 467–68, 682
Myroides, 467–68, 534, 632
myxobacteria, 63–65
Novosphingobium, 609
Oceanitherums, 807, 808
Opitutus, 882
Ornithobacterium, 467–68, 471, 701
Oscillochloris, 828–29
Pasteurella, 701
Pelosigma, 1003
Pirellula, 780
Planctomyces, 775, 777
Polaribacter, 467–68, 682
Porphyromonas, 428, 431, 437
Prosthecobacter, 882
Psychroflexus, 467–68, 682
Psychroserpens, 467–68, 682
Rhodothermus, 712, 720–21
Riemerella, 467–68, 471, 648, 700, 701
Robiginitalea, 682
Roseiflexus, 817, 822, 827, 829
Salegentibacter, 467–68, 682
Salinibacter, 712, 729, 731, 734
Saprospira, 599
Sphingobacterium, 534
Sphingobium, 609
Sphingomonads, 613, 615
Sphingomonas, 609
Sphingopyrix, 609
Spirochaeta, 202–5
Sporocytophaga, 555
Tenacibaculum, 467–68, 682
Thermocrinis, 927–30
Thermoleophilum, 845
Thermomicrobium, 849–51
Thermonema, 712, 723
Thermus, 798–99, 805–6
Thiovulum, 949
Ulvibacter, 467, 682
Verrucomicrobium, 882, 886
Victivallis, 882
Vitellibacter, 467, 682
Vulcanitherums, 807, 808
Weeksella, 468, 471, 534, 539
Zobellia, 468, 682
Zoogloea, 963
Pillotina, 213, 971–81
Pillotina calotermitidis, 971, 978
Pillotinaceae, 971
Pinta, 211–12, 225
Piperacillin resistance/sensitivity
Bacteroides, 407
Chryseobacterium, 668
Elizabethkingia, 667
Pirellula, 760–72, 775, 778–81, 784
Pirellula marina, 758–59, 761, 763–64, 767, 769–72, 779–80
Pirellula staleyi, 759, 767, 770, 776, 780–81
Planctomyces, 760–78, 784
Planctomyces bekefii, 758, 760, 762–63, 772–73, 775–78
Planctomyces brasiliensis, 759, 763–64, 774–77
Planctomyces condensatus, 763
Planctomyces crassus, 760
Planctomyces gracilis, 760
Planctomyces guttaeformis, 760, 763, 773, 776–78
Planctomyces limnophilus, 759, 762–64, 766, 771–72, 774–77
Planctomyces maris, 759, 761, 764, 767, 769, 775–77
Planctomyces staleyi, 713, 780. *See also Pirellula staleyi*
Planctomyces stranskae, 760, 763, 773, 776–78
Planctomycetales, 757–88, 881, 900
Plant habitat. *See also specific* plants
Bdellovibrio, 14–16
Chryseobacterium, 643
Cytophaga, 560
Cytophagale, 553
Flavobacterium, 487, 510, 540
Myroides, 631
Novosphingobium, 611
Sphingomonads, 613, 616, 621
Sphingomonas, 610
Sporocytophaga, 560
Plasmids
Bacteroides, 392–95, 411
Borrelia, 249–54
Brachyspira, 342
Flavobacterium, 510
Herpetosiphon, 873
Leptospira, 302
Planctomycetales, 771
Rhodothermus, 722
Sphingomonads, 618
Thermotoga, 915
Plasmin, *Borrelia*, 276–77
Plasminogen
Borrelia, 276–77
Chryseobacterium, 669
Plasminogen activation system, *Borrelia*, 276–77
Plating technique, myxobacteria, 45
Plesiocystis, 85
Pneumonia
Bergeyella, 536
Chlamydia, 741, 746–49, 752
Chryseobacterium, 667
Elizabethkingia, 666
Ornithobacterium, 695–96, 704
Riemerella, 703
Pneumonitis
Chlamydia, 747
Cytophagale, 554
Flavobacterium, 510–11
Podangium. See Melittangium
Podangium erectum. See Stigmatella erecta

Polaribacter, 455, 462, 466–69, 471, 481, 679, 681–83, 689
Polaribacter butkevichii, 458
Polaribacter filamentus, 458, 462, 679–80, 697
Polaribacter franzmannii, 458, 462, 679–80
Polaribacter glomeratus, 458, 462, 679–80
Polaribacter irgensii, 458, 462, 466, 679–80
Polyangium, 33, 36, 39, 41–43, 51–52, 56, 63, 71, 73, 76–77, 81–82, 85–86, 88–90
Polyangium aureum, 90
Polyangium fumosum, 63, 90, 99
Polyangium luteum, 90
Polyangium minus, 89
Polyangium parasiticum, 90
Polyangium spumosum, 90, 99
Polyangium vitellinum, 34, 73, 81, 90
Poly-β-hydroxybutyrate (PHB), *Zoogloea*, 965–67
Polychlorinated biphenyls (PCB)
  Sphingomonads, 612–13, 616
  Sphingomonas, 605–6
Polychlorophenol metabolism, *Novosphingobium*, 620
Polyethylene glycol utilization
  Pelobacter, 5–7, 9–10
  Planctomycetales, 765
Polymyxin B resistance/sensitivity
  Chryseobacterium, 664, 667
  Cytophaga, 561
  Elizabethkingia, 667
  Helicobacter, 156
  Hymenobacter, 728
  Leptospira, 304
  Riemerella, 705
  Sphingomonads, 618
Polymyxin resistance
  Cytophagale, 575
  Flavobacterium, 490, 492
  Ornithobacterium, 700
  Treponema, 221–22
Polyphosphate granule
  Helicobacter, 139
  myxobacteria, 60
Polyprenol, Cytophagale, 571
Polysaccharide metabolism
  Bacteroides, 381–86, 398
  Cytophagale, 574
  Flavobacteriaceae, 684
  Flavobacterium, 501–2, 510
  Rhodothermus, 715, 718
  Sphingomonads, 615–17
Polysaccharide production
  myxobacteria, 60, 70
  Porphyromonas, 438–39
  Sphingomonads, 621
  Zoogloea, 960–63, 966
Polysaccharide utilization
  Fervidobacterium, 905
  Geotoga, 905
  Marinitoga, 905
  Petrotoga, 905
  Streptobacillus, 984
  Thermosipho, 905
  Thermotoga, 905
Polysomes, *Chondromyces*, 60
Polysulfide reduction, *Wolinella*, 178, 182, 184–88, 185–86
Porphyrin production, *Porphyromonas*, 428
Porphyrin utilization, *Cytophaga*, 539
Porphyrobacter, 606
Porphyromonas, 393, 412, 428–48, 459
Porphyromonas asaccharolytica, 429
Porphyromonas cangingivalis, 429
Porphyromonas canoris, 429
Porphyromonas cansulci, 429
Porphyromonas catoniae, 382, 429
Porphyromonas circumdentaria, 429
Porphyromonas crevioricanis, 429
Porphyromonas endodontalis, 429
Porphyromonas gingivalis, 382, 389, 411, 428–43, 473
Porphyromonas gingivicanis, 429
Porphyromonas gulae, 429
Porphyromonas levii, 429, 431
Porphyromonas macacae, 428–29, 431
Porphyromonas salivosa, 429
Potassium requirement, *Bacteroides*, 383
Poultry. *See* Fowl
Pox virus, *Borrelia*, 251
Prawn habitat
  Pirellula, 763, 780
  Planctomyces, 763
Prevotella, 393, 411–12, 429, 459
Prevotella intermedia, 411, 428, 432, 473
Prevotella melaninogenica, 382, 390–91
Prevotella ruminicola, 382, 389, 410
Prevotella tannerae, 382
Primates. *See also specific* primates
  Brachyspira, 331
  Fusobacterium, 1017
  Helicobacter, 160
  Porphyromonas, 428–29, 447
  Treponema, 217
Promyxobacterium flavum, 482, 486–87, 489, 492
1,2-Propanediol utilization, *Pelobacter*, 7
1,3-Propanediol production, *Pelobacter*, 9
Propanol production, *Pelobacter*, 8–9
Propanol utilization, *Pelobacter*, 7
Propionate production
  Bacteroides, 387, 414
  Chlorobiaceae, 372
  Fusobacterium, 1019, 1022
  Opitutus, 891
  Pelobacter, 8–10
  Porphyromonas, 428
  Propionigenium, 955–57
  Ultramicrobacteria, 891
Propionate utilization. *See* Organic acid utilization
Propionibacterium, 398
Propionigenium, 955–58
Propionigenium maris, 955–58
Propionigenium modestum, 955–58
Prosthecae
  Prosthecobacter, 882, 885, 887–88
  Verrucomicrobium, 882, 885–86, 888
Prosthecobacter, 881, 887–88
Prosthecobacter debontii, 883, 888
Prosthecobacter dejongeii, 882–83, 888
Prosthecobacter FC-2, 881
Prosthecobacter fusiformis, 881, 883, 888
Prosthecobacter vanneervenii, 883, 888
Prosthecochloris, 360, 363, 885
Prosthecochloris aestuarii, 360–63, 367–68
Prosthecochloris phaeoasteroidea, 361
Prosthecomicrobium, 885–86
Prosthetic infections, *Campylobacter*, 132
Protease activity
  Bacteroides, 398–99, 405
  Chryseobacterium, 669
  Cytophaga, 573
  Flavobacterium, 513
Protease sensitivity
  Bacteroides, 386, 389
  Borrelia, 244–45
Protein hydrolysates, *Porphyromonas*, 429
Protein hydrolysis, Flavobacteriaceae, 684
Proteobacteria
  alpha subclass, 605
  delta subclass, 5–99
  epsilon subclass, 119–88
Proteus, 405, 1021
Protoheme production, *Porphyromonas*, 428
Protoporphyrin utilization, *Bacteroides*, 384
Protozoa, termite gut, 318–19
Pseudanabaena, 785
Pseudoalteromonas piscicida, 519
Pseudocytophaga algae, 458
Pseudogenes, *Borrelia*, 254–55
Pseudomonadaceae, 960
Pseudomonas, 17, 520, 605–6, 621, 669, 965
Pseudomonas aeruginosa, 205, 699
Pseudomonas azotocoligens, 606. *See also Sphingomonas trueperi*
Pseudomonas bipunctata, 946. *See also Macromonas bipunctata*
Pseudomonas cepacia, 620
Pseudomonas denitrificans, 965
Pseudomonas echinoides, 606. *See also Sphingomonas echinoides*
Pseudomonas fluorescens, 26
Pseudomonas glycinea, 26
Pseudomonas paucimobilis, 620–21. *See also Sphingomonas paucimobilis*
Pseudomonas piscicida, 519. *See also Pseudoalteromonas piscicida*

Pseudomonas saccharophila, 606. *See also Sphingomonas subarctica*
Pseudomonas testosteroni, 14
Psychroflexus, 455, 462, 466–69, 471, 481, 484, 679
Psychroflexus gondwanensis, 458, 462, 679–80, 683
Psychroflexus torquis, 458, 462, 679–83, 685, 687, 690, 697
Psychroflexus tropicus, 458, 679–80, 683
Psychrophile, myxobacteria, 37
Psychroserpens, 455, 462, 466, 471, 481, 690
Psychroserpens burtonensis, 458, 462, 466–69, 679–80, 682–83, 697
P. syringae, 17
Pullularia pullulans, 83
Pulmonary environment, *Chlamydia*, 752
Purine, *Leptospira*, 298
Puromycin, *Bacteroides*, 411
Purple sulfur bacteria, 815–16, 823, 833–34, 900, 999
Pyrazin amidase, *Campylobacter*, 125–26
Pyrimidine, *Leptospira*, 298
Pyrogallol utilization, *Pelobacter*, 7
Pyruvate pathway
  Bacteroides, 387
  Leptospira, 298
Pyruvate production, *Chloroflexus*, 831
Pyruvate utilization. *See* Organic acid utilization
Pyxicoccus, 85–88
Pyxicoccus fallax, 77, 94

Q

Quinolone resistance/sensitivity
  Bacteroides, 407, 409, 411
  Capnocytophaga, 581
  Chryseobacterium, 667
  Elizabethkingia, 667
  Flavobacterium, 508
  Myroides, 633
Quinone
  Flavobacteriaceae, 466
  myxobacteria, 65

R

Rabbit habitat
  Borrelia, 239, 272
  Fusobacterium, 1017
  Leptospira, 302
  Treponema, 211, 218–19
Radiation therapy. *See* Chemotherapy
Radioimmunoassay, *Leptospira*, 310
Random amplified polymorphic DNA (RAPD), *Flavobacterium*, 506, 517
Rat bite fever, *Streptobacillus*, 983
Rat habitat
  Bacteroides, 402–4
  Borrelia, 238, 257, 272
  Brachyspira, 331
  Capnocytophaga, 578
  Fusobacterium, 1017
  Helicobacter, 140, 153, 156
  Leptospira, 305
  Porphyromonas, 438, 447
  Streptobacillus, 983
  Treponema, 212
16S rDNA sequence
  Aquifex, 925
  Bacteroides, 415
  Borrelia, 265
  Brachyspira, 330, 332–33, 336
  Flavobacteriaceae, 456
  Flavobacterium, 505–6
  Helicobacter, 145–46, 162, 164–65
  Hydrogenobacter, 927
  Hydrogenobaculum, 927
  Hydrogenothermus, 927
  Meiothermus, 801, 807
  Opitutus, 888–89
  Planctomycetales, 757
  Prosthecobacter, 887–88
  termite gut spirochete, 320, 326
  Thermocrinis, 927
  Thermus, 797, 801, 807
  Treponema, 226
  Verrucomicrobia, 881, 883–84
  Verrucomicrobium, 885
  Wolinella, 178, 181
  Xiphinematobacter, 892–93
23S rDNA sequence, *Borrelia*, 265
Rectal cultures, *Helicobacter*, 153, 162
Relapsing fever. *See also* Louse-borne relapsing fever; Tick-borne relapsing fever
  Borrelia, 256–57, 273, 275, 277
  Myroides, 634
Renal. *See* Kidney
Renobacter vacuolatum, 999
Respiratory tract
  Bacteroides, 402
  Bergeyella, 536
  Chlamydia, 747–49, 752
  Coenonia, 695, 699, 703
  Empedobacter, 461, 631
  Flavobacterium, 488, 510–11
  Fusobacterium, 1016, 1018
  Ornithobacterium, 462, 695–96, 702
  Riemerella, 695–96, 703
  Sphingomonas, 621
Rhamnose utilization. *See* Monosaccharide utilization
Rheas, *Brachyspira*, 331, 347
Rhesus habitat
  Brachyspira, 334
  Helicobacter, 148, 153
Rheumatological disease, *Chlamydia*, 751
Rhinitis, *Fusobacterium*, 1019
Rhizobium, 20
Rhizobium radiobacter, 713
Rhizoctonia, 83
Rhizomonas, 606
Rhizomonas suberifaciens, 606–7, 621. *See also Sphingomonas suberifaciens*
Rhodococcus, 9
Rhodococcus rhodochrous, 874
Rhodocyclus, 999
Rhodopseudomonas palutris, 804
Rhodopseudomonas sphaeroides, 846–47
Rhodospirilum rubrum, 21, 713
Rhodothermus, 712–22, 729–30
Rhodothermus marinus, 712–18, 721–22, 731
Rhodothermus obamensis, 712–14, 716, 721
Ribose utilization. *See* Monosaccharide utilization
Ribosomal DNA, *Treponema*, 222
Ribosomal RNA
  Anaeromyxobacter, 38
  Bacteroides, 392
  Bdellovibrio, 14
  Borrelia, 235
  Campylobacter, 128
  Flavobacterium, 505
  Helicobacter, 145–46
  Isosphaera, 772
  myxobacteria, 32–34
  Pelobacter, 5
  Planctomycetales, 757, 765, 772
  Porphyromonas, 428
Riemerella, 455, 462, 466–71, 473, 509, 639, 655, 695–706
Riemerella anatipestifer, 458, 462, 466, 639, 641, 647–51, 657, 680, 695–706
Riemerella columbina, 458, 462, 639, 647–51, 695–706
Rifampicin resistance/sensitivity
  Borrelia, 241
  Chryseobacterium, 667
  Elizabethkingia, 667–68
  Fusobacterium, 1020
  Hydrogenobacter, 930
  Leptospira, 304
  Salinibacter, 733
  Spirochaeta, 199–201
  Thermotogales, 908
  Treponema, 220–22
Rifamycin-fosfomycin solution, termite gut spirochete, 324
Rifamycin resistance/sensitivity
  Bacteroides, 413
  termite gut spirochete, 323
River environment. *See* Freshwater environment
Robiginitalea, 481
Robiginitalea biformata, 679–80, 688
Rocky Mountain spotted fever, *Borrelia*, 263
Rodents. *See also specific* rodents
  Bacteroides, 397
  Borrelia, 237–39, 257, 262
  Leptospira, 305, 307
  Porphyromonas, 447
Roseiflexus, 818, 820, 822–23, 827–29, 834, 867, 870

Roseiflexus castenholzii, 815, 817, 819–20, 822–23, 827, 829–33, 835, 870
Rose plant, *Novosphingobium*, 611–12
Rosettes
Cristispira, 976, 978
Gemmata, 781
Pirellula, 760, 779
Planctomyces, 758, 760, 763, 773, 775–77
Planctomycetales, 757, 760, 762–63
spirochetes, symbiotic, 976
5S rRNA sequence
Gemmata, 781, 783
Herpetosiphon, 870
Isosphaera, 784
Leptospira, 301
Pirellula, 780
Planctomycetales, 757
Thermomicrobium, 849
Verrucomicrobium, 885
14S rRNA sequence, *Leptospira*, 301
16S rRNA sequence
Aquificales, 925–26, 930
Bacteroides, 381–82, 396, 413, 415
Borrelia, 242
Brachyspira, 332, 337, 342
Capnocytophaga, 578, 696, 709
Chlamydia, 742
Chlorobiaceae, 359–60, 364–65, 372
Chloroflexaceae, 815, 817, 820
Chloroflexus, 818, 820, 828, 870
Chloronema, 816
Chryseobacterium, 638–39, 642–44, 646, 660–61, 663–64
Coenonia, 696
Croceibacter, 688
Cytophaga, 572, 578, 680–81
Cytophagale, 549, 554, 575–76, 578
Elizabethkingia, 638–39, 643, 660–61, 663–64
Epixenosomes, 892
Flavobacteriaceae, 456–59, 465, 473, 677, 680, 688–89
Flavobacterium, 481–82, 484, 487–88, 490, 501, 505–6, 508, 510, 520
Flexibacter, 577–78, 681
Gemmata, 767, 781, 784
Helicobacter, 146, 148, 150–61, 163–64
Heliothrix, 817, 820, 829
Herpetosiphon, 855, 863, 870–72
Hymenobacter, 725
Isosphaera, 764, 767, 784, 788
Leptospira, 301, 303
Meiothermus, 801
Novosphingobium, 609
Ornithobacterium, 696, 702
Oscillochloris, 817
Pirellula, 763, 767, 780
Planctomyces, 763–65, 767, 776–78
Planctomycetales, 757–61, 763, 766–68, 772, 899
Porphyromonas, 428
Propionigenium, 957
Rhodothermus, 712–13, 722, 730
Riemerella, 695–96
Robiginitalea, 688
Roseiflexus, 822, 829, 870
Salinibacter, 714, 728–32, 734
Saprospira, 591
Sphingobium, 609
Sphingomonads, 615
Sphingomonas, 606, 609
Sphingopyrix, 609
Spirochaeta, 195, 199
spirochetes, symbiotic, 980–81
Sporocytophaga, 572
Taxeobacter, 578
Tenacibaculum, 681
termite gut spirochete, 319–20, 322
Thermocrinis, 927–28
Thermoleophilum, 843
Thermonema, 723
Thermotoga, 899–900
Thermotogales, 899, 902
Thermus, 801
Treponema, 211, 213–15
Victivallis, 893
17S rRNA sequence, *Leptospira*, 301
23S rRNA sequence
Bacteroides, 408, 413, 415
Borrelia, 242
Brachyspira, 332, 337, 342, 349
Helicobacter, 145–46
Leptospira, 301, 303
Planctomycetales, 757, 772
Thermotoga, 899
Rubredoxin, *Spirochaeta*, 207
Rumen
Bacteroides, 382
Treponema, 212–13, 215
Wolinella, 178

S

Saccharolytic activity
Bergeyella, 534
Chryseobacterium, 534
Myroides, 534
Sphingobacterium, 534
Weeksella, 534
Saccharomyces cerevisiae, 42, 83
Saccharose utilization
Cytophagale, 563
Riemerella, 700
Salegentibacter, 455, 462, 466, 471, 481, 484
Salegentibacter salegens, 458, 462, 466–69, 679–80, 682, 697
Salicin utilization
Bergeyella, 649
Brachyspira, 414
Chryseobacterium, 649, 654, 656
Elizabethkingia, 649, 654
Riemerella, 649
Verrucomicrobium, 887
Saline environment. *See also* Marine environment
Achromatium, 944, 946
Chloroflexaceae, 815–16, 818, 820, 823
Chloroflexus, 818
Chloronema, 818
Oscillochloris, 818
Pirellula, 778
Planctomyces, 773, 777
Planctomycetales, 762
Thermus, 797–98, 799, 802
Salinibacter, 728–34
Salinibacter ruber, 682, 721, 729–34
Saliva, *Porphyromonas*, 430
Salmon
Chryseobacterium, 644
Flavobacterium, 485–86, 488, 491, 512, 520
Tenacibaculum, 687
Salmonella, 252, 265
Salmonella enterica, 131
Salmonella enteritidis, 703
Salmonella typhimurium, 404, 438, 772
Salmonid. *See also* Salmon; Trout
Cytophagale, 554
Flavobacterium, 487–88, 491, 502, 511–14, 516, 518
Salt requirement. *See also specific* salts
Chlorobiaceae, 360–61, 364
Flavobacteriaceae, 472–73
Marinithermus, 807
Oceanitherums, 807
Rhodothermus, 712, 714–15, 718
Salinibacter, 728–29, 731–32, 734
Vulcanitherums, 807
Salt sensitivity
Thermotoga, 914
Thermotogales, 914
Saprophyte, *Leptospira*, 294–95, 302
Saprospira, 549, 569, 591–600
Saprospira albida, 591–99
Saprospira flammula, 593, 595, 597
Saprospira flexuosa, 591, 593, 597
Saprospira gigantea, 593
Saprospira grandis, 591–600
Saprospira lepta, 591, 593
Saprospira nana, 591, 593
Saprospira puncta, 591, 593
Saprospira spiroidea, 593, 597
Saprospira thermalis, 592–93, 595–600
Saprospira toviformis, 593, 595, 600
Sardines
Chryseobacterium, 644
Flavobacterium, 488
Tenacibaculum, 687
Scalindua, 773
Scours. *See* Dysentery
SDS-polyacrylamide gel, *Wolinella*, 180–81
Sea environment. *See* Marine environment
Sebaldella, 957
Sebaldella termitidis, 957
Sediment environment. *See also* Estuarine environment; Freshwater environment; Marine environment
Arenibacter, 678

Bdellovibrio, 14–16
Cellulophaga, 460, 678
Chlorobiaceae, 359, 362–63
Chloroflexaceae, 820
Chloroflexus, 825
Chryseobacterium, 643
Cytophagale, 553
Flavobacteriaceae, 689
Flavobacterium, 461, 485, 487–88, 540
Muricauda, 462, 679
Novosphingobium, 610–12, 620
Pelobacter, 5–6, 10
Propionigenium, 955
Sphingomonads, 613
Spirochaeta, 195, 198, 201, 207
Thermosipho, 903
Verrucomicrobia, 881
Zobellia, 679
Sejongia, 639–40, 643, 647
Sejongia antarctica, 657
Sejongia jeonii, 657
Selenite reduction
Bergeyella, 535
Weeksella, 535
Sepsis, *Bacteroides*, 415
Septicemia
Bergeyella, 536
Fusobacterium, 1018
Serine utilization
Brachyspira, 344
Meiothermus, 802
Serology
Bdellovibrio, 14
Flavobacterium, 507
Leptospira, 302–3, 310–11
Serovar
Brachyspira, 346
Chlamydia, 747
Leptospira, 294–95, 302–3, 305–6, 308, 310–11
Riemerella, 701, 705
Serpens flexibilis, 203
Serpulina. See Brachyspira
Sewage environment
Herpetosiphon, 854, 858–59, 862, 868, 872–73
Planctomycetales, 762
Prosthecobacter, 882, 887
Sphingomonas, 610
Verrucomicrobia, 884
Zoogloea, 960, 963, 967–68
Sewage sludge
Bacteroides, 397
Bdellovibrio, 14–16
Chryseobacterium, 640
Elizabethkingia, 640
Flavobacteriaceae, 474
Flavobacterium, 496–97
Pelobacter, 5–6, 10
Saprospira, 592
Wolinella, 178
Sheath
Fervidobacterium, 906
Herpetosiphon, 864–67
Thermosipho, 906
Thermotogales, 906
Sheep. *See* Ovine
Shellfish
Campylobacter, 122
Cytophagale, 553
Shigella, 745
Shigella flexneri, 26, 252
Shrew, *Helicobacter*, 142
Shrimp, *Treponema*, 212
Siderobacter, 1006
Siderobacter brevis, 1006
Siderobacter gracilis, 1006
Siderobacter latus, 1006
Siderocapsa, 986, 1005–14
Siderocapsa anulata, 1006–7, 1009, 1011
Siderocapsa arlbergensis, 1006, 1011
Siderocapsa botryoides, 1006
Siderocapsaceae, 1005–14
Siderocapsa coronata, 1006, 1011
Siderocapsa eusphaera, 1006–7, 1009, 1011
Siderocapsa geminata, 1006–7, 1009, 1011, 1013–14
Siderocapsa major, 1006–7
Siderocapsa monoica, 1006–7
Siderocapsa treubii, 1006–9, 1011–12
Siderococcus, 996, 1006, 1009, 1011
Siderococcus communis, 1009
Siderococcus limoniticus, 1006–7, 1009, 1011, 1014
Siderocystis, 1006, 1009, 1011
Siderocystis confervarum, 1006–9, 1011, 1014
Siderocystis vulgaris, 1005–7, 1009, 1011–12
Silver staining methods
Borrelia, 263
Leptospira, 294, 310
Treponema, 219
Single-strand conformation polymorphism (SSCP), *Borrelia*, 264
Sinuse habitat
Bacteroides, 402
Chlamydia, 752
Skin disease, *Treponema*, 212
Skin habitat
Bacteroides, 402
Borrelia, 236, 240–42, 263
Chryseobacterium, 643–45, 663–64
Cytophagale, 561
Elizabethkingia, 643
Flavobacterium, 502, 506, 512–20
Fusobacterium, 1017
Leptospira, 305–6
S-layer
Flexibacter, 568
Meiothermus, 805
Snakes
Borrelia, 257
Elizabethkingia, 664
Fusobacterium, 1017
Sneathia, 957
Sodium bicarbonate (NaHCO3) utilization, *Spirochaeta*, 205
Sodium carbonate (Na2CO3) utilization, *Spirochaeta*, 205
Sodium chloride requirement
Aquifex, 929
Fervidobacterium, 904
Geotoga, 904
Hydrogenobacter, 929
Hydrogenobaculum, 929
Hydrogenothermus, 929
Marinitoga, 904
Petrotoga, 904
Propionigenium, 956
Salinibacter, 712
Thermocrinis, 929
Thermonema, 723–24
Thermosipho, 904
Thermotoga, 904
Sodium chloride sensitivity/tolerance
Bergeyella, 533
Campylobacter, 121, 125–26
Chryseobacterium, 472, 643, 662
Cytophaga, 561
Cytophagale, 563
Elizabethkingia, 643, 662
Flavobacteriaceae, 472
Flavobacterium, 489, 496
Helicobacter, 149–59, 162
Herpetosiphon, 869
Hymenobacter, 728
Planctomyces, 777
Salinibacter, 712
Sphingomonads, 614, 616–17
Ultramicrobacteria, 891
Verrucomicrobium, 886
Weeksella, 533
Wolinella, 180
Sodium chloride utilization
Haliangium, 36, 91
Myxococcus, 57
Saprospira, 595
Spirochaeta, 198, 205
Sodium requirement
Bacteroides, 383
Flavobacteriaceae, 682
Propionigenium, 955–58
Psychroflexus, 682
Salegentibacter, 682
Spirochaeta, 205
Soft tick, *Borrelia*, 235–38
Soil environment
Archangium, 36
Bdellovibrio, 14–16
Brachyarcus, 998
Chondromyces, 36
Chryseobacterium, 460, 638, 640, 643, 645
Corallococcus, 36
Cystobacter, 36
Cytophaga, 555, 560
Cytophagale, 550, 552, 553, 557, 576
Elizabethkingia, 638, 640, 643
Flavobacteriaceae, 470, 474
Flavobacterium, 461, 486–88, 490, 493, 495, 497, 519–20, 540–41
Flexibacter, 553, 555
Gemmata, 781, 782
Herpetosiphon, 854–55, 858–59, 862
Hymenobacter, 712, 724–26
Leptospira, 305
Melittangium, 36
myxobacteria, 36–37, 39–40, 48, 93
Myxococcus, 36

Nannocystis, 36
Naumanniella, 1006
Opitutus, 882, 888–89
Planctomycetales, 762, 764–65
Polyangium, 36
Saprospira, 592, 594
Siderocapsa, 1005, 1006
Sideroccoccus, 1006
Sorangium, 36
Sphingobium, 610, 612
Sphingomonads, 613
Sphingomonas, 610–12
Sphingopyxis, 611
Sporocytophaga, 555, 560
Verrucomicrobia, 884
Weeksella, 533
Sorangicin, 92
Sorangium, 36–37, 39, 42, 47, 49, 52, 55–57, 59–63, 71, 73, 76–77, 80, 82, 85–86, 88, 90
Sorangium cellulosum, 36, 53, 56, 59–60, 62, 64, 66, 76–79, 80–82, 84, 90, 98
Sorangium compositum, 32, 84, 90
Sorangium nigrum, 90, 98
Sorbitol utilization. *See* Alcohol utilization
Sorbose utilization, *Riemerella*, 700
Southern blotting
Borrelia, 276
Campylobacter, 124, 128
Southern hybridization, *Borrelia*, 254, 269–70
Sparfloxacin, *Bacteroides*, 411
Sparrow, *Helicobacter*, 142
Spectinomycin, *Bacteroides*, 411
Sphaerobacter thermophilus, 817
Sphaerocytophaga, 561, 567, 581, 709
Sphaeromyxa, 567
Sphaerotilus, 604, 986
Sphaerotilus natans, 604
Sphingans, Sphingomonads, 616–17, 621
Sphingobacterium, 39, 459, 482, 484, 534, 539–45, 641, 652, 667
Sphingobacterium spiritovorum, 482, 540, 543–44, 713
Sphingobium, 605–22
Sphingobium chlorophenolicum, 607, 610, 612. *See also Sphingomonas chlorophenolica*
Sphingobium herbicidovorans, 607, 610. *See also Sphingomonas herbicidovorans*
Sphingobium yanoikuyae, 607, 610, 612. *See also Sphingomonas yanoikuyae*
Sphingomonads, 605–22
applications, 621
cultivation methods, 614
ecology, 619–20
genetics, 617–18
genomics, 618–19
identification procedure, 615
isolation procedure, 613–14
physiology, 615–17
preservation methods, 615
Sphingomonas, 510, 605–22
Sphingomonas adhaesiva, 605, 607, 610, 621
Sphingomonas alaskensis, 607, 609–11, 614, 617–19
Sphingomonas aquatilis, 607, 609–10, 613
Sphingomonas aromaticivorans, 606–7, 621
Sphingomonas asaccharolytica, 605, 607, 610
Sphingomonas capsulata, 605, 607
Sphingomonas chlorophenolica, 605, 607
Sphingomonas chungbukensis, 607, 609–10
Sphingomonas cloacae, 607, 609–10
Sphingomonas echinoides, 606–7, 610
Sphingomonas elodea, 617
Sphingomonas herbicidovorans, 606–7
Sphingomonas koreensis, 607, 609–10, 613
Sphingomonas macrogoltabidus, 605, 607
Sphingomonas mali, 605, 607, 610–11
Sphingomonas natatoria, 606–7, 610
Sphingomonas parapaucimobilis, 605, 607, 611, 621
Sphingomonas paucimobilis, 482, 605, 607, 610–11, 613–14, 616–18, 620–21
Sphingomonas pituitosa, 607, 609–10
Sphingomonas pruni, 605, 607, 610
Sphingomonas rosa, 605, 607
Sphingomonas roseiflava, 607, 609–10
Sphingomonas sanguis, 605, 607, 610, 621
Sphingomonas stygia, 606, 607
Sphingomonas subarctica, 606–7
Sphingomonas suberifaciens, 606–7, 610, 621
Sphingomonas subterranea, 606–7
Sphingomonas taejonensis, 608–10, 613
Sphingomonas terrae, 605, 607
Sphingomonas trueperi, 606–7, 610
Sphingomonas ursincola, 606–7. *See also Blastomonas ursincola*
Sphingomonas wittichii, 607, 609–10
Sphingomonas xenophaga, 607, 609–10, 619
Sphingomonas yanoikuyae, 605, 607, 619, 621
Sphingopyxis, 605–22
Sphingopyxis macrogoltabida, 607, 611–12. *See also Sphingomonas macrogoltabidus*
Sphingopyxis terrae, 607, 611. *See also Sphingomonas terrae*
Spiramycin sensitivity, *Riemerella*, 704
Spirillum bipunctatum, 948. *See also Thiospira bipunctata*
Spirochaeta, 195–208, 213, 215, 235, 321–22
Spirochaeta africana, 197–99, 201, 204–6
Spirochaeta alkalica, 197–99, 201, 204–6
Spirochaeta asiatica, 197–99, 201, 204, 206
Spirochaeta aurantia, 196–98, 203–7, 216, 326
Spirochaeta bajacaliforniensis, 197–98, 204
Spirochaeta caldaria, 195, 197–99, 204, 206–7, 214–15, 320
Spirochaeta gigantea, 591. *See also Saprospira*
Spirochaeta halophila, 196–99, 201–3, 205, 207, 214, 326
Spirochaeta isovalerica, 197–98, 204, 206, 208, 214
Spirochaeta litoralis, 196–98, 202, 204, 207
Spirochaeta plicatilis, 195–98, 200, 205, 208
Spirochaeta smaragdinae, 197–98, 204, 206
Spirochaeta stenostrepta, 195–99, 201–2, 204, 207, 214–15, 320, 326
Spirochaeta thermophila, 197–99, 204, 206–7
Spirochaeta zuelzerae, 195–98, 204, 206, 214–15, 322, 326
Spirochete
Brachyspira, 330–49
symbiotic, 971–81
termite gut, 318–27
Spirochetosis, *Brachyspira*, 334, 345, 348
Spiromonas, 1001. *See also Pelosigma*
Spiromonas cohnii, 1001. *See also Pelosigma cohnii*
Spironema, 195, 197, 213, 235
Spironema culicis, 197, 214
Spirulina, 591
Spirulina alba, 591. *See also Saprospira*
Spirulina albida, 591. *See also Saprospira*
Spirulina subsalsa, 714
Spleen habitat
Borrelia, 241
Chlamydia, 747
Cytophagale, 561
Flavobacterium, 512–13
Sponges, Planctomycetales, 763
Sporangioles, myxobacteria, 39, 44, 73–81, 83
Sporocytophaga, 549, 555, 565, 567, 569–70, 573, 576, 579
Sporocytophaga cauliformis, 482–84, 486–87, 489, 492, 505, 577
Sporocytophaga ellipsospora, 559
Sporocytophaga myxococcoides, 549, 551, 559, 568–72, 574, 579
Squalene production, myxobacteria, 63
Squirrel habitat
Borrelia, 237, 257
Streptobacillus, 983

SSCP. *See* Single-strand conformation polymorphism
S system motility
Flavobacterium, 499
myxobacteria, 68–69
Staining, *Treponema*, 211
Staphylococcus, 84, 580
Staphylococcus aureus, 256
Staphylococcus epidermis, 580
Starch hydrolysis
Aequorivita, 467
Arenibacter, 467
Bacteroides, 384–86, 398
Bergeyella, 467, 535, 649
Capnocytophaga, 467
Cellulophaga, 467
Chryseobacterium, 467, 649
Coenonia, 467
Croceibacter, 467
Cytophaga, 467, 539
Cytophagale, 574, 579
Elizabethkingia, 649
Empedobacter, 467
Flavobacterium, 467, 493–95, 542, 543
Fucobacter, 467
Gelidibacter, 468, 469
Haliscomenobacter, 604
Herpetosiphon, 869
Hymenobacter, 728
Meiothermus, 802
Mesonia, 468, 469
Muricauda, 468, 469
Myroides, 468, 469
Opitutus, 891
Ornithobacterium, 468, 469
Pirellula, 780
Polaribacter, 468, 469
Psychroflexus, 468, 469
Psychroserpens, 468, 469
Rhodothermus, 715
Riemerella, 468, 469, 649
Salegentibacter, 468, 469
Salinibacter, 733
Saprospira, 599
Tenacibaculum, 468, 469
Thermus, 799
Ultramicrobacteria, 891
Ulvibacter, 468
Vitellibacter, 468
Weeksella, 469, 535
Zobellia, 469
Stella, 885
Stenotrophomonas maltophilia, 372
Stigmatella, 39, 43–44, 60, 64, 71, 73, 81, 85–86, 89
Stigmatella aurantiaca, 32, 34, 36–37, 43, 49–53, 56, 63–64, 66, 72, 75–77, 79–82, 84, 89
Stigmatella erecta, 33, 36–37, 43, 63, 66, 72, 76–77, 80, 84, 89
Stigmatellin, 92
Stool. *See* Feces
Streptobacillus, 957, 983–84
Streptobacillus moniliformis, 983–84
Streptococcus, 345, 580
Streptococcus gordonii, 224
Streptococcus mutans, 869
Streptococcus pneumoniae, 752, 836
Streptomyces, 769
Streptomyces albus, 874
Streptomyces ambofaciens, 926
Streptomyces griseus, 865
Streptomycin resistance/sensitivity
Bacteroides, 411
Cytophaga, 572
Flavobacterium, 545
Gemmata, 782
Helicobacter, 153
Rhodothermus, 721
Riemerella, 704
Salinibacter, 733
Succinate production
Bacteroides, 387–88, 390, 406, 414
Capnocytophaga, 578, 710
Flavobacterium, 509
Porphyromonas, 428
Spirochaeta, 204, 206
Ultramicrobacteria, 891
Wolinella, 180, 183, 186–87
Succinate utilization. *See* Organic acid utilization
Sucrase, *Brachyspira*, 339–40
Sucrose utilization. *See* Di- and trisaccharide utilization
Sugar fermentation
Bacteroides, 382–83, 386–88, 413
Brachyspira, 330
Chloroflexus, 830
Corallococcus, 49
Cystobacter, 49
myxobacteria, 49
Pelobacter, 5
Salinibacter, 733
Spirochaeta, 200
Stigmatella, 49
termite gut spirochete, 325
Thermotoga, 916
Sulbactam, *Bacteroides*, 407
Sulfamethazine sensitivity, *Ornithobacterium*, 705
Sulfamethoxazole-trimethoprim resistance/sensitivity
Bergeyella, 534–35
Weeksella, 534–35
Sulfate production, Chlorobiaceae, 368, 371
Sulfate utilization, *Chloroflexus*, 831
Sulfide oxidation
Bacteroides, 383
Chlorobiaceae, 364, 368–69, 371–72
Chloroflexus, 830, 831
Oscillochloris, 830, 831
Sulfide production, *Propionigenium*, 957
Sulfite sensitivity, Chlorobiaceae, 368
Sulfitobacter mediterraneus, 768
Sulfitobacter pontiacus, 768
Sulfolobus, 941
Sulfonamides resistance/sensitivity
Chryseobacterium, 664
Flavobacterium, 515, 518
Ornithobacterium, 705
Riemerella, 704
Sulfur metabolism
Achromatium, 941–46
Bacteroides, 383
Brachyarcus, 998–99
Chlorobiaceae, 359, 364, 368, 371
Chloroflexus, 831
Hydrogenobacter, 934
Hydrogenobaculum, 934
Macromonas, 941–42, 946–47
Thermus, 805
Thiobacterium, 941–42, 945, 947
Thiospira, 941–42, 947–48
Thiovulum, 941–42, 948–52
Sulfurospirillum, 119–20, 178
Sulfurospirillum arcachonense, 120
Sulfurospirillum arsenophilum, 120
Sulfurospirillum barnesii, 120, 179
Sulfurospirillum delyianum, 120, 179
Sulfur-oxidizing eubacteria, 941–52
Sulfur reducing
Desulfuromonas, 5
Spirochaeta, 204, 206
Sulfur spring, Chlorobiaceae, 362
Superoxide dismutase (SOD)
Aquifex, 932
Bacteroides, 391
Campylobacter, 123
Porphyromonas, 440
Thermoleophilum, 846
Thermomicrobium, 852
Swamp environment
Gallionella, 990
Siderocapsa, 1005–6
Siderocystis, 1006
Swarms
Cytophagale, 549, 554–55, 573
Herpetosiphon, 854, 865–67
myxobacteria, 31–35, 38–44, 48, 70–72, 83
Thiovulum, 949
Swine
Bacteroides, 382, 397
Bergeyella, 533
Brachyspira, 330–31, 334–35, 345–49
Campylobacter, 122
Chryseobacterium, 645
Coenonia, 695
Fusobacterium, 1017–18
Helicobacter, 140, 142, 155, 160
Hymenobacter, 724, 726, 728
Leptospira, 305, 307
Riemerella, 701, 703
Streptobacillus, 983
Treponema, 212–13
Weeksella, 533
Synangium. See Chondromyces lanuginosus
Synechocystis, 388
Syphilis, 211–12, 224–26, 263, 277

T

Tamarin, *Helicobacter*, 142, 163
Taq polymerase
Chloroflexus, 836
Thermus, 809
Taxeobacter, 41, 549–50, 555, 567, 576, 578, 713, 724–26, 728–29
Taxeobacter chitinovorans, 726

Taxeobacter gelupurpurascens, 725–26
Taxeobacter ocellatus, 552, 565, 572, 726
Tazobactam resistance/sensitivity
Bacteroides, 407
Chryseobacterium, 668
T cells, *Porphyromonas*, 431, 441–44
Temperature tolerance
Aequorivita, 467, 683
Aquifex, 929
Aquificales, 925
Arenibacter, 467, 683
Bacteroides, 395–96
Bergeyella, 467, 534–35, 648
Borrelia, 271
Brachyarcus, 998–99
Brachyspira, 346
Campylobacter, 121–22, 124
Capnocytophaga, 467
Cellulophaga, 467, 683
Chlorobiaceae, 364
Chloroflexus, 819
Chloronema, 819
Chryseobacterium, 467, 647, 648, 656
Coenonia, 467
Croceibacter, 467, 683
Cytophaga, 467, 683
Cytophagale, 565
Elizabethkingia, 647, 648
Empedobacter, 467, 633
Fervidobacterium, 904
Flavobacteriaceae, 466, 472, 682
Flavobacterium, 467, 484–87, 489, 491, 493, 494–97, 502–3, 515, 519, 543–44
Formosa, 683
Fucobacter, 467
Fusobacterium, 1023
Gallionella, 990
Gelidibacter, 468, 683
Gemmata, 781
Geotoga, 904
Gillisia, 683
Helicobacter, 141, 143, 150–66
Heliothrix, 819
Herpetosiphon, 859–60, 869
Hydrogenobacter, 929
Hydrogenobaculum, 929
Hydrogenothermus, 929
Hymenobacter, 712
Isosphaera, 786
Leptospira, 297
Maribacter, 683
Marinitoga, 904
Meiothermus, 805
Mesonia, 468, 683
Muricauda, 468, 683
Myroides, 468, 633
myxobacteria, 82
Ornithobacterium, 468
Oscillochloris, 818, 819
Petrotoga, 904
Planctomyces, 777
Polaribacter, 468, 683
Porphyromonas, 440
Psychroflexus, 468, 683
Psychroserpens, 468, 683
Rhodothermus, 712, 721–22
Riemerella, 468, 648, 699
Robiginitalea, 683
Roseiflexus, 819
Salegentibacter, 468, 683
Salinibacter, 733
Saprospira, 592, 595–96
Sphingomonads, 614
Sphingomonas, 617
Spirochaeta, 197–204
Sporocytophagas, 559
Tenacibaculum, 468, 683
Thermocrinis, 928, 929
Thermoleophilum, 844
Thermomicrobium, 850–51
Thermonema, 712
Thermosipho, 904
Thermotoga, 904, 907
Thermus, 805
Toxothrix, 986–88
Ultramicrobacteria, 891
Ulvibacter, 468, 683
Verrucomicrobium, 886
Victivallis, 894
Vitellibacter, 468, 683
Weeksella, 468, 534–35, 544
Wolinella, 180
Zobellia, 468, 683
Zoogloea, 962
Tenacibaculum, 456, 462, 466–69, 481, 679
Tenacibaculum amyloliticum, 458, 462, 679–80
Tenacibaculum cellulophagum, 458
Tenacibaculum maritimum, 458, 462, 472, 501, 515, 679–81, 687, 697
Tenacibaculum mesophilum, 458, 462, 679–80
Tenacibaculum ovolyticum, 458, 462, 679–81, 687
Tenacibaculum skaggerakense, 679–80, 683
Terminal restriction fragment length polymorphism (T-RFLP), *Flavobacterium*, 506
Termite gut spirochete, 318–27
isolation procedure, 322–25
phylogeny, 320–22
Termites
Bacteroides, 382
Blattabacterium, 465
Clevelandina, 975, 977–78
Diplocalyx, 975, 979
Hollandina, 975, 979
Pillotina, 975, 978, 979
spirochetes, symbiotic, 974–75, 981
termite gut spirochete, 318–27
Treponema, 212–13, 215
Tern, *Helicobacter*, 155
Tetracycline resistance/sensitivity
Bacteroides, 406, 409–10
Bergeyella, 534–35
Borrelia, 277
Capnocytophaga, 581
Chlamydia, 747–48, 750
Chryseobacterium, 667, 668
Cytophagale, 581
Elizabethkingia, 667, 668
Flavobacterium, 514–15
Helicobacter, 162, 167
Hymenobacter, 728
Leptospira, 307
Myroides, 633
Ornithobacterium, 705
Porphyromonas, 432–33
Rhodothermus, 721
Riemerella, 704
Salinibacter, 733
Weeksella, 534–35
Tetrathionate reducing, Chlorobiaceae, 368
Thermocrinis, 925–30
Thermocrinis albus, 926–27, 929–31
Thermocrinis ruber, 926–31, 934
Thermofilum pendens, 926
Thermoleophilum, 843–47
Thermoleophilum album, 843–47, 850
Thermoleophilum minutum, 843–47
Thermomicrobium, 815, 844, 849–52, 874
Thermomicrobium roseum, 798, 806–7, 817, 849–52
Thermonema, 722–24
Thermonema lapsum, 713, 722–24
Thermophile
Aquificales, 925, 934
Aquificales, 899
Chloroflexus, 815, 820–22, 828, 835–36
Hydrogenobacter, 930
Isosphaera, 764, 784–85
Marinithermus, 807
Oceanitherums, 807
Pirellula, 778
Rhodothermus, 712, 714–15
Thermocrinis, 928
Thermoleophilum, 843–44, 847
Thermomicrobium, 849–50
Thermonema, 722–23
Thermotoga, 899–900
Thermotogales, 899–900, 902
Thermus, 797–98, 799
Vulcanitherums, 807
Thermosipho, 899, 903, 906, 914
Thermosipho africanus, 901–6, 908
Thermosipho geolei, 901–5, 915
Thermosipho japonicus, 901–5
Thermosipho melanesiensis, 901–5
Thermosphaera aggregans, 902, 926
Thermotoga, 772, 844, 899, 902, 907, 914–16
Thermotoga elfii, 901–2, 904–5, 908
Thermotoga hypgea, 901–2, 904–5
Thermotogales, 899–917
Thermotoga lettingae, 901–2, 904–5
Thermotoga maritima, 899–902, 904–8, 914–17, 926
Thermotoga naphthophila, 899–902, 904–5
Thermotoga neapolitana, 899–902, 904–5, 908, 914–16
Thermotoga petrophila, 899–902, 904–5
Thermotoga subterranea, 901–2, 904–5, 914
Thermotoga thermarum, 900–902, 904–5, 908

Thermovibrio rubrum, 926
Thermus, 772, 797–807, 809, 851
Thermus antranikianii, 797–99
Thermus aquaticus, 797–99, 801–2, 804, 807, 809, 850
Thermus brockianus, 797–99, 801
Thermus filiformis, 797–99, 804, 806
Thermus igniterrae, 797–99
Thermus oshimai, 797–99
Thermus rossianum, 722–24
Thermus ruber, 801. *See also Meiothermus ruber*
Thermus scotoductus, 797–99, 801–2, 804–6
Thermus thermophilus, 713, 797–99, 802–5, 807
Thiamine requirement
  Cytophagale, 565
  Haliscomenobacter, 604
  Leptospira, 299
  myxobacteria, 56
Thiobacillus, 941, 943
Thiobacterium, 941–42, 945, 947
Thiobacterium bovista, 942, 947
Thiocapsa, 823
Thiocystis violacea, 998, 1001
Thiodendron mucosum, 947. *See also Thiobacterium bovista*
Thiomicrospira, 941
Thiopedia rosea, 371
Thiophysa macrophysa, 945. *See also Achromatium volutans*
Thiophysa volutans, 945. *See also Achromatium volutans*
Thioploca, 941
Thiospira, 941–42, 947–48
Thiospira bipunctata, 942, 948
Thiospira winogradskyi, 942, 948
Thiospirillum winogradskii, 948. *See also Thiospira winogradskyi*
Thiosulfate oxidation, *Meiothermus*, 805
Thiosulfate reducing
  Chlorobiaceae, 368, 372
  Spirochaeta, 204, 206
Thiosulfate utilization, *Chloroflexus*, 831
Thiothrix, 941
Thiovulum, 941–42, 948–52
Thiovulum majus, 949
Thiovulum minus, 949. *See also Thiovulum majus*
Thiovulum muelleri, 949. *See also Thiovulum majus*
Thiovulumspora, 998
Threonine pathway, *Leptospira*, 298
Threonine utilization
  Fusobacterium, 1022
  Saprospira, 599
Thrombin production, *Porphyromonas*, 435
Tick-borne relapsing fever, *Borrelia*, 237–38, 240, 242, 260–62
Tick habitat
  Babesia, 279
  Borrelia, 235–36, 239–42, 252, 264–66, 269–75, 279
Tiger habitat, *Helicobacter*, 151, 161
Tobramycin resistance, *Flavobacterium*, 490–91, 545
Tomato plant habitat, *Flavobacterium*, 487
Tongue, *Porphyromonas*, 430
Tonsils, *Porphyromonas*, 430
Torulopsidosira filamentosa, 785
Toxothrix, 986–89
Toxothrix ferruginea, 986. *See also Toxothrix trichogenes*
Toxothrix trichogenes, 986–88
Trachoma, *Chlamydia*, 741, 746, 748–49
Transposon mutagenesis
  Bacteroides, 392–94, 411
  Elizabethkingia, 663
  Flavobacterium, 510
  myxobacteria, 67
  Porphyromonas, 440
  Sphingomonads, 618
Trees. *See also specific* trees
  Sphingomonas, 610
Trehalose production, *Myroides*, 635
Trehalose utilization. *See* Di- and trisaccharide utilization
Treponema, 195, 197, 211–27, 235, 244, 255, 320–22
Treponema amylovorum, 213–14
Treponema brennaborense, 213–14, 216, 222
Treponema bryantti, 213–14, 326, 345
Treponema calligyrum, 214
Treponema carateum, 211–12, 217
Treponema denticola, 197, 213–14, 217, 220, 222–24, 326, 342, 412, 428
Treponema hyodysenteriae. See Brachyspira hyodysenteriae; Brachyspira innocens
Treponema lecithinolyticum, 213–14, 219–20
Treponema maltophilum, 213–14, 217, 219–20, 222
Treponema minutum, 212–13, 222
Treponema pallidum, 197, 211, 214, 216–18, 223–26, 244, 246, 249, 261, 301, 326, 342, 713
Treponema pallidum endemicum, 211–12, 217
Treponema pallidum pallidum, 211–12, 215–18, 223
Treponema pallidum pertenue, 211–12, 214, 216–17, 226
Treponema paraluis-cuniculi, 211, 217
Treponema parvum, 213–14, 220
Treponema pectinovorum, 214, 220, 326
Treponema phagedenis, 212–14, 217, 220, 222–23
Treponema refringens, 212–14, 222–23
Treponema saccharophilium, 213–14, 971
Treponema skoliodontum, 213
Treponema socranskii, 213–14, 220, 224
Treponema socranskii buccale, 220
Treponema socranskii paredis, 220
Treponema socranskii socranskii, 220
Treponema succinifaciens, 213–14, 326, 345
Treponema vincentii, 213–14, 217, 220, 222, 224
Treponema ZAS-1, 322–26
Treponema ZAS-2, 322–26
Treponema ZAS-9, 322–26
Tributyrin hydrolysis, *Herpetosiphon*, 869
Tricarboxylic acid cycle
  Chlorobiaceae, 368–69, 372–73
  Chloroflexus, 830
  Leptospira, 298
  myxobacteria, 59
  Thermoleophilum, 847
Trichiasis, *Chlamydia*, 741, 748
Trichome, *Toxothrix*, 986–89
Trihydroxybenzenoids utilization, *Pelobacter*, 5, 7–8
Trimethoprim sensitivity/tolerance
  Campylobacter, 123
  Chryseobacterium, 667
  Elizabethkingia, 667–68
  Helicobacter, 153, 163
  Myroides, 633
  Ornithobacterium, 705
2,3,5-Triphenyltetrazolium chloride reduction, *Helicobacter*, 150, 152–53, 156, 162
Trospectomycin, *Bacteroides*, 411
Trout
  Chryseobacterium, 663–64
  Cytophagale, 561
  Flavobacterium, 502, 509, 512–17
Trovafloxacin, *Bacteroides*, 407–8, 411
Trypanosoma balbiani, 971. *See also Cristispira pectinis*
Trypticase soy broth agar (TSB agar)
  Capnocytophaga, 562, 563–64, 709–10
  Chryseobacterium, 646–47, 662
  Coenonia, 696, 699
  Elizabethkingia, 653, 662
  Empedobacter, 632
  Flavobacterium, 489–90, 494–95
  Myroides, 632
  Ornithobacterium, 696
  Riemerella, 696, 699
  Sphingomonads, 615
  Thermomicrobium, 850
TSB agar. *See* Trypticase soy broth agar
Tumor necrosis factor-α (TNF-α)
  Chlamydia, 746
  Porphyromonas, 434, 438, 442–43, 446–47
Tumors, *Bacteroides*, 402
Turbot habitat, *Chryseobacterium*, 663–64
Turkey habitat
  Borrelia, 279
  Chlamydia, 747
  Ornithobacterium, 462, 695–96, 703–5

Riemerella, 695, 703–4
Streptobacillus, 983
Turneria, 294
Turneria parva, 294
Turtle habitat
Chryseobacterium, 646
Elizabethkingia, 664
Tween
Aequorivita, 685
Bergeyella, 649
Chryseobacterium, 649
Elizabethkingia, 649
Psychroserpens, 683
Riemerella, 649
Thermus, 799
Tween utilization
Bergeyella, 535
Flavobacterium, 494–95
Leptospira, 299
Weeksella, 535
Tylosin, *Brachyspira*, 349
Typhlitis
Brachyspira, 345, 347–48
Helicobacter, 159
Tyrosine hydrolysis
Bergeyella, 649
Chloroflexus, 831
Chryseobacterium, 649
Elizabethkingia, 649
Myroides, 633
Riemerella, 649

U

Ubiquinol, *Aquifex*, 932
Ubiquinone
Novosphingobium, 609
Sphingobium, 609
Sphingomonads, 615
Sphingomonas, 605, 609
Sphingopyrix, 609
Ultramicrobacteria, 881, 888–92
Ulvibacter, 456, 463, 466, 471, 481
Ulvibacter litoralis, 458, 463, 467–68, 679
Ungulates. *See also specific* ungulates
Terponema, 222
Urea degradation
Bergeyella, 649
Chryseobacterium, 649
Elizabethkingia, 649
Flavobacterium, 494–96, 542
Riemerella, 649
Verrucomicrobium, 887
Urease activity
Aequorivita, 467, 685
Arenibacter, 467, 685
Bergeyella, 467, 534, 535
Campylobacter, 121, 125–26
Capnocytophaga, 467
Cellulophaga, 467, 685
Chryseobacterium, 467, 656, 701
Coenonia, 467, 701
Croceibacter, 467, 685
Cytophaga, 467, 685
Empedobacter, 467, 633
Flavobacterium, 467, 543–44
Formosa, 685
Fucobacter, 467
Gelidibacter, 468, 469, 685
Gillisia, 685
Helicobacter, 139, 141, 143, 148–67
Herpetosiphon, 869, 871
Maribacter, 685
Mesonia, 468, 469, 685
Muricauda, 468, 469, 685
Myroides, 468, 469, 633
Ornithobacterium, 468, 469, 701–2
Pasteurella, 701
Polaribacter, 468, 469, 685
Psychroflexus, 468, 469, 685
Psychroserpens, 468, 469, 685
Riemerella, 468, 469, 701
Robiginitalea, 685
Salegentibacter, 468, 469, 685
Tenacibaculum, 468, 469, 685
Ulvibacter, 468, 685
Vitellibacter, 468, 685
Weeksella, 469, 534, 535, 544
Zobellia, 469, 685
Urease production, *Verrucomicrobium*, 887
Urethritis, *Chlamydia*, 751
Urine
Borrelia, 241
Empedobacter, 631
Flavobacterium, 541
Leptospira, 305, 307, 310
Myroides, 631
Sphingomonas, 610–11, 621
Streptobacillus, 983
Weeksella, 463, 533
Urogenital disease, *Chlamydia*, 749–51
Urogenital tract
Campylobacter, 122
Chlamydia, 746–47, 749–51
Uterine mucosa, *Campylobacter*, 122
U-tube method, *Treponema*, 221
UV light resistance, myxobacteria, 82

V

Vaccines
Chlamydia, 746
Coenonia, 703
Flavobacterium, 503, 514–15, 518
Leptospira, 308–9
Ornithobacterium, 706
Porphyromonas, 439
Riemerella, 705
Valine fermentation
Chloroflexus, 831
Leptospira, 298
Saprospira, 599
Spirochaete, 206, 208
Vancomycin resistance/sensitivity
Campylobacter, 123
Chryseobacterium, 667–68
Elizabethkingia, 667–68
Fusobacterium, 1020
Hymenobacter, 728
Leptospira, 304
myxobacteria, 42
Thermonema, 724
Vegetative cell
Cytophagale, 576
myxobacteria, 31–32, 42, 51, 57–60, 65–67, 76–77, 80, 82, 84
Veillonella, 1021
Veillonella alcalescens, 955
Veillonella parvula, 955
Verrucomicrobia, 881–94
Verrucomicrobium, 881–82, 885–88
Verrucomicrobium spinosum, 881–82, 885, 887
Verruco spinostum, 759
Viable counting, myxobacteria, 50–51
Vibrio cholera, 131
Vibrio coli. See Campylobacter coli
Vibrio fetus. See Campylobacter fetus
Vibrio jejuni. See Campylobacter jejuni
Vibrio parahaemolyticus, 17–18
Vibrio succinogenes. See Wolinella succinogenes
Victivallis, 881, 893–94
Victivallis vadensis, 881, 883, 893–94
Virulence factors
Bacteroides, 400–406
Leptospira, 307–8
Porphyromonas, 433–39
Vitamin B_{12} requirement
Bacteroides, 383, 390
Chlorobiaceae, 361, 367
Haliscomenobacter, 604
myxobacteria, 56
Vitellibacter, 456, 463, 466, 471, 481, 685
Vitellibacter vladivostokensis, 463, 467–68, 679–80, 697
Vitreoscilla, 855
Vulcanitherums, 797, 807–9
Vulcanitherums mediatlanticus, 798, 808–9

W

Weeksella, 456, 463, 466, 470, 473, 488, 532–36, 539–45, 632, 641, 645, 655
Weeksella virosa, 458, 463, 468–69, 471, 532–33, 535–36, 540–41, 543–44, 639, 641, 657, 664, 680, 697
Weeksella zoohelcum. See Bergeyella zoohelcum
Weils disease, *Leptospira*, 294, 305
Well environment
Gallionella, 990
Naumanniella, 1006
Siderobacter, 1006
Siderocapsa, 1005
Siderocapsaceae, 1007
Well-plate method, *Treponema*, 221
Western blot, *Borrelia*, 257
Whale, *Helicobacter*, 165

Wolbachia pipentis, 893
Wolinella, 119–20, 178–88
Wolinella curva. See Campylobacter curvus
Wolinella rectus. See Campylobacter rectus
Wolinella succinogenes, 178–88
Woodchuck, *Helicobacter*, 142, 164–65
Wood environment
  Chondomyces, 36
  Corallococcus, 36
  Haploangium, 36
  myxobacteria, 36–37, 40
  Myxococcus, 36
  Sorangium, 36–37
  Stigmatella, 36–37

X

Xanthomonas, 605
Xanthomonas amaranthicola, 874
Xanthomonas oryzae, 17, 26
Xiphinematobacter, 881, 892–93
Xiphinematobacter americani, 883, 893
Xiphinematobacter brevicoli, 883, 893
Xiphinematobacter rivesi, 883, 893
Xylan degradation
  Flavobacteriaceae, 470
  Flavobacterium, 501, 520
  Herpetosiphon, 869
  myxobacteria, 84
  Opitutus, 891
  Rhodothermus, 715, 722
  Spirochaeta, 207
  Ultramicrobacteria, 891
Xylan utilization, *Bacteroides*, 384–86, 398, 414
m-Xylene catabolism, Sphingomonads, 619
Xylose utilization. *See* Monosaccharide utilization

Y

Yaws, 211–12, 224–26
Yeast extract
  Fusobacterium, 1023
  Propionigenium, 957
  Thermomicrobium, 850

Z

Zobellia, 456, 463, 466, 468–69, 471, 481, 484, 679, 683
Zobellia galactanivorans, 458, 463, 679–80, 688, 697
Zobellia uliginosa, 463, 679–81
Zoogloea, 960–68
Zoogloea filipendula, 960, 963, 965–66
Zoogloeal matrix
  Caulococcus, 997
  Zoogloea, 960–63, 966
Zoogloea ramigera, 960–68