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*Editors*

# The Prokaryotes

## Prokaryotic Biology and Symbiotic Associations

*Fourth Edition*

Springer Reference

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With 183 Figures and 62 Tables

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# 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 11 volumes (planned) 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 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. The 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, that is, 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!

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# Preface

During most of the twentieth century, microbiologists studied pure cultures under defined laboratory conditions in order to uncover the causative agents of disease and subsequently as ideal model systems to discover the fundamental principles of genetics and biochemistry. Microbiology as a discipline onto itself, e.g., microbial ecology, diversity, and evolution-based taxonomy, has only recently been the subject of general interest, partly because of the realization that microorganisms play a key role in the environment. The development and application of powerful culture-independent molecular techniques and bioinformatics tools has made this development possible. The fourth edition of *the Handbook of the Prokaryotes* has been updated and expanded in order to reflect this new era of microbiology.

The first five volumes of the fourth edition contain 34 updated and 43 entirely new chapters. Most of the new chapters are in the two new sections: Prokaryotic Communities and Bacteria in Human Health and Disease. A collection of microorganisms occupying the same physical habitat is called a "community," and several examples of bacterial communities are presented in the Prokaryotic Communities section, organized by Edward F. DeLong. Over the last decade, important advances in molecular biology and bioinformatics have led to the development of innovative culture-independent approaches for describing microbial communities. These new strategies, based on the analysis of DNA directly extracted from environmental samples, circumvent the steps of isolation and culturing of microorganisms, which are known for their selectivity leading to a nonrepresentative view of prokaryotic diversity. Describing bacterial communities is the first step in understanding the complex, interacting microbial systems in the natural world.

The section on Bacteria in Human Health and Disease, organized by Stephen Lory, contains chapters on most of the important bacterial diseases, each written by an expert in the field. In addition, there are separate general chapters on identification of pathogens by classical and non-culturing molecular techniques and virulence mechanisms, such as adhesion and bacterial toxins. In recognition of the recent important research on beneficial bacteria in human health, the section also includes chapters on gut microbiota, prebiotics, and probiotics. Together with the updated and expanded chapter on Bacterial Pharmaceutical Products, this section is a valuable resource to graduate students, teachers, and researchers interested in medical microbiology.

Volumes 6–11, organized by Erko Stackebrandt and Fabiano Thompson, contain chapters on each of the ca. 300 known prokaryotic families. Each chapter presents both the historical and current taxonomy of higher taxa, mostly above the genus level; molecular analyses (e.g., DDH, MLSA, riboprinting, and MALDI-TOF); genomic and phenetic properties of the taxa covered; genome analyses including nonchromosomal genetic elements; phenotypic analyses; methods for the enrichment, isolation, and maintenance of members of the family; ecological studies; clinical relevance; and applications.

As in the third edition, the volumes in the fourth edition are available both as hard copies and e-books, and as eReferences. The advantages of the online version include no restriction of color illustrations, the possibility of updating chapters continuously and, most importantly, libraries can place their subscribed copies on their servers, making it available to their community in offices and laboratories. The editors thank all the chapter authors and the editorial staff of Springer, especially Hanna Hensler-Fritton, Isabel Ullmann, Daniel Quiñones, Alejandra Kudo, and Audrey Wong, for making this contribution possible.

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**Eugene Rosenberg** holds a Ph.D. in biochemistry from Columbia University (1961) where he described the chemical structures of the capsules of *Hemophilus influenzae*, types B, E, and F. His postdoctoral research was performed in organic chemistry under the guidance of Lord Todd in Cambridge University. He was an assistant and associate professor of microbiology at the University of California at Los Angeles from 1962 to 1970, where he worked on the biochemistry of *Myxococcus xanthus*. Since 1970, he has been in the Department of Molecular Microbiology and Biotechnology, Tel Aviv University, as an associate professor (1970–1974), full professor (1975–2005), and professor emeritus (2006–present). He has held the Gol Chair in Applied and Environmental Microbiology since 1989. He is a member of the American Academy of Microbiology and European Academy of Microbiology. He has been awarded a Guggenheim Fellowship, a Fogarty International Scholar of the NIH, the Pan Lab Prize of the Society of Industrial Microbiology, the Proctor & Gamble Prize of the ASM, the Sakov Prize, the Landau Prize, and the Israel Prize for a "Beautiful Israel."

His research has focused on myxobacteriology; hydrocarbon microbiology; surface-active polymers from *Acinetobacter*; bioremediation; coral microbiology; and the role of symbiotic microorganisms in the adaptation, development, behavior, and evolution of animals and plants. He is the author of about 250 research papers and reviews, 9 books, and 16 patents.

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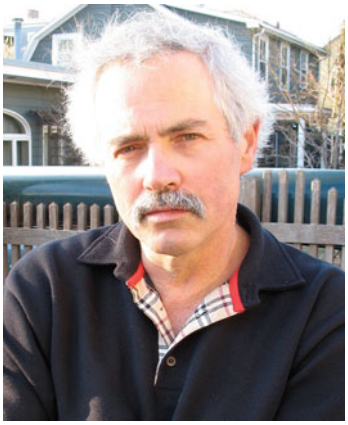
**Edward DeLong** received his bachelor of science in bacteriology at the University of California, Davis, and his Ph.D. in marine biology at Scripps Institute of Oceanography at the University of California, San Diego. He was a professor at the University of California, Santa Barbara, in the Department of Ecology for 7 years, before moving to the Monterey Bay Aquarium Research Institute where he was a senior scientist and chair of the science department, also for 7 years. He now serves as a professor at the Massachusetts Institute of Technology in the Department of Biological Engineering, where he holds the Morton and Claire Goulder Family Professorship in Environmental Systems. DeLong's scientific interests focus primarily on central questions in marine microbial genomics, biogeochemistry, ecology, and evolution. A large part of DeLong's efforts have been devoted to the study of microbes and microbial processes in the ocean, combining laboratory and field-based approaches. Development and application of genomic, biochemical, and metabolic approaches to study and exploit microbial communities and processes is his another area of interest. DeLong is a fellow in the American Academy of Arts and Science, the U.S. National Academy of Science, and the American Association for the Advancement of Science.



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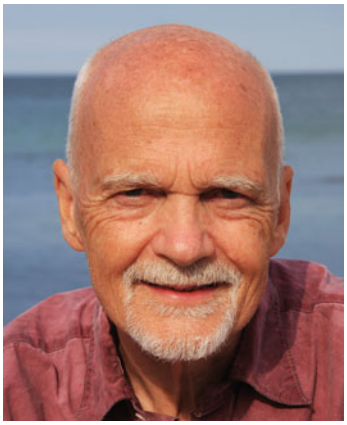
**Stephen Lory** received his Ph.D. degree in Microbiology from the University of California in Los Angeles in 1980. The topic of his doctoral thesis was the structure-activity relationships of bacterial exotoxins. He carried out his postdoctoral research on the basic mechanism of protein secretion by Gram-negative bacteria in the Bacterial Physiology Unit at Harvard Medical School. In 1984, he was appointed assistant professor in the Department of Microbiology at the University of Washington in Seattle, becoming full professor in 1995. While at the University of Washington, he developed an active research program in host-pathogen interactions including the role of bacterial adhesion to mammalian cells in virulence and regulation of gene expression by bacterial pathogens. In 2000, he returned to Harvard Medical School where he is currently a professor of microbiology and immunobiology. He is a regular reviewer of research projects on various scientific panels of governmental and private funding agencies and served for four years on the Scientific Council of Institute Pasteur in Paris. His current research interests include evolution of bacterial virulence, studies on post-translational regulation of gene expression in *Pseudomonas*, and the development of novel antibiotics targeting multi-drug-resistant opportunistic pathogens.



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**Erko Stackebrandt** holds a Ph.D. in microbiology from the Ludwig-Maximilians University Munich (1974). During his postdoctoral research, he worked at the German Culture Collection in Munich (1972–1977), 1978 with Carl Woese at the University of Illinois, Urbana Champaign, and from 1979 to 1983 he was a member of Karl Schleifer's research group at the Technical University, Munich. He habilitated in 1983 and was appointed head of the Departments of Microbiology at the University of Kiel (1984–1990), at the University of Queensland, Brisbane, Australia (1990–1993), and at the Technical University Braunschweig, where he also was the director of the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (1993–2009). He is involved in systematics, and molecular phylogeny and ecology of Archaea and Bacteria for more than 40 years. He has been involved in many research projects funded by the German Science Foundation, German Ministry for Science and Technology, and the European Union, working on pure cultures and microbial communities. His projects include work in soil and peat, Mediterranean coastal waters, North Sea and Baltic Sea, Antarctic Lakes, Australian soil and artesian wells, formation of stromatolites, as well as on giant ants, holothurians, rumen of cows, and the digestive tract of koalas. He has been involved in the description and taxonomic revision of more than 650 bacteria taxa of various ranks. He received a Heisenberg stipend (1982–1983) and his work has been awarded by the Academy of Science at Göttingen, Bergey's Trust (Bergey's Award and Bergey's Medal), the Technical University Munich, the Australian Society for Microbiology, and the American Society for Microbiology. He held teaching positions in Kunming, China; Budapest, Hungary; and Florence, Italy. He has published more than 600 papers in refereed journals and has written more than 80 book chapters. He is the editor of two Springer journals and served as an associate editor of several international journals and books as well as on national and international scientific and review panels of the German Research Council, European Science Foundation, European Space Agency, and the Organisation for Economic Co-Operation and Development.

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**Fabiano Thompson** became the director of research at the Institute of Biology, Federal University of Rio de Janeiro (UFRJ), in 2012. He was an oceanographer at the Federal University of Rio Grande (Brazil) in 1997. He received his Ph.D. in biochemistry from Ghent University (Belgium) in 2003, with emphasis on marine microbial taxonomy and biodiversity. Thompson was an associate researcher in the BCCM/LMG Bacteria Collection (Ghent University) in 2004; professor of genetics in 2006 at the Institute of Biology, UFRJ; and professor of marine biology in 2011 at the same university. He has been a representative of UFRJ in the National Institute of Metrology (INMETRO) since 2009. Thompson is the president of the subcommittee on the Systematics of Vibrionaceae–IUMS and an associate editor of *BMC Genomics* and *Microbial Ecology*. The Thompson Lab in Rio currently performs research on marine microbiology in the Blue Amazon, the realm in the southwestern Atlantic that encompasses a variety of systems, including deep sea, Cabo Frio upwelling area, Amazonia river-plume continuum, mesophotic reefs, Abrolhos coral reef bank, and Oceanic Islands (Fernando de Noronha, Saint Peter and Saint Paul, and Trindade).

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# Biology of Bacteria and Archaea

# 1 How We Do, Don't, and Should Look at Bacteria and Bacteriology

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## Background

Microbiology today has a new-found wealth far greater than any it possessed before. The source of that wealth is the universal phylogenetic tree—the framework essential for understanding organismal relationships. The power that flows from phylogenetic ordering permeates the field. Microbiologists now accomplish with ease things that were previously impossible and approach bacteria in ways that 20 years ago were unthinkable. Microbial ecology is no longer the *faux* ecology it had been—when defining a niche in organismal terms was not an option. Today, the field rests on a par with plant and animal ecology and exceeds them in importance, for it is in the microbial realm that the base and fount of the global ecosystem lie. Studying microbial diversity used to be the equivalent of hunting through antique shops for curios—which resulted in a collection of species no more connected to one another than the items in a bower bird's nest. Now all organisms sit on the well-ordered tips of branches on the universal phylogenetic tree (Woese 1987; Olsen et al. 1994), and the study of one, far from being an isolated adventure, can contribute to the study of all. An interest in bacterial evolution used to be perceived as metaphysical and worthless. Today, evolutionary relationships are the foundation and motive force behind a new and resurgent microbiology and hence biology as a whole.

Microbial genomes can be sequenced today in their entirety, and when measured against the information gained, the cost of

To try to plan for the future without a sense of the past is like trying to plant cut flowers.
(Daniel Boorstein)

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so doing is actually small—and that cost is continually decreasing. An exploration of a given bacterium can now start with knowledge of all of its genes. Not only are we (potentially) privy to the full range of the organism's biochemistry, but we possess a partial but very useful record of its evolutionary past. This historical record, it turns out, extends so far back into biological antiquity that the study of bacterial evolution fuses with the study of the origins of modern cells (Woese 2002).

Unfortunately, the universal phylogenetic tree, bacteriology's essential framework, arrived rather late in the course of events. Its belated arrival adversely affected not only the development of microbiology but that of all biology (see below). Consequently, the introductory chapter to the third edition of *The Prokaryotes* is largely devoted to understanding the historical development of bacteriology, for in its twists and turns lie insights into much of what microbiology and biology are today, as well as a guide to their future development.

The editors of the second edition of *The Prokaryotes* wisely decided to give the volume phylogenetic underpinnings (as best they could at the time). In this third edition, the trend continues, and the book's phylogenetic bent is more pronounced. Bacterial taxonomy, which only two decades ago was a dry subject whose main if not only purpose was determinative classification, identification of species, has now blossomed into an intriguing, meaty study in its own right. As you use this third edition of *The Prokaryotes*, realize that the book is not merely a manual for identification, cultivation, and determinative classification. Use it for what it really is, a long overdue treatise on comparative bacteriology. If you let your imaginations follow the evolutionary trails the book lays out, they will lead you to the edge of microbiology's future. Most of all, let this work by its structure open your eyes to the emerging world of bacterial evolution and the challenge that poses to all of biology. This is not the evolution that Darwin and the classical evolutionists had in mind. It is centered on physiologies rather than forms, on molecules rather than gross morphologies. And it extends the scientific reach much further into the past than was ever before possible. But to begin charting the future, we need a map of the past. As in so many walks of life, a knowledge of history is the best guide we have.

## Microbiology's Halting Development

Prior to its phylogenetic liberation, microbiology had long been mired down conceptually, sinking ever deeper as twentieth-century biology unfolded. This descent into innocence started long before that, however, when microbiologists failed to develop a phylogenetic, or "natural," classification system for bacteria based upon the classical characteristics available to them. As a consequence, bacteriology's development became increasingly one-sided, so much so that from an organismal perspective, the discipline developed not at all. The condition was chronic, progressing over several generations of microbiologists, and so, was scarcely felt, and to the extent that it was felt, the condition was accepted as normal.

One of the rare (if not only) times the problem was brought to the fore was in 1962 when Stanier and van Niel lamented: ". . .the abiding intellectual scandal of bacteriology has been the absence of a clear concept of a bacterium." Unfortunately, the authors' suggested remedy for the malaise was untenable, as we shall see, and as a consequence served to encourage the unfortunate changeover from the name "bacteriology" to "microbiology," a change that dogs microbiology to this day. A label that had denoted the study of what seemed a naturally defined grouping of organisms, "bacteriology," was replaced by one that encompassed an organismal *potpourri*. One can in principle have a biologically valid, unified concept of "bacteria," but a comparable unifying concept of the artificial grouping "microorganisms" is impossible. The semantic thimblerig involved in this name change was one of the factors that helped to push the problem of a "concept of a bacterium" off the table. From a basic scientific perspective, bacteriology in 1992 was worse off than it had been in 1962. But by 1992, bacteriology had a powerful remedy for the situation.

## What Is Bacteriology?

Whatever else it is, bacteriology is first and foremost an *organismal science*, just as are zoology and botany. As such, its focus is understanding particular naturally defined groupings of organisms. A critical difference between bacteriology and the other two organismal sciences, however, is that the other two could develop as organismal sciences in the absence of molecular characterizations, whereas bacteriology could not. Bacterial morphologies are too simple and variable to have much phylogenetic significance, but phylogeny is embedded in the complex morphologies and variations on morphological themes characteristic of animal and plant life. In the bacterial world, it is physiology (not morphology) that predominates and distinguishes. But, physiological characteristics have so far proven phylogenetically intractable. Whereas among eukaryotes, organization manifests itself predominately in structure, organization in the bacterial world occurs mainly in the shifting and subtle biochemical states of the system—something the eye cannot directly apprehend. In the absence of a knowledge of phylogenetic relationships, a concept of bacteria was unattainable.

What exactly is an organismal science? Simply put, one that seeks to understand naturally defined groups of organisms in *biological terms*. Such an understanding has four principal components: (1) structure/function—how the organisms in the group are built (organized) and how they work; (2) diversity—how many and what kinds of organisms the group comprises, and the ways in which the various kinds are similar and different; (3) ecology—how the organisms interact with their environments (including other organisms); and (4) evolution—the origins of the group and how the organisms therein are ancestrally related. This last, evolution, is what underlies and enables the development of a biological concept of a group. As Dobzhansky famously put it, ". . .nothing in biology makes sense except in the light of evolution" (Dobzhansky 1963). And it was the lack of this

essential evolutionary framework that prevented bacteriology from developing as an organismal science.

With no understanding of phylogenetic relationships, there can be no effective study of ecology or diversity. Ecological niches cannot be defined in organismal terms, and studies in bacterial diversity amount to no more than a catalog of disconnected vignettes. It is inconceivable that a zoologist or botanist could go into the field and not be able to distinguish the animals from the plants. Yet would-be microbial ecologists were in this exact position or worse all the time: they couldn't distinguish their "animals" from their "plants" or (with a few exceptions) from representatives of any others of the many kingdom-level bacterial taxa.

Blocked from developing into a full-fledged organismal science throughout most of the twentieth century, bacteriology wandered aimlessly, not knowing what it was, where it came from, or what it should become. The field was driven by the winds of scientific fad and other outside influences. Any development our science did undergo was confined to its structure/function aspect and its applied side. With exceptions too rare to impact, twentieth-century microbiologists (and biologists in general) remained unaware of microbiology's "identity crisis."

## Dumbing Down

What more than anything served to obscure bacteriology's structural problem was the molecular perspective dominating twentieth-century biology. Molecular biology embodies a reductionist fundamentalism that rules out any holistic perspective. The paradigm assumed that the age-old problem of biological organization would be automatically solved when comprehensive molecular parts-lists for cells were generated. Yet even with the parts-lists now in hand, the problem remains with us, awaiting a fresh, constructive, and holistic outlook. The molecular perspective took evolution for granted, found it intellectually wanting, and dismissed its study as trivial. In such a fundamentalist milieu, the organism per se has only a secondary existence, shadowy and temporal. For molecular biology, the organism lies essentially in its collective parts (which molecular biologists felt no compulsion to reassemble into a whole). Little wonder that under the molecular aegis, bacteriology felt no need to develop into an organismal science, even if it *could* have done so. Instead, microbiology followed molecular biology's lead and slipped into a mechanistic reductionism.

Much of the important biochemistry of the last century was done in bacterial systems. Bacteria—with their enormous population numbers, rapid growth rates, and general ease of handling—also proved well suited to many of the contemporary studies in genetics and molecular biology. In one sense, great progress occurred in microbiology over the last five or so decades. None of it, however, contributed to the development of a concept of bacteria.

## Bacteriology's Wandering Course

It is difficult to know where you are going if you don't know where you came from. And if you don't know where you are going, the saying goes, any path will take you there. The future course of bacteriology (microbiology) is not something that can be left to the vagaries of chance and necessity. How bacteriology now develops is a matter of utmost concern. *The future of all biology turns upon it.*

Although Leeuwenhoek discovered the microbial world during the time of Newton, microbiology did not emerge as an effective science until two centuries later. In the last half of the nineteenth century, the works of scientists like Cohn, Koch, and Pasteur laid the groundwork. And with the next generation of microbiologists, the Beijerincks, Winogradskys, and Orla-Jensens, the study of microorganisms began in earnest. The grouping of microbiologists that grew around Beijerinck and then Kluyver, known as the Delft School, became the dominant influence in microbiology for most of the twentieth century. Among the school's members, one finds articulate spokesmen with an overview of their discipline. So, it is to the Delft School that we mainly turn to learn the early history of microbiology.

Martinus Beijerinck is the actual founder of the Delft School, although Leeuwenhoek, who had lived in Delft, is portrayed as its spiritual father (van Niel 1949). Beijerinck is credited, along with Winogradsky, with the development of enrichment culturing, through which methodology microbiologists could begin a major exploration of the nature and scope of the microbial world. Beijerinck's contributions were many and varied (van Iterson et al. 1940): he added greatly to the understanding of bacterial physiology and diversity. Through his studies on iron bacteria and the like, he was one of the first to appreciate the intimate role bacteria play in geologic processes. He was keenly aware of developments in genetics (in the higher forms) and was perhaps first to suggest that some of the variation seen in the microbial world was of mutational origin. His discovery of a filterable factor associated with tobacco mosaic disease made him a pioneer in virology. And he was keenly aware of Darwin. How did this enlightened Dutch scientist view the study of microorganisms? On the occasion of his being awarded the Leeuwenhoek Medal (microbiology's highest and his most prized honor), Beijerinck addressed the question:

> [M]icrobial ecology. . .is the most necessary and fruitful direction to guide us in organizing our knowledge of that part of nature which deals with the lowest limits of the organic world, and which constantly keeps before our minds the profound problem of the origin of life itself (Beijerinck 1905; van Iterson et al. 1940; translated by van Niel 1949).

This is a sophisticated view of microbiology. The importance of the microbe-environment relationship is foremost, with (microbial) evolution constantly in the background, shaping and deepening the outlook. To me, Beijerinck's concept of the microbial world seems deeper and more holistic than any that followed until the present day, when the realities of genomics are bringing us once more to a similarly broad and inspiring perspective on bacteria and their world.

In this quote (and in his studies), Beijerinck did not (to my knowledge) speak to the importance of determining the natural relationships among microorganisms. It is possible he did not

believe (as others of his day did not) that bacteria evolved in a Darwinian fashion, as animals and plants do. I would prefer to believe, however—especially given his familiarity with Darwin's writings (van Iterson et al. 1940)—that Beijerinck saw no way to determine these relationships given the primitive state of bacteriology in his day. Only with the later accumulation of large numbers of bacterial species (mainly through enrichment culturing) would the problem of their classification become a choking one, and only then, when traditional taxonomic approaches failed to yield a satisfactory (natural) classification system, would the problem become genuinely acute (Stanier and van Niel 1962).

Beijerinck's successor at Delft was Albert Jan Kluyver. A brilliant and sophisticated scientist, Kluyver was by training a biochemist, not a microbiologist. Only upon assuming Beijerinck's chair did he transform himself into the latter (Kamp et al. 1959). It is in Kluyver that I see the beginning of bacteriology's drift away from striving to become an organismal discipline. Kluyver is noted, and rightly so, for his fundamental, unifying contributions to biochemistry (Kluyver and Donker 1926; Kluyver 1931). He pioneered what he called "comparative biochemistry," a field he envisioned as ". . .benefit[ing] biochemistry in a manner similar to that in which the concept of "comparative anatomy" had helped to bring order into [anatomy]" (Kluyver 1931; van Niel 1949). Yet in one way, the analogy deceives. Comparative anatomy is basically an organismal, evolutionary pursuit. Comparative biochemistry is not. The latter is simply a way to bring some chemical order to the plethora of biochemistries that abound in nature. Kluyver's simile here veils the important distinction between the organism and its parts. My assertion that Kluyver represented (or came to represent) the biochemical dissection of bacteria rather than an organismal comprehension thereof finds indirect support in van Niel's historical account of the Delft School, which details Kluyver's contributions to biochemistry while failing once to mention Kluyver's (and his own) concerns with developing a natural bacterial classification (van Niel 1949).

## The Gordian Knot of Bacterial Classification

The Linnaean system had proven extremely useful in structuring our knowledge of zoology and botany, making both into respectable organismal sciences even before Darwin's time. Darwin's theory did not change the Linnaean classification of animals and plants all that much; it merely provided it theoretical justification (Darwin 1859).

Early microbiologists were aware of the benefits of "natural" classification. Yet I have never satisfied myself as to the degree of their commitment to or the depth of their appreciation of evolution. Was a natural system merely the most useful classification, or were bacteria given the evolutionary significance Beijerinck (quoted above) seemed to accord them? Clearly, most microbiologists of the time simply wanted some kind, any kind, of useful pigeonholing system. *Bergey's Manual*, a (much criticized though popular) determinative system, provided precisely that (Stanier and van Niel 1941).

Kluyver and van Niel's classic paper of 1936 on bacterial classification represents the Delft School outlook. The paper was a reasoned discussion of the problems faced in contemporary bacterial classification, in the context of which the authors then proposed a system of their own, which they hoped would overcome some of the problems and, so, move bacteriology closer to a genuine natural bacterial system. At the time, classifications were largely morphological and their purpose for the most part utilitarian. A salient exception to these approaches had been the system of Orla-Jensen (see Kluyver and van Niel 1936), which was based on an evolutionary conjecture, namely, that the first organisms to evolve were necessarily autotrophs (Oparin and his ocean had yet to enter the picture). Like Beijerinck, Orla-Jensen appeared to believe that microorganisms held the key to understanding life's origin, and he constructed a classification that hopefully would help to bring out the origin of metabolism. At this time, Kluyver and van Niel seemed to share Beijerinck's belief in the importance of bacteria as evolutionary beacons; at least they said: "A true reconstruction of the course of evolution is the ideal of every taxonomist" (Kluyver and van Niel 1936).

In the Kluyver-van Niel article, one readily senses bacteriology's ongoing struggle regarding the bases upon which to develop bacterial classification. Because zoological and botanical classifications are morphologically based, a strong precedent existed for putting bacterial classification on a similar footing—despite the fact that microbiologists intuitively knew that bacteria are as fundamentally physiological as animals and plants are morphological. On what levels, to what extent, in what ways, then, are the morphological and various physiological properties of bacteria taxonomically significant? In the following, we see Kluyver and van Niel worry the problem (Kluyver and van Niel 1936, pp. 370, 371):

> The question then arises in which [bacterial] characters phylogeny expresses itself. There is no doubt that in this respect morphology remains the first and most reliable guide . . .[although] the indispensability of physiological characters for the purpose of classification has also been generally accepted....
>
> A lack of insight in the fundamentals of metabolism has thus far been the great stumbling block for a rational application of physiological characteristics in taxonomy, and it also explains the horror with which many systematicians have witnessed their ever increasing use.... The fundamental nature of the energy providing processes justifies the view that they should be rated first amongst the physiological characters suitable for classification....

Because the classification sought was a natural one, the kinds of choices Kluyver and van Niel were entertaining required the answers to certain evolutionary questions. But there were no a priori answers—even hints of them. Natural classification then had to rest on a foundation of conjecture—bolstered by the hope that if the scheme were anywhere near phylogenetically valid, it could bootstrap itself into a true natural classification. (Only today is knowledge of evolutionary relationships independent of [and so can precede the construction of] a taxonomy rather than follow from it.)

There is little point in detailing Kluyver and van Niel's attempt at a natural classification. But since the tenor of their thinking is so instructive as to the microbiological gestalt of their day, it is worth sampling a bit more. After debating the relative significance and utility of morphological and physiological characteristics in bacterial classification, the authors (tentatively) opt for "...the use of morphological criteria as [the] main guiding principle...above the rank of genera" (Kluyver and van Niel 1936).

It is basic to biological thought that the complex tends to evolve from the simple, and given the presumed primitive nature of bacteria, Kluyver and van Niel carried this notion to the extreme:

- It seems acceptable that the diversity of bacterial forms is the outcome of various independent morphological evolutions which have had their starting-point in the simplest form both existent and conceivable: the sphere (Kluyver and van Niel 1936, p. 387).

From this starting assumption, they picture the aboriginal spherical bacterium as (somehow) giving rise to four basic morphological types: (1) coccoid (the Micrococcaceae), (2) cells with polar flagella (Pseudomonadaceae), (3) cells with peritrichous flagella (Bacteriaceae), and (4) a nonmotile line whose beginnings are streptococci. Each of the four primary lineages in turn evolves in a quasi-ontogenetic fashion through stages of increasing complexity to some "highest stage of development," for example, the coccoid forms giving rise to Sarcinae (packets of cocci), and hence Sarcinae that form spores, or the "streptococcal" lineage developing through short Gram-positive rods to mycobacteria and ultimately the complex actinomycetes (Kluyver and van Niel 1936).

Within each main line of morphological descent, morphology increasingly gives way to physiological characters in defining the lower taxonomic levels (Kluyver and van Niel 1936). In retrospect, unfortunately, bacterial classification in the early twentieth century comes to be little more than a tapestry of *Just So Stories*.

## The Beginning of the End

We need to consider one final attempt to devise a natural bacterial system, which was proposed by Stanier and van Niel 5 years after the Kluyver and van Niel system (Stanier and van Niel 1941). Again our reason for doing so does not lie in the system itself but in the discussion that accompanied it. Theirs was the last blush of enthusiasm among microbiologists for developing a comprehensive natural bacterial classification, and the last time that having an evolutionary overview of the bacteria of any kind was vigorously defended (Stanier and van Niel 1941).

Bacterial taxonomists had long been split into two camps: the *idealists*, represented especially by members of the Delft School, and the *realists*, represented by the majority of other bacteriologists, for example, the board of *Bergey's Manual* (Breed 1939; Stanier and van Niel 1941). The overarching perspective of the idealists recognized the importance to the discipline's future of a phylogenetically based taxonomy. The realists' practical perspective was content with the intellectual pauper's gruel provided by a taxonomy merely enabling species identification and a convenient pigeonholing system. Stanier and van Niel deplored this mercantile mentality (Stanier and van Niel 1941):

- In most biological fields it is considered a truism to state that the only satisfactory basis for the construction of a rational system of classification is the phylogenetic one. Nevertheless, "realistic" bacteriologists show a curious aversion to the attempted use of phylogeny in bacterial systematics.... To what may we ascribe this distrust of phylogeny? In part it is undoubtedly due to the unsatisfactory nature of certain systems, purportedly based on phylogeny, which have been proposed in the past. However, the mere fact that a particular phylogenetic scheme has been shown to be unsound by later work is not a valid reason for total rejection of the phylogenetic approach.

The authors then turn to the drawbacks of the alternative:

- ...there is good reason to prefer an admittedly imperfect natural system to a purely empirical one. A phylogenetic system has at least a rational basis, and can be altered and improved as new facts come to light; its very weaknesses will suggest the type of experimental work necessary for improvement. On the other hand, an empirical system is largely unmodifiable because the differential characters employed are arbitrarily chosen and usually cannot be altered to any great extent without disrupting the whole system... [When the] wide separation of closely related groups caused by the use of arbitrary differential characters...makes it impossible to tell with certainty in what order a given organism belongs, an empirical system loses its value....

This is incisive commentary! But unfortunately, it is just about the last one hears the idealist perspective, the last time the attitude "if it can't be solved today, then we will try anew tomorrow," is expressed.

That final time seems to be 1946, when van Niel gave a major address concerning bacterial classification at that year's memorable postwar Cold Spring Harbor Symposium. In the address, he analyzed in detail the failures of current and past attempts at natural bacterial classification. He pointed out the difference between the successful use of morphological characters in plant and animal classification and their lack of utility in the bacterial case (as mentioned above). He detailed the frustrations bacteriologists had experienced in trying to use physiological characteristics for classificatory purposes, concluding that there is no way one can determine natural relationships in the bacterial world as things then stood (van Niel 1946). But at the end of this depressing critique, van Niel held firm to the idealist position: "[Thus, since] the morphology of a bacterium is of no more use in [classification] than is its physiology...the search for a basis upon which a 'natural system' can be constructed must continue" (van Niel 1946, p. 290).

## Where to Play? The Sand Castles Have All Been Washed Away

The year was 1955 when van Niel returned for the last time (to my knowledge) to the subject of natural bacterial classification (van Niel 1955). By that time, biochemists, following Kluyver's pioneering synthesis of microbial biochemistry, had turned their attentions in earnest to bacterial systems; sexual recombination had been discovered in bacteria by Lederberg and Tatum (1946), and geneticists were poised to take advantage, and (as mentioned) molecular biologists too were finding bacterial systems highly suitable for their studies. The emphasis in bacteriology was definitely shifting strongly in the reductionist structure/function direction. And van Niel returned to his topic, with a changed, jaded outlook.

Van Niel's still hopeful (and scientifically proper) perspective of 1946—"it hasn't been done yet, so we must continue"—had now become the pessimistic (and scientifically unacceptable)—"it hasn't been done yet, so it can't be done," which he expressed this way:

- What made Winogradsky. . .grant that the systematics of plants and animals on the basis of the Linnean system is defensible, while contending that a similar classification of bacteria is out of the question? The answer must be obvious to those who recognize in the former an increasingly successful attempt at reconstructing a phylogenetic history of the higher plants and animals. . .and who feel that comparable efforts in the realm of the bacteria (and bluegreen algae) are doomed to failure because it does not appear likely that criteria of truly phylogenetic significance can be devised for these organisms (van Niel 1955, pp. 101–102).

The idealists were now in full retreat and in the process took an untenable fallback position, namely, that the concept of a bacterium could be developed *without* resort to phylogenetic relationships. It could be developed simply by knowing the structure/function differences between eukaryotes and "prokaryotes." A little thought shows the folly in this. As emphasized above, an organismal science *must* be founded on evolutionary relationships. Yet, that is far from what the next generation of microbiologists were taught. In *The Microbial World* first edition, 1957:

- An eminent contemporary bacteriologist, van Niel, who is noted for his taxonomic studies on several groups of bacteria, has expressed the opinion that it is a waste of time to attempt a natural system of classification for bacteria, and that bacteriologists should concentrate instead on the more humble practical task of devising *determinative keys* (Stanier et al. 1957, p. 296).

In *Archives of Microbiology*, 1962:

- It is now clear that among organisms there are two different organizational patterns of cells. . .the eucaryotic and procaryotic type. *The distinctive property of bacteria and blue-green algae is the procaryotic nature of their cells*.... The remaining pages of this essay will be devoted to a discussion of the essential differences between these two cell types, upon which rests our only hope of more clearly formulating a "concept of a bacterium" (Stanier and van Niel 1962, pp. 20–21).

In *The Microbial World* second edition, 1963:

- All [bacteria] share the distinctive structural properties associated with the procaryotic cell, and we can therefore safely infer a common origin for the whole group in the remote evolutionary past; we can also discern four principal sub-groups, blue-green algae, myxobacteria, spirochetes, and eubacteria, which seem to be distinct from one another.... Beyond this point, however, any systematic attempt to construct a detailed scheme of natural relationships becomes the purest speculation, completely unsupported by any sort of evidence. The only possible conclusion is, accordingly, that *the ultimate scientific goal of biological classification cannot be achieved in the case of bacteria* (Stanier et al. 1963, p. 409).

A similarly dark and contrived picture was painted in the third, 1970, edition of *The Microbial World* and carried through essentially unchanged into the fourth (Stanier et al. 1970, 1976).

## Here Comes the (Molecular) Cavalry

The sad irony in all this is that while the idealist position was in retreat, with defeat masquerading as victory, and while new recruits were taught not to look back, as the importance of a "concept of a bacterium" (and with it any interest in bacterial evolution) slipped from view, the ground for a genuine phylogeny of bacteria, which could transform the discipline, was simultaneously being prepared. In the early 1950s, Sanger had sequenced the first proteins (Sanger and Tuppy 1951; Sanger and Thompson 1953), and the notion that molecular sequence comparisons were a rich source of evolutionary information was beginning to take hold:

- Biologists should realize that before long we shall have a subject which might be called "protein taxonomy"—the study of amino acid sequences of proteins of an organism and the comparison of them between species. It can be argued that these sequences are the most delicate expression possible of the phenotype of an organism and that vast amounts of evolutionary information may be hidden away within them (Crick 1958, p. 142).

However, among microbiologists Crick's prescience fell on deafened ears. The molecular and bacteriology paradigms were somehow on different conceptual planes. However, some "macrobiologists" did see the new molecular royal road to phylogeny (Zuckerkandl and Pauling 1965), and a cottage industry arose around confirming and (slightly) extending the classical phylogenetic tree through sequence comparisons of cytochrome *c*s and of a few other molecules (Fitch and Margoliash 1967). Yet the record shows that even among these first-generation molecular evolutionists, there was no recognition of the importance of the larger and far more important problem of using molecular sequence comparisons to infer

*bacterial* phylogenies, which would have opened the door to the universal phylogenetic tree.

Microbiologists did get on the molecular evolution bandwagon but belatedly and half-heartedly. In the main, their efforts relied upon simple and relatively uninformative methods, such as nucleic acid hybridization (Gillespie and Spiegelman 1965), and their interests were confined largely to weeding out misclassified species from genera and in properly grouping genera into families. A few protein sequences were attempted and superficially analyzed, but those responsible saw nothing of evolutionary value in the comparisons (Ambler et al. 1979a, b; Meyer et al. 1986)! The compelling vision of the idealists, the overriding concern with building a concept of bacteria on a comprehensive natural bacterial taxonomy, was gone. (Two prominent exceptions to the above characterization of microbiologists' attitudes toward bacterial taxonomy appear to be expressed by the Europeans J. De Ley and O. Kandler, in whose works and writings the candle of hope still flickered for a broad reaching, if not comprehensive, natural bacterial system [Heberlein et al. 1967; Schleifer and Kandler 1972].)

## Cleaving the Gordian Knot

It was only a matter of time before the sleeping giant of bacterial phylogeny would be roused by a dose of molecular medicine. By the mid-1960s Sanger's "oligonucleotide cataloging" method had come along (Sanger et al. 1965). By 1970, my laboratory was using it on ribosomal RNAs (Sogin et al. 1971; Woese et al. 1974). The universal distribution of rRNA, its large size, its high degree of sequence conservation, and a presumed refractoriness of rDNA to horizontal gene displacement argued that the molecule could be used to derive a universal phylogeny (Fox et al. 1977b; Woese 1987). By 1975, we had characterized about 30 rRNAs (mainly bacterial) by the method (Woese et al. 1975), a number that approached a 100 by the end of the decade. In the 1980s, newer and faster methods permitted effectively full sequencing of an rRNA molecule (Lane et al. 1985), which significantly sharpened branching orders in the universal tree. Today, the sequencing of rRNA (or rDNA) has become trivial, and the collection of rRNA sequences now numbers in the tens of thousands (Maidak et al. 2001).

The most important findings to come out of this new and revolutionary approach to bacterial taxonomy (and taxonomy in general) were that, yes, phylogenetic relationships among bacteria (distant or not) could be determined, and so, developing a clear concept of a bacterium was now feasible, second in importance only to the determining of the universal phylogenetic tree itself (Fox et al. 1980)—one of the great problems that the nineteenth century in biology bequeathed to the twentieth. As Darwin had said (Burkhardt and Smith 1990): "The time will come I believe, though I shall not live to see it, when we shall have very fairly true genealogical trees of each great kingdom of nature" (Darwin Corresp. 6:456). The discovery of the Archaea (Fox et al. 1977a; Woese and Fox 1977b) once and for all exploded the prokaryote/eukaryote phylogenetic myth. The universal tree shows prokaryotes to comprise not one, but two, unrelated major organismal groups (primary lines of descent), the Archaea and the Eubacteria. The new perception of microbial diversity that came out of the universal tree, in addition to the unexpected discovery of a third highest level grouping of organisms, found biology organizationally unprepared. Academic department structures, their organization and courses, did not and still do not reflect the new reality. Most textbooks do not do so adequately even today. And the funding priorities for governmental supported biology remain structured in a way that recognizes a bipartite, not tripartite, division of life.

## Reaching into the Unknown

While rRNA phylogenies put bacteriology on the road to becoming a full-fledged organismal discipline, they alone did not completely resolve bacterial ecology's core problem: a phylogenetic framework is a necessary but not sufficient condition for developing bacterial ecology. To identify a species, the bacterial ecologist has first to detect and isolate it. Proper identification of a bacterial species was firmly believed to require its cultivation (in pure culture) in the laboratory. Yet the vast majority of bacterial species in the typical niche go undetected, and among those that can be detected, most defy laboratory (pure) culture. (Just how severe a problem this was did not become apparent until relatively recently, well after the fact.)

But, contrary to established belief, isolation of bacteria in laboratory culture is not an absolute requirement for bacterial identification and classification. Since bacteria can now be identified and related merely through the sequence of one molecule, rRNA, only that molecule needs to be isolated from the environment to detect, identify, and classify organisms. This realization seems first to have occurred to Norman Pace, in the early 1980s. Over the next several years, he and his coworkers published a series of articles pointing out in principle and demonstrating in practice the new molecular sequence-based approach to microbial ecology (Stahl et al. 1985; Olsen et al. 1986). The crippling limitations that organismal detection and laboratory isolation had previously imposed on microbial ecology had vanished!

Pace and coworkers went further, showing that for certain purposes it was not even necessary to *isolate* a molecule (or its gene) from the environment: "phylogenetic stains" (sets of fluorescent nucleic acid probes for rRNA) could be developed to identify individual cells microscopically in situ *by species, genus, family*, etc. (DeLong et al. 1989). Using this powerful new methodology, a microbiologist could identify any and virtually all microbial species in a given niche in terms of phylogenetic relationships and morphology (and that, obviously, is only the beginning for such a methodology). At last, a genuine bacterial ecology is emerging! It is impossible to overestimate the importance of phylogeny and direct detection of genes to bacterial ecology's development.

## Perspective

Throughout its history, molecular biology has been guided in one way or another by "the problem of the gene," which basically defined twentieth-century biology. What would molecular biology have been like had the gene not been its focus? I ask this simply to provide perspective in posing a second question: what would bacteriology have been like if developing "a concept of a bacterium" *had* been its central focus and it had succeeded in doing so—allowing bacteriology to become a full-fledged organismal science?

Early on, microbiologists were not averse to asking evolutionary questions: are all bacteria at base specifically related to one another? Did bacterial evolution follow a course primarily laid out by morphological or physiological development? Were the first bacteria heterotrophs or autotrophs? Were they mesophiles or thermophiles? Are all photosynthetic bacteria related to one another to the exclusion of nonphotosynthetic species? How did the many diverse bacterial metabolisms come into being? How many times did this or that phenotypic feature evolve? Does this or that common feature signify relationship or convergent evolution? Unfortunately, all such questions (necessarily) were answered only by (untestable) conjectures—not a very reliable foundation upon which to base a concept of bacteria. A fabric of conjecture soon comes to be viewed as metascience (Stanier 1970), and this has the effect (most unfortunate in microbiology's case) of squelching inquiry. In this way, bacteriology came to abandon its evolutionary curiosity—much to its and biology's detriment.

Just as unsubstantiated conjecture leads to stifling curiosity, phylogenetic knowledge would have done the opposite. Fairly reliable responses to the early microbiologists' evolutionary musings would have led to the asking of new, more detailed questions. Had a reasonable natural bacterial classification come on the scene early enough, the spirit of evolutionary inquiry would not have withered but intensified, and a genuine concept of bacteria would naturally have followed. The development of all twentieth-century biology would have been different, retaining to some extent a focus on the organism as a whole and its evolution. Ah well....

## Archaea and Eubacteria

As discussed above, the character of a prokaryotic type of cell is expressed far more in physiology (dynamic pattern) than in morphology (static pattern), which underlies why bacterial morphologies and the like are so uninformative (even deceiving) phylogenetically. It also accounts for why the two prokaryotic cell types appear so similar upon superficial analysis. At the molecular level, however, this impression of kinship between the two quickly evaporates. In their histories, the two prokaryotic types have followed very different paths that from time to time have crossed, leading to the occasional transfer of genes (especially early on), which accounts for much of the similarity often taken to signify specific relationship at the organismal level. Yet in looking at the core functionality of the cell, the enormous gulf between archaea and eubacteria leaps out.

## Metabolism, Membranes, and Walls

Let us begin a comparison of the two types with their metabolisms, wherein the differences are relatively few, but still significant. The eubacteria are far and away the most metabolically diverse and versatile group of organisms on the planet. The metabolic uniqueness of the archaea is most prominently displayed in methanogenesis, a metabolism confined to the Euryarchaeota. The most interesting thing about archaeal metabolism may be the unique set of cofactors it employs (mainly in methanogenesis), for example, the C-1 carriers, coenzyme M, methanofuran, and methanopterin; $F_{420}$, an electron carrier analogous to NAD; $F_{430}$, a nickel-containing porphyrin akin to heme, vitamin $B_{12}$, and chlorophyll; and methanophenazine, a membrane-bound carrier that functions like a quinone (Wolfe 1992; Beifuss et al. 2000). Viewed metabolically, these cofactors are interesting but not all that unusual in the reaction types they catalyze. What is unusual and intriguing, however, are their biosyntheses (the study of which is still in its beginning phases). While the pathways in archaeal cofactor synthesis themselves tend to be fairly standard in a chemical sense, the enzymes catalyzing the reactions more often than not have no homologs outside of the archaea. As suggested by Graham and White (2002), the unity of biochemistry we have known since the time of Kluyver may be as much a matter of evolutionary convergence on common biochemical themes as it is retention of ancestral metabolic ways.

Archaeal membrane structure is unique as well. It is based upon ether-linked branched-chain lipids, whereas in the eubacterial and eukaryotic cases, straight-chain ester-linked lipids predominate (Kates 1964; Kates et al. 1968; Tornabene and Langworthy 1979). Cell wall structures differ too. The eubacterial wall characteristically comprises murein (peptidoglycan), while archaeal walls are most often proteinaceous (Kandler and Hippe 1977; Kandler and König 1985). In Methanobacteriales species, however, walls comprise pseudomurein, a compound that, as the name suggests, resembles eubacterial murein (König and Kandler 1979). Yet the sugars from which murein and pseudomurein are built are largely different, and when the biosynthetic mechanisms involved in wall formation are considered, it is clear that murein and pseudomurein are another example of biochemical convergence (Hartmann and König 1990).

## Translation

It is in information processing that the differences between eubacterial and archaeal cell designs appear in their full glory. Of the three main information processing systems (translation, transcription, and genome replication), translation exhibits the most universality in its componentry, the most homology between archaea and eubacteria (and eukaryotes). Ribosomal

RNAs, elongation factors, ribosomal proteins, and aminoacyl-tRNA synthetases are all basically universal. However, among these universal protein components, a clear distinction exists between the eubacterial and archaeal versions. The distinction is characteristically so blatant that it tends to stand out even upon gross inspection of a sequence alignment (Woese et al. 2000). (For example, the eubacterial versions of a given sequence often contain moderate to large blocks of amino acids [located terminally or in the interior of the molecule] not seen in the archaeal versions and vice versa. In addition, for those columns in an alignment where composition is highly conserved, one often sees that the characteristic eubacterial composition differs from that characteristic of the archaea.) This nearly qualitative distinction between archaeal and eubacterial versions of a sequence, this difference in genre between the two, has been called the "canonical phylogenetic pattern" (Woese et al. 2000). Differences this extreme are never encountered among the various eubacterial taxa or among different archaeal taxa (although in each case there have been three billion years or so, most of this planet's history, in which such differences could have arisen). When eubacterial and archaeal sequences show these differences in genre, their eukaryotic counterparts are almost always of the archaeal genre (Woese et al. 2000).

A minority of ribosomal proteins, however, are not universally distributed. A relatively small cadre are characteristic of and found only in eubacteria, while a somewhat larger set is common and confined to the archaea and eukaryotes, with yet a few others being found only in eukaryotic ribosomes. Overall, though, translation gives the impression of a system that existed in basically modern form at the root of the phylogenetic tree.

## Transcription

Transcription presents a similar evolutionary picture but with more, and more pronounced, exceptions to universality (Langer and Zillig 1995). The two largest (the catalytic) subunits of the DNA-dependent RNA polymerase, β and β′ in eubacterial nomenclature, are clearly universal and show the canonical pattern (with again eukaryotic sequences being of the archaeal genre). But that's about it for homology. For the remaining main subunit of the eubacterial RNA polymerase (α), homology between the eubacterial and archaeal versions is only partial. Eubacterial α occurs in two copies in the holoenzyme, whereas its archaeal counterpart (found also in eukaryotes) comprises two separate proteins of very different size, each present in single copy in the holoenzyme, with parts of each showing homology to (somewhat) different parts of eubacterial α (Langer and Zillig, 1995). The archaeal transcription polymerase has a number of additional (smaller) subunits, all of which occur in the eukaryotic enzyme(s), but not in the eubacterial. And as in the case of translation, the eukaryotic version(s) of the transcription polymerase contain(s) a few additional subunits specific to themselves (Tjian 1996). When it comes to transcription initiation, one sees no homology between the eubacteria and the archaea: the two mechanisms are different and use different componentry (Bell and Jackson 2001). Once again, the eukaryotes possess an embellished archaeal version.

## Genome Replication

Genome replication is the extreme example of archaeal/eubacterial difference. The closely related archaeal and eukaryotic systems are to a first approximation totally distinct from their eubacterial counterpart (Olsen and Woese 1996). And, as now might be expected, the mechanism of initiation of chromosome replication in the eubacteria is fundamentally different from that basically common to the archaea and eukaryotes (Kelman 2000). In the past, it was common to draw phylogenetic significance (i.e., specific relationship) from the fact that archaea and eubacteria both have circular chromosomes, in distinction to eukaryotic chromosomes, which are linear. In light of the similarity between the archaea and eukaryotes in both chromosome replication and the initiation thereof, that conclusion needs revisiting.

It is also of interest that both the eukaryotic and archaeal (euryarchaeal) chromosomes show nucleosome organization—the single archaeal histone responsible being a homolog of the four (related) histones that structure the eukaryotic nucleosome (Reeve et al. 1997). This is an example of a theme that repeatedly occurs in the chromosome replication mechanism and elsewhere, namely, a job is done by a particular multimeric protein complex. The many subunits therein, however, represent only one or a few gene families. In the eukaryotic case, a number of different members of a family tend to be represented in the complex, each present in *single* copy. In the archaeal case, there tends to be only one member of the corresponding family and that present in *multiple* copies.

Comparisons between the information processing systems of Archaea and Eukarya are what give rise to the strong impression of fundamental relationship between the two. However, that specific relationship is reflected in relatively few molecules other than those involved in information processing, one example being HSP-60. Overall, the relationships among the three basic cell types present a complex, fascinating conundrum—a delightful challenge.

The close resemblance between archaeal and eukaryotic versions of the three information processing systems has been the source of much speculation as to the origin of the eukaryotic cell, speculation that usually takes the form of invoking a fusion of a particular bacterial cell (such as an α proteobacterium) with a particular archaeal cell (say, a methanogen; Martin and Mueller 1998) or sees some hypothetical ancient "protoeukaryotic" cell as horizontally acquiring certain metabolic capabilities from the eubacteria and information processing capabilities from the archaea (Hartman 1984). I do not like either type of explanation. The first type gives the eukaryotic cell no character of its own before it emerges from the fusion of the two prokaryotic types. In the second, the protoeukaryotic cell suddenly acquires information processing systems that it has never before encountered, and its basic cellular design is suddenly altered. All such models

miss the essential point in cellular evolution: the core machinery of the three primary cell types evolved and became established in each case well before any cells achieved their "modern" type of organization (Woese 2002). Evolution does not proceed by suddenly and drastically altering a given cell design (at least a fairly advanced one). These models ignore the possibility (likelihood) that a cell design is stable, homeostatic, in an evolutionary sense.

## Horizontal Gene Transfer: How Cells Evolved

It might seem that horizontal gene transfer (HGT) would be a complicating factor in understanding how the various major cell types evolved. Yet, far from being a complicating factor, HGT turns out to be the essence of the process (Woese 2000). As is now well known, HGT appears to have had (at certain times in evolution) the potential not only to introduce entirely new functions into cells but also to displace any constituent (gene) in the cell with an alien equivalent. What effect has HGT had on the genetic record of organismal descent? What is its relationship to canonical pattern? Understanding here pivots on what the characteristics of HGT are and what factors determine them.

The interested reader will find a more extensive and useful discussion of the major problem of evolving cellular organization and its relevance to the universal phylogenetic tree structure in Woese (Woese 2000, 2002).

The most obvious thing about HGT is its extreme variability, in both frequency and phylogenetic range, from one gene to another, from one major taxon to another, and *likely* from one evolutionary stage to another (Woese et al. 2000). It turns out that the dominant factor shaping HGT is the organization (design) of the potential recipient cell (Woese 2002). The various components of a cell are in one way or another, to one extent or another, interconnected, variously integrated into the cell's "design fabric." Some of them are weakly and simply integrated into the fabric, others tightly and complexly so (Woese and Fox 1977a; Woese et al. 2000). Components of the first type are in effect modular: functionally and structurally, they are pretty much self-contained and self-defining; that is, their interactions with the rest of the cell componentry are minimal. It stands to reason that such a modular, loosely coupled, component is far more likely to undergo horizontal gene displacement by some alien equivalent than is highly and idiosyncratically integrated componentry.

An ideal system for assessing the character of HGT is the translation apparatus, for while most of its componentry, for example, the ribosomal proteins and elongation factors, are tightly coupled into the rest of the mechanism (and hence the cell fabric), the tRNA charging enzymes (aminoacyl-tRNA synthetases) are not; the latter are modular and are loosely coupled into the cellular fabric. And as might then be expected, the ribosomal proteins and elongation factors have been largely unaffected by HGT, whereas the aminoacyl-tRNA synthetases have been quite highly buffeted by it, both in terms of the frequency with which HGT has affected them and the (broad) phylogenetic range of many of the alien displacements that have occurred (Woese 2000). It is common, for example, to see the archaeal genre of a given aminoacyl-tRNA synthetase in one or a few eubacterial lineages (Woese et al. 2000).

A detailed phylogenetic analysis of the 20-odd aminoacyl-tRNA synthetases (Woese et al. 2000) shows (1) that about two-thirds of the tRNA charging enzymes exhibit the canonical pattern typical of the other components of the cellular information processing systems and (2) that in all these cases the canonical pattern has to one extent or another been eroded by HGT, but (3) that the degree to which and the ways in which that pattern has been eroded are idiosyncratic, qualitatively different for each of the enzymes. From these facts, it was concluded that the canonical phylogenetic pattern itself was not produced by HGT but reflects some earlier evolutionary dynamic (Woese et al. 2000). Thus, the canonical pattern represents a (partial) genetic record of organismal histories that extends back to the root of the universal tree. In other words, while HGT has been a powerful, pervasive, and shaping force in organismal evolution (especially early on), it has not completely obliterated the ancestral organismal trace that the universal tree represents.

As the reader can see, I cannot discuss similarities and differences among the major organismal types without the discussion shifting into an evolutionary framework. This, however, is no peculiarity of mine but rather the fact that such matters cannot be productively discussed in any other context. So, like it or not, microbiology is going to be in the center of evolutionary study in the future—and vice versa.

## Microbiology on the Move

A great deal has happened in bacteriology in the last decade or so. The four pillars upon which a true bacteriology must be built are now in place, and the long sought "concept of bacteria" is beginning to take shape. Bacteria are now routinely seen in terms of their natural (phylogenetic) relationships; discussions often turn on various aspects of their evolution. The sleeping giant of microbial ecology has finally awakened, and we are beginning to see the microbial base of the biosphere. Geomicrobiology, which increasingly ties bacteria to geological processes, is a burgeoning field. Marine microbial ecology is bringing to the fore the important role that bacteria play in determining the character of the oceans. Genomic microbiology is beginning to tackle some of the knotty problems associated with bacterial evolution, adaptation to environments, and the evolution of the global ecosystem itself. These and similar developments are the potential building blocks of a new microbiology. But the concrescence of twenty-first-century microbiology will not occur unless we, its practitioners, understand *what bacteriology is and, thus, what its future goals are.*

To me, microbiology today resembles a peasant who has suddenly become wealthy. The chains of poverty are gone, but the cobwebs that are the habits of poverty remain and hold him fast. Obviously, there are no longer scientific barriers to bacteriology's becoming the organismal discipline it should

long ago have become. But, who cares anymore? The idealists among us, whose crucial role it had been to define and develop the character of bacteriology, to keep the discipline on track, are long gone. The character of bacteriology (like that of the peasant) is essentially defined by overlords, external circumstances, be they scientific or practical (societal). Contrast bacteriology to disciplines such as genetics and molecular biology, disciplines that once were strong in being defined from within—disciplines whose paradigms embodied major scientific goals, disciplines that had a grand vision. Microbiology was never that. Today's microbiologists are, unfortunately, the realists of yesteryear. The field is without overview, without a concept of itself, and its goals remain shaped by practical considerations and influences from the stronger disciplines. Only an agonizing awareness of the situation will change that.

## Where Is Our Essence?

It is time to take stock. Microbiology today is not the discipline microbiologists knew 30 years ago. Everything is new—the technology, the problems that the discipline faces, our training, our conceptual framework, and the way microbiology is perceived by the other sciences. Only the organisms remain the same. But is this last even true? The organisms per se may be the same, but our perception of them surely isn't. What is a bacterium to us except a synthesis of the various ways in which we experience it? That surely has changed, dramatically so. (Is one's concept of Bacteroides and Cytophagas, for example, the same as it previously was once one knows that these two phenotypically dissimilar types are actually specifically related to one another? What are *Deinococcus* and *Thermus* doing in the same stable? Are the myxobacteria that we know today as members of the δ-proteobacteria the same as the classical myxobacteria, which if they had any affiliation at all, it was to the cyanobacteria?) The only things that remain at all constant are the prejudices we have inherited from our intellectual forbears. Time to clear the table!

There are two fundamental challenges that microbiology faces today, challenges deep and basic enough that they speak to the whole of biology. First is the age-old question "where do we come from," which can now be addressed in the form of how cellular organization evolved. The other is the problem of the biosphere: what is this incredibly complex yet smooth functioning web of interactions among this incredibly diverse collection of organisms—this coherent, self-sustaining, overarching state of the whole? Actually these two problems are at base the same. And the understanding of both lies in the microbial world, in its diversity, in the (functional) structure of microbial communities, in the organization of microbial cells, in evolutionary and other adaptive responses of microorganisms to environmental perturbations, and in the incredible interconnectedness of microbial life in general. In a way, twenty-first-century microbiology is a return to microbiology's roots, a rediscovered and development of the grand view of microorganisms that Beijerinck and other early microbiologists shared.

## The View from the Top

We have discussed above the major features that distinguish the Archaea and Eubacteria. What remains to be done is provide an organismal overview of the individual domains (see Chaps. 7–9, in Vol. 1). We will go about this in a top-down fashion, starting with the major eubacterial and archaeal taxa, leaving the details and definitions of the lower, subordinate taxa to you and the next generation of microbiologists. I do not claim that these high-level taxa as currently defined are entirely correct (are genuine naturally defined groupings) in all cases, but most of them surely are, and, therefore, our discussing them at this point is useful. Let us start with the following:

## The Organismal Structure of the Archaea

As we have seen above (and elsewhere), the Archaea represent a unique cell design (Graham et al. 2000). In their cellular organization, they stand apart from the Eubacteria and, of course, the Eukarya. The Archaea are also unique in their overall phylogenetic structure: the domain comprises two characterized kingdoms, the Euryarchaeota and the Crenarchaeota—with the possibility of a third kingdom, the "Korarchaeota," lurking among the unwashed masses of species that have been identified only through direct isolation of rRNA genes from the environment (Barns et al. 1996). Even a fourth archaeal kingdom is suggested by the recent discovery of a bizarre nanosized exosymbiont associated with particular ones of the Crenarchaeota (Huber et al. 2002). What makes archaeal phylogeny stand apart from that of the Eubacteria and Eukarya is the pronounced phenotypic and genotypic distinction between the kingdoms Euryarchaeota and Crenarchaeota—the depth of their phylogenetic split, the genomic differences between them. Interkingdom differences of this magnitude are not seen in the other two domains.

The Euryarchaeota appear phenotypically constituted (designed) around a methanogenic metabolism. They are also characteristically thermophilic and show relatively high intracellular concentrations of monovalent ions. To our present knowledge, the kingdom has spawned four main methanogenic branches (phyla), the *Methanococcales*, the *Methanobacteriales*, the *Methanomicrobiales*, and the *Methanopyrales* (Olsen et al. 1994).

The euryarchaeal kingdom also harbors other, non-methanogenic, phenotypes. Interestingly, all but one of these are offshoots of the lineage that gives rise to the Methanomicrobiales. The non-methanogenic phenotypes in question are the extreme halophiles, the sulfate-reducing archaea (Archaeoglobales), and the Thermoplasmatales. The lone non-methanogen group not stemming from this lineage is the Thermococcales (which comprises the genera *Thermococcus* and *Pyrococcus*).

The extreme halophiles are facultative aerobes that grow in highly saline environments and have been known to microbiologists for ages: they are known for putrefying salted fish among other

things. The extreme halophiles are also notable for the reddish pigment(s) they produce, which are in part responsible for the striking red color in salt evaporation ponds. Among these pigments is bacterial (now archaeal) rhodopsin, which provides them a photochemistry. Archaeal rhodopsin is central to a light-driven transmembrane pump, by means of which the extreme halophiles can derive energy.

Beyond their extremely high (5 M) intracellular potassium ion levels and their photochemical ability (unique among cultured bacteria), the extreme halophiles offer little of interest, unless it be the fact that as an organismal group, they are phylogenetically very tightly clustered—and so, of relatively recent origin.

One naturally assumes, given the specific (though somewhat distant) phylogenetic relationship to the Methanomicrobiales, that the metabolism of the halophiles is probably a derived, degenerate form of the metabolism characteristic of the Methanomicrobiales. But, the halophile genome shows something different. It is rife with bacterial genes and others that have no relationships functionally or phylogenetically to methanogens and methanogenesis.

The second grouping in the Methanomicrobiales cluster comprises the anaerobic sulfate-reducing organisms of the family Archaeoglobales. Iron reduction is seen in the genus *Ferroglobus* (Hafenbradl et al. 1996). The members of this order, though anaerobic, produce no methane, except for a trickle thereof. The biochemical reason is obvious: the gene for the terminal enzyme in the methanogenic pathway, methyl CoM reductase, is missing from their genomes. Yet the rest of the methanogenic pathway appears intact in these organisms. However, the *Archaeoglobus* sp. runs the pathway backward (oxidatively), producing carbon dioxide as the end product. The Archaeoglobales are highly thermophilic, with no known exceptions, which distinguishes them from their sister lineages, the Methanomicrobiales and extreme halophiles, lineages that do not appear to harbor hard-core thermophiles.

The final aberrant phenotype within the Methanomicrobiales segment of the euryarchaeal tree is the Thermoplasmatales. They are ostensibly wall-less thermophiles (unique among the archaea), whose thermoacidophilic phenotype superficially resembles that of the Crenarchaeota. And again, iron metabolism surfaces in the genus Ferroplasma (Golyshina et al. 2000). Direct environmental sampling for rRNA genes reveals that the Thermoplasmatales also encompass a low-temperature marine grouping (DeLong 1992)—whose phenotype is otherwise unknown. In their genomic makeup, these organisms are the most phylogenetically cosmopolitan of all the archaea.

I find it intriguing that among all Euryarchaeota, it is only the Methanomicrobiales (and their ancestral lineage) that have proven so evolutionarily inventive. In addition to spawning the above-described aberrant phenotypes, the Methanomicrobiales proper have been inventive in terms of methanogenesis itself. These are the only methanogens that utilize certain carbon sources other than carbon dioxide (e.g., acetate) in methanogenesis. And they seem to be able to utilize the methanogenesis pathway in both directions—which they do in metabolizing acetate. Interestingly, their genomes are quite large by archaeal standards (Deppenmeier et al. 2002; Galagan et al. 2002)—and contain a number of non-archaeal genes!

Among the euryarchaeotes, one finds both autotrophic and heterotrophic organisms (which is typical of large organismal groupings, of course). In the present case, the phylogenetic arrangement and genomic analysis of species strongly suggest that the heterotrophs are derived from the autotrophs, not the reverse.

The Crenarchaeota, the other of the (classically recognized) archaeal kingdoms, cannot be usefully characterized at this point in time. The reason is twofold. For one, the group has been relatively poorly studied in the laboratory. For another, the crenarchaeal species isolated in laboratory culture form only a phylogenetically restricted and nonrepresentative subset of the kingdom as a whole: direct environmental gene probing has shown that the crenarchaeal tree contains many branchings that are far more deeply placed than those of the cultured crenarchaeal species (DeLong 1992; Barns et al. 1994). I will leave you with this impression: Crenarchaeota are basically thermophilic organisms whose metabolisms somehow feature sulfur prominently. They are basically anaerobic, though can be facultatively aerobic in some cases. And, as an evolutionist might expect, mesophilic (even psychrophilic) phenotypes also exist. Indeed, one of the major phenotypes in the oceans—the largest biotope on the planet—represents one particular crenarchaeal group, the Order Cenarchaeales, which abounds throughout the breadth and depth of the oceans, being remarkably in evidence in Antarctic waters (DeLong 1997).

I have one closing thought concerning the archaea. The depth of the phylogenetic split between the two archaeal kingdoms seems the tip of an evolutionary iceberg. We have much to learn evolutionarily from this group of organisms. It saddens me to see how little interest goes into studying the archaea as an organismal group. And the fact that there exists an entire archaeal kingdom, the Korarchaeota, not a single representative of which has been isolated, is doubly distressing.

## The Organismal Structure of the Eubacteria

The eubacteria are the consummate metabolists: sheer metabolic power and versatility. They are the masters of metabolic theme and variation—just as eukaryotes are masters of structural theme and variation. Someday, we will understand just how eubacteria could have acquired such a varied metabolic repertoire. Their versatility is the more interesting when one realizes (from genomic analysis) that much of the more limited metabolic repertoire of the Archaea beyond that characteristic of the methanogens and methanogenesis is (as alluded to above) the product of metabolic capabilities (enzymes and pathways) imported from the eubacterial world.

The phylogenetic structure of the Eubacteria is rather different from the archaeal one just discussed. Viewed from afar, the

main eubacterial lineages appear to radiate out from a "crown group," with a few atavistic forerunners branching from the eubacterial stem slightly earlier (examples being the Aquificales and the Thermotogales). (It is the crown radiation that spawns the phenotypic richness we characteristically associate with the Eubacteria.)

Among the major (kingdom level) eubacterial taxa—those dominant by virtue of numbers, phylogenetic diversity, and ecological impact—there exist several kingdoms and phyla about which we know next to nothing. Only a few, if any, cultured species represent them, their evolutionary breadth and ecological prominence being demonstrated by the large numbers of environmentally derived rRNA sequences from the various groupings (Pace 1997). The lesson that has yet to sink in is that microbiologists *know relatively little about the bacterial world*. We clearly have no useful idea of what some of the major environmentally important bacterial groups are and are doing. You would think that a great deal of work would now be going into remedying this situation. This is not the case, however. Ask yourselves: do microbiologists have better things to do?

Let us begin with the major (most significant) eubacterial kingdoms and phyla and then, when appropriate, extend to their subordinate classes. I more or less lump kingdoms and phyla here, because—until the proper genomic criteria have been developed to define taxonomic rank on the basis of the properties of naturally defined organismal groups—it is not in some cases possible to distinguish the two.

## The Proteobacteria

One of the most, if not the most, ecologically dominant eubacterial kingdoms is the Proteobacteria. The kingdom comprises five phyla, distinguished by the Greek letters α ε. The α, β, and γ phyla obviously cluster, which easily emerges by any number of different phylogenetic analyses, while δ and ε, which may be specifically related, stand apart—but still within the proteobacterial kingdom. The β-phylum is actually a major branching within γ. I still consider β to be of phylum rank because of the apparent phenotypic diversity within it and its branching from a section of the γ-tree which, except for the β-branch, contains no photosynthetic representatives. (A taxonomic precedent for such a ranking is given by the class of mammals arising from within the class of reptiles.)

The α-phylum to a first approximation comprises (purple) photosynthetic lineages, nonphotosynthetic derivatives thereof, and a variety of pathogenic groups, mainly intracellular pathogens. The most notable of the latter is the rickettsial group (which comprises the genera *Rickettsia*, *Wolbachia*, *Ehrlichia*, *Anaplasma*, and *Cowdria*). What seem to be free-living relatives of these pathogens have been detected by rRNA analyses from marine habitats, although none have been cultured.

While the closely related agrobacteria and rhizobacteria, which play such important roles in the plant kingdom, are not members of the rickettsial group proper, the mitochondria are (Gray et al. 2001). At this point, I'm going to leave further exploration of this interesting eubacterial phylum to the tastes of the reader—but not without first mentioning an evolutionary peculiarity of the α-proteobacteria. The α-phylum appears (anecdotally) to be (to have been) more prone to the horizontal import of genes than are most or all other proteobacterial phyla, as judged by the number of archaeal genre aminoacyl-tRNA synthetase genes they contain (Woese et al. 2000). A systematic study of HGT in the α-phylum is definitely called for, especially an accounting of when in the phylum's evolutionary history the bulk of these transfers occurred.

Little more needs be said than already has been about the β-phylum. The photosynthetic phenotype therein (represented in several lineages) is unlike the photosynthesis found in the γ-phylum (the latter are the purple sulfur bacteria) but akin to the purple nonsulfur types seen in the α-phylum. The β-phylum is again a haven for pathogens but also for a varied collection of metabolisms. I am inclined to believe, on the basis of the relative uniqueness of the characteristic β-rRNA sequence, that the grouping may have begun as a rapidly evolving offshoot of the γ-lineage.

The γ-proteobacteria do live up to the implication of the designation "proteo-"; a great deal of morphological and other phenotypic variation is housed therein. Thus, one can make few generalizations about the phylum as a whole. The purple photosynthesis characteristic of the γ-phylum is, as just mentioned, biochemically unique, and the lineages harboring it, the genera *Chromatium*, *Ectothiorhodospira*, and closely related kin branch early from the γ-stem. These photosynthetic γ-proteobacteria deserve far more genomic attention than they have been given to date. I stress this because if photosynthesis is ancestral to the proteobacteria, at least to the α-β-γ cluster therein, then a genomic understanding of the photosynthetic representatives of the kingdom should prove key in the longer term to an understanding of the group's evolution.

This leaves the δ- and -phyla of the proteobacteria. Again, a lack of significant genomic coverage of this assemblage—except, of course, for a notable preoccupation with the pathogenic helicobacteria—leaves little to be said. Perhaps the most significant phenotypic characteristic of the group is a lack of photosynthetic representatives. The reduction of sulfur compounds is a predominant theme among the δ-lineages. One would like to see a representative genomic sweep of the δ-phylum and some genomic emphasis on the nonpathogenic members of the -phylum, such as *Wolinella* and *Thiovulum*.

Mention must be made, of course, of the myxobacteria, which are one of the four major classically recognized bacterial groups (see quote from *The Microbial World* second edition, above). Under molecular scrutiny, however, the myxobacteria turn out to be subordinate to, a lineage within, the δ-proteobacteria. Yet, their complex and beautiful morphologies and complicated life cycles give cause to wonder. How could such morphologically rich and metabolically versatile entities phylogenetically reside among the otherwise morphologically drab δ-proteobacteria? The genomes of the myxobacteria are inordinately large, much larger than other δ-proteobacteria. Within their excess DNA must lie the answer.

## The Gram-Positive Eubacteria

This ecologically and phylogenetically major eubacterial kingdom is one of the few classical eubacterial groupings to survive relatively unscathed the molecular phylogenetic assault on bacterial taxonomy. A fair bit of taxonomic reorganization has occurred within the kingdom, and some new members that do not possess typical Gram-positive walls have been added. But the two classical traits, endospore formation and Gram-positive wall, turn out to be phylogenetically valid characteristics. The only real taxonomic surprise has been the addition of the photosynthetic heliobacteria to the group (Woese et al. 1985a).

The main phylogenetic split among the Gram-positive eubacteria separates the "high G+C" group (the actinomycetes, mycobacteria, and a number of others) from the "low G+C" group. The phylogenetic split between the two is great enough that their specific relationship might be questioned. While the coherence of the entire kingdom was apparent in phylogenetic analyses of the small-subunit rRNAs (Olsen et al. 1994), it cannot be reliably demonstrated in my experience using the large subunit rRNAs. What is called for here is an appropriate test of the coherence of this kingdom based upon genomic analysis of the proper Gram-positive genomes—key ones of which have yet to be sequenced, however.

While most eubacterial genomes are sequenced for medical reasons—and this is surely true in the Gram-positive kingdom—one would still not single out the kingdom as an evolutionary training ground for pathogens. Metabolic versatility is its hallmark. The clostridia (not a genus or family, but a phylogenetically widely dispersed agglomerate of many different low G+C Gram-positive lineages) are classically notorious for the variety of degradative biochemistries of which they are collectively capable. Several clostridial genomes have now been sequenced, but many more are needed, and I would hope chosen in a systematic, phylogenetically comprehensive manner.

## The Cyanobacterial Kingdom

This grouping is one of the very few high-level eubacterial taxa that are anywhere near phenotypically uniform. Morphological variety abounds (as expected for a high-level taxon), but chlorophyll-based photosynthesis appears a constant metabolic theme in all. Classically isolated cyanobacteria contained only chlorophyll *a*, whereas the chloroplasts of plants, which are domesticated cyanobacteria, contain both chlorophylls *a* and *b*. Cyanobacteria that contain both chlorophylls have more recently been found (Lewin 1984). Although the chloroplast lineage(s) traces to the general cyanobacterial cluster, no lineage representing a free-living cyanobacterium shows a specific relationship to the chloroplast lineage. Indeed the cyanobacterial phylogenetic tree has a remarkably bushy appearance. Why this is so is again the type of question that an evolutionarily oriented bacteriology finds interesting. Did the invention of oxygenic photosynthesis in the ancestor of the whole group kick off a major evolutionary radiation, open a vast new evolutionary world for exploration?

It should be noted that chlorophyll *g*, characteristic of the Gram-positive photosynthetic heliobacteria, is the closest in structure to the cyanobacterial chlorophylls of all the eubacterial chlorophylls. Tantalizing molecular clues have also suggested a specific relationship between the Gram-positive eubacteria and the cyanobacteria—none as yet convincing, however.

## The Chlorobium-Cytophaga Kingdom

This grouping and the drawing together of the Cytophaga-Bacteroides phylum (by whatever name) is one of the more significant triumphs of rRNA-based molecular phylogeny of the bacteria. As one can infer from the glut of generic names that constitute the phylum (*Cytophaga*, *Flexibacter*, *Flavobacterium*, *Microscilla*, *Bacteroides*, and a host of lesser known genera), the grouping lay in separate taxonomic pieces before the advent of this molecular approach. The bringing together of the aerobic cytophagas and their ilk with the anaerobic bacteroides was an impressive join. Interestingly, the phylum does not comprise two major classes, split along aerobic/anaerobic lines. The bacteroides cluster forms a rather superficial branching from the main stem of the phylum and has close specific relatives having a cytophaga phenotype. (None of the latter has piqued the interest of genome sequencers yet.) I would note that the rRNA analysis reveals the coherence of this phylum very clearly: the members of the group exhibit a clean rRNA signature (Woese et al. 1985b).

The specific relationship between cytophagas and the green photosynthetic *Chlorobium* species (the latter an unexpectedly tight phylogenetic cluster for a phylum) was only weakly suggested by the small-subunit rRNA comparisons. To demonstrate the relationship convincingly, we had to resort to sequencing the large subunit rRNA (Burggraf et al. 1991). And although expected, it is most reassuring to see the coherence of the kingdom spelled out in full genomic detail by the comparison of the genome of a *Chlorobium* species (Eisen et al. 2002) with that of *Porphyromonas*, a member of the bacteroides contingent (Paster et al. 1994). In its entirety, this kingdom, with its spectacular admixture of phenotypes, demands some sort of evolutionary rationalization. So, as you might by now expect, I would encourage a concerted, systematic genomic assault on the problem.

## The Spirochetes

Since bacteriology's beginnings, this kingdom has been recognized as a major taxon (again see above quote from the second edition of *The Microbial World*). The unusual morphology of these organisms together with their unique mode of locomotion makes them stand out among all bacteria. The kingdom is phenotypically (morphologically) pure, monolithic—with one minor exception, an organism originally described as mycoplasma-like but otherwise uncharacterized (K. Stetter, unpublished

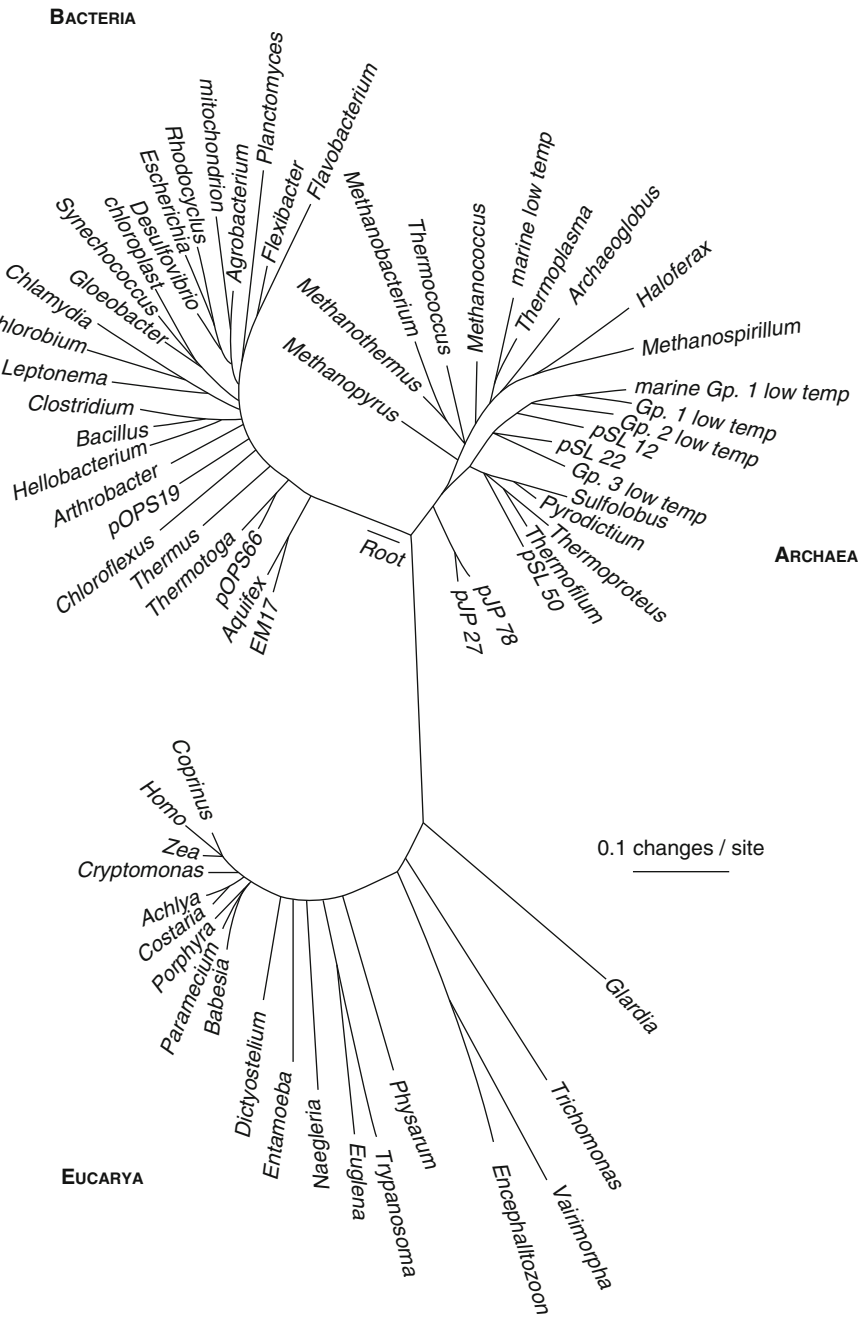


**Fig. 1.1**

**Universal phylogenetic tree based on small-subunit (SSU) rRNA sequence. Sixty-four rRNA sequences representative of all known phylogenetic domains were aligned and a tree was produced using FASTD NAML. That tree was modified, resulting in the composite one shown, by trimming lineages and adjusting branch points to incorporate results of other analyses. The scale bar corresponds to 0.1 changes per nucleotide**

observation) except for an rRNA sequence that places it clearly among the spirochetes (Maidak et al. 2001).

The spirochete grouping is deeply split phylogenetically into two major lineages, phyla. The one encompasses the more typical spirochetes such as *Spirochaeta halophila* (and houses the spirochete pathogens to which so much genomic attention has been paid, i.e., *Borrelia* and *Treponema*). The other is basically a lineage of aerobic spirochetes, the leptospiras and their relatives (some pathogenic).

At some early point in the evolutionary history of the spirochete kingdom (exactly how early cannot be pinned down yet because genome sequences are not available for the leptospira lineages), the spirochetes experienced a considerable amount of HGT. This assertion, however, is based on a very small sampling

of genes, namely, the aminoacyl-tRNA synthetases. The spirochetes have replaced more of their ancestral aminoacyl-tRNA synthetases by synthetases of the archaeal genre than has any other eubacterial group (Woese et al. 2000).

## The Planctomycetes, Chlamydiae and Verrucomicrobia

These three phenotypically disparate and phylogenetic distant groupings are combined here because fragmentary (unpublished) evidence suggests that they may well turn out to be specifically related to one another (at the kingdom level) when the appropriate genomes are available. Only the chlamydiae have been given significant attention to date (again for reasons of their pathogenicity). The reader is invited to peruse this third edition of *The Prokaryotes* for evidence of this relationship.

As their name suggests, the *Planctomyces* were originally thought to be eukaryotic. Their walls, as are the walls of chlamydiae and verrucomicrobia, are not typically eubacterial. It is unfortunate that so few examples of the verrucomicrobia exist in culture, for in nature they are clearly a major and ecologically important group of organisms. The planctomyces and relatives also appear to be playing a significant environmental role(s), although perhaps not at the level of the verrucomicrobia.

It has recently been discovered that the *Prostheocobacterium* subgroup of the verrucomicrobia carries two tubulin genes, the only known bacterial sources of these molecules, heretofore considered unique and essential components of the eukaryote cytoskeleton (Jenkins et al. 2002).

## The Deinococcus-Thermus and Chloroflexus Group

Here again are two phylum level taxa that, I feel, will someday be united into a eubacterial kingdom. The phylogenetic grouping of Deinococcus and Thermus, two disparate phenotypes in their own right, is another triumph of molecular (rRNA-based) phylogenetics (Weisberg et al. 1989). And the genomic sequence data now available more than confirm the relationship. The *Chloroflexus* grouping likewise was pulled together through rRNA analysis (Olsen et al. 1994). A specific relationship between the two phyla, however, is not convincingly demonstrated on this level, merely vaguely suggested. So far, a lack of sufficient genomic sequence data prevents the hypothesis being put to critical test—but early results from the genomic front are encouraging, however (R. Overbeek, personal communication).

The ecological significance of these two phyla is still being explored (by default as it were, because no one knows a priori what will be uncovered when new niches are probed by Pace's methodology). Environmental sampling of rRNA sequences shows there to exist additional major lineages in both the Deinococcus-Thermus and the Chloroflexus phyla, none as yet with isolated representatives (N. Pace, personal communication).

## The Remaining Eubacterial Kingdoms and Phyla

With the above, I have come nowhere near to covering the full range of eubacterial kingdoms and phyla. However, those that remain fall into one or both of two categories (1) demonstrated to be ecologically and phylogenetically major groupings by direct rRNA analyses of niches, but not represented by any (or very few) cultivated examples, and (2) represented by few directly isolated rRNA clones and no or few cultivated species. These bear little comment at this point in time. A phylogenetic tree (*Fig. 1.1*) generated by N. Pace and colleagues gives some idea of the overall scope of the major eubacterial lineages (Pace et al. 1997).

I would end this brief, incomplete survey of the major organismal groupings by mentioning two eubacterial kingdoms that have rather limited ecological distribution but are represented by genome sequences. Both are among the deepest branching eubacterial lineages (see *Fig. 1.1*). The first is represented by *Aquifex* (Huber et al. 1992; Deckert et al. 1998). The second comprises what are now called the *Thermotogales* (Huber et al. 1986; Nelson et al. 1999). The number of generic names in the group, *Thermotoga*, *Geotoga*, *Petrotoga*, and so on, does suggest a somewhat varied ecological distribution for the various members of the kingdom.

Now, on to the feast that awaits you in the rest of this third edition of *The Prokaryotes*.

## References


Ambler RP, Daniel M, Hermoso J, Meyer TE, Bartsch RG, Kamen MD (1979a) Cytochrome $c_2$ sequence variation among the recognised species of purple nonsulphur photosynthetic bacteria. Nature 278:659–660

Ambler RP, Meyer TE, Kamen MD (1979b) Anomalies in amino acid sequence of small cytochromes c and cytochromes c' from two species of purple photosynthetic bacteria. Nature 278:661–662

Barns SM, Fundyga RE, Jeffries MW, Pace NR (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci USA 91:1609–1613

Barns SM, Delwiche CF, Palmer JD, Pace NR (1996) Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proc Natl Acad Sci USA 93:9188–9193

Beifuss U, Tietze M, Baumer S, Deppenmeier U (2000) Methanophenazine: structure, total synthesis, and function of a new cofactor from methanogenic Archaea. Angew Chem Int Ed Engl 39:2470–2472

Beijerinck MW (1905) Versl K Akad Wet Amsterdam 14:168–169

Bell SD, Jackson SP (2001) Mechanism and regulation of transcription in archaea. Curr Opin Microbiol 4:208–213

Breed RS (1939) Bergey's manual of systematic bacteriology, 2nd edn. Williams & Wilkins, Baltimore

Burggraf S, Ching A, Stetter KO, Woese CR (1991) The sequence of *Methanospirillum hungatei* 23S rRNA confirms the specific relationship between the extreme halophiles and the Methanomicrobiales. Curr Opin Microbiol 14:358–363

Burkhardt F, Smith S (1990) The correspondence of Charles Darwin, vol 6. Cambridge University Press, Cambridge, UK, pp 1856–1857

Crick FHC (1958) The biological replication of macromolecules. Symp Soc Exp Biol 12:138–163

Darwin C (1859) The origin of species by means of natural selection, or the preservation of favoured races in the struggle for life. John Murray, London, p 69

Deckert G, Warren PV, Gaasterland T, Young WG, Lenox AL, Graham DE, Overbeek R, Snead MA, Keller M, Aujay M, Huber R, Feldman RA, Short JM, Olsen GJ, Swanson RV (1998) The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. Nature 392:353–358

DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci USA 89:5685–5689

DeLong EF (1997) Marine microbial diversity: the tip of the iceberg. Trends Biotechnol 15:203–207

DeLong EF, Wickham GS, Pace NR (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243:1360–1363

Deppenmeier U, Johann A, Hartsch T, Merkl R, Schmitz RA, Martinez-Arias R, Henne A, Wiezer A, Baumer S, Jacobi C, Bruggemann H, Lienard T, Christmann A, Bomeke M, Steckel S, Bhattacharyya A, Lykidis A, Overbeek R, Klenk HP, Gunsalus RP, Fritz HJ, Gottschalk G (2002) The genome of *Methanosarcina mazei*: evidence for lateral gene transfer between bacteria and archaea. J Mol Microbiol Biotechnol 4:453–461

Dobzhansky T (1963) Evolution, genetics and man. Wiley, New York

Eisen JA, Nelson KE, Paulsen IT, Heidelberg JF, Wu M, Dodson RJ, Deboy R, Gwinn ML, Nelson WC, Haft DH, Hickey EK, Peterson JD, Durkin AS, Kolonay JL, Yang F, Holt I, Umayam LA, Mason T, Brenner M, Shea TP, Parksey D, Nierman WC, Feldblyum TV, Hansen CL, Craven MB, Radune D, Vamathevan J, Khouri H, White O, Gruber TM, Ketchum KA, Venter JC, Tettelin H, Bryant DA, Fraser CM (2002) The complete genome sequence of *Chlorobium tepidum* TLS, a photosynthetic, anaerobic, green-sulfur bacterium. Proc Natl Acad Sci USA 99:9509–9514

Fitch WM, Margoliash E (1967) Construction of phylogenetic trees. Science 155:279–284

Fox GE, Magrum LJ, Balch WE, Wolfe RS, Woese CR (1977a) Classification of methanogenic bacteria by 16S ribosomal RNA characterization. Proc Natl Acad Sci USA 74:4537–4541

Fox GE, Pechman KR, Woese CR (1977b) Comparative cataloging of 16S ribosomal ribonucleic acid: molecular approach to procaryotic systematics. Int J Syst Bacteriol 27:44–57

Fox G, Stackebrandt E, Hespell RB, Gibson J, Maniloff J, Dyer TA, Wolfe RS, Balch WE, Tanner RS, Magrum LJ, Zablen LB, Blakemore R, Gupta R, Bonen L, Lewis BJ, Stahl DA, Luehrsen KR, Chen KN, Woese CR (1980) The phylogeny of prokaryotes. Science 209:457–463

Galagan JE, Nusbaum C, Roy A, Endrizzi MG, Macdonald P, FitzHugh W, Calvo S, Engels R, Smirnov S, Atnoor D, Brown A, Allen N, Naylor J, Stange-Thomann N, DeArellano K, Johnson R, Linton L, McEwan P, McKernan K, Talamas J, Tirrell A, Ye W, Zimmer A, Barber RD, Cann I, Graham DE, Grahame DA, Guss AM, Hedderich R, Ingram-Smith C, Kuettner HC, Krzycki JA, Leigh JA, Li W, Liu J, Mukhopadhyay B, Reeve JN, Smith K, Springer TA, Umayam LA, White O, White RH, Conway de Macario E, Ferry JG, Jarrell KF, Jing H, Macario AJ, Paulsen I, Pritchett M, Sowers KR, Swanson RV, Zinder SH, Lander E, Metcalf WW, Birren B (2002) The genome of *M. acetivorans* reveals extensive metabolic and physiological diversity. Genome Res 12:532–542

Gillespie D, Spiegelman S (1965) A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J Mol Biol 12:829–842

Golyshina OV, Pivovarova TA, Karavaiko GI, Kondrateva TF, Moore ER, Abraham WR, Lunsdorf H, Timmis KN, Yakimov MM, Golyshin PN (2000) *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the Ferroplasmaceae fam. nov., comprising a distinct lineage of the Archaea. Int J Syst Evol Microbiol 50:997–1006

Graham DE, White RH (2002) Elucidation of methanogenic coenzyme biosyntheses: from spectroscopy to genomics. Nat Prod Rep 19:133–147

Graham DE, Overbeek R, Olsen GJ, Woese CR (2000) An archaeal genomic signature. Proc Natl Acad Sci USA 97:3304–3308

Gray MW, Burger G, Lang BF (2001) The origin and early evolution of mitochondria (Reviews). Genome Biol 2:1018

Hafenbradl D, Keller M, Dirmeier R, Rachel R, Rossnagel P, Burggraf S, Huber H, Stetter KO (1996) *Ferroglobus placidus* gen. nov., sp. nov., a novel hyperthermophilic archaeum that oxidizes $Fe^{2+}$ at neutral pH under anoxic conditions. Arch Microbiol 166:308–314

Hartman H (1984) The origin of the eukaryotic cell. Speculations Sci Technol 7:77–81

Hartman H, Fedorov A (2002) The origin of the eukaryotic cell: a genomic investigation. Proc Natl Acad Sci USA 99:1420–1425

Hartmann E, König H (1990) Comparison of the biosynthesis of the methanobacterial pseudomurein and the eubacterial murein. Naturwissenschaften 77:472–475

Heberlein GT, De Ley J, Tijtgat R (1967) Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium*, and *Chromobacterium*. J Bacteriol 94:116–124

Huber R, Langworthy TA, Konig H, Thomm M, Woese CR, Sleytr UB, Stetter KO (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, Wilharm T, Huber D, Trincone A, Burggraf S, Konig H, Rachel R, Rockinger I, Fricke H, Stetter KO (1992) *Aquifex pyrophilus* gen. nov., sp. nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. Syst Appl Microbiol 15:340–351

Huber H, Hohn MJ, Rachel R, Fuchs T, Wimmer VC, Stetter KO (2002) A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. Nature 417:63–67

Jenkins C, Samudrala R, Anderson I, Hedlund BP, Petroni G, Michailova N, Pinel N, Overbeek R, Rosati G, Staley JT (2002) Genes for the cytoskeletal protein tubulin in the bacterial genus *Prosthecobacter*. Proc Natl Acad Sci USA 99:17049–17054

Kamp AF, La Riviere JWM, Verhoeven W (eds) (1959) Albert Jan Kluyver: his life and works. North Holland, Amsterdam

Kandler O, Hippe H (1977) Lack of peptidoglycan in the cell walls of *Methanosarcina barkeri*. Arch Microbiol 113:57–60

Kandler O, König H (1985) Cell envelopes of archaebacteria. In: Woese CR, Wolfe RS (eds) The bacteria, vol 8, Archaebacteria. Academic, New York, pp 413–458

Kates M (1964) Bacterial lipids. Adv Lipid Res 2:17–90

Kates M, Wassef MK, Kushner DJ (1968) Radioisotopic studies on the biosynthesis of the glyceryl diether lipids of *Halobacterium cutirubrum*. Can J Biochem 46:971–977

Kelman Z (2000) DNA replication in the third domain (of life). Curr Protein Pept Sci 1:139–154

Kluyver AJ (1931) The chemical activities of microorganisms. University Press, London

Kluyver AJ, Donker HJL (1926) Die Einheit in der Biochemie. Chem Zell Gewiss 13:134–190

Kluyver AJ, van Niel CB (1936) Prospects for a natural system of classification of bacteria. Zentralbl Bakteriol Parasitenkd Infektionskrankh 94:369–403

König H, Kandler O (1979) The amino acid sequence of the peptide moiety of the pseudomurein from *Methanobacterium thermoautotrophicum*. Arch Microbiol 121:271–275

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA 82:6955–6959

Langer D, Hain J, Thuriaux P, Zillig W (1995) Transcription in archaea: similarity to that in eucarya. Proc Natl Acad Sci USA 92:5768–5772

Lederberg J, Tatum EL (1946) Gene recombination in *E. coli*. Nature 158:558

Lewin RA (1984) Prochloron—a status report. Phycologia 23:203–208

Maidak BL, Cole JR, Lilburn TG, Parker CY Jr, Saxman PR, Farris RJ, Garrity GM, Olsen GJ, Schmidt TM, Tiedje JM (2001) The RDP-II (ribosomal database project). Nucleic Acids Res 29:173–174

Martin W, Mueller M (1998) The hydrogen hypothesis for the first eukaryote. Nature 392:37–41

Meyer TE, Cusanovich MA, Kamen MD (1986) Evidence against use of bacterial amino acid sequence data for construction of all-inclusive phylogenetic trees. Proc Natl Acad Sci USA 83:217–220

Nelson KE, Clayton RA, Gill SR, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Nelson WC, Ketchum KA, McDonald L, Utterback TR, Malek JA, Linher KD, Garrett MM, Stewart AM, Cotton MD, Pratt MS, Phillips CA, Richardson D, Heidelberg J, Sutton GG, Fleischmann RD, Eisen JA, Fraser CM et al (1999) Evidence for lateral gene transfer between Archaea

and bacteria from genome sequence of *Thermotoga maritima*. Nature 399: 323–329

Olsen GJ, Woese CR (1996) Lessons from an archaeal genome: what are we learning from *Methanococcus jannaschii*. Trends Genet 12:377–379

Olsen GJ, Lane DJ, Giovannoni SJ, Pace NR, Stahl DA (1986) Microbial ecology and evolution: a ribosomal RNA approach. Annu Rev Microbiol 40:337–365

Olsen GJ, Woese CR, Overbeek R (1994) The winds of (evolutionary) change: breathing new life into microbiology. J Bacteriol 176:1–6

Pace NR (1997) A molecular view of microbial diversity and the biosphere. Science 276:734–740

Paster BJ, Dewhirst FE, Olsen GJ, Fraser I (1994) Phylogeny of *Bacteroides*, *Prevotella*, and *Porphyromonas* spp. and related bacteria. J Bacteriol 176:725–732

Reeve JN, Sandman K, Daniels CJ (1997) Archaeal histones, nucleosomes, and transcription initiation. Cell 89:999–1002

Sanger F, Thompson EOP (1953) The amino-acid sequence in the glycyl chain of insulin. Soc Gen Microbiol Symp 53:353–374

Sanger F, Tuppy H (1951) The amino-acid sequence in the phenylalanyl chain of insulin. Biochem J 49:481–490

Sanger F, Brownlee GG, Barrell BG (1965) A two-dimensional fractionation procedure for radioactive nucleotides. J Mol Biol 13:373–398

Schleifer K-H, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36:407–477

Sogin SJ, Sogin ML, Woese CR (1971) Phylogenetic measurement in procaryotes by primary structural characterization. J Mol Evol 1:173–184

Stahl DA, Lane DJ, Olsen GJ, Pace NR (1985) Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. Appl Environ Microbiol 49:1379–1384

Stanier RY (1970) Organization and control in prokaryotic and eukaryotic cells. In: Charles HP, Knight BCJG (eds) Society for general microbiology symposium 20. Cambridge University Press, Cambridge, UK, pp 1–38

Stanier RY, van Niel CB (1941) The main outlines of bacterial classification. J Bacteriol 42:437–466

Stanier RY, van Niel CB (1962) The concept of a bacterium. Arch Mikrobiol 42:17–35

Stanier RY, Doudoroff M, Adelberg EA (1957) The microbial world, 1st edn. Prentice-Hall, Engelwood Cliffs

Stanier RY, Doudoroff M, Adelberg EA (1963) The microbial world, 2nd edn. Prentice-Hall, Engelwood Cliffs

Stanier RY, Doudoroff M, Adelberg EA (1970) The microbial world, 3rd edn. Prentice-Hall, Engelwood Cliffs

Stanier RY, Adelberg EA, Ingraham JL (1976) The microbial world, 4th edn. Prentice-Hall, Engelwood Cliffs

Tjian R (1996) The biochemistry of transcription in eukaryotes: a paradigm for multisubunit regulatory complexes. Philos Trans R Soc Lond B 51:491–499

Tornabene TG, Langworthy TA (1979) Diphytanyl and dibiphytanyl glycerol ether lipids of methanogenic archaebacteria. Science 203:51–53

van Iterson Jr G, den Dooren de Jong LE, Kluyver AJ (1940) Martinus Willem Beijerinck: his life and his work. Delftsch Hoogeschoolfonds, Delft

van Niel CB (1946) The classification and natural relationships of bacteria. Cold Spring Harb Symp Quant Biol 11:285–301

van Niel CB (1949) The "Delft School" and the rise of general microbiology. Bacteriol Rev 13:161–174

van Niel CB (1955) Classification and taxonomy of the bacteria and blue green algae. A century of progress in the natural sciences 1853–1953. California Academy of Sciences, San Francisco, pp 89–114

Weisberg WG, Giovannoni SJ, Woese CR (1989) The Deinococcus-Thermus phylum and the effect of mRNA composition on phylogenetic tree construction. Syst Appl Microbiol 11:128–134

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271

Woese CR (2000) Interpreting the universal phylogenetic tree. Proc Natl Acad Sci USA 97:8392–8396

Woese CR (2002) On the evolution of cells. Proc Natl Acad Sci USA 99: 8742–8747

Woese CR, Fox GE (1977a) The concept of cellular evolution. J Mol Evol 10:1–6

Woese CR, Fox GE (1977b) The phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci USA 74:5088–5090

Woese CR, Sogin ML, Sutton LA (1974) Procaryote phylogeny. I: concerning the relatedness of *Aerobacter aerogenes* to *Escherichia coli*. J Mol Evol 3:293–299

Woese CR, Fox GE, Zablen L, Uchida T, Bonen L, Pechman K, Lewis BJ, Stahl D (1975) Conservation of primary structure in 16S ribosomal RNA. Nature 254:83–96

Woese CR, Debrunner-Vossbrinck BA, Oyaizu H, Stackebrandt E, Ludwig W (1985a) Gram-positive bacteria: possible photosynthetic ancestry. Science 229:762–765

Woese CR, Stackebrandt E, Macke TJ, Fox GE (1985b) A phylogenetic definition of the major eubacterial taxa. Syst Appl Microbiol 6:143–151

Woese CR, Olsen GJ, Ibba M, Söll D (2000) Aminoacyl-tRNA synthetases, the genetic code, and the evolutionary process. Microbiol Mol Biol Rev 64:202–236

Wolfe RS (1992) Biochemistry of methanogenesis. Biochem Soc Symp 58:41–49

Zuckerkandl E, Pauling L (1965) Molecules as documents of evolutionary history. J Theor Biol 8:357–366

# 2 What Is a Prokaryote?

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It is easy enough to collect scientific data on *Escherichia coli* 0157:H7 or *Sulfolobus solfataricus* strain P2 and to pronounce them "prokaryotes." But it is not so easy to say what the word "prokaryote" refers to, in any general sense. The issue is ontological. Does "prokaryote"' refer to a category of things existing independently of our categorizing, perhaps a "natural kind" to which any organism can be surely said to belong or not belong? And if "prokaryote" *is* such a natural kind, is it defined by its essential properties or by its genealogical relatedness to other such kinds (O'Hara 1998)? Or does the word instead denote merely a human concept, initially constructed on incomplete knowledge and possibly now hopelessly out-of-date? When we ask whether an organism belongs to some recognized and named taxon, such as Archaea, Aves, or *E. coli*, we at least have in mind both a system of classification and an understanding of a coherent theory (evolution) that gives us faith in the legitimacy of that system. But is the same true for "prokaryotes"? For "Prokaryota"?

These may seem to be abstract philosophical questions, of little relevance to practicing scientists. And yet microbiologists debate fiercely and tediously not only over what "prokaryote" means but whether or not the word should be used at all. In just the last 2 or 3 years, the dispute has taken on new heat, such that some leading scientists would surely question whether there is any reason other than misguided adherence to tradition for a volume like this, dealing with Bacteria and Archaea as if they were one kind of thing. Not least, the concerns are pedagogical. Norman Pace, an unquestioned leader in microbial evolution and systematics, has written:

> Because it has long been used by all texts of biology, it is hard to stop using the word, prokaryote. But the next time you are inclined to do so, think what you teach your students: a wrong idea. (Pace 2006, p. 289)

This chapter, then, hopes to explain why such questions remain alive, and—to some of us at least—still interesting. Our goal is exploration and explication, not resolution, because we believe that the question "What is a prokaryote?" *has* no simple answer. What Evelyn Fox-Keller has recently written about the nature versus nurture debate applies with equal force to the two domain versus three domain contretemps (and perhaps in most areas of protracted biological contestation).

> There is no single answer to this question, for a number of different questions take refuge under its umbrella. Some of the questions express legitimate and meaningful concerns that can be addressed scientifically, others may be legitimate and meaningful, but perhaps not answerable, and still others simply make no sense ... all these different questions are tangled together into an insoluble knot, making it all but impossible for us to stay clearly focused on a single, well-defined, and meaningful question. (Fox-Keller 2010, p. 1)

## Some Tangled-Together Questions About "Prokaryote"

> The lessons of the three-domain tree are profound. Instead of two kinds of organism, prokaryotes and eukaryotes, there are three: bacteria, eukarya (eukaryotes) and archaea. (Pace 2006, p. 289)

At issue is whether living organisms on this planet are of two kinds (prokaryotes and eukaryotes) or instead, three (Bacteria, Archaea, and Eukarya). Begged in this pronouncement by Pace, and in fact the thorniest question taking refuge under the umbrella, is "What do we mean by *kinds of organism*"? Failure to agree on this keeps the debate alive, and no data will bring it to an end because the issue cannot be addressed scientifically, only philosophically.

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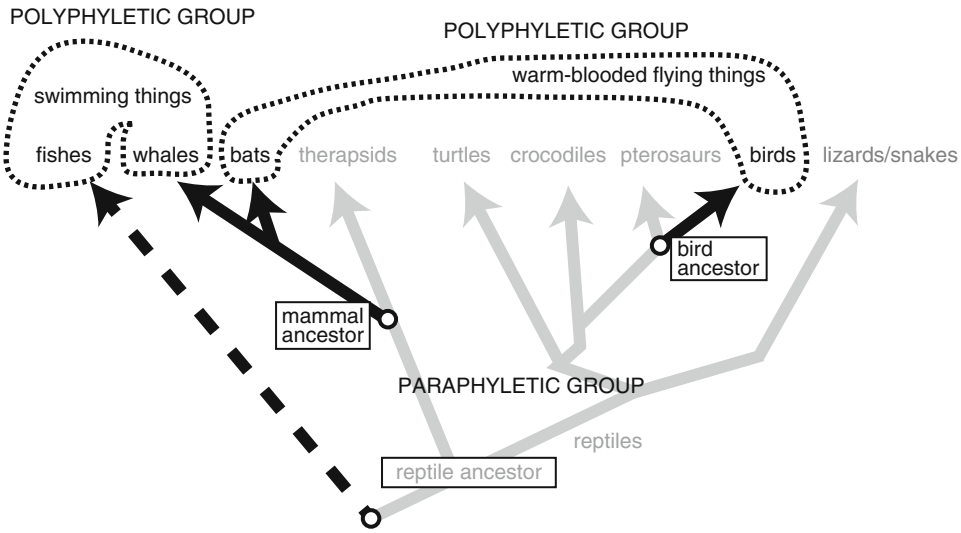


**Fig. 2.1**
***Polyphyly and paraphyly*. See text for explanation**

There are two general sorts of things we *might* mean by "kind" in this context, and of course an enormous relevant philosophical/biological literature, inadequately cited in the summary that follows. First, we might be thinking only in terms of current properties, as in a *phenetic* classification. We might consider prokaryotes and eukaryotes, or alternatively Bacteria, Archaea, and Eukarya, to be candidate *natural kinds*, real in a way that a category comprising all organisms taller than 1 m or whose genus name begins with the letter "E" would *not* be (Dupré 1981; Hacking 1991; Bird and Tobin 2010). They could be thought natural because they are defined by shared essences or essential properties, found in all and only representatives of the kind and somehow determinative of other characteristics. Some of the rhetoric around the two domain versus three domain controversy seems to embrace such a view, with "informational genes," especially those of translation, embodying such essential properties.

Essence-based natural kind thinking is out of fashion generally, however, and a more relaxed but still phenetic, evolution-independent, or ahistorical alternative would be that biological taxa, from species up to domains, are "cluster" kinds, which share . . .

> ▶ families of properties [that] cluster together over time either because the presence of some properties in the family favors the presence of others or because there are underlying internal mechanisms and/or extrinsic contextual mechanisms that tend to secure the co-occurrence of the properties . . . Cluster kind realists will readily concede that, depending on the case, environmental pressures may affect and alter the set of properties associated with a kind over time. Therefore, in such cases, none of the properties . . . themselves need be individually necessary for kind membership. (Bird and Tobin 2010)

Although pre-Darwinian systematists were perhaps not so wedded to an essentialistic classification as many of us were taught to believe by Ernst Mayr (Winsor 2006; McOuat 2009), it was unquestionably Darwin's *Origin of Species* that redefined the basis of naturalness in systematics, from phenetics to phylogenetics.

> ▶ All the foregoing rules and aids and difficulties in classification are explained, if I do not greatly deceive myself, on the view that the natural system is founded on descent with modification, that the characters which naturalists consider as showing true affinity between any two or more species are those which have been inherited from a common parent, and, in so far, all true classification is genealogical, that community of descent is the hidden bond, which naturalists have been unconsciously seeking, and not some unknown plan of creation, or the enunciation of general propositions, and the mere putting together and separating objects more or less alike. (Darwin 1859, p. 420)

This second, *phylogenetic* rather than phenetic way of seeing kinds as natural and real is what worked for Darwin and for most biologists today. The primary guarantor of legitimacy for any claim about how many "kinds of organism" there might be is community of descent, *not* shared current properties. We and chimpanzees are primates together because we descend from a common ancestral species that would have been considered a primate. Our many phenetic similarities with chimps justify putting us in the same order because (and *only* because) they are taken as evidence of that genealogical relationship. This second, historical or phylogenetic, way of being a natural kind might be called "tree-essentialism" and seen as a consequence of what Robert O'Hara (1997) calls "tree-thinking."

Often, classifications based on phenetic similarities and common descent or phylogeny converge. *Indeed, that they generally do was at the very heart of Darwin's case for evolution.* But they may sometimes come in conflict, in one of two ways (◉ *Fig. 2.1*). First is *polyphyly.* Insectivorous warm-blooded flying vertebrates include some birds and bats. As a taxon,

"Insectivorous warm-blooded flying vertebrates" would be called *polyphyletic*, because the most recent common ancestor of birds and bats was neither warm-blooded nor flying, and possibly not insectivorous. Such polyphyletic taxa represent a serious violation of the principle of genealogical classification, and are unacceptable to biologists as "natural" taxa, however similar the organisms so aggregated might be. Whales as fishes would be an even more egregious example. If Bacteria and Archaea do *not* share a common ancestor that we could consider a prokaryote, Pace's objection to the use of "prokaryote" (as explained below) would carry considerable weight.

The second sort of phenetic/phylogenetic conflict is *paraphyly*. Birds and bats are both members of *monophyletic* taxa, in that the classes Aves and Mammalia descend from common ancestors we would consider to be class members (the last common ancestral bird and the last common ancestral mammal, respectively). Moreover, no non-birds or non-mammals descend from those respective ancestors. But the class Reptilia, from which both Aves and Mammalia arose then reveals itself to be a *paraphyletic* taxon. It includes an ancestor (the last common ancestral reptile) and some but not all of its descendants because others of those descendants are birds and others still are mammals. Cladists consider such taxa illegitimate, instead requiring that we recognize two kinds of what once were called reptiles, synapsids (including mammals) and archosaurians (including dinosaurs and birds).

But unlike polyphyletic groupings, paraphyletic taxa do not result in the false unification of species that do not share community of descent, and thus do hardly any violence to Darwin's understanding of the evolutionary foundations of natural systematics. Indeed, by recognizing that because of variations in tempo and mode some of an ancestor's descendants resemble it far less than others, the acceptance of paraphyletic taxa, coupled with knowledge of their origins, adds explanatory value to classification. Many traditional systematists, including most prominently Ernst Mayr, incorporate such considerations of what Darwin called "degree of difference" into their practice. They deploy and defend paraphyletic groupings, given adequate justification (Ashlock 1974; Grant 2003).

Birds and mammals, with respect to their reptilian ancestors, and eukaryotes with respect to prokaryotes, represent what Mayr called evolutionary *grades*.

> ... the anagenetic component of evolution often leads to the development of definite "grades", or levels of evolutionary change, which must receive recognition in classification. The objection raised by the cladists that this would introduce subjectivity into classification has been rejected by the evolutionary taxonomist .... (Mayr 1982, p. 234)

As we hope to show, the argument over "prokaryote" survives in no small part because its protagonists claim to be disagreeing over scientifically addressable facts when in actuality they are arguing about different notions of what are "natural" groups, sometimes indeed from conflicting positions taken simultaneously. The grade/clade distinction is a large part of what is at stake here, but there is more.

## History and Fact

> Historical narratives in which science appears to advance steadily in the direction of greater accumulations of factual knowledge are now widely scorned as "whig history." ... like the stories the Whig political historians used to tell about the steady growth of English liberty. Today's historians are more likely to set themselves the goal of understanding the past "in its own terms" (whatever that might mean) rather than in the light of subsequent developments. This has yielded histories in which knowledge, rather than continuously increasing, has undergone radical discontinuities and transformations, and in which what subsequently come to be seen as forward movements are deeply rooted in contexts that are quite foreign from a modern perspective. (Golinski 1998, p. 4)

Regrettably, much history of science written by scientists is unabashedly and joyfully of the "whiggish" variety scorned by professional historians, portraying an inevitable advance (albeit with occasional detours through back alleys) from ignorance and error into truth, seldom admitting that today's knowledge may well be seen as relative ignorance tomorrow, and often overemphasizing the authors' own roles in that advance. Perhaps to working scientists accuracy and independent confirmation are essential for dealing with experimental data, but for historical "data" requiring nuanced interpretation, not so much.

Many false stories are propagated by repetitious citation, the academic equivalent of "urban myths." Worse, historical narratives can be deliberately constructed as foils against which the value of a favored new observation or hypothesis will shine out. Ernst Mayr's magisterial tome *The Growth of Biological Thought*, as we can see quite clearly now, was no disinterested exercise in pure historical scholarship (O'Malley 2010)! Moreover, even truly disinterested practitioners of a discipline will inevitably have come to whatever is their current understanding of the accumulated factual knowledge by different routes, in all honesty remembered quite differently. Outsiders cannot but be influenced most by those practitioners who write most persuasively or most often, no matter how idiosyncratic their views.

Not surprisingly, much of the dispute over the use of "prokaryote" derives from different readings of that term's intended meaning and of the common understandings of microbiologists who have used it over the last 50 years. A crucial element of the recent argument against "prokaryote" has become that it was intended – *or has come to be understood by most of its users* – to denote a phylogenetic division rather than (or as well as) a cell type, a clade rather than a grade, or a level of organization. If the former, then we would expect that many of the features in which Bacteria and Archaea are judged to be similar are genuinely homologous: if the latter, it is possible that many such features are convergent or merely analogous. Considered as a clade

designation, the "prokaryote/eukaryote dichotomy" imposed upon microbiology a misleadingly reductionist, falsely unitary, and dangerously non-evolutionary worldview, its detractors claim (Pace 2006, 2009; Pace et al. 2012).

Perhaps, but a complete reading of the microbiology's past as it affects its present is surely more complex. In our view, the important and still open question about "prokaryote" concerns its value as a descriptor of a grade or level of cellular organization. That it might be also taken or mistaken as the name of a monophyletic clade (the so-called Prokaryota) is an interesting side issue. Moreover, whether or not monophyly is properly attributed to prokaryotes depends rather much on what "monophyly" itself is taken to mean, as does the assumption that any of the three domains *is* actually "monophyletic."

## The Concept of a Bacterium

Jan Sapp, a professional historian unusually well-read in the microbial evolutionary/systematic literature and unusually well acquainted with the discipline's key figures, has recently highlighted some of the "urban mythology" concerning "prokaryote," while providing what seems a balanced intellectual history of the concept in "its own terms" (Sapp 2006, 2009a, b). The 1999 Berkeley Ph.D. thesis of Susan Spath (1999) earlier provided important details backgrounding the 1962 publication by Roger Stanier and Cornelius van Niel of "The Concept of a Bacterium" in *Archiv für Mikroboliogie.*

This paper is widely considered to be the cornerstone on which the belief that the most natural way to divide living things into two kinds is as "prokaryotes and eukaryotes" rested—for almost half a century. In it, Stanier and van Niel wrote:

> It is now clear that among organisms there are two different organizational patterns of cells, which Chatton (1937) called, with singular prescience, the eucaryotic and procaryotic type. *The distinctive property of bacteria and blue-green algae is the prokaryotic nature of their cells*. It is on this basis that they can be clearly segregated from all other protists (namely, other algae, protozoa, and fungi), which have eucaryotic cells. (Stanier and van Niel 1962, pp. 20–21)

Sapp shows Chatton's "singular prescience," since lauded and repetitively cited by many of us who cannot read French, to be mythic. The terms were first coined quite casually by Chatton and came to Stanier and van Niel via his most successful student, André Lwoff. But once accepted, Spath notes:

> The terminology introduced by Stanier and van Niel appears to have diffused widely through all branches of biology with little discussion . . . From the 1960s to the 1980s, the term procaryote and eucaryote were usually defined without acknowledgement of their origin. They became as much a part of standard biological discourse as 'molecule' or 'DNA.' (Spath 1999, p. 51)

Indeed, the prokaryote:eukaryote division seemed to satisfy many needs at once. Stanier and his former mentor van Niel were at pains to protect general bacteriology as a science and

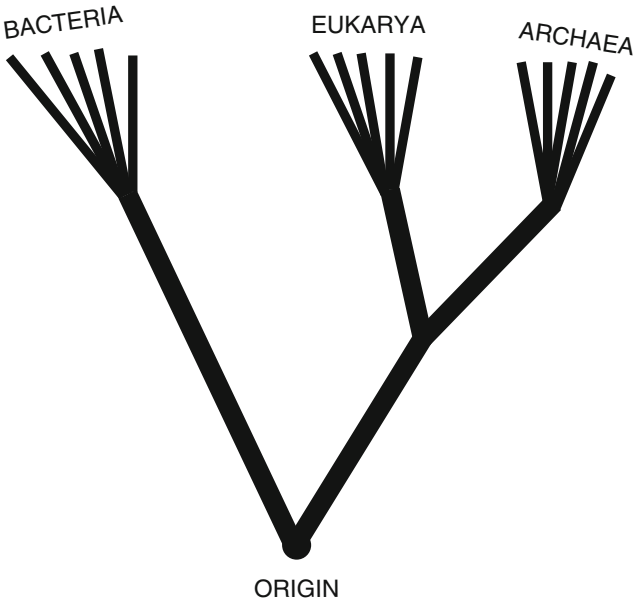


**Fig. 2.2**
***The three-domain tree, redrawn from* Pace (2006). Structure of the universal Tree of Life on which Pace (2006) bases his case against the use of "prokaryote"**

bacteria as a subject of study from the narrowing focus of infectious disease researchers and the reduction to mere model organisms threatened by the successes of molecular biologists who saw Bacteria primarily as tools. They hoped to draw a line above the Bacteria (separating them from eukaryotes) just as Lwoff, in his 1957 "The Concept of a Virus" had drawn a line below. Moreover, they intended to quash the false belief, supported by some microscopists, that Bacteria have nuclei. And lastly they hoped to codify a long-standing but still contested intuition that "blue-green algae"—in spite of their photobiochemical affinities to "higher" algae and green plants (and widespread speculation about their close evolutionary links thereto)— were just another kind of bacterium (indeed, "cyanobacteria").

Almost as frequently quoted as representative of Stanier and van Niel's formulation are these words from the second (1963) edition of the classic text *The Microbial World*, coauthored by Stanier with Michael Doudoroff and Edward Adelberg, which must have been written at about the same time.

> In fact, this basic divergence in cellular structure, *which separates the bacteria and blue-green algae from all other cellular organisms*, probably represents the greatest single evolutionary discontinuity to be found in the present day world. (Stanier et al. 1963, p. 85)

*Figures 2.3a–d* illustrate the various ways in which these words might together be interpreted. That great "evolutionary discontinuity" might be seen (and was described in Stanier and van Niel's "The Concept of a Bacterium") as primarily a structural one, of course the product of evolution (as is all of

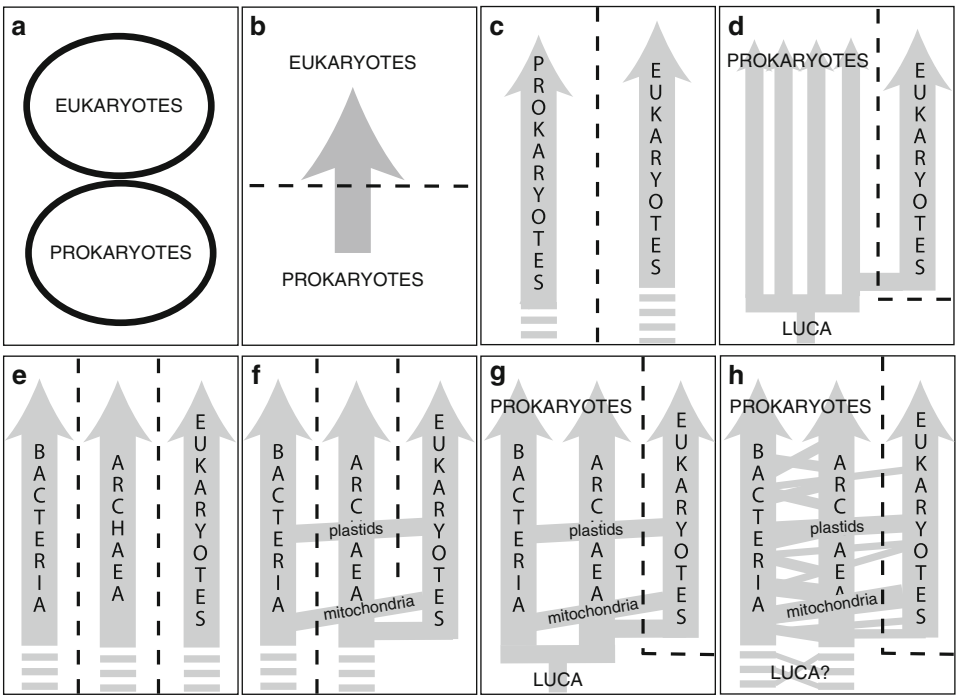


**Fig. 2.3**

***Eight representations of the possible relationship between prokaryotes and eukaryotes*. (a) Two distinct types of cellular organization (without implications about evolutionary relationship); (b) relationship between types, assuming that all life has a common origin and that simple precedes complex; (c) prokaryotes and eukaryotes as separately evolving clades, their common ancestor being pre-cellular, possibly as per Ernst Haeckel; (d) likely the most common view in the mid-to-late twentieth century, the Last Universal Common Ancestor (LUCA) being a cell or species of the prokaryotic type. (e) Bacteria, Archaea, and eukaryotes as separately evolving clades, their common ancestor being pre-cellular or "progenotic"; (f) as (e), but recognizing a specific Archaeal/eukaryotic ancestor, and taking into account endosymbioses giving rise to mitochondria and plastids: (g), as (f) but with the Last Universal Common Ancestor (LUCA) being a cell or species of the prokaryotic type, and Bacteria and Archaea considered a paraphyletic taxon ("Prokaryotes"); (h), as (f), but recognizing extensive interdomain lateral gene transfer (LGT). (h) seems most consistent with publications by Woese or Pace cited herein, although it is (g) that seems to be evoked in** ❯ ***Fig. 2.2*** **above**

biology), but entailing no particular phylogenetic scenario (❯ *Fig. 2.3a*). However, if one believes that simpler cells inevitably come before more complex ones, then Stanier and van Niel's line of cell-structure demarcation had to have been crossed at least once: the prokaryotic cell type having given rise to the eukaryotic cell type (❯ *Fig. 2.3b*). Indeed, they thought that too, and the passage quoted above continues . . .

> ▸ It is not too unreasonable to consider that the bacteria and blue-green algae represent vestiges of a stage in the evolution of cells which, once it achieved a eukaryotic structure in the ancestors of the present-day higher protists, did not undergo any further changes through the entire subsequent course of biological evolution. (Stanier et al. 1963, p. 85)

Possibly, one might consider existing prokaryotes and eukaryotes as the tips of separate evolutionary lineages, or rather clades of lineages, each with its own history and, possibly, essential characters (❯ *Fig. 2.3c*). But if one cannot deny (as Stanier and van Niel could not) that all living things are related and believes (as they did) that prokaryotes preceded and gave rise to eukaryotes, then that "evolutionary discontinuity" also represents a deep branch in the Universal Tree of Life (❯ *Fig. 2.3d*). The Tree's root (LUCA, or the Last Universal Common Ancestor) must have been a prokaryote.

Spath and Sapp seem to disagree on how much we are to understand "The Concept of a Bacterium" to be making a phylogenetic claim (along the lines of ❯ *Fig. 2.3d*) on top of the structural differentiation (❯ *Fig. 2.3a*) that was so clearly its main intent. The former argues that:

> ▸ The procaryote/eucaryote distinction implicitly expressed an important taxonomic proposition about the deepest phylogenetic division among living things. At the time it was formulated, it seemed obvious to Stanier and van Niel that the procaryotes, though diverse, belonged to a monophyletic category, as did the eucaryotes. The distinction provided the basis for ending a conviction about the natural world formalized by Aristotle in the fifth century B.C. and made sacred by Linnaeus in the eighteenth century: namely, that all organisms were either plants or animals. Though challenges to that conviction had been launched since the middle of the nineteenth century, none had

> achieved wide acceptance. The procaryote/eucaryote distinction, in contrast, ended the reign of the plant and animal kingdoms as the fundamental bifurcation of living things. When compared at the cellular level, it became evident that plants and animals were much more like each other than they were like procaryotes. (Spath 1999)

Sapp (2006), on the other hand, writes that:

> Stanier and van Niel's distinction was neither an evolutionary nor a taxonomic one – at least not as they drew it. In fact, their attitude toward an evolutionary-based classification of bacteria had taken a sudden change of course prior to 1962. (Sapp 2006, p. 165)

Sapp here refers to the well-documented and often decried mid-century abandonment by these two most influential figures in microbial systematics of all hope for a satisfactorily complete phylogenetic framework for bacteriology, leaving microbial taxonomists with at best a useful "determinative," identification-directed, system of classification (see Sapp 2006; Pace et al. 2012 and the next chapter in this volume). Probably a balanced reading of "The Concept of a Bacterium" is as a sort of "at least there is this" proposition: we may never be able to sort out the tree of bacterial evolution, but we can (at least) clearly distinguish prokaryotes (of which all were then considered Bacteria) from eukaryotes, which (probably) arose from within them.

Whatever the deeper convictions of Stanier and van Niel were in 1962, many microbiologists post-1962 were indeed happy to accept the prokaryote:eukaryote split as a phylogenetic division as well as cell-structural dichotomy, as sketched in ❯ *Fig. 2.3d*. It would not however have been seen (*pace* Pace and Spath) as the deepest division in the Tree of Life, as long as one held that eukaryotes likely arose from *within* the prokaryotes, that is, from one or more already established prokaryotic lineages. So (we believe) the default notion throughout most of the last half of the last century was that it was the concomitant radical structural reorganization that made the prokaryote: eukaryote divergence into the "greatest single evolutionary discontinuity to be found in the present day living world," *not* the topology of the implicit Tree of Life (❯ *Fig. 2.3d*). For those who cared about such niceties, prokaryotes were already paraphyletic.

In any case, "The Concept of a Bacterium" taken "in its own terms" is relatively noncommittal about the specific evolutionary connection between prokaryotes and eukaryotes, while admitting to parallel evolutionary processes.

> The evolutionary diversification of the prokaryotic protists is expressed in: (1). gross organization, leading to the existence of unicellular, multicellular, and coencytic groups; (2). mode of cellular locomotion; (3). mode of cell division; and (4). major patterns of energy-yielding metabolism . . . With respect to all these features, there are parallel modes of evolutionary diversification among the eukaryotic protists (i.e. other groups of algae, protozoa and fungi). Consequently, if we look at the microbial world in its entirety, we can now see that evolutionary diversification through time has taken place on two distinct levels of cellular organization, each of which embodied, with certain limits, the same kinds of evolutionary potentialities. (Stanier and van Niel 1962, p. 33)

*The Microbial World* does express faith in the ability to "safely infer a common origin for the whole group [prokaryotes] in the remote evolutionary past" (Stanier et al. 1963, p. 409). Pace et al. 2012 take this as a specific inference about prokaryotic monophyly but more likely Stanier and colleagues had in mind something like ❯ *Fig. 2.3d*. They were not cladists.

One could also complain (as Pace and Woese have) that the notion that eukaryotes come from prokaryotes (❯ *Fig. 2.3b*) is progressivist and reflects an incomplete commitment to tree-thinking. Molecular biologists in particular still talk and write about a progression in complexity from prebiotic chemistry-to-bacteria-to-yeast-to-*Drosophila*-to-*Homo sapiens* in ways that call to mind *The Great Chain of Being* (Lovejoy 1936), and ignore the obvious fact that all extant lineages have equally long evolutionary histories. That prokaryotes and eukaryotes are successive rungs on the ladder of life is just another example of that pre-Darwinian way of thinking, it might be claimed. But, as argued below, if one objection to "prokaryote" is that it means nothing more than "not-eukaryote" (Pace 2006, 2009), then all that ❯ *Fig. 2.3b* or ❯ *Fig. 2.3d* imply is that eukaryotes arose from ancestors we would not now call eukaryotes. Though some authors (even one of us at one time; Darnell and Doolittle (1986)) have suggested that the first cells were eukaryote-like, with prokaryotes being their streamlined descendants (Penny and Poole 1999), this seems now an irresponsibly wild conjecture.

## Three Domains Versus Two: The Debate with Ernst Mayr

As Woese masterfully summarizes in the next chapter, the proposal that living things are fundamentally of three kinds, not two, did not have the same easy ride to acceptance as did Stanier and van Niel's dichotomy—in no small part because of the widespread, almost culturally ingrained acceptance of the latter. Many of us, biologists, felt or still feel deeply that prokaryotes versus eukaryotes is "just the way it is," not from any direct observational experience or dispassionate consideration of the "accumulation of factual knowledge," but because it seems foundational to the biology we were taught and the institutional and economic structuring of our disciplines, even our social lives!

Somewhat belatedly, Ernst Mayr (1990, 1991, 1998) vigorously expounded a traditional(ist) objection to Woese's three domain view, based both on his (Mayr's) different perspective on how classification *should* be done and what he thought the Archaea were *really* like. Mayr contrasts his favored mode of taxonomic practice, "Darwinian classification" (aka "evolutionary taxonomy"), to the "Hennigian cladification" to which he thinks Woese largely, if incompletely, adheres. With the former . . .

- organisms are grouped into taxa on the basis of two criteria, similarity and genealogy. A higher taxon recognized by these criteria is composed of a group of similar and/or related species descended from their nearest common ancestor. Such a taxon is called monophyletic. In a cladification, favored by cladists, only genealogy is considered. It recognizes branches (clades) of the phylogenetic tree, comprised of the stem species of such a branch together with *all* its descendants. (Mayr 1998, p. 9721, Emphasis ours)

The important difference indeed is in the word "all." Citing the already familiar example of reptiles, birds, and mammals Mayr (1998) continues . . .

- In both cases, the cladist removes the branches that gave rise to the mammals or birds from the reptiles, thereby making the reptiles, a taxon used in our every-day grouping of animals, a "paraphyletic group," not permissible as a formal taxon in a strictly cladistic arrangement. In both cases, the Darwinian taxonomist, who deals with groups rather than with branches, retains the ancestral groups within the Reptilia and recognizes as mammals or birds only those assemblages of species which by their diagnosis are characterized as mammals or birds. It was on this basis that Stanier and van Niel recognized two empires, the prokaryotes and the eukaryotes. (Mayr 1998, p. 9721)

Mayr, like Woese and most biologists today, accepted the phylogeny displayed in ❯ *Fig. 2.3f* or ❯ *Fig. 2.3g*. But he felt that the similarities between Bacteria and Archaea were so great and their collective similarity to eukaryotes so small that the former two are legitimately classified into one superkingdom (Prokaryota, as shown in ❯ *Fig. 2.3g*) and the last into another (Eukaryota). It was a matter of recognizing that—as a grade—the eukaryote lineage had undergone massive, presumably selected, changes in the organization of its informational and especially cellular machinery. Moreover, he pointed out that Woese was not a very thorough-going cladist, because by those principles (and with the branching pattern shown in ❯ *Fig. 2.3g*) Woese *should* recognize only two primary domains, Bacteria and a second (called Neomura by Cavalier-Smith (2002)) comprising two sub-domains, Archaea and Eukarya. Mayr charitably attributed these failings to Woese's naïveté, as he expressed in a letter to WFD, dated January 31, 2000, that . . .

- Woese came into microbiology from outside of biology and did not (and still does not) understand what classification is all about. Biologists trained in classification and evolution would like to express the fact [of] how different the Prokaryotes are from the Eukaryotes.

But, one could easily rebut, Mayr did not understand what Archaea were all about! Woese, in the next chapter, emphasizes the many ways in which Archaea and Bacteria differ in basic cell and molecular biology. And in his immediate response to Mayr's 1998 attack, he cogently noted . . .

- Diversity can be of many types. It can be at the level of structure and organization; it can be anabolic or catabolic enzymatic diversity; it can be environmental adaptation at the molecular/biochemical level; it can be in the basic information processing systems of the cell; and so on. Clearly the vast diversity among birds and among insects is structural diversity, whereas that among the Bacteria or the Archaea is necessarily of the other types. Dr. Mayr's is an eye-of-the-beholder type of diversity. It rests on the incredible capacity of the human eye to distinguish minute differences in pattern. But almost all microbial diversity cannot be sensed visually, which means that subtle variations in pattern almost always go undetected. . . When he compares plant and animal diversity to microbial diversity, Dr. Mayr is comparing apples and oranges, and his attempt to apply globally a parochial and subjectively defined concept of diversity serves only to reveal the futility in such an approach. (Woese 1998a, p. 11045)

## Time for a Change?

In 2006, Norman Pace took up the attack on "prokaryote" with renewed vigor. In an pithy one page editorial entitled "Time for a Change" appearing in *Nature* (Pace 2006), he drew attention to what he considered the implications for systematics of the rooted version of the three-domain rRNA tree (Woese et al. 1990), in which Archaea and Eukarya are sister taxa, splitting later than Bacteria. His argument as presented then and reiterated 3 years later (Pace 2009) was fourfold.

1. How, he asks, can we consider Bacteria and Archaea to form a single group (the Prokaryota) when in fact one of its two subgroups, the Archaea, is more closely related to the eukaryotes? (See ❯ *Fig. 2.2*, reproduced from Pace 2006). This would be to recognize a paraphyletic taxon (like Reptilia), anathema to cladists.
2. The pro- and eu- prefixes themselves imply that prokaryotes gave rise to eukaryotes. But "the nuclear line of descent is as ancient as the archaeal line and not derived from either archaea or bacteria."
3. Prokaryotes are defined only negatively, by those eukaryotic features that they lack. "No one can define what is a prokaryote, only what it is not."
4. "Lumping bacteria and archaea conceptually discounts fundamental differences between these two kinds of organism . . ."

Thus, he concluded that "prokaryote" embodies a scientifically disprovable false idea . . .

- Prokaryote: gene-sequence comparisons show the tree of life consists of Bacteria, Eukarya, and Archaea. The use of the term "prokaryote" fails to recognize that an idea about life's origins has been proved wrong. (Pace 2006)

There is much that can be said in rebuttal and was, for instance, in correspondences to Nature by Martin and Koonin (2006), Dolan and Margulis (2007), and Cavalier-Smith (2007) and later by Whitman (2009) in the *Journal of Bacteriology*. An excellent recounting of the published and unpublished

contestation following "Time for a Change" is provided by Sapp (2009a, b). Each of Pace's four complaints is debatable at length, and is the subject of one of the next four sections of this essay.

## Paraphyly

Pace's first argument seems to be that *prokaryotes comprise a paraphyletic group.* This should thoroughly distress only cladists, which neither Pace nor Woese in other contexts appears to be. Their above-mentioned refusal to recognize or name Neomura as a clade shows that they are not strict Hennigians, as does their insistence on the meaningfulness of "domain" status. Degree of difference (especially in the informational transcription, translation, and replication machineries) and the fact that Archaea and Bacteria are separated by deep and long branches in the rRNA tree both figure in Pace and Woese's case for the domain status of either, and are most consistent with the practices Mayr styles "Darwinian classification."

Most microbial systematists, and indeed most biologists who care at all about microbes would be aware of the benefits and dangers of the grade/clade distinction. But many of them would argue that the elaboration of the cytoskeleton and endomembrane systems and the uncoupling of transcription and translation that seemed to have occurred rapidly and simultaneously in the formation of LECA (the last universal eukaryote ancestor), however that came about, were so radical and of such consequence as to make the prokaryote-eukaryote divide still "the greatest single evolutionary discontinuity to be found in the present day world" (Embley and Martin 2006; Field and Dacks 2009). Thus, as Whitman (2009) writes,

> Even though the prokaryotes are not monophyletic and the evolutionary processes they have experienced are extremely complex, this classification strategy remains useful and knowledge of the evolutionary processes which formed modern organisms provides a great deal of insight into their biological properties. (Whitman 2009, p. 2003)

## "Pro" Implies Before

Pace's second objection, *that the "pro" in prokaryotes is misleading because it implies an ancestral relationship to eukaryotes*, seems inconsistent with his first, almost disingenuously so. To call a taxon paraphyletic is to admit that another taxon emerged from within it. It is of course commonly understood that contemporary taxa did not evolve from each other. Humans did not evolve from chimpanzees: they instead share with chimps a common hominid ancestor, which was neither chimp nor human. But one could be almost certain it was hairier than us, because all the outgroup apes are. Similarly, the common ancestor of Archaea and eukaryotes was by definition neither one nor the other. But simple parsimony together with the accepted three-domain tree dictates that features found in eukaryotes, but not in Archaea and Bacteria, are more likely to have been invented in the eukaryotic branch than lost in the archaeal branch. And if, as Pace observes, all it takes to be a prokaryote is to lack eukaryotic defining traits, then the common archaeal/eukaryote ancestor surely *was* a prokaryote, although (if Archaea and Eukarya are indeed sisters) more Archaea-like than Bacteria-like.

Moreover, it seems now equally possible that the eukaryotic "nuclear line of descent" is not in fact "as ancient as the archaeal line," but arose from within it! Such an alternative view has been around almost as long as Archaea have been recognized, James Lake having suggested in the early 1980s, on the basis of ribosome structure, that eukaryotes show a particular affinity to one of the two then recognized archaeal subdomains, the Crenarchaeota, which he called "eocytes" (Lake et al. 1984) Martin Embley and coworkers (Cox et al. 2008; Foster et al. 2009) find that sophisticated and conservative phylogenetic methods that compensate for compositional and rate heterogeneity across sites and across trees are indeed prone to produce the "eocyte tree" in preference to the accepted three-domain tree, when applied to rRNA gene sequences or to a set of several dozen highly conserved and "core" genes of translation, transcription, and replication.

Taking into account also the presence or absence of certain key genes—several ribosomal protein genes, two RNA polymerase subunit genes, genes for components of the cell division machinery and a ubiquitin modifying system, and most excitingly a cell-shape-determining actin ortholog—Guy and Ettema (2011) recently argued for a specific placement of eukaryotes within what they call the TACK superphylum of Archaea. This comprises Crenarchaeota, Thaumarchaeota, Korarchaeota, and the recently proposed Aigarchaeota, and specifically excludes Euryarchaeota and Nanoarchaeota. Given the recent and still unstable taxonomic treatment of an expanding archaeal data set, we take this as being a modern version of Lake's "eocyte hypothesis." If this view gains wide acceptance and Archaea themselves become paraphyletic, Pace's phylogenetic/cladistic argument against prokaryotes would also deconstruct Archaea. At the very least we should remain agnostic as to the relationship between Archaea and the eukaryotic nuclear lineage (Gribaldo et al. 2010).

## Negative Definition

Pace's third argument, *that prokaryotes are defined only negatively*, has more bite, and requires more discussion. Stanier and van Niel's definition of "prokaryote" was indeed dismayingly minimalistic.

> The principal distinguishing features of the procaryotic cell are: 1. absence of internal membranes which separate the resting nucleus from the cytoplasm, and isolate the enzymatic machinery of photosynthesis and of respiration in specific organelles; 2. nuclear division by fission, not by mitosis, a character possibly

> related to the presence of a single structure which carries all the genetic information of the cell; and 3. the presence of a cell wall which contains a specific mucopeptide as its strengthening element. (Stanier and van Niel 1962, pp. 32–33)

Even these "negative" descriptors now seem problematic, since none is thought true of *all* prokaryotes. The specific peptidoglycan mucopeptide referred to is absent from eukaryotes (except in some photosynthetic organelles of cyanobacterial origin), but also from many bacterial and all archaeal walls, and there is much compositional variation in the walls of both prokaryotic domains (Whitman 2009). Fission is not the only way either Bacteria or Archaea divide (Angert 2005; Makarova et al. 2010). And although no prokaryote has internal membranes that are likely to be homologous to eukaryotic membranes, it is the case that within the planctomycetes there are remarkable *analogs* thereof (Fuerst and Sagulenko 2011), arising by covergence or gene transfer (McInerney et al. 2011).

Still, it is not always so easy to tell negative from positive in such general descriptions of organismal characteristics. The absence of functionally differentiated internal membranes has, for instance, been effectively re-cast positively, as the multifunctionality of the prokaryotic cytoplasmic membrane, by Whitman (2009).

> ► The cytoplasmic membrane is multifunctional in procaryotes and represents the defining structure of the cell. A proton motive force is generated on the cytoplasmic membrane by respiration, photosynthesis, or ATP hydrolysis to empower key cellular processes such as ATP biosynthesis, $NAD^+$ reduction by reverse electron transport, nutrient uptake, motility, and secretion. Procaryotes utilize membrane transporters on the cell surface to assimilate nutrients dissolved in their environment. In many procaryotes, the cytoplasmic membrane possesses a complex topology composed of lamellae, tubules, or other cytoplasmic intrusions. In contrast, the cytoplasmic membrane of eucaryotes is very different in structure and function. (Whitman 2009, p. 2000)

Similarly, Martin and Koonin (2006), in an immediate response to Pace's challenge in *Nature*, point out that the absence of nuclear membranes makes possible the coupling of transcription and translation and several prokaryote-specific types of regulation, a seemingly "positive" feature. Coupling might necessitate the absence of spliceosomal introns, which could be "positivized" for prokaryotes as "having continuous genes." Coupling might also mandate more direct forms of regulating gene expression (Payankaulam et al. 2010). Indeed, there is considerable literature indicating a common prokaryotic repertoire of transcriptional regulatory factors (Bell and Jackson 2001; Peeters and Charlier 2010).

There is also an enormous shared prokaryote-specific pool of "operational" genes, as illustrated in ❯ *Fig. 2.4* and previously in Walsh and Doolittle (2005). Thus, though (1) Bacteria and Archaea are indeed distinguishable from eukaryotes and each other by the character of the "informational genes" of transcription, translation, and replication, and (2) for many of *these* genes a specific archaeal/eukaryote relationship may be demonstrated (Cox et al. 2008; Cotton and McInerney 2010), it is still true (3) that a much larger number, many hundreds, of "operational" genes make up a shared resource that is common to Bacteria and Archaea. Such genes are often (on an evolutionary time scale) laterally transferred within and between them, and generally absent from eukaryotes. Indeed, one might define "prokaryotes" "positively" on this basis. They are that community of organisms that, over an evolutionary timescale, draws on a vast shared pool of genes encoding diverse metabolic functions seldom if ever used by eukaryotes. In a sense, evolution by LGT remains an important defining feature of prokaryotes, just as Woese would have it have been for progenotes, a feature which eukaryotes lack, relatively speaking (Woese 1998b, see below).

In any case, the key systematics question is not so much whether there are "non-negative" features uniting Bacteria and Archaea as whether there is *any* reason to believe that they derive from a common ancestor that might itself be called a prokaryote, so that they might together be considered a legitimate clade—albeit a paraphyletic one. There are two different questions embedded here: (1) what do we mean by common *ancestor* (versus common *ancestry*), and (2) do we have a sufficiently refined definition of "prokaryote" to be able to say whether or not the common ancestor, if it existed, was one? As with nature versus nurture, "these different questions are tangled together into an insoluble knot" (Fox-Keller 2010).

In several earlier and recent writings, Woese has argued that the common ancestor of all three domains was not a prokaryote or even a population of prokaryotes, but rather "the progenote," an inchoate collection of entities still in the throes of evolving the genotype/phenotype coupling. The manifest differences in information processing machinery between Bacteria, Archaea, and eukaryotes tell us about that early period, he feels, and suggests a model in which the three domains are best thought of as arising independently from an ancestral "state" rather than an ancestral cell or species, as in ❯ *Fig. 2.3e*. Of that state, he wrote (Woese 1998b)...

> ► Organismal lineages, and so organisms as we know them, did not exist at these early stages. The universal phylogenetic tree, therefore, is not an organismal tree at its base but gradually becomes one as its peripheral branchings emerge. The universal ancestor is not a discrete entity. It is, rather, a diverse community of cells that survives and evolves as a biological unit. This communal ancestor has a physical history but not a genealogical one. Over time, this ancestor refined into a smaller number of increasingly complex cell types with the ancestors of the three primary groupings of organisms arising as a result. (Woese 1998b, p. 6854)

It surely is the case that evolution *was* different in tempo and mode in the ancient pre-cellular past. One of us (WFD; Doolittle and Brown 1994), called this the period of "progressive Darwinian evolution" because many improvements in the speed, accuracy, and efficiency of replication, transcription, and translation that would have tightened the genotype-phenotype coupling and helped cells adapt to environmental

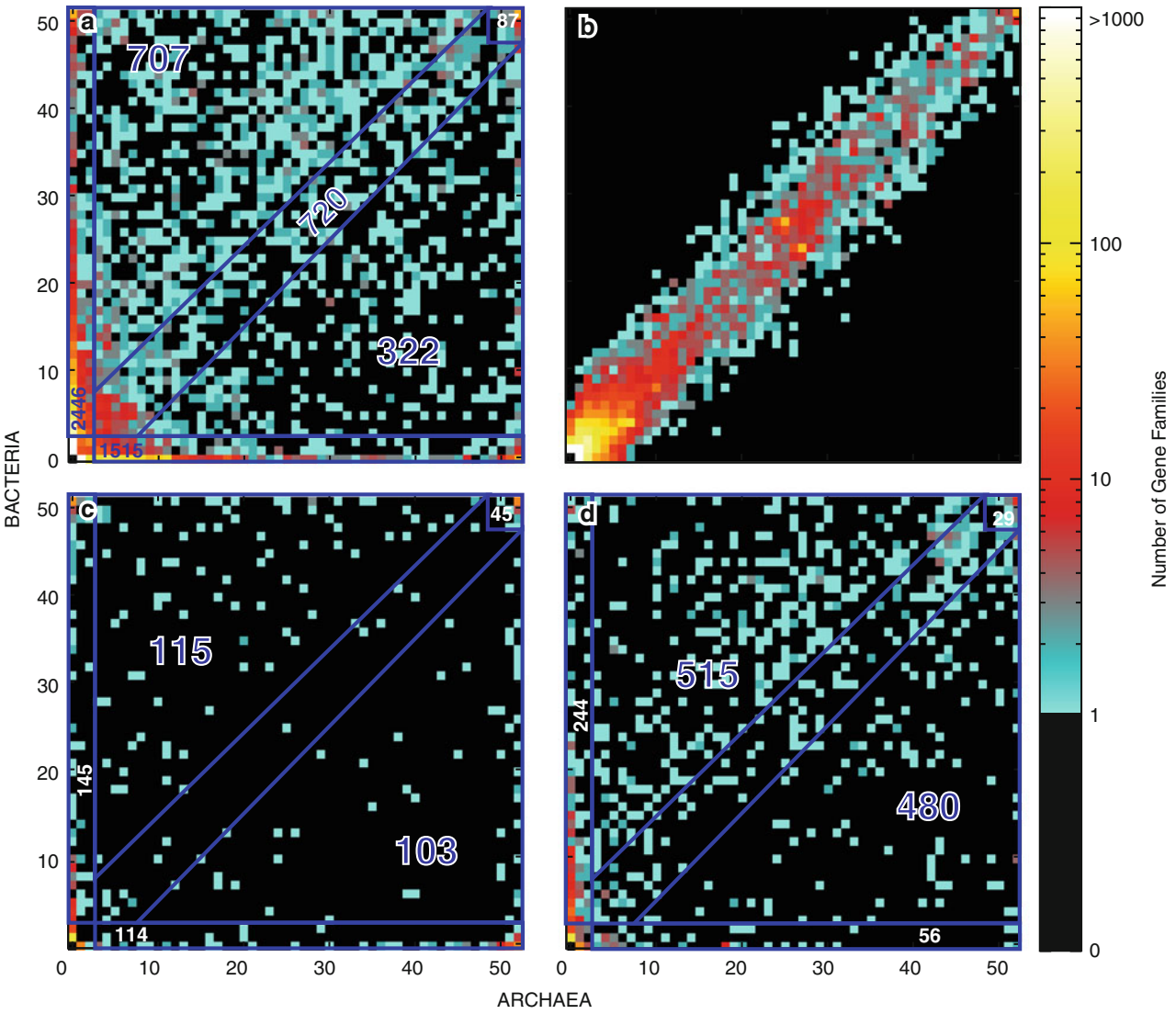


**Fig. 2.4**

***Distribution of gene families in representative archaeal and bacterial genomes***. To reconstruct gene families, we performed all-against-all BLASTP (E-value cutoff of $10^{-4}$) searches (Altschul et al. 1997) of open reading frames (ORFs) in 103 selected genomes. Normalized bitscores from pairwise BLASTP comparisons were used to cluster ORFs into superfamilies using MCL algorithm (inflation parameter I = 1.1; Enright et al. 2002). Superfamilies with more than four members were further divided into gene families using the BRANCHCLUST pipeline (Poptsova and Gogarten 2007). To select representative genomes among completed sequenced genomes available in GenBank we used the following criteria: (a) Only genomes larger than 1 Megabases were considered; (b) The largest representative per Archaeal genus was taken, resulting in 52 genomes; (c) The largest two representatives per bacterial phylum representing at least different genera were chosen. For broadly represented phyla (such as proteobacteria and cyanobacteria, among others), additional genomes were added until the number of selected bacterial genomes was comparable to the number of Archaeal ones (total of 51 genomes). The obtained gene families' distribution is visualized in a heat map, where the number of gene families present in *x* archaeal and *y* bacterial genomes, shown on X and Y axes, respectively, is color coded on a logarithmic scale (see color legend on the *right* of the figure; *black* values corresponds to 0 families, and *white* to >1,000 families). *Numbers* depicted over a heat map correspond to the total number of gene families in regions, perimeters of which are delineated by *blue lines*. *Panel A*. Comparison of all gene families. Near-universal core consists of 87 gene families. While the bulk of gene families present in at least four genomes can be called domain-specific (2,446 for Bacteria and 1,515 for Archaea), only very small fraction of those is universally present in the majority of the genomes of each domain. A substantial number of genes (1749) are patchily distributed (shared) across the two domains. *Panel B*. Randomization of 103 genomes (one of five replicates is shown in this panel) into two groups resulted in a different distribution of genes, suggesting that the distribution of gene families among the archaeal and bacterial domains is not random. *Panel C*. Subset of ''informational'' gene families (as defined by ''Information Storage and Processing'' functional category of the Clusters of Orthologous Groups, or COG, database (Tatusov et al. 2003)). *Panel D*. Subset of ''operational'' gene families (as defined by ''Metabolism'' functional category of the COG database)

challenges of all sorts should have been under positive selection then. True "progess" was being made: organisms would have been getting better just at being organisms. After that (after crossing what Woese calls "the Darwinian threshold"), adaptations would have become more ecology-driven, tracking environmental change—even "reversing" themselves, and progressive only in a highly contingent and local context.

But whenever and at whatever stage we consider the common *ancestor* of Bacteria and Archaea to have appeared, or indeed even if we think there was no "discrete entity" deserving of that name, there is no denying that the two domains share a common *ancestry*. The universality of the genetic code of the ribosome, and of many of its constituent proteins (Fox 2010), of demonstrably homologous RNA polymerase subunits (Werner and Grohmann 2011), and of so many operational enzymes and the biochemistries they promote make it impossible to imagine an independent origin of Bacteria and Archaea. That is, they could not have gone from prebiotic chemistry through to the first cellular forms that we might consider Bacteria or Archaea without extensive pooling of information. Even Bill Martin and Mike Russell (2003)—whose origin-of-life scenario entails advancement to the stage of ribosomes and the universal code among *pre-cellular* systems maintained in FeS compartments—must invoke extensive information exchange.

Vetsigian et al. (2006) describe the code as the product of "collective evolution" via LGT...

> The phylogenetic expression of ambiguity is reticulate evolution. In reticulate evolution, there is no unique notion of genealogical descent: genetic content can be distributed collectively... The players are cell-like entities still in early stages of their evolutions. The evolutionary dynamic (the "rules") involves communal descent. The key element in this dynamic is innovation-sharing, an evolutionary protocol whereby descent with variation from one "generation" to the next is not genealogically traceable but is a descent of a cellular community as a whole. Even if an organismal ancestry were to some extent traceable, it would have no significance, because it is the community as a unit, not the individual organismal lineages therein, that varies in descent... The central conjecture in our model is that innovation-sharing, which involves horizontal transfer of genes and perhaps other complex elements among the evolving entities [a dynamic far more rampant and pervasive than our current perception of horizontal gene transfer (HGT)], is required to bring the evolving translation apparatus, its code, and by implication the cell itself to their current condition. (Vetsigian et al. 2006, p. 10696)

Importantly, and perhaps ironically, this "dynamic" is also how one might describe the evolution of a complex trait within a modern multicellular sexually reproducing species, ours for instance. Because of recombination, to which mammalian reproduction is inextricably tied, any multigenic advance is similarly going to be the product of "innovation sharing." Though each contributing mutant allele should be traceable to a last common ancestor (its "coalescent") arising earlier in the population, the complex trait as a whole will not be "genealogically traceable," and is indeed the achievement of the "community as a whole." The difference of course is that with *Homo sapiens* it is homologous recombination *within* the species that underwrites the process, whereas for prokaryotes or the "cell-like entities" comprising the progenote, it is LGT (alternatively HGT) *between* "species."

Both within-species recombination and between-species LGT/HGT complicate the concept of "ancestor" (as opposed to ancestry), in a way not often appreciated by microbial phylogeneticists, who tend to equate gene trees with species trees. In a recombining sexually reproducing species, alleles at different loci find their last common ancestors (their coalescents) in different genomes present at different times in the population's past. "Gene trees" are uncoupled from "species trees" and sexually reproducing organisms have organismal ancestors from whom they have received no genetic information. (We receive half our alleles from each parent but it is possible if ridiculously improbable that we received none from one of our grandparents, and in fact certain that, many more generations back, we have reproductive ancestors from whom we received no alleles.)

Similarly, because of LGT, the last common ancestral versions of the many gene families found distributed in Bacteria and Archaea today will trace to common ancestral versions that existed in different genomes at different times in the history of their ancestral global prokaryotic population. The last common ancestral prokaryotic cell quite possibly housed none of them. Some researchers think that a small core (as few a 1%) of all genes, mostly involved in translation, have never been subjected to LGT, and thus do find their last common ancestral versions in the genome of a single cellular Last Universal Common Ancestor, or LUCA (Ciccarreli et al. 2007). Others, who may doubt the existence of any such never-transferred genes but still hope to give a traditional meaning to the Tree of Life, assert that, rather than being a single individual or even a single species, "LUCA was a population" (or ancestral "state"), indeed a heterogeneous population extended through time, possibly over the many tens or hundreds of millions of years consumed by "progenotic," or "progressive Darwinian" evolution (Koonin 2009; Glansdorff et al. 2008, 2009; Di Giulio 2011).

This seems a radically different use of the word and concept of "ancestor," which is more commonly understood to single out *individuals* in earlier populations. And we need not buy into such a redefinition (or indeed any definition of common *ancestor*) in order to accept a general model of common *ancestry*, in which the various features of modern cells evolved "collectively" (i.e., in no single genomic lineage), in a globally communicating (via LGT) super-population of entities becoming, over the course of hundreds of millions of years, ever more like contemporary prokaryotic cells.

A recasting of the question asked earlier, whether there is *any* reason to believe that Bacteria and Archaea derive from a common ancestor that might itself be called a prokaryote, is this. "Would that ancestral cell or cells in which the last common ancestral versions of genes that (it is assumed) have never been laterally transferred – in particular ribosomal RNA and protein

genes – be more "primitive" in terms of its informational machineries (their accuracy, efficiency or general character) than are contemporary prokaryotes?" This is a formulation that we think most authors who embrace either a single-cell or heterogenous population notion of LUCA might accept. We submit that we do not, in spite of the great appeal of the progenote concept and the various ingenious but tortured arguments put forth in favor a primitive, even RNA-genomed ancestral state (Koonin 2009; Glansdorff et al. 2008, 2009; Di Giulio 2011), know the answer to it.

To be sure, Woese's observation that the many structural differences between the informational machineries of Bacteria and Archaea, particularly the ribosomes, reflect their independent evolutionary refinement from a more primitive (less accurate and efficient) ancestral form is the best-articulated argument along these lines. In concluding a recent survey of domain-specific features ("signatures") of bacterial and archaeal ribosomes, he and colleagues (Roberts et al. 2008) wrote . . .

> Through our analysis of ribosomal signatures, we have provided a glimpse into the evolutionary past, at the "base" of the [universal phylogenetic tree]. This study has identified the ribosomal signatures and provided examples of how they are helpful in understanding the evolutionary dynamic by which the ribosome arose. These signatures give each phylogenetic domain a distinctive character and bespeak stages through which the evolution of the ribosome must have proceeded, both before the emergence of the individual lineages themselves (in the universal ancestral state) and subsequently, separately within each primary lineage. (Roberts et al. 2008, p. 13958)

But the differences between archaeal and bacterial ribosomes and the very long branches between bacterial and archaeal domains in rRNA and many other gene trees are not *necessarily* evidence of a different mode and tempo of evolution or of a primitive LUCA at the root of any gene tree. All bifurcating trees must have a deepest branching that divides them into two subtrees. On average (assuming a random birth-death model) the two branches will themselves appear unbranched halfway up the tree, this being the null model under coalescence theory (Zhaxybayeva and Gogarten 2004). Unusually rapid evolutionary rates and ancestral primitivity need not be invoked.

Moreover, there are selective (Gupta 2000) and nonselective (Lukes et al. 2011) forces other than improvement from a primitive state that might radically transform the translational machinery. Mitochondrial ribosomes, for instance, are vastly different in composition and structure from their alpha-proteobacterial ancestors (O'Brien 2002), and eukaryote ribosomes are larger and more complex than their presumed archaea-like predecessors. If we accept the scenario in *Fig. 2.3e*, these latter differences might bespeak separately evolved refinements of a primitive progenotic ancestral ribosome. But most would favor phylogenies more like those in *Fig. 2.3f–h*. We can infer from the similarity across bacterial phyla that the bacterial ribosome was already in the final form at the time LECA (the last eukaryote common ancestor) welcomed on board the alpha-proteobacterial symbiont that was to become the mitochondrion. And evidence favoring the "eocyte" hypothesis (Cox et al. 2008; Foster et al. 2009) also implies that LECA's ribosomes were already fully functional archaeal types.

We cannot from comparative ribosomology alone decide whether the ancestral cell or cells in which the last common ancestral versions of ribosomal RNA and ribosomal protein genes resided were "progenotes" or "prokaryotes." If progenotes, then "prokaryote" becomes polyphyletic, as well as paraphyletic, and the word would have two strikes against it. But this second strike depends entirely on how we define both "prokaryote" and "progenote," not "meaningful concerns that can be addressed scientifically" and on the existence and nature of LUCA, which is similarly problematic.

## Bacteria and Archaea, and the Fundamental Differences Between Them

Pace's fourth argument is that *disregard of these differences "reinforces an incorrect understanding of biological organization and evolution"* (Pace 2006). To be sure (rephrasing Stanier et al. 1963) the "basic divergence in cellular structure which separates the bacteria [and archaea] from each other represents the greatest single evolutionary discontinuity to be found in the present day [prokaryotic world]." In the next chapter, Woese summarizes those differences—in membrane composition and the machineries of translation, transcription, and replication—that have long been considered diagnostic of the bacterial/archaeal discontinuity. It is also the case that "operational" genes (primarily those of catabolism and anabolism), though widely shared between Bacteria and Archaea, are nevertheless often preferentially associated with one or the other domain. The gene family presence/absence data presented in *Fig. 2.4* and the comparison to a random partitioning of genes make this abundantly clear.

The "incorrect understanding" that concerns Pace is the presumption of uniformity among prokaryotes. Stanier and van Niel were themselves cognizant and celebratory of bacterial diversity (Spath 1999; Sapp 2006, 2009a, b), though they were not aware of the "discontinuity" between Bacteria and Archaea (nor was anyone at the time). It seems unlikely that they would have been dismissive of the kinds of evidence Woese brings to bear. Indeed, Woese lays much of the blame for what he sees as a woeful neglect of difference on molecular biologists, whose reductionist paradigm led them to think that what is true for *E. coli* is true for elephants and (coincidentally) for *Bacillus subtilis* and *Sulfolobus solfataricus*.

It is not just the bacterial/archaeal distinction that is painted over by the broad brush of molecular biology, however. There are fungi whose molecular genetics are wildly unlike that of *Saccharomyces cerevisiae*, insects whose population genetics are not like that of *Drosphila melanogaster*, vertebrates whose development is strikingly different from that of *Gallus domesticus*, and primates who do not behave much like us. It is not surprising that biologists who have spent their lives with one model organism want their results to be seen as more generally valid and that

cell and molecular and cell biology textbooks follow their lead, while teachers of evolution and diversity defend differences but reach fewer students.

As Woese himself noted in rebuttal to Mayr, "Diversity can be of many types," and what we emphasize will depend on our experience, disciplinary focus, and research goals. That Bacteria and Archaea appear different in many fundamental biological features is beyond question: whether this degree of difference is dwarfed by that between prokaryotes and eukaryotes is not something that science can decide. This is, as Woese noted in the passage quoted earlier, to compare apples to oranges.

## Reticulation, the Bête Noire of Cladistics

Trees based on rRNA are unrootable, because there is no outgroup. The accepted rooted version of the three-domain rRNA tree (as in *Fig. 2.2*) was initially based on a few widely distributed paralogous protein-coding gene families, assumed to be the products of gene duplications that predated LUCA (Iwabe et al. 1989; Gogarten et al. 1989; Brown and Doolittle 1994). The tree based on sequences of one of the duplicates can be used to root that of the other and vice versa, the two trees being in principle congruent with each other and with the true Tree of Life (assuming no LGT). Such duplication-based rootings generally showed eukaryotes and Archaea to be sisters, an inference supported by strong and specific similarities between elements of the archaeal and eukaryotic replication, transcription, and translation machineries (Pace et al. 2012).

Of course it was known since the early seventies that there would be *some* bacterial contribution to the eukaryotic nuclear genome, via the mitochondrial symbiosis and organelle-to-nucleus gene transfer. The initial assumption that this contribution would be small—and perhaps a prejudice that it is the phylogeny of the host that matters most—led to the now largely unquestioned representation of eukaryotes as sister to Archaea.

But unexpectedly, estimates of the bacterial contribution have grown enormously as genomic data pour in. In 2004, Esser et al., described a surprising result. . .

> . . . approximately 75% of yeast genes having homologues among the present prokaryotic sample share greater amino acid sequence identity to eubacterial than to archaebacterial homologues. At high stringency comparisons, only the eubacterial component of the yeast genome is detectable. Our findings indicate that at the levels of overall amino acid sequence identity and gene content, yeast shares a sister-group relationship with eubacteria, not with archaebacteria, in contrast to the current phylogenetic paradigm based on ribosomal RNA. (Esser et al. 2004, p. 1643)

More recently, Pisani et al. (2007), using multiple (including photosynthetic) eukaryotic genomes and a larger collection of bacterial and archaeal genomes reached a similarly startling conclusion. . .

> . . . there are three distinct phylogenetic signals in eukaryotic genomes. In order of strength, these link eukaryotes with the Cyanobacteria, the Proteobacteria, and the Thermoplasmatales, an archaebacterial (euryarchaeotes) group. These signals correspond to distinct symbiotic partners involved in eukaryote evolution: plastids, mitochondria, and the elusive host lineage. (Pisani et al. 2007, p. 1752)

Why, if the Archaeal signal is the weakest, is the Archaeal/eukaryote sister relationship enshrined in textbooks, and the concepts of paraphyly employed so enthusiastically by Pace in his effort to discredit "prokaryote"? Of course, even if the preponderance of data were all that mattered, prokaryotes would still be paraphyletic, with eukaryotes seen as sisters to the Cyanobacteria or Proteobacteria. But the more we come to see eukaryotes as a chimeric clade, established by symbiosis, cell fusion, and LGT, through the active participation of several or many prokaryotic lines, the more aptly descriptive the "eu" and "pro" in "eukaryotes" and "prokaryotes" begin to look. This would have been all along the position of the late Lynn Margulis, coincidentally (Margulis 1996).

In any case, the language of cladistics and the tree-like model on which it is based seem almost irrelevant in the face of such reticulation, or non-tree like signal.

> Reticulation is thus the *bête noire* for cladistics, as initially recognized by Hennig. There are a number of sources of homoplasy (incongruency between certain character distributions and the cladogram based on maximum parsimony), such as adaptive convergence, gene conversion, developmental constraints, mistaken coding, and reticulation. The last-named factor is the most problematical because it involves the fundamental model of reality underlying cladistic analysis. (Mishler and Theriot 2000, p. 50)

Indeed, in 1975, Peter Sneath, a microbiologist and leading theoretical systematist, noted that "reticulate evolution requires consideration of ways to represent the descent of genes, instead of entire genomes as in simple branching cladograms" (Sneath 1975). In 1992, the systematist David Mindell eloquently defended

> . . . the idea that the history of no one symbiont should take precedence over another in assessing genealogy (monophyly) and classification, regardless of size, weight, status as host, or other measure of relative dominance . . . To discount one symbiont within a holobiont when reconstructing the holobiont's overall phylogenetic history and proposing a classification . . . is equivalent to disposal of data. (Mindell 1992, p. 57)

If monophyly is to retain its meaning as containing all descendants of a single ancestor and that ancestor—*and* no data are to be disposed of—then eukaryotes are not monophyletic, nor are Bacteria or Archaea, because of LGT.

A compromise that might allow for a principled if not unarguably "natural" classification in the face of reticulation was suggested in 2007 by David Baum, a noted plant systematist.

> A primary concordance tree, a tree built from clades that are true of a plurality of the genome, provides a useful summary of the dominant phylogenetic history for a group of organisms.

> Residual historical signals can also be extracted in the form of secondary, tertiary, etc. concordance trees, which are built from clades that are present in the genome but contradict clades on the primary concordance tree. (Baum 2007, p. 417)

Most of us would react with horror to a proposal to consider reclassifying non-photosynthesizing eukaryotes as sister to proteobacteria and photosynthetic eukaryotes as sister to cyanobacteria (though this last view was common until the mid-twentieth century). But this would be arguably more consistent with Darwin's call for *genealogical* classification, and would be based on the same facts and the same understanding of what has happened during the evolution of genes, genomes, and organisms as is the "phylogenetic classification" enshrined in the hegemonic three-domain tree.

It is only by "disposal of data"—allowing rRNA and a few associated informational genes to stand in for an evolutionary lineage as a whole—that inferences about monophyly, polyphyly, or paraphyly of the three domains can be made to appear clean. Two assumptions lie behind this maneuver. First is that informational genes are less likely to be transferred, especially across large phylogenetic distances, because of the complexity of the interaction of their products with those of other genes (Jain et al. 1999). Thus, they track the history of individual cell divisions and speciation events better than other genes. The second, more presumption than assumption, is that it is in this history that a true genealogical classification most naturally rests.

The first is indeed plausible from several perspectives. Interestingly, Cotton and McInerney (2010) recently published a paper whose title says much about the privileging of the archaeal trace. In "Eukaryotic genes of archaebacterial origin are more important than the more numerous eubacterial genes, irrespective of function" they show that those (quantitatively) fewer yeast eukaryotic nuclear genes that show archaeal sisterhood are disproportionately "essential to yeast viability, are more highly expressed, and are significantly more highly connected and more central in the yeast protein interaction network," as might be expected if the archaeal heritage were more stably associated with the less frangible aspects of cell biology.

The second, that even when a majority of genes contradict the informational signal, it is the latter we should rely on in classification, is not a question of science, answerable by experiment. Classification has less to do with identifying "natural groups" and more to do with the philosophy, history, and politics of the idea of "natural" than we generally like to admit. There is much intellectual momentum behind this approach, but there *are* serious alternatives (Cavalier-Smith 2002, 2007) that cannot be dismissed out of hand, simply because they have fewer adherents. There is in fact no principled way, given massive reticulation, of deciding what is the most "natural" way to classify organisms, or divide all living things up into "kinds." The various positions taken by microbiology's leaders, though bolstered by appeals to scientific evidence, are in the end rhetorical.

## Coda

Natural kind thinking has been part of biological classification for millennia, of course, as many philosophers of biology and biologists who practice philosophy have noted. Ernst Mayr called it "typological thinking." The most extreme form of such thinking has been described (and then rejected) by the philosopher Ian Hacking as the belief that: . . .

> ► There is a unique best taxonomy in terms of natural kinds, that represents nature as it is, and reflects the network of causal laws. We do not have nor could we have a final taxonomy of anything, but any objective classification is right or wrong according as it captures part of the structure of the one true taxonomy of the universe. (Hacking 1991, p. 111)

Such a view, which we too reject, underlies the debate about whether there are three rather than two kinds of living things in this world. To be sure, there are more or less reasonable ways to look at diversity. But as to how many kinds there *actually* are, there should be no more definitely true answer than there would be to "How many kinds of people are there in North America?" or "How many kinds of books are there in my town's public library?"

And yet, such natural kind thinking comes naturally to biologists, generating much of the heat in our arguments. Insistence that the grade distinction between prokaryotes and eukaryotes must be discarded because it conflicts with the division of living things into clades reflects a belief that "the one true taxonomy of the universe" is the Tree of Life—to which concept, as loyal Darwinists, we feel we must cling. And yet, because of chimerism and extensive LGT, that Tree means much less as a description of what organisms are like and how they came to be that way than Darwin himself would have ever imagined (Doolittle 1999; Martin 2011).

Furthermore, the Tree unambiguously depicts three discretely defined monophyletic clades if and only if we accept as settled many highly arguable propositions about how evolution proceeds, about what the data say in that regard, and about how classification is properly to be pursued. Some of these propositions, reviewed here, are that (1) in spite of the testimony of most of their genes, the "one true" way to look at Eukarya is as sisters to or emerging from within Archaea; (2) in spite of very sophisticated recent analyses of those genes thought to be most truthful, sisterhood (rather than emergence from within) is the right way to understand this relationship; (3) Bacteria and Archaea are themselves properly described as monophyletic clades, even though it is but a few percent of their genes that tell us this, with the preponderance of genes saying that they comprise a single, albeit highly structured and admittedly *very* slowly mixing population; (4) that it makes sense to speak of LUCA, either as a single cell or species or (alternatively) as a heterogeneous population extended over time; (5) that it makes sense to describe LUCA (of whatever type) as something other than a prokaryote. None of these is provably wrong, but none will ever be proved right, and all are matters of opinion, not fact. Definition-dependent, supposedly logic-driven

arguments on the use of "prokaryote" seem doomed to failure by the same criteria with which they are undertaken, coupled with the vast underdetermination of any of our current theories about early cellular evolution (Vesteg and Krajcovic 2011; Forterre 2011).

Moreover, much of the debate is about the definition of terms, of which Popper wrote:

> In science, we take care that the statements we make should never *depend* on the meaning of our terms. Even where the terms are defined, we never try to derive any information from the definition, or to base any argument upon it. . . . We are always conscious that our terms are a little vague (since we have learned to use them only in practical applications) and we reach precision not by reducing their penumbra of vagueness, but rather by keeping well within it, by carefully phrasing our sentences in such a way that the possible shades of meaning of our terms do not matter. This is how we avoid quarrelling about words. (Popper 2003, pp. 21–22)

So, in a more relaxed context, but still one in which we strive to keep well within Popper's "penumbra of vagueness," is there anything sensible to say in defense of "prokaryote"?

As a phenetically based grade distinction, the prokaryote: eukaryote dichotomy resonates so strongly largely because eukaryotes seem so different from either Archaea or Bacteria, and all so much like each other, at the level of the cell. Four decades ago, the prevailing view was that eukaryotes arose from within prokaryotes by the gradual evolution of cytoskeleton and endomembrane systems—driven by selection for the acquisition of phagotrophy and followed by the establishment of the endosymbionts destined to become mitochondria and plastids (Gray and Doolittle 1982). Theory predicted that there might be some surviving primitively amitochondriate phagotrophs still out there. But so far none has been found, and most of the complexification of internal systems believed to be characteristically eukaryotic were apparently already in place in LECA. Arguably, there was very rapid, selection-directed modification of many archaeal-type genes (of the "important" kind identified by Cotton and McInerey), such that many now are hard to align with their still-prokaryotic homologs. To cell biologists working on the eukaryote side of that "great discontinuity," it is the "pro" in prokaryotes that gives meaning to the term. Especially for symbionticists like Lynn Margulis (1996), but even for more conservative theorists like John Maynard Smith and Eors Szathamary (1995), the chimeric fusion event(s) that forged eu- from prokaryotes was a profoundly important evolutionary transition.

Whether archaeal/bacterial differences are comparable in importance to the eukaryote:prokaryote distinction is indeed a matter of "comparing apples to oranges." Woese in rebutting Mayr asserted that his (Mayr's) "attempt to apply globally a parochial and subjectively defined concept of diversity serves only to reveal the futility in such an approach." But it is hard to imagine concepts of phenotypic diversity applicable to both prokaryotes and eukaryotes that are not "parochial and subjectively defined," at the very least insofar as they involve choices as to differential relevance of datasets. For the cell biologist of the school of classification Mayr called "Darwinian," focusing on the translational machinery is parochial. And for the microbiologist working with Bacteria or Archaea, although there are important differences that should inform experiments on gene structure and function, in the study of metabolism, regulation, population genetics, and ecology, this is much less the case. Without knowing in advance it would, in many instances, be hard to say whether a particular published paper on these topics had a bacterium or an archaeon as its study object.

It is not necessary to imagine that the world holds some particular number of basic types in order to understand it, or advance biological knowledge. Almost certainly "prokaryote" glosses over the many differences between Bacteria and Archaea, but "Bacteria" glosses over many differences between mycoplasmas and planctomycetes, and by what criteria can we judge one glossing-over more egregious than the other? Prokaryota is indeed not a legitimate taxonomic group by the principles of cladists, nor are Eukarya, Bacteria, and Archaea, unless we ignore or rework those principles to the point that they are no longer recognizable or useful. To avoid quarreling about words, we need first to realize that that is what we are doing.

The continued use of "prokaryote" with a small "P" seems to us justified by history and utility. If indeed it confuses students to teach them this "wrong idea," this will be because we have dumbed our science too far down. Those who come to see that "prokaryote" is neither right nor wrong and that its meaning is contextual will achieve a deeper understanding of microbiology, and of science as a human endeavor.

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## References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Angert ER (2005) Alternatives to binary fission in bacteria. Nat Rev Microbiol 3:214–224

Ashlock PD (1974) The uses of cladistics. Ann Rev Ecol Syst 5:81–99

Baum DA (2007) Concordance trees, concordance factors, and the exploration of reticulate genealogy. Taxon 56:417–426

Bell SD, Jackson SP (2001) Mechanism and regulation of transcription in archaea. Curr Opin Microbiol 4:208–213

Bird A, Tobin E (2010) Natural kinds. In: Zalta EN (ed) The Stanford encyclopedia of philosophy, summer 2010 edn. http://plato.stanford.edu/archives/sum2010/entries/natural-kinds/

Brown JR, Doolittle WF (1994) Root of the universal tree based on aminoacyl-tRNA gene duplications. Proc Natl Acad Sci USA 92:2441–2445

Cavalier-Smith T (2002) The neomuran origin of archarebacteria, the negibacterial root of the universal tree and bacterial megaclassification. Int J Syst Evol Microbiol 52:7–76

Cavalier-Smith T (2007) Concept of a bacterium still valid in prokaryote debate. Nature 446:257

Chatton E (1937) Titres et travaux scientifiques. Sette, Sottano, Italy

Cotton JA, McInerney JO (2010) Eukaryotic genes of archaebacterial origin are more important than the more numerous eubacterial genes, irrespective of origin. Proc Natl Acad Sci USA 107:17252–17255

Cox CJ, Foster PG, Hirt RP, Harris SR, Embley TM (2008) The archaebacterial origin of eukaryotes. Proc Natl Acad Sci USA 105:20356–20361

Darnell JE, Doolittle WF (1986) Speculations on the early course of evolution. Proc Natl Acad Sci USA 83:1271–1275

Darwin CR (1859) On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life, 1st edn. John Murray, London

Di Giulio M (2011) The Last Universal Common Ancestor (LUCA) and the ancestors of Archaea and Bacteria were progenotes. J Mol Evol 72:119–126

Dolan MF, Margulis L (2007) Advances in biology reveal truth about prokaryotes. Nature 445:21

Doolittle WF (1999) Phylogenetic classification and the universal tree. Science 284:2124–2129

Doolittle WF, Brown JR (1994) Tempo, mode, the progenote, and the universal root. Proc Natl Acad Sci USA 91:6721–6728

Dupré J (1981) Natural kinds and biological taxa. Philos Rev 90:66–90

Embley TM, Martin W (2006) Eukaryote evolution, changes and challenges. Nature 440:623–630

Enright AJ, Van Dongen S, Ouzounis CA (2002) An efficient algorithm for large-scale detection of protein families. Nucl Acids Res 30:1575–1584

Esser C, Ahmadinejad N, Wiegand C, Rotte C, Sebastiani F, Gelius-Dietrich G, Henze K, Kretschmann E, Richly E, Leister D, Bryant D, Steel MA, Lockhart PJ, Penny D, Martin W (2004) A genome phylogeny for mitochondria among alpha-proteobacteria and a predominantly eubacterial ancestry of yeast nuclear genes. Mol Biol Evol 21:1643–1660

Field MC, Dacks JB (2009) First and last ancestors: reconstructing evolution of the endomembrane system with ESCRTs, vesicle coat proteins, and nuclear pore complexes. Curr Opin Cell Biol 21:4–13

Forterre P (2011) A new fusion hypothesis for the origin of Eukarya: better than previous ones, but probably also wrong. Res Microbiol 162:77–91

Foster PG, Cox CJ, Embley TM (2009) The primary divisions of life: a phylogenomic approach employing composition-heterogeneous methods. Philos Trans Roy Soc B 364:2197–2207

Fox GE (2010) Origin and evolution of the ribosome. Cold Spr Harbor Perspect Biol 2010:a003483

Fox-Keller E (2010) The mirage of a space between nature and nurture. Duke University Press, Durham

Fuerst JA, Sagulenko E (2011) Beyond the bacterium: planctomycetes challenge our concepts of microbial structure and function. Nat Rev Microbiol 9:403–415

Glansdorff N, Xu Y, Labedan B (2008) The Last Universal Common AncestoR: emergence, constitution and genetic legacy of an elusive forerunner. Biol Direct 3:29

Glansdorff N, Xu Y, Labedan B (2009) The origin of life and the last universal common ancestor: do we need a change of perspective? Res Microbiol 160:522–528

Gogarten JP, Kibak H, Dittrich P, Taiz L, Bowman EJ, Bowman BJ, Manolson MF, Poole RF, Date T, Oshima T, Konishi J, Denda K, Yoshida M (1989) Evolution of the vacuolar ATPases: implications for the origin of eukaryotes. Proc Natl Acad Sci USA 86:6661–6665

Golinski J (1998) Making natural knowledge: Constructivism and the history of science. University of Chicago Press, Chicago

Grant V (2003) Incongruence between cladistic and taxonomic systems. Am J Bot 90:1263–1270

Gray MW, Doolittle WF (1982) Has the endosymbiont hypothesis been proven? Microbiol Rev 46:1–42

Gribaldo S, Poole AM, Daubin V, Forterre P, Brochier-Armanet C (2010) The origin of eukaryotes and their relationship with the Archaea: are we at a phylogenomic impasse? Nat Rev Microbiol 8:743–752

Gupta RS (2000) The natural evolutionary relationships among prokaryotes. Crit Rev Microbiol 26:111–131

Guy L, Ettema TJG (2011) The archaeal "TACK" superphylum and the origin of eukaryotes. Trends Microbiol 19:580–587

Hacking I (1991) A tradition of natural kinds. Philos Stud 61:109–126

Iwabe N, Kuma K, Hasegawa M, Osawa S, Miyata T (1989) Evolutionary relationship of archaebacteria, eubacteria and eukaryotes inferred from phylogenetic trees of duplicated genes. Proc Natl Acad Sci USA 86:9355–9459

Jain R, Rivera MC, Lake JA (1999) Horizontal gene transfer among genomes: the complexity hypothesis. Proc Natl Acad Sci USA 96:3801–3806

Koonin EV (2009) On the origin of cells and viruses: primordial virus world scenario. Ann NY Acad Sci 1178:47–64

Lake JA, Henderson E, Oakes M, Clark MW (1984) Eocytes: a new ribosome structure indicates a kingdom with a close relationship to eukaryotes. Proc Natl Acad Sci USA 81:3786–3790

Lovejoy AO (1936) The great chain of being. Harvard University Press, Cambridge

Lukes J, Archibald JM, Keeling PJ, Doolittle WF, Gray MW (2011) How a neutral evolutionary ratchet can build cellular complexity. IUBMB Life 63:528–537

Makarova KS, Yutin N, Bell SD, Koonin EV (2010) Evolution of diverse cell division and vesicle formations systems in Archaea. Nat Rev Microbiol 8:731–741

Margulis L (1996) Archaeal-eubacterial mergers in the origin of Eukarya: phylogenetic classification of life. Proc Natl Acad Sci USA 93:1071–1076

Martin WF (2011) Early evolution without a tree of life. Biol Direct 6:36

Martin W, Koonin E (2006) A positive definition of prokaryotes. Nature 442:24

Martin WF, Russell MJ (2003) On the origins of cells: a hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautotrophic prokaryotes, and from prokaryotes to nucleated cells. Philos Trans R Soc Lond B Biol 358:59–63

Maynard Smith J, Szathmary E (1995) The major transitions in evolution. Oxford University Press, Oxford

Mayr E (1982) The growth of biological thought. Harvard University Press, Cambridge, MA

Mayr E (1990) A natural system of organisms. Nature 348:491

Mayr E (1991) More natural classification. Nature 353:122

Mayr E (1998) Two empires or three? Proc Natl Acad Sci USA 95:9720–9723

McInerney JO, Martin WF, Koonin EV, Allen JF, Galperin MY, Lane N, Archibald JM, Embley TM (2011) Plactomycetes and eukaryotes: a case of analogy not homology. Bioessays 33:810–817

McOuat G (2009) The origins of "natural kinds": keeping "essentialism" at bay in the Age of Reform. Intell Hist Rev 19:211–230

Mindell DP (1992) Phylogenetic consequences of symbioses: Eukarya and eubacteria are not monophyletic taxa. Biosystems 27:53–62

Mishler BD, Theriot EC (2000) The phylogenetic species concept (*sensu* Mishler and Theriot): monophyly, apomorphy and phylogenetic species concepts. In: Wheeler QD, Meier R (eds) Species concepts and phylogenetic theory. Columbia University Press, New York

O'Brien TW (2002) Evolution of a protein-rich mitochondrial ribosome: implications for human genetic disease. Gene 286:73–79

O'Hara, Robert J (1997) Population thinking and tree thinking in systematics. Zool Scr 26(4):323–329

O'Hara RJ (1998) Population thinking and tree thinking in systematics. Zoologica Scripta 26:323–329

O'Malley MA (2010) Ersnt Mayr, the tree of life, and philosophy of biology. Biol Philos 25:529–552

Pace NR (2006) Time for a change. Nature 441:289

Pace NR (2009) Rebuttal: the modern concept of the prokaryote. J Bacteriol 191:2006–2007

Pace NR, Sapp J, Goldenfeld N (2012) Phylogeny and beyond: scientific, historical, and conceptual significance of the first tree of life. Proc Natl Acad Sci USA. Early edition on line

Payankaulam S, Li ML, Arnosti DN (2010) Transcriptional repression: conserved and evolved features. Curr Biol 20:R764–R771

Peeters E, Charlier D (2010) The Lrp family of transcription regulators in archaea. Archaea 2010:750457

Penny D, Poole A (1999) The nature of the last universal common ancestor. Curr Opin Genet Dev 9:672–677

Pisani D, Cotton JA, McInereny JO (2007) Supertrees disentangle the chimerical origin of eukaryotic genomes. Mol Biol Evol 24:1752–1760

Popper K (2003) The open society and its enemies, vol 2. Routledge, London (first published 1945)

Poptsova MS, Gogarten JP (2007) BranchClust: a phylogenetic algorithm for selecting gene families. BMC Bioinformatics 8:120

Roberts E, Sethi A, Montoya J, Woese CR, Luthey-Schluten Z (2008) Molecular signatures of ribosomal evolution. Proc Natl Acad Sci USA 105:13953–13958

Sapp J (2006) Two faces of the prokaryote concept. Int Microbiol 9:163–172

Sapp J (2009a) The prokaryote-eukaryote dichotomy: meanings and mythology. Microbiol Mol Biol Rev 69:292–305

Sapp J (2009b) The new foundations of evolution. Oxford University Press, Oxford

Sneath PHA (1975) Cladistic representation of reticulate evolution. Syst Zool 24:360–368

Spath SB (1999) C.B. van Niel and the culture of microbiology, 1920–1965. PhD thesis, History, University of California, Berkeley

Stanier RY, Doudoroff M, Adelberg EA (1963) The microbial world. Prentice-Hall, Engelwood Cliffs, NJ

Stanier RY, van Niel CB (1962) The concept of a bacterium. Arch Microbiol 42:17–35

Tatusov RL, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B, Koonin EV, Krylov DM, Mazumder R, Mekhedov SL, Nikolskaya AN, Rao BS, Smirnov S, Sverdlov AV, Vasudevan S, Wolf YI, Yin JJ, Natale DA (2003) The COG database: an updated version includes eukaryotes. BMC Bioinformatics 4:41

Vesteg M, Krajcovic J (2011) The falsifiability of the models for the origin of eukaryotes. Curr Genet 57:367–390

Vetsigian K, Woese D, Goldenfeld N (2006) Collective evolution and the genetic code. Proc Natl Acad Sci USA 103:10696–10701

Walsh DA, Doolittle WF (2005) The real 'domains' of life. Curr Biol 15: R237–R240

Werner F, Grohmann D (2011) Evolution of multisubunit RNA polymerases in the three domains of life. Nat Rev Microbiol 9:85–98

Whitman WB (2009) The modern concept of the prokaryote. J Bacteriol 191:2000–2005

Winsor MP (2006) Linnaeus's biology was not essentialist. Ann Missouri Botanical Garden 93:2–7

Woese CR (1998a) Default taxonomy: Ernst Mayr's view of the microbial world. Proc Natl Acad Sci USA 95:11043–11046

Woese C (1998b) The universal ancestor. Proc Natl Acad Sci USA 95:6854–6859

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucarya. Proc Natl Acad Sci USA 87:4576–4579

Zhaxybayeva O, Gogarten JP (2004) Cladogenesis, coalescence and the evolution of three domains of life. Trends Genet 20:182–187

# 3 Prokaryotes and Their Habitats

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Prokaryotes are well recognized as essential members of the biosphere. They inhabit all possible locations in which life exists from those offering ideal conditions for growth and reproduction to those representing extreme environments at the borderline of abiotic conditions.

The ubiquity of microorganisms is based on three major properties: their small size for easy dispersal by air and water, their metabolic versatility and flexibility, and their ability to tolerate unfavorable conditions. A predominant population is commonly composed of species able to grow under the particular conditions of a habitat. Many other species may also be present but in low numbers of individuals. As a rule, ecosystems of indistinct physicochemical and nutritional characteristics, such as many soils or seawater, which neither suppress nor specifically support microbial growth, usually carry low numbers of microorganisms but a high diversity of species. In contrast, ecosystems of strong environmental characteristics, such as acid mine waters, salt brines, and hot springs, commonly contain high cell numbers of very few species.

Experimental enrichment procedures bring about the predominance of certain species by controlling the supply of specific nutrients or the use of certain physicochemical conditions. If the growth conditions of a particular microorganism are known and reproducible, enrichment and isolation usually pose no problem. But if the particular requirements for growth of an organism are unknown, isolation procedures may be difficult to discover (Pfennig 1961; Schlegel and Pfennig 1961). For that reason, a number of organisms long known from microscopical observations, such a *Thiovulum* or *Achromatium*, have not yet been isolated in pure culture. Furthermore, organisms that have hitherto unknown growth characteristics and that are too small and inconspicuous for easy microscopical recognition have often escaped detection. An excellent example is *Desulfuromonas acetoxidans* (Pfennig and Biebl 1976).

In characterizing an ecosystem microbiologically, it is important to distinguish between (1) organisms introduced incidentally by air, soil runoff, etc., physiologically just making the best of it, and (2) organisms typically adapted to the particular habitat and not occurring in any other except in the form of survival stages. An example of the former is *Escherichia coli*, as frequently found in polluted waters. An example of the latter is the above-mentioned *Thiovulum* sp., whose need for dissolved oxygen and hydrogen sulfide at the same time requires a high motility combined with chemotactic orientation in an aquatic oxic/anoxic interface.

Although their morphological differentiation is limited, prokaryotes have evolved a number of structural and chemical mechanisms that enable them to inhabit various extreme environments. The presence of a specified pigment, for instance, protects a cell against detrimental radiation or may provide for the absorption of light energy at specific wavelengths encountered in deeper water. Some filamentous cyanobacteria show a certain degree of cell differentiation, a feature that permits the fixation of elemental nitrogen concomitantly with oxygenic photosynthesis in oligotrophic environments. More importantly, however, the metabolic versatility of the prokaryotes, which reflects the development of metabolism during the evolution of life, enables them to live in many parts of the biosphere, including several where eukaryotes are not able to exist.

The vegetative microbial cell, with its relatively large reactive surface, responds quickly to changing physicochemical conditions of its immediate surrounding. As a consequence, the effective habitat of a microorganism is its microhabitat, the immediate surrounding of the cell in a compatible scale of space and time as determined by the radius of its metabolic action and interaction.

Naming microorganisms for their occurrence in certain characteristic macrohabitats, for example, soil and water bacteria, is of limited use. The two apparently very different habitats, soil and water, can be characterized as representing different proportions of the two phases, solid surface and water. The continuum of habitats ranges from highly arid desert soil with no or firmly bound pore water to offshore pelagic seawater containing a minimum of suspended particulate matter. Within the range of suitable physicochemical conditions, the abundance of microorganisms in an ecosystem is determined by the availability of the required energy and carbon sources and essential nutrients. All the more or less specific environments—for example, the surface of leaves or skin, intestinal tracts, and symbiotically or parasitically invaded tissues—conform to this general description.

The concept of microenvironments eliminates the sharp dividing lines between aquatic, terrestrial, and even medical microbiology. Indeed, in ecological research, the distinction between these academic disciplines is now more and more deemphasized by encompassing them under the label of environmental or biogeochemical microbiology. This chapter does not try to cover all the habitats of all organisms treated in this handbook; the individual habitats and their characteristics are considered chapter by chapter for single species or physiological groups of prokaryotes. This chapter reviews the versatility of prokaryotic metabolism in relation to a few principles that determine the distribution of prokaryotes in nature.

The principal methods for the enrichment and isolation of the major metabolic types of microorganisms were discovered within a relatively short time. Details of the techniques developed by Winogradsky and Beijerinck are dispersed through the journals. Their collected papers (Beijerinck 1921–1940; Winogradsky 1949) are treasure troves for microbiologists; only one contemporary compilation exists (Stockhausen 1907). Since the enrichment principles and methods were the subject of a symposium (Schlegel 1965), several reviews have appeared (Aaronson 1970; Schlegel and Jannasch 1967; Veldkamp 1970).

The aim of this handbook is to encourage biologists to continue and intensify the search for bacteria in their natural environments, define habitats and ecological niches, and understand the flux of matter and energy through the biosphere. One may remember that in many soil and water samples, there are more kinds of bacteria present than we can cultivate. Furthermore, much data on the flux of carbon and of trace gases through ecosystems cannot yet be accounted for by the bacteria cultivated so far. The gaps need to be filled by laboratory and field studies.

## Ecological Terminology

### Ecosystems

"The ultimate aim of ecology is to understand the relationships of all organisms to their environment" (Hungate 1962). The principles of ecology were developed independently for the sciences dealing with plants, animals, and microorganisms. Identical terms are used to designate ecological units and parameters when dealing with macro- and microorganisms, but the implications of these terms are slightly different. The differences sometimes lead to considerable confusion (Whittaker et al. 1973). Furthermore, microorganisms, especially bacteria, exhibit properties not encountered in higher forms of life. The ecology of microorganisms has, therefore, been studied separately, and emphasis has been given to those microorganisms and their habitats that live under conditions not tolerated by the majority of the higher forms of life (Alexander 1971, 1977; Hungate 1962; Kushner 1978). The recognition of the principles of microbial ecology is not yet complete and requires studies in many areas of experimental science (Atlas and Bartha 1987; Belkin et al. 1986a, b, 1987; Stetter 1986, 1989; Megusar and Gantar 1986). Therefore, the usage of terms in microbial ecology is not uniform and partially deviates from that used for the ecology of macroorganisms. A short survey of the present usage of some terms found in this chapter is presented below.

The ecology of microorganisms is concerned with the relationships between different species of microorganisms and between microorganisms and the environment. The basic unit of ecology is the community or biocoenosis. The community has to be considered in relation to the physical and chemical characteristics of the site. Both the biotic components—the community—and the abiotic, physicochemical components make up the ecosystem. The abiotic component of the ecosystem is frequently referred to as the environment, although when speaking about a particular member of the community, for example, a species or its population, the term environment is often used to designate both the physicochemical and the biotic components of the ecosystem. In this case, the term environment is used synonymously with "habitat."

Ecosystems differ from each other with respect to their extension. Ecosystems may be as large as a pond, a lake, a river, or a fir forest, or they may be as small as the rumen, the intestinal tract of an earthworm, or the rhizosphere of a plant. Some ecosystems, such as a cultivated field or the human skin, are characterized by weak (normal) environmental factors. Other ecosystems, such as hot springs or solar salt ponds, are characterized by strong environmental factors which cause a strict selection among potential inhabitants.

### Habitats

Within an ecosystem, a habitat for each species can be defined. The habitat is the location or dwelling place of a particular organism. For each microorganism, at least one habitat can be defined where it lives and grows and from which it may be recovered and isolated. Within a distinct ecosystem, a microorganism usually has only one kind of habitat. However, a microorganism may have two or more habitats, each in a different ecosystem. For example, many rhizobia are able to grow in the soil as well as intracellularly in root nodules; opportunist pathogenic bacteria such as *Pseudomonas aeruginosa*, *P. pseudomallei*, and *Erwinia* live in aquatic habitats as well as in mammalian hosts; and luminescent bacteria are met as free-living forms as well as living symbiotically in light organs of fish and invertebrates. Some bacteria are associated with both plant and animal hosts. A particular species of cellulolytic bacteria or of methanogenic bacteria may have its habitat in a lake bottom sediment, in the rumen, and in the sewage-sludge fermenter. In other words, the habitat designates the street and house number of a particular organism; some organisms may have several addresses.

When a species or a particular group of microorganisms is discussed, the word habitat is often used to denote an ecosystem, that is, the entirety of the biotic and abiotic factors to which the particular microorganism is exposed in its dwelling place. This meaning of the term is in general usage in bacterial ecology. Since the operational area of many bacteria is small, the term "microhabitat" is frequently used synonymously with "microecosystem" or with "microenvironment."

## Inhabitants of an Ecosystem

Each ecosystem harbors a variety of diverse microorganisms. Two principal categories of microorganisms are distinguished: (1) autochthonous or indigenous (resident) microorganisms and (2) zymogenous, allochthonous, or nonindigenous (transient) microorganisms. This concept of two large groups of microorganisms, which are different with respect to their function, was first suggested by Winogradsky (1925). This concept, originally developed in soil microbiology (Winogradsky 1925, 1926, 1947, 1949), has been adopted in general ecology (Alexander 1971; Savage 1977a). Autochthonous microorganisms in the strict sense are those that are native to a distinct soil (e.g., humic soil) and are always present. The population of autochthonous bacteria does not depend on exterior organic matter or on periodic increases of nutrients. Instead it reflects a more or less constant flux of nutrients. Indigenous microorganisms are known for many ecosystems, such as humus soil, the intestinal tract of animals, the rumen, the skin, and plant leaves.

The terms zymogenous, allochthonous, or nonindigenous refer to microorganisms that are dependent on occasional increases of nutrient concentrations or the occurrence of unusual substrates. As aliens and transients, they may persist in the absence of their required substrate or substrate concentration in dormant or starvation-resistant cell stages.

The ecosystem may be occupied by metabolically highly specialized organisms that are restricted to a distinct environment. These microorganisms are easily recognized as belonging to the indigenous flora of the ecosystem. Various

obligately parasitic bacteria are specialized to certain animals or plants or even to certain tissues; some bacteria have only been isolated from hot springs, from sewage, or from other extreme environments. At the opposite extreme are the ubiquitous bacteria that are able to grow in distinctly different environments. Examples are soil and water bacteria that occasionally become opportunistic pathogens in animals (*Pseudomonas aeruginosa*, *P. pseudomallei*) or that may switch from plants to animals (*Erwinia*).

## Ecological Niches

The term ecological niche was originally used in the sense of an area or location, the properties of which enabled an organism to live there. The functional concept of the niche led to some confusion. The different meanings were discussed by Whittaker, Levin, and Root (1973). Now "ecologists use the term ecological niche to mean the role that the organisms play in the ecosystem" (Odum 1977). The ecological niche means the function of a species in a community of species in the ecosystem. Each species or strain of a bacterium has particular nutritional requirements, kinetic properties, biochemical abilities, and structural particularities; furthermore, it has distinct degrees of tolerance to environmental conditions. The ability or inability to fulfill a particular function in a distinct ecosystem is dependent on those properties.

For example, many cellulose-hydrolyzing bacteria are not highly specialized. They occupy a broad niche. However, in some ecosystems, they may be restricted to cellulose utilization only, because they cannot compete with other organotrophic bacteria in performing other functions such as glucose fermentation. In these ecosystems, they occupy a narrow niche. This description agrees with the general observation that the actual distribution of a species or population is usually more limited than its predicted one. In other words, real niches are always smaller than potential niches. Secondary factors determine the predominance of one species among those which could potentially occupy the niche. In the rumen, for example, among the cellulolytic bacteria, only those that are able to hydrolyze cellulose under anaerobic conditions and to generate energy for growth by fermentation will be suited to fulfill the function of cellulose hydrolysis. Furthermore, cellulose degradation in the rumen requires tolerance to the particular environmental conditions in the rumen, such as temperatures up to 39–40 °C and presence of various fatty acids, enzymes, ammonia, and gases. Finally, the function of the cellulolytic population of the rumen is influenced by the activities of other members of the community. In some rumen bacteria, the metabolic processes are either impeded or modified if a product of their fermentation, hydrogen, is not removed by methanogenic bacteria. In this case, interspecies hydrogen transfer may be involved, as demonstrated for the S-organism and strain MoH of *Methanobacterium omelianskii* (Reddy et al. 1972a), *Ruminococcus albus* and *Vibrio succinogenes* (Iannotti et al. 1973), *Ruminococcus flavefaciens* and *Methanobacterium ruminantium* (Latham and Wolin 1977), and *Clostridium thermocellum* and *Methanobacterium thermoautotrophicum* (Winfrey and Zeikus 1977). Therefore, the organism fulfilling a certain function in a particular ecosystem is determined by many physical and chemical factors and by all of the organisms constituting the community.

Studies on bacteria in pure culture attempt to define their entire ecological potential. The conclusions derived from pure-culture studies can be used to describe the potential niche of the particular bacterium. In nature, the predicted niche may be narrowed by competition. For example, it is possible to grow a pure culture of *Chromatium vinosum* in the laboratory in a medium containing malate as the sole electron donor (Thiele 1968; van Niel 1932, 1936). In its natural habitat, *Chromatium vinosum* would not dominate on this medium; members of the Rhodospirillaceae would outgrow the Chromatiaceae.

Finding the explanation for the dominance of a particular species in the habitat requires nutritional and kinetic pure-culture studies. For example, in aquatic environments where hydrogen sulfide is generated in the bottom sediment, bright purple or salmon-colored suspensions of the phototrophic bacterium *Chromatium okenii* or *Thiospirillum jenense* are often seen. Microscopic inspection reveals the presence of several small species of the Chromatiaceae in addition to the large species. Both types evidently coexist in their habitat. However, when samples of these natural enrichments are used for inoculating enrichment cultures in the laboratory, the dominant large bacterial species are overgrown by small species of the same physiological group, such as *C. vinosum* (Pfennig 1965; Schlegel and Pfennig 1961). The dominance of the large species in the natural habitat was not understood until Van Gemerden (1974) studied the competition of a representative of the large species, *C. weissei*, and a representative of the small species, *C. vinosum*, for the same substrate, hydrogen sulfide, in chemostat culture.

The dominance of *C. weissei* in the natural habitat turned out to depend upon the diurnal rhythm of illumination. The two species differ from each other with respect to the affinity (the $K_s$ of intact cells) for hydrogen sulfide and to the rate of its uptake and oxidation to sulfur globules stored intracellularly. The maximal rate of hydrogen sulfide uptake (on the basis of total cell number) by *C. weissei* is about two and a half times higher than that by *C. vinosum*. In contrast, *C. vinosum* has a lower $K_s$ for hydrogen sulfide and has a higher maximum growth rate. On the basis of experimental evidence, the dominance or the coexistence of *C. weissei* in the natural habitat can be explained: when both strains are growing in continuous light, most of the hydrogen sulfide will be consumed by *C. vinosum* due to its high growth rate and high affinity for hydrogen sulfide. In the dark, hydrogen sulfide accumulates; on illumination, the major amount of the accumulated hydrogen sulfide is oxidized by *C. weissei*, and sulfur is stored intracellularly, allowing at least moderate growth for a few hours. Under intermittent illumination, both species can coexist. The example demonstrates that competition in the natural habitat can be based on kinetic properties. It further emphasizes the necessity

of pure-culture studies for understanding the ecological niches of some microorganisms.

The importance of the substrate affinity, expressed in the $K_s$ constant, can be easily demonstrated by growing mixed bacterial populations in the chemostat at varying dilution rates. At high dilution rates with substrate excess, the fast-growing bacteria are favored. At low dilution rates, when the substrate limits growth, the organism endowed with high substrate affinity (low $K_s$) will successfully compete. Continuous culture enrichment procedures with natural populations are based on these species-specific substrate affinities (Jannasch 1967). Competition studies were conducted with psychro- and mesophilic bacteria (Harder and Veldkamp 1971), a spirillum, and a rod-shaped bacterium in phosphate-limited medium (Kuenen et al. 1977). Such studies have been used to describe the characteristics of prokaryotes living in habitats where growth is largely limited by carbon compounds utilized by a large number of competing species. The use of the chemostat and the kinetics of microbial growth at characteristic ecological niches have been repeatedly reviewed (Jannasch and Mateles 1974; Veldkamp 1976; Veldkamp and Jannasch 1972).

Understanding the ecological niche of a bacterial species is necessary to design enrichment culture conditions. Elective enrichment requires knowing more about a bacterium than its basic features. The knowledge of general growth conditions is not sufficient. For elective enrichment, only those properties can be considered which allow successful competition within a mixed population. The determining feature for occupying the actual narrow niche may be as obvious as in the case of the dinitrogen-fixing *Azotobacter*, abundantly growing and dominating in a sample of soil to which sucrose has been added without providing a source of combined nitrogen. The determining feature, however, may still be hidden, as in the case of enriching for green sulfur bacteria (Chlorobiaceae), which compete successfully at low light intensities but succumb to purple sulfur bacteria at high light intensities (Biebl and Pfennig 1978; Pfennig and Cohen-Bazire 1967).

## Bacterial Metabolism as an Ecological Determinant

Many ecosystems, both natural and artificial, are almost exclusively occupied by prokaryotes. Environments characterized by the absence of eukaryotes are the strictly anaerobic regions of freshwater lakes and of marine estuary sediments and any organic material fermented or decomposed under the exclusion of air (sauerkraut, silage, biodigestion with methane production). Prokaryotic cells have also adapted almost exclusively to certain extreme conditions of temperature, acidity, and salinity.

The exclusiveness of bacteria is based on unique metabolic capabilities either absent from or only rudimentarily present in eukaryotes. The uniqueness of bacteria pertains to their modes of energy conversion, to their wide range of growth substrates, and to their tolerance toward extreme environmental conditions.

## Modes of Energy Conversion

All forms of life use energy for maintenance as well as for biosynthesis of cell material. Biochemical energy is generated by metabolic reactions. Energy sources are organic or inorganic substrates that are taken up from the environment. In the cell, they are converted via a series of metabolic pathways. These pathways fulfill two essential functions: they provide precursors for the macromolecular cell constituents and they make energy available for biosynthetic and other energy-requiring processes.

The principle of "unity in biochemistry" (Kluyver and Donker 1925, 1926) is one of the few lasting dogmas of this century. Although the original concept referred to the basic energy relationships of organisms, it is now clear that "unity" expresses the assumption that the biochemistry of all organisms on this planet follows a few basic principles, for example, the uniformity of cell constituents, the universality of ATP as the principal carrier of biological energy, the universality of the genetic code, and the distribution of the degradative pathways, the respiratory chain, and the basic mechanisms of cellular energy conversion. Even the main metabolic pathways are almost identical in all organisms. Only a few groups of bacteria have modified patterns of central metabolic routes. The metabolic pathways arose during evolution. The biochemical apparatus that is typical for aerobic organisms developed when oxygen became available.

The prokaryotes developed billions of years before the atmosphere became aerobic and before carbohydrates became abundant products of primary biomass production. They may be regarded as relics of the evolution of life. During their early evolution, the prokaryotes learned to exploit a multitude of sources of energy and cell carbon different from the predominant present-day nutrient sources. The morphological uniformity of prokaryotes contrasts astonishingly with their versatility in substrate utilization, peripheral metabolic pathways, and modes of energy conversion. The most outstanding metabolic capacities, which are either restricted to only a few eukaryotes or completely lacking among eukaryotes, are anaerobic growth, use of inorganic electron donors for growth, fixation of molecular nitrogen, and the utilization of methane and a few polymers.

## Anaerobic Growth

The ability to grow indefinitely under anaerobic conditions is almost exclusively confined to prokaryotes (see Chap. 13, "The Anaerobic Way of Life" in Vol. 2). In the majority of eukaryotes, anaerobic energy generation is only a transient process occurring during intense activity and exposure to hypoxic environments (Bennett 1978). There are a few exceptions; among the protozoa, two ciliate groups, holotrichs and entodiniomorphs, live in the rumen (Hungate 1975). *Entamoeba*, *Diplomonas*, and *Trichomonas* lack mitochondria and grow under strictly anaerobic conditions (Bauchop 1971; Müller 1975; Steinbüchel 1986; Williams 1986; Stumm and Zwart 1986;

**Table 3.1**
**Groups of anaerobic phototrophic bacteria**

| Bacterial group | Typical species | Growth | | Photosynthetic electron donors | Sulfur deposited |
|---|---|---|---|---|---|
| | | Anaerobically in light | Aerobically in dark | | |
| Nonsulfur purple bacteria | ***Rhodospirillum rubrum*** | + | (+) | $H_2$, organic, ($S^{2-}$) | (Extracellularly)[a] |
| Purple sulfur bacteria | *Chromatium okenii* | + | – | $S^{2-}$, $S^0$, $S_2O_3^{2-}$, $H_2$ | Intracellularly |
| Green sulfur bacteria | *Chlorobium limicola* | + | – | $S^{2-}$, $S^0$, $S_2O_3^{2-}$, $H_2$ | Extracellularly |
| *Chloroflexus* group | *Chloroflexus aurantiacus* | + | + | Organic ($S^{2-}$) | (Extracellularly) |
| Heliobacterium | *Heliobacterium chlorum* | + | – | Organic | – |

[a]Parentheses indicate substrates used by only a few strains or species

Hobson 1988). Among the helminths, there are various facultative anaerobes, such as *Ascaris lumbricoides*, *Trichuris vulpis*, *Trichinella spiralis* larvae, various tapeworms, and *Schistosoma mansoni* (Fairbairn 1970; Hochachka and Mustafa 1972; Hochachka and Somero 1973). The question of whether fungi are able to grow anaerobically has now been definitely settled. An early statement ["One of the major metabolic differences between fungi and bacteria is that there are no anaerobic moulds either obligate or facultative" (Foster 1949)] has been shown to be wrong by experimental evidence for anaerobic growth of species of *Mucor* (Bartnicki-Garcia and Nickerson 1962) and *Fusarium* (Gunner and Alexander 1964), *Aqualinderella fermentans* (Held 1970; Held et al. 1969), and *Neocallimastix frontalis* (Mountfort and Asher 1983; Orpin and Joblin 1988). The latter belongs to the Chytridiomycetes and ferments polysaccharides, cellulose included, with the formation of lactic and acetic acids and $H_2$ and $CO_2$.

The contribution of eukaryotes to anaerobic decomposition appears to be negligibly small. The presence of various protozoa, fungi, and lower metazoa in the anaerobic layers of coastal marine sediments, especially within the sulfide system, and their disappearance when the sediment becomes oxidized suggest that the number of eukaryotes able to live under anaerobic conditions is large (Fenchel 1969; Fenchel and Riedl 1970; Schroff and Schöttler 1977; Zebe 1977).

The forms of anaerobic metabolism are briefly discussed here because of the uniqueness of anaerobic metabolism in prokaryotes and its function in whole ecosystems. The energy for bacterial growth under anaerobic conditions can be provided by three fundamental types of anaerobic energy generation: (1) anoxygenic photosynthesis, (2) anaerobic respiratory energy generation, and (3) fermentative energy generation.

Anoxygenic photosynthesis is the light-driven process that anaerobic phototrophic bacteria use to generate energy (*Table 3.1*). Unlike green plants, these bacteria are not able to use water as the ultimate reductant; consequently, they do not evolve oxygen, and they require the presence of other reduced compounds such as organic acids, alcohols, carbohydrates, hydrogen sulfide, sulfur, or hydrogen (Gromet-Elhanan 1977; Jones 1977). Two major groups are different by their pigmentation: the purple and the green bacteria. On the basis of their source of reducing power, the purple and green bacteria are subdivided into sulfur and nonsulfur bacteria. The purple and green sulfur bacteria can use reduced sulfur compounds and oxidize them via elemental sulfur to sulfate. The nonsulfur purple bacteria require reduced organic compounds or, when using hydrogen sulfide, oxidize it to sulfur or sulfate. In contrast to the purple sulfur bacteria, they lack the ability to oxidize elemental sulfur. The green bacteria comprise two groups, the *Chlorobium* group and the *Chloroflexus* group. The chlorobia use hydrogen sulfide as reductant and comprise strictly anaerobic bacteria; the latter are facultative and versatile (Pfennig 1979; Trüper 1976). The majority of phototrophic bacteria require light for growth; a few species can equally well obtain energy via aerobic respiration.

Anaerobic respiratory energy conversion is similar to aerobic respiration with respect to the electron donors, which may be either organic or inorganic compounds. Oxygen serves as the ultimate electron acceptor in aerobic respiratory energy generation, but anaerobic respiration depends on the presence of inorganic compounds, which, under anaerobic conditions, are reduced. The physiological groups listed in *Table 3.2* are differentiated with respect to the electron acceptors used and to respective end products of the respiratory process. Each of these physiological groups comprises strains and species belonging to various taxonomic groups.

Fermentative energy generation depends on organic compounds serving as electron donors and as electron acceptors. These compounds are usually two different metabolites derived from sugar by cleavage or, in a few cases, derived from two different compounds. Fermentation is accompanied by the production of more or less reduced compounds, such as alcohols, organic acids, ammonia and hydrogen and of carbon dioxide as the oxidized product.

The fermentative bacteria are usually separated into groups on the basis of one or several fermentation products which reflect their metabolic pathways (*Table 3.3*).

Table 3.2
**Physiological groups of bacteria able to grow under anaerobic conditions using external electron acceptors for electron transport ("aerobic respiration")**

| Bacterial group | Typical species | Metabolic process | Electron acceptor | Reduction products(s) |
|---|---|---|---|---|
| Denitrifiers | ***Pseudomonas denitrificans*** | Nitrate respiration | $NO_3^-$ | $N_2$, $N_2O$, $NO_2^-$ |
| Sulfate reducers | ***Desulfovibrio vulgaris*** | Sulfate respiration | $SO_4^{2-}$ | $S^{2-}$ |
| Sulfur reducers | ***Desulfuromonas acetoxidans*** | Sulfur respiration | $S^0$ | $S^{2-}$ |
| Methanogenic bacteria | *Methanobacterium thermoautotrophicum* | Carbonate respiration | $CO_2$ | $CH_4$ |
| Acetogenic bacteria | ***Acetobacterium woodii*** | Carbonate respiration | $CO_2$ | $CH_3$–COOH |
| Succinogenic bacteria | ***Wolinella succinogenes*** | Fumarate respiration | Fumarate | Succinate |
| Iron reducers | ***Pseudomonas*** GS-15 | Iron respiration | $Fe^{3+}$ | $Fe^{2+}$ |

## Growth with Inorganic Electron Donors

The ability to use inorganic compounds as electron donors for growth, called lithotrophy, is exclusively restricted to prokaryotes (see Chap. 14, "The Chemolithotrophic Prokaryotes" in Vol. 2; and Chap. 4, "The $H_2$-Metabolizing Prokaryotes"; Chap. 15, "The Colorless Sulfur Bacteria" and Chap. 3, "Oxidation of Inorganic Nitrogen Compounds as an Energy Source" in Vol. 3). The electrons are used for electron transport phosphorylation, either aerobically with oxygen or anaerobically with inorganic compounds (nitrate, sulfate, carbonate) as the ultimate electron acceptors. The electrons also serve to reduce carbon dioxide, which is the common carbon source for a physiological group of bacteria called chemolithoautotrophs. These bacteria are usually separated into groups on the basis of their electron donors (*Table 3.4*). In memory of S.N. Winogradsky, who developed the concept of chemolithotrophy and autotrophy in 1887–1891, a symposium on lithoautotrophy was held in 1987 (Schlegel and Bowien 1989).

## Fixation of Molecular Nitrogen

Only prokaryotes are able to fix molecular nitrogen (dinitrogen = $N_2$) and to grow in the absence of a source of combined nitrogen. Dinitrogen fixation requires the presence of a special enzyme system, nitrogenase, and particular environmental conditions. The ability to fix dinitrogen is distributed among many species of oxygenic and anoxygenic phototrophic bacteria, chemoautotrophic and chemoheterotrophic bacteria, and both aerobic and anaerobic bacteria—in short, in all major physiological groups. There is almost no correlation with the taxonomic unit. A recent symposium considered all aspects of research in this area (Bothe et al. 1988).

## Range of Organic Substrates for Growth

The bacteria as a whole are considered to be omnipotent with respect to their substrate spectrum. All natural (biosynthetic) compounds are subject to degradation by microorganisms. There are a few compounds that can be utilized exclusively by prokaryotes. Among the low-molecular-weight compounds, methane is oxidized only by highly specialized bacteria, the methylotrophs. Some of these bacteria are obligate methylotrophs, being able to use methane, methanol, and dimethyl ether as substrates.

No macromolecular compounds are used only by prokaryotes. Cellulose, hemicelluloses, pectins, xylanes, galactan, mannans, chitin, and others are subject to degradation by bacteria and fungi; some polymers are hydrolyzed by protozoa and a few metazoa. Under anaerobic conditions, however, the degradation of polysaccharides is almost exclusively confined to prokaryotes.

## Extremes of Environmental Conditions Allowing Bacterial Growth and Survival

Room or body temperature, the oxygen partial pressure of the atmosphere, neutrality of pH, and abundant nutrients supporting luxurious growth of the kinds of organisms studied in the laboratory are generally considered to be "normal" conditions for growth of microorganisms. Any conditions substantially deviating therefrom are regarded as being extreme. The ecologically minded biologist will define those conditions under which the greatest species diversity develops as normal. Environmental conditions different from the norm have an elective effect on organisms; as a rule, the species diversity decreases with the increase of environmental adversity. The greater the severity of an adverse environmental factor, the smaller is the number of species of actively growing microorganisms. In addition, among the organisms adapted to extreme conditions, the abundance of physiological types is restricted. However, this trend of limitation of species and number of individuals may not be as pronounced as is presently assumed. It may just reflect reluctance to examine extreme environments and to isolate new organisms, many of which cannot easily be grown in laboratory cultures.

In general, the microorganisms that tolerate and grow under the most extreme conditions are obligately adapted to their particular environment and fail to grow at lower intensities of the same environmental factor. Such an organism has acquired

**Table 3.3**

**Groups of fermentative bacteria able to grow under anaerobic conditions and their fermentation products**

| Fermentation characterizing bacterial groups | Typical species | Substrate | Fermentation product | |
|---|---|---|---|---|
| | | | Major | Minor |
| Ethanol fermentation | ***Zymomonas mobilis*** | Glucose | Ethanol | $CO_2$ |
| Lactate fermentation: | | | | |
| Homofermentative | ***Lactobacillus casei*** | Glucose | Lactate | |
| Heterofermentative | ***Leuconostoc mesenteroides*** | Glucose | Lactate | Ethanol, $CO_2$ |
| Heterofermentative | ***Bifidobacterium bifidum*** | Glucose | Acetate | Lactate |
| Butyrate fermentation | ***Clostridium butyricum*** | Glucose | Butyrate | Acetate + $H_2$ + $CO_2$ |
| | ***Clostridium acetobutylicum*** | Glucose | Butyrate, butanol | Acetone, 2-propanol |
| | ***Clostridium kluyveri*** | Ethanol + acetate | Butyrate | Caproate, $H_2$ |
| Homoacetate fermentation | ***Clostridium aceticum*** | Fructose | Acetate | |
| Propionate and succinate fermentation | ***Propionibacterium pentosaceum*** | Sugars, lactate | Propionate | Succinate |
| | ***Veillonella alcalescens*** | Lactate | Propionate | Acetate, $H_2$, $CO_2$ |
| | ***Bacteroides ruminicola*** | Sugars | Propionate | |
| Mixed acid and butanediol fermentation | ***Escherichia coli*** | Glucose | Lactate, ethanol, acetate | Formate, $H_2$ + $CO_2$, succinate |
| | ***Enterobacter aerogenes*** | Glucose | 2,3-Butanediol, ethanol | Formate, $H_2$ + $CO_2$ |
| Nitrogenous compounds fermentation | ***Clostridium tetanomorphum*** | Glutamate | Butyrate | Acetate, $CO_2$, $NH_3$ |
| | ***Clostridium sticklandii*** | Lysine | Butyrate | Acetate, $NH_3$ |
| | ***Clostridium oroticum*** | Orotate | Acetate | $CO_2$, $NH_3$ |

**Table 3.4**

**Groups of bacteria able to use inorganic electron donors for growth (''chemolithoautotrophs'')**

| Bacterial group | Typical species | Metabolic process | Electron donor | Electron acceptor | Carbon source | Product |
|---|---|---|---|---|---|---|
| Hydrogen-oxidizing bacteria | ***Alcaligenes eutrophus*** | $H_2$ oxidation | $H_2$ | $O_2$ | $CO_2$ | $H_2O$ |
| Carbon monoxide-oxidizing bacteria | ***Pseudomonas carboxydovorans*** | CO oxidation | CO | $O_2$ | $CO_2$ | $CO_2$ |
| Ammonium-oxidizing bacteria | ***Nitrosomonas europaea*** | Ammonium oxidation | $NH_4^+$ | $O_2$ | $CO_2$ | $NO_2^-$ |
| Nitrite-oxidizing bacteria | ***Nitrobacter winogradskyi*** | Nitrite oxidation | $NO_2^-$ | $O_2$ | $CO_2$ | $NO_3^-$ |
| Sulfur-oxidizing bacteria | ***Thiobacillus thiooxidans*** | Sulfur oxidation | S, $S_2O_3^{2-}$ | $O_2$ | $CO_2$ | $SO_4^{2-}$ |
| Iron-oxidizing bacteria | ***Thiobacillus ferrooxidans*** | Iron oxidation | $Fe^{2+}$ | $O_2$ | $CO_2$ | $Fe^{3+}$ |
| Methanogenic bacteria | *Methanobacterium thermoautotrophicum* | Methanogenesis | $H_2$ | $CO_2$ | $CO_2$ | $CH_4$ |
| Acetogenic bacteria | ***Acetobacterium woodii*** | Acetogenesis | $H_2$ | $CO_2$ | $CO_2$ | $CH_3$-COOH |

the ability to grow in one extreme environment at the expense of its ability to grow in others. Less rigid or extreme environmental conditions are tolerated by a greater number of organisms; some of these are obligately bound to these conditions, and others grow there facultatively. There are several examples of low numbers of species and of high specialization in habitats of extreme conditions, such as low and high temperature, high salt concentrations, low moisture, low pH, and low-nutrient concentrations. Much attention has been given during recent years both to microorganisms that either tolerate or are

dependent on extreme conditions and to their habitats. Life in extreme environments was the subject of several symposia, and emphasis was paid to the microorganisms, to the regulatory or molecular mechanisms that make life under adverse conditions possible, and to the possible existence of life on other planets (Alexander 1971, 1976; Brock 1969; Ellwood et al. 1980; Heinen 1974; Hochachka and Somero 1973; Kushner 1971, 1978; Shilo 1979; Brock, 1986a, b, Stetter 1986, 1989; Larsen 1986; Tindall and Trüper 1986).

Thanks to the amount of attention that the "extremophiles" and their habitats have received during recent years, there is no need to add another exhaustive review. However, it is likely that more as yet unidentified microorganisms representing various metabolic groups are present in extreme environments. They can only be discovered by experimental approaches based on new ideas on possibly existing organisms. The following short survey is meant to encourage relevant research.

## Low Temperatures

The temperature of the natural environments of water and soil bacteria is distinctly lower than their optimum growth temperature. The average soil temperature in the temperature climate zone is 12 °C, and 90 % of the ocean water is 5 °C or colder. Many mesophilic bacteria do not find their optimum growth temperature in their natural habitats; they are, however, able to tolerate the seasonal fluctuations of temperature, which in the summer can easily span 30 °C or more. This tolerance is lacking in psychrophilic bacteria. As defined by Morita (1975), "the psychrophiles are those organisms having an optimum growth temperature of 15 °C or lower, a maximal temperature for growth at about 20 °C and a minimal temperature for growth at 0 °C or lower." Many bacteria that have been isolated from Arctic and Antarctic Ocean waters and sediments have optimal growth temperatures of about 10 °C but do not survive exposure to 20 °C. True psychrophiles will, therefore, be found only in habitats that never become warmer than 20 °C. At temperatures below 10 °C, psychrophiles do have selective advantage as demonstrated in continuous culture experiments (Harder and Veldkamp 1968, 1971). One of the ecological niches of psychrophiles in the ocean is chitin degradation. The vast majority of psychrophilic bacteria are members of *Pseudomonas*, *Flavobacterium*, *Achromobacter*, and *Alcaligenes* (Rose 1968).

Food preservation added another habitat for psychrophilic bacteria (Schmidt-Lorenz 1967). However, the majority of bacteria that grow on food near the freezing point are apparently facultative psychrophiles or psychrotrophs that have temperature maxima for growth above 20 °C but are able to grow at low temperatures also. With respect to growth at temperatures down to −18 °C, the capacities of prokaryotes do not seem to exceed those of the eukaryotes.

Microbial life at low temperatures has been comprehensively reviewed and discussed on the basis of ecological aspects (Baross and Morita 1978) and molecular aspects (Inniss 1975; Inniss and Ingraham 1978).

## High Temperatures

Growth at temperatures higher than about 60 °C is restricted to the prokaryotes. Habitats with such temperatures include piles of self-heating hay, compost, or other organic materials (Hussain 1973), circulating hot or cooling water in industrial plants, and hot springs and other geothermal sources.

The ecology of hot springs has been especially well studied and reviewed (Brock 1967, 1970, 1978, 1986; Castenholz 1969, 1979). The runoff of a hot spring is an ideal location to study the upper temperatures for continual growth of organisms. As in most extreme habitats, the species diversity decreases with increasing severity of the environmental factor. When the temperature gradient rises from 50 °C to 70 °C, thermophilic bacteria such as the yellow-pigmented *Thermus aquaticus* (Brock and Freeze 1969), which has an optimum temperature for growth at 70 °C and a maximum at 79 °C, can be found. In many runoff channels, thick mats consisting of the cyanobacterium *Synechococcus lividus* and the phototrophic, gliding, filamentous bacterium *Chloroflexus aurantiacus* are present (Pierson and Castenholz 1974). In these mats, several layers of *Chloroflexus* are covered by a thin surface layer containing a mixture of *Synechococcus* and *Chloroflexus* (Bauld and Brock 1973; Madigan and Brock 1977).

Several strains of the facultative autotroph *Sulfolobus acidocaldarius*, with a growth pH range of 0.9–5.8 and temperature optimum of 70–75 °C, and of an extreme thermophile growing at 85 °C have been isolated from acid, hot, aqueous, and soil habitats (Brock et al. 1971, 1972; Fliermans and Brock 1972). Thermophilic *Thiobacillus*-type bacteria growing at 60 °C or 75 °C were found in Icelandic thermal areas (Le Roux et al. 1977). The habitats par excellence of these bacteria are volcanic hot springs where magmatic hydrogen sulfide is oxidized to elemental sulfur and sulfuric acid. Thermophilic iron- and sulfur-oxidizing bacteria may be involved in metal leaching from low-grade ore (Brierley 1977; Brierley and Lockwood 1977; Brierley 1978; Golovacheva and Karavaiko 1978; Levi and Linkletter 1989; Hughes and Poole 1989).

Yellow-pigmented *Thermus* strains have also been isolated from Icelandic hot springs (Pask-Hughes and Williams 1977). Nonpigmented thermophilic bacteria, otherwise similar to *Thermus aquaticus*, were found in laundry heaters (Brock and Boylen 1973), hot tap water (Pask-Hughes and Williams 1975), and a stream receiving hot-water effluents (Ramaley and Hixson 1970). The range between 50 °C and 70 °C is occupied by a multitude of bacteria, among them members of the genera *Bacillus*, *Thermoactinomyces* (Cross 1968), *Methanobacterium* (Zeikus and Wolfe 1972), *Methylococcus*, and others. Several thermophilic bacteria find excellent growth conditions in canned foods (Gillespy and Thorpe 1968) and sugar (Scarr 1968).

Our knowledge of microbial life at high temperatures has been greatly extended by studies of continental and marine hot springs of volcanic origin. A considerable number of organisms have been isolated that cover a range for optimal growth at 85–105 °C and are generally termed extreme thermophiles

or hyperthermophiles (Stetter 1986; Stetter et al. 1990). These organisms are mainly archaebacteria and appear to be so well adapted to these high temperatures that they do not grow below 60 °C, and some of them do not grow below 80 °C. Acidic terrestrial solfataric environments have primarily yielded acidophilic, hyperthermophilic autotrophs of the order Sulfolobales that aerobically oxidize $H_2S$ and S°, with the exception of *Acidianus*, which is able to grow anaerobically on $H_2$ and S° forming $H_2S$ (Huber et al. 1987a). The same is true for some neutrophilic isolates that exist in neutral and anaerobic zones of terrestrial solfataras and are members of the genera *Thermoproteus* and *Pyrobaculum* (Zillig et al. 1981, 1982; Stetter and Zillig 1985). A facultatively autotrophic isolate (*Pyrobaculum islandicum*) grows by sulfur respiration of organic matter (Huber et al. 1987a).

Another habitat that has recently yielded a number of extremely thermophilic bacterial isolates is the deep-sea hydrothermal hot vents found at depths from 1,800 to 3,700 m. The high pressures make possible the presence of liquid water at temperatures far beyond the 1-atm boiling point of water, that is, at a depth of 2,500 m, extruding vent water will not boil below approximately 460 °C. This habitat provides an ideal situation to look for the upper temperature limit of prokaryotic life. Early indications that growth might occur at 250 °C (Baross and Deming 1983) have not yet been confirmed, although active growth at 110 °C has been found in a culture of a new group of methanogenic archaebacteria (*Methanopyrus*, Huber et al. 1989). While this organism grows with its optimum doubling time of about 1 h just below 100 °C, isolates of the genus *Pyrodictium* (Stetter et al. 1983) grow optimally at 105 °C. *Pyrodictium*, as well as a large number of physiologically diverse hyperthermophilic archaebacteria, was isolated from a shallow marine hot spring (Stetter 1986) and an erupting volcano (Huber et al. 1990).

Using $^{35}SO_4^{2-}$, hyperthermophilic sulfate reduction has been demonstrated in sediment cores freshly collected from a hydrothermal vent field (Jørgensen et al. 1990), and the organisms responsible (*Archaeoglobus profundus*, Burggraf et al. 1990) could be isolated at the same time. This particular vent field, located in the Gulf of California at a depth of 2,000 m, is overlayed by 3–400 m of organic-rich sediment (Jannasch 1989). In the upper 60 cm of these sediments, downward temperature gradients of 3–180 °C have been measured, overlayed by ambient bottom water with a temperature of 2.1 °C. In these sediments, methanogenic hyperthermophiles of the genus *Methanococcus* (Jones et al. 1983, 1989; Zhao et al. 1988) appear to be common in addition to those of the above-mentioned *Methanopyrus*. The majority of the newly described hyperthermophiles were found in both the deep and the shallow marine hot vents. This includes the genera *Pyrococcus*, *Pyrodictium*, and *Staphylothermus* and the only eubacterial genus of extreme thermophiles, *Thermotoga* (Fiala et al. 1986; Belkin et al. 1986; Huber et al. 1986; Jannasch et al. 1988; Windberger et al. 1989). Some of them depend on the reduction of elemental sulfur, while others conduct an unknown type of fermentation.

Although many thermophilic water and soil bacteria were reported to be present only in samples of thermal habitats, experience indicates that they are much more widespread. The isolation of thermophilic, hydrogen-oxidizing bacteria from hot springs and the failure to isolate them from other places indicated a very narrow distribution of these highly specialized bacteria (Goto et al. 1977). Surprisingly, attempts to isolate thermophilic, hydrogen-oxidizing bacteria from cold lake sediments (Aragno 1978; Schenk and Aragno 1979) and from the oxidation ponds of a sugar factory (Schlegel unpublished) were successful. The search for thermophiles among other highly specialized bacteria resulted in equally encouraging findings, for example, thermophilic, nitrifying bacteria (Golovacheva 1976), iron-oxidizing bacteria (Brierley 1978), sulfur-oxidizing bacilli (Golovacheva and Karavaiko 1978), and carbon monoxide-oxidizing bacteria (Meyer unpublished). These recent findings indicate that the ability to grow at temperatures higher than 50 °C is not confined to only a few metabolic types.

Research in the ecology of thermophilic microorganisms has recently yielded spectacular results; they are summarized by Brock (1978), Tansey and Brock (1978), Brock (1986), and Castenholz (1979).

## Water Stress

Without water, life is not possible. The normal environment of microorganisms is an aqueous solution. The water body may differ in size, and the water may be free or adsorbed to external or internal surfaces of materials. The availability of water to microorganisms is a function not only of the water content of a material but also of solution and adsorptive factors. In order to compare solutions and solid materials with respect to available water, the parameters "water activity" ($a_w$, ranging from 0 to 1.0) and "relative humidity" (expressed as a percentage) are used. These parameters express the ratio of water in the vapor phase to the amount of water the air would contain when vapor-saturated at a given temperature. Another parameter, the "water potential," is based on the free energy of molecules in water and makes differentiation of the matric and solute components of the system easy (Brown 1978; Griffin and Luard 1979; Smith 1978; Griffin 1981).

Microbial growth is possible in the range of water activity between 0.998 and 0.6 (Duckworth 1975; Mossel 1975; Mossel and Ingram 1955). Bacteria are not very successful at extracting water from environments of lowest water activities. Fungi lead the list of organisms arranged with respect to the lowest water activity at which they can grow. The osmotolerant yeast, *Saccharomyces rouxii*, and the osmophilic mold, *Xeromyces bisporus*, are able to grow down to $a_w = 0.60$; even *Aspergillus glaucus* can survive at $a_w = 0.80$. In contrast, the majority of bacteria need water activities higher than 0.98 (which is the $a_w$ of seawater at 25 °C); only a few bacteria grow at 0.95–0.91. The halophilic bacteria are exceptions, growing at $a_w = 0.75$. The data are in accordance with the results of measurements on the microbial degradation of plant residues and straw mixed with field soil.

If the soil was kept in equilibrium with pure water, the rate of carbon dioxide evolution was maximal. With decreasing humidity, the activity of bacteria decreased and reached zero at 92 % relative humidity.

One has to distinguish between two effects of humidity, that on the activity and that on the viability of microorganisms. Some bacterial species are more resistant to drying than others. After some weeks of air-drying in the laboratory, the remaining viable population consists of bacteria that are able to form endospores, cysts, and other resting stages resistant to desiccation (Boylen 1973; Robinson et al. 1965). However, under certain conditions, even vegetative cells survive (Clark 1967). Survival of vegetative cells depends on the type of soil, the velocity of the drying process, and the water activity. Several reports indicate that processes like nitrification, sulfur oxidation, and nonsymbiotic nitrogen fixation proceeded after rewetting without any soil reinoculation. Apparently, the endospores of some bacilli can survive for hundreds of years. On the basis of viable counts made on soil granules from the roots of plants gathered and dried in 1640 (Herbarium, Royal Botanical Gardens, Kew), "one can estimate that a ton of dry soil would still contain a few viable spores even after 1,000 years" (Sneath 1962).

### Salinity

High salt concentrations represent a special case of low water activity. Seawater ($a_w = 0.98$) is not tolerated by the majority of bacteria living in soil and in freshwater. Ecosystems containing salt (sodium chloride) at saturating concentrations are inhabited by only a few organisms. Many bacteria (Briston et al. 1974) as well as the flagellated alga *Dunaliella viridis* and the brine shrimp *Artemia salina* can tolerate such high salt concentrations, but only members of the prokaryotes find their optimum growth conditions there. The extreme halophiles have their best growth at 20–30 % salt, moderate halophiles at 5–20 %, and slight halophiles at 2–5 % (Dundas 1977; Larsen 1967, 1971, 1973, 1986; Tindall and Trüper 1986).

Extremely halophilic bacteria, such as *Halobacterium cutirubrum*, *H. salinarum*, and *Halococcus morrhuae*, are distributed in evaporation ponds for the production of solar salt and occasionally on salted fish and hides. They are easily recognized by their red color caused by carotenoids. Moderately halophilic bacteria live in similar habitats, namely, salt brines and mud, and are often found in curing brines for meat, fish, and vegetables. About a dozen different bacteria are reported to find their optimum growth conditions in the range of 5–20 % sodium chloride. The majority of the bacteria inhabiting the sea and marine mud require 2–5 % sodium chloride; some of these slight halophiles fail to grow at lower salt concentrations.

The general aspects of halophilism and life in high salt concentrations have been recently discussed by Kushner (1978); Lanyi (1979); Brock (1979); Bayley and Morton (1978, 1979); and Csonka (1989).

From the ecological point of view, salinity plays a more important role than the water potential suggests. The following differences between freshwater and saline ecosystems cannot be overemphasized. As long as a saline water body or a water-soaked sediment is aerobic, the water potential may be the dominating factor among the selective environmental conditions for the growth of bacteria. The sequence of dominance of selective factors changes as soon as the location becomes anaerobic. Many saline waters, either marine or salt-polluted inland waters, contain sulfate; in the absence of oxygen, sulfate is the preferred terminal electron acceptor in anoxic environments and gives rise to the generation of hydrogen sulfide. The concentration of hydrogen sulfide may then exert a selective pressure on the bacterial flora in the location and govern the composition of the anaerobic food chain. In many freshwater lakes, the sulfate concentration is just high enough to provide sufficient hydrogen sulfide for securing the redox potential necessary for methanogens to grow, but in marine ecosystems, such as sublittoral and estuarine sediments, the concentration of sulfate and, in consequence, that of hydrogen sulfide exceed the threshold where methanogenesis is possible (Cappenberg 1974a, b; Winfrey and Zeikus 1977).

## Environments of Extreme pH Values

Hydrogen and hydroxyl ions are the most mobile of all ions. The concentration of these ions affects the growth of microorganisms either directly or indirectly via its influence on the ionic state and the availability of many inorganic ions and metabolites to the cells. The majority of bacteria prefer neutral or slightly alkaline conditions.

### Acid Environments

The hydrogen ion concentrations of the ocean and the major part of the land vary only within a narrow range, allowing growth of both fungi and bacteria. Only volcanic lakes and soils of recently claimed land have drastically reduced pH values. An extreme aquatic habitat of low pH is represented by the drainage of coal mines and coal mine refuse piles; the acidity is due to the oxidation of pyritic minerals, reduced iron, and sulfur compounds associated with coal (Colmer et al. 1950; Leathen et al. 1953). Sulfuric acid produced by thiobacilli causes a decrease of the pH down to 2.0 or even 0.7. Iron- and sulfur-oxidizing bacteria are present wherever mine water enters a stream. *Thiobacillus ferrooxidans*, involved in metal-leaching processes (Tuovinen and Kelly 1972), can tolerate even pH 1.0, as well as ions of copper, cobalt, zinc, nickel, and iron up to extremely high concentrations (Torma 1977). Organic acids are excreted in laboratory cultures (Schnaitman and Lundgren 1965); their ecological significance is unknown. In acidic water, most Gram-positive, aerobic and anaerobic, heterotrophic bacteria die quickly, while yeasts and molds predominate (Marchlewitz and Schwartz 1961). Among the heterotrophic bacteria, only *Bacillus*, *Pseudomonas*, *Achromobacter* (Tuttle et al. 1968), *Flavobacterium acidurans* (Millar 1973),

and other slime-forming bacteria (Dugan et al. 1970) were encountered. Waters of acid hot springs are further habitats of acidophilic bacteria, such as thermophilic bacteria related to *Sulfolobus acidocaldarius* (Brierley and Brierley 1973; Brock et al. 1971; De Rosaet et al. 1975) and, at lower temperatures, of mesophilic thiobacilli.

Many lakes, bogs, pine forests, and tea soils are slightly acidic, with pH values between 3 and 5.5. Bacterial life in these locations is scarce, and, especially under anoxic conditions, degradation of plant polymers is slow. Nitrification proceeds slowly in acidic soils, and the question of whether nitrification in these areas is partly due to autotrophic nitrifying bacteria (Bhuiya and Walker 1977) and to heterotrophs is still open (Focht and Verstraete 1977). Acid bogs apparently offer the marginal conditions under which some rarely encountered bacteria, such as *Planctomyces*, *Bactoderma*, *Caulobacter*, and *Microcyclus* (Hirsch and Pankratz 1970), as well as other stalked and prosthecate bacteria, can thrive (Henrici and Johnson 1935; Schmidt 1971).

### Alkaline Environments

The environment where human or animal urine undergoes a urea fermentation cannot be ignored because of the penetrating odor of ammonia. The causative agents of this noticeable process were first studied by Pasteur in 1862 and designated *Torule ammoniacale*. The ureolytic bacterium, *Bacillus pasteurii*, is an alkalophile that requires pH values of 8.5 or higher and high ammonia concentrations (Wiley and Stokes 1963). *Sporosarcina ureae* grows at lower alkalinities (pH 8.5) (Mazanec et al. 1965).

The isolation of alkaliphilic bacteria, able to grow in culture media of pH 10–11, is apparently rather easy. Such variants growing up to pH 11 were isolated after adapting *Bacillus circulans* (Chislett and Kushner 1961). *Bacillus alcalophilus* growing at pH 8–10 was isolated from human feces (Vedder 1934) and from dried sewage sludge (Boyer et al. 1973). Several *Bacillus* strains originated from projects on the production of alkali-tolerant enzymes and were isolated from indigo balls and plain soil (Ohta et al. 1975).

The szik (salt and alkali) soils with pH values of 8–9 in the Hungarian lowlands have been extensively studied with regard to soil ecology and agriculture; however, their bacterial flora was not characterized in detail (Bokor 1933). Alkali soils in India were reported to contain only few microorganisms, the total count decreasing drastically with increasing pH (Rupela and Tauro 1973). With these exceptions, alkaline environments have not attracted much attention. Investigations of strictly alkaline springs in northern California led to the isolation of an anaerobic spore-former and an aerobic, pigmented bacterium. The latter grew between pH 8 and 11.4 with the optimum within the range of pH 9–9.5 (Souza and Deal 1977; Souza et al. 1974). The lakes of the Wadi el Natrun in Egypt are most interesting aquatic habitats, with pH values ranging from 9 to 11. Topographically and geochemically described by Schweinfurth and Lewin in 1898, these shallow salt pans harbor a rich population of halophilic and phototrophic microorganisms. Sodium is the major cation of the saturated brine (98 %); the major anions are chloride, sulfate, and carbonate/bicarbonate (56, 26, and 17 %, respectively; Jannasch 1957). Species of the genus *Ectothiorhodospira* and other phototrophic bacteria have been isolated from these and other alkaline waters (Grant et al. 1979; Imhoff and Trüper 1977). Alkaline lakes such as those in Ethiopia and Anatolia, with ambient values of pH 10–11, are also worth study.

Life at extreme pH values has been discussed by Langworthy (1978), Horikoshi and Akiba (1982), and Krulwich and Guffanti (1983).

## Oxygen

When considering oxygen as an environmental factor, at first glance, the anoxic environment appears to be the extreme one. However, if the peculiarities of generating biochemical energy under anaerobic conditions are not considered, the anoxic environment is not difficult for life. Oxygen causes troubles for living cells. It plays a dual role: it acts as an effective electron acceptor, making energy conversion with high efficiency possible; however, oxygen can be considered toxic to life processes that depend on slow and thoroughly controlled oxidations. The toxic effect is intensified in light. Oxygen is reduced by univalent or single electron steps, and by the uptake of one electron, the superoxide anion radical (or simply the superoxide) is formed, which by further reactions gives rise to hydrogen peroxide and to the hydroxyl radical; this radical is the most reactive and, therefore, the most detrimental product. From the onset of photosynthetic oxygen production in the early atmosphere of this planet, it was the major task of biochemical evolution to develop an enzyme system for tetravalent reduction of oxygen, cytochrome oxidase, which does not release any toxic intermediates, and to develop elaborate detoxification systems (Fridovich 1974, 1975, 1976). Although forms of life are still susceptible to oxygen toxicity (Gottlieb 1971), there now exist a variety of defense mechanisms in different organisms (Hassan and Fridovich 1979; Morris 1975, 1976, 1978, 1979).

The rise of the oxygen level in the early atmosphere may have started not only the evolution of aerobic organisms, but also that of modestly oxygen-tolerant, anaerobic, fermentative bacteria as well. There is a wide spectrum of degrees of oxygen tolerance, starting with the strict anaerobes such as *Bacteroides*, *Butyrivibrio*, *Fusobacterium*, *Megasphaera*, *Peptococcus*, *Ruminococcus*, *Selenomonas*, *Succinivibrio*, *Methanobacterium*, *Methanococcus*, *Methanospirillum*, and *Methanosarcina* and continuing to the moderately oxygen-tolerant clostridia (*C. tetani*, *C sporogenes*) and the highly oxygen-tolerant clostridia (*C. perfringens*, *C. acetobutylicum*) and to the majority of the lactic acid bacteria, which can grow in the presence of air (Loesche 1969; Morris and O'Brien 1971; O'Brien and O' Morris 1971; Uesugi and Yajima 1978).

Although the obligately anaerobic bacteria have in common a hypersensitivity to oxygen, they are apparently not restricted to places like mud and intestinal tracts but can also be found in seemingly aerobic locations. The wide distribution of *Clostridium* can be explained by their possession of oxygen-insensitive spores. However, non-spore-forming, strictly anaerobic bacteria are similarly widely dispersed, perhaps because under field conditions, the anaerobes are associated with oxygen-consuming bacteria that keep the local oxygen concentration low. These apparent discrepancies caution one not to overemphasize the results of pure-culture studies when extrapolating to the behavior of microorganisms in their natural environment.

In some cases, the oxygen concentration is the deciding factor in the ability of the organism to develop potential metabolic activities. The oxygen concentration may determine whether various nitrogen-fixing bacteria can occupy a niche in an environment low in combined nitrogen. In contrast to members of the *Azotobacter* group, which fix nitrogen in the presence of air (Mulder and Brotonegoro 1974), the nitrogen-fixing, hydrogen-oxidizing bacterium *Xanthobacter autotrophicus* (Berndt et al. 1976; Wiegel and Schlegel 1976; Wiegel et al. 1978) is able to grow with molecular nitrogen as the sole nitrogen source only at oxygen concentrations below 2% oxygen (by volume), while in the presence of combined nitrogen, the bacterium grows under air. Other nitrogen-fixing bacteria, such as *Azospirillum lipoferum* (Okon et al. 1976), *Aquaspirillum fasciculus* (Strength et al. 1976), and *Methylosinus* species (De De Bont and Mulder 1974), respond to oxygen in a similar manner. A number of aerobic marine bacteria have been demonstrated to be microaerophilic; they survive but do not grow in air-saturated seawater medium (Jannasch 1977).

Whether there are differences among microorganisms with respect to their tolerance to oxygen at higher concentrations than that of air-saturated water is not known. Many strictly aerobic bacteria that form colonies from single cells on petri dishes exposed to air can tolerate gas mixtures up to 40% oxygen by volume but fail to grow at 50%. Also, 100% oxygen is usually considered to suppress growth; however, many cyanobacteria that form blooms are certainly tolerant to high oxygen concentrations. During photosynthesis in the sun, blooms of cyanobacteria and algal mats near the water surface, as well as the lawn of submerged water plants like *Elodea canadensis*, *Chara fragilis*, or *Ranunculus aquaticus* in ditches, shallow ponds, and estuaries, are covered with bubbles of oxygen. This growth indicates that the water body must contain higher concentrations of oxygen than in equilibrium with air and that the epiphytic and other bacteria are exposed to oxygen concentrations supersaturated with respect to oxygen. It is not known whether bacteria exist able to grow at these oxygen concentrations of almost 100 %. Furthermore, it would be interesting to know whether among the variety of bacteria a continuum of thresholds of oxygen tolerance exists or whether there are certain discontinuities of threshold concentrations for certain species or metabolic types, for example, at 2%, 20%, 40%, and 100% oxygen and whether correlations between oxygen-tolerance thresholds and habitats are encountered.

## Low-Nutrient Environments

The most commonly cited low-nutrient environments are desert soils, oligotrophic lakes, and, most prominently, the oceans. Considering suspended detrital matter, decaying plankton organisms, and fecal pellets, isolated high-nutrient habitats abound in various parts of the sea. However, if this amount is divided by the vast volume of the ocean, which amounts to about 90% of the biosphere, the dissolved organic carbon in seawater is rarely more than 1 mg/l. Particulate organic carbon commonly amounts to 10–20% of the total organic carbon. A large fraction of the latter is "refractory," that is, unavailable for microbial attack (Barber 1968; Menzel and Ryther 1970). The description of low-nutrient habitats is, therefore, more realistically based on the flux of nutrients across the ecosystem rather than on the standing nutrient concentrations (Hirsch 1979).

Many prokaryotes will survive in such conditions, as amply documented by the fact that poor soils and natural waters have often been used as a source for the isolation of microorganisms. Low-nutrient habitats may be extreme with respect to stress exerted toward some vegetative cells, but these conditions are hardly selective.

Vegetatively growing cells may be carried into the ocean from land runoff or be exposed to low-nutrient levels after separation from relatively nutrient-rich particles. These cells may adopt one of a variety of strategies in order to cope with starvation conditions. Two general strategies have been pinpointed whereby: (1) the survival of the individual cell is achieved by the most efficient use of the limited amount of food available or (2) the survival of the species is achieved by producing the maximum amount of progeny in the form of dormant stages capable of immediate growth and multiplication at the renewed availability of nutrients.

In terms of population dynamics studied in continuous culture, the "K-strategists" have been described as organisms adapted to highly efficient growth or uptake at low substrate concentrations, as indicated by a low substrate saturation constant ($K[s]$). In contrast, $\mu_{max}$-strategists ($\mu_{max}$ = maximum growth rate, in nonprokaryotes also called r-strategists) will outgrow the former in the presence of relatively high-nutrient concentrations (Koch 1979).

Early continuous culture studies on the synthesis and activity of prokaryotic enzyme systems led to the discovery of catabolic derepression by Gorini (1960) in the presence of extremely low concentrations of the growth-limiting substrate. Subsequently, the mechanisms of adaptation to low-nutrient environments have been intensively studied (Tempest and Neijssel 1976, 1979). These studies resulted in the discovery of the high-affinity mechanism for assimilating ammonia into glutamate via glutamine synthetase and glutamate synthase

(Tempest et al. 1970, 1973) and of dual mechanisms for the assimilation of glycerol: the glycerol kinase route at carbon-limiting conditions and the glycerol dehydrogenase route in glycerol-sufficient environments (Neijssel et al. 1975). Matin (1979) reported the onset of a multiple substrate utilization technique as a response to decreasing dilution rates and pools of intracellular metabolites. Adaptation of distinctive species to low-nutrient environments is well illustrated by a comparison with respect to starvation and survival of a freshwater *Spirillum* sp., apparently belonging to the oligotrophic flora, with a *Pseudomonas* sp. adapted to environments richer in nutrients (Matin and Veldkamp 1978). The spirillum accumulated poly-β-hydroxybutyric acid (PHB) during carbon-limited growth in the chemostat; the stored amount was highest at the lowest dilution rate examined. After growth at $D = 0.03–0.05\ h^{-1}$, the spirillum was much more resistant to starvation than the pseudomonad that did not accumulate PHB (Matin et al. 1979). The survival value of storage materials and the regulation of their synthesis in various types of bacteria may assist the understanding of adaptation to low-nutrient environments.

One of the most important characteristics of low-nutrient habitats is the strong influence of solid surfaces in the colonizing of microbial films and layers (Marshall 1976). In a summary of work done on the effect of inert particulate material suspended in low-nutrient aquatic habitats, Jannasch and Pritchard (1972) included studies using the chemostat for the enrichment of bacterial strains that exhibit a tendency for specific attachment mechanisms. In Caulobacteriales, typical low-nutrient organisms, Poindexter (1979) postulates a morphological–physiological mechanism that regulates an efficient metabolic response to low-nutrient conditions, including the role of holdfast organelles.

Life under conditions of low-nutrient concentrations was a topic of a Dahlem Konferenz (Jannasch 1979; Koch 1979; Poindexter 1979; Rittenberg 1979) and an international symposium on microbial ecology (Hattori et al. 1989).

## Resting Stages and Survival

In order to survive under adverse environmental conditions, a few bacterial groups are able to form resting cells, which are more resistant to deleterious environmental conditions than their vegetative cells. The transformation occurs when the metabolic activities decline because of nutrient depletion or transfer to a growth-limiting environment. The resting cells are characterized by thick, frequently multilayered walls. Typical is endospore formation by the genera *Bacillus* and *Clostridium*. Many endospores are extremely resistant to heat, desiccation, radiation, and chemical agents. The formation of exospores is, as far as is known, restricted to the methane-utilizing bacterium, *Methylosinus trichosporium*; the thermoresistant exospores are formed by a process similar to budding of vegetative cells (Whittenbury et al. 1970).

The rod-shaped cells of the genus *Azotobacter* frequently turn into spherically shaped cells called cysts. Unlike endospores, the vegetative rod as a whole is transformed into a cyst. These cysts share with endospores structural rigidity and resistance to desiccation and to ultraviolet radiation; however, they lack heat resistance (Sadoff 1975). Similarly, myxobacteria (*Myxococcus*) turn into spherical cells called myxospores (Sadoff 1973; Voelz and Dworkin 1962). In the genus *Methylocystis*, the rodlike cells round up and turn into desiccation-resistant cysts similar to those of *Azotobacter* species. *Methylomonas* and *Methylococcus* form spherical cells that are neither thick walled nor resistant to desiccation (Whittenbury et al. 1970). A modest resistance to desiccation is shown by *Arthrobacter* coccoid cells (Boylen 1973; Ensign and Wolfe 1964; Veldkamp et al. 1963).

Bacteria that are not able to transform into resting stages are less apt to survive under adverse environmental conditions. Their resistance, however, varies from species to species; it may be high as in mycobacteria or arthrobacters, which survive desiccation for several weeks, or low as in *Neisseria gonorrhoea* or *Treponema pallida*, which respond to desiccation by immediate death. Furthermore, maintenance of the viability of vegetative cells under a mild environmental stress is a function of a multitude of factors, such as growth conditions, content of storage materials, speed of transition from growth to resting conditions, and many others (Strange 1976). Many observations on the effect of drying and rehydration of vegetative bacteria have been made during long-term preservation in culture collections (Bousfield and MacKenzie 1976; Lapage et al. 1970; Martin 1964; Miller and Simons 1962). These observations indicate that the survival of bacteria depends on many factors and may vary among species and environments. Many aspects were reviewed by Kjelleberg et al. (1987) and Matin et al. (1989).

## Light as an Extreme Environment

The well-known predominance of pigmented colonies on nutrient-agar plates exposed for some time to air, compared to plates inoculated with soil, indicates the selective action of sunlight and air and the survival value of pigments. Yellow, orange, and red pigments point to members of the genera *Micrococcus*, *Corynebacterium*, *Mycobacterium*, and *Nocardia*, which contain carotenoids. In nonphototrophic bacteria, there is a good negative correlation between the presence of carotenoids in the cell and the anaerobic way of life; carotenoids are rarely found in strict anaerobes except the phototrophic bacteria. Selective effects similar to those observed in air are exerted in several sunlight-exposed environments, such as leaf surfaces, freshwaters, and salt brine. The radiation resistance of pigmented bacteria has been studied with *Halobacterium salinarium* (Dundas and Larsen 1962), *Rhodospirillum rubrum*, *Corynebacterium poinsettiae* (Cohen-Bazire and Stainer 1958), and other microorganisms.

Resistance to radiation also seems to be positively correlated with a high GC content of the cellular DNA; this correlation agrees with the conclusion that inactivation of cells by ultraviolet

light is mainly due to thymine dimerization (Singer and Ames 1970). The hypothesis was in accordance with the distribution of GC contents of the purple and green bacteria, the majority of which possess DNAs with 60–70 mol% GC (Mandel et al. 1971). However, in contrast to the anoxygenic phototrophic bacteria, the cyanobacteria show a markedly different mean DNA base composition, the majority of species having only 40–50 mol% GC (Herdman et al. 1979). Cyanobacteria obviously need solar radiation and are more exposed to ultraviolet light than most bacteria. One would have expected them to possess a high GC content, if the hypothesis of Singer and Ames holds true. Solar radiation should, therefore, not be considered as the selective factor that led to the great divergence of DNA base composition among prokaryotes. The effect of high irradiation, mainly of ultraviolet-light damage to DNA and its repair, has been recently reviewed by Nasim and James (1978).

The bactericidal action of diffuse sunlight has not gained much attention. The growth-retarding and lethal action of wavelengths of light not absorbed by DNA has been shown for *Serratia marcescens* (Swart-Füchtbauer and Rippel-Baldes 1951), *Nitrobacter winogradskyi* (Müller-Neuglück and Engel 1961), and *Nitrosomonas europaea* (Schön and Engel 1962). The inhibitory effect is apparently most pronounced and therefore easily recognizable with slowly growing bacteria. Further studies were reviewed by Jagger (1983) and Cornax et al. (1990).

Light exerts its killing effect on microorganisms primarily in the presence of oxygen; in its absence, the effect is smaller by several orders of magnitude. The killing effect is apparently due to photooxidation reactions that are mediated by singlet oxygen. This highly toxic oxygen type is produced by photosensitized activation of triplet oxygen. Protection against single oxygen is readily provided by carotenoids that possess more than seven conjugated double bonds. These carotenoids are able to quench singlet oxygen as well as excited photosensitizers very effectively. Experimental results and a literature review "on the mechanisms for coping with the stress of photosensitized oxidations" have been presented by Krinsky (1979).

The information on the tolerance to intense solar radiation is still scarce. The generalizations on the protective effect of carotenoids just outlined are mainly based on studies of anaerobic, phototrophic bacteria. Among the aerobic bacteria, only those containing carotenoids were considered. There are many more bacteria characterized by remarkable pigments different from carotenoids, such as pyocyanine (*Pseudomonas aeruginosa*), prodigiosin (*Serratia marcescens*) and related pigments (Gerber 1975), indochromes, violacein, xanthomonadines, and others. It would be interesting to know the ecological significance of these pigments in the natural habitats of the bacteria containing them.

The sensitivity of bacteria and protozoa to visible light and oxygen can be increased by the addition to the medium of sensitizing pigments such as methylene blue, toluidine blue, eosin, or acridine orange. Although this photodynamic effect has long been known and is advantageously applied on animal farms to keep the microbial number in drinking water low (Acher and Juven 1977), the ecological significance of photosensitization by natural pigments liberated by autolysis of part of the population is far from clear. Carotenoid pigments also exert a protective effect against photosensitized killing, as has been shown for *Micrococcus luteus* (Anwar et al. 1977; Matthews and Sistrom 1959; Prebble and Huda 1977).

## Surfaces as Habitats

The microbiologist dealing with homogeneous suspensions of bacteria for physiological studies usually pays little attention to the growth of bacteria on surfaces. In nature, however, the growth of bacteria as a surface film may be more frequent and important than growth in homogeneous suspensions (Marshall 1976, 1984; Fletcher and Marshall 1982) (see also Chap. 11, "Planktonic Versus Sessile Life of Prokaryotes" in Vol. 2). Three kinds of interfaces have to be considered: (1) liquid–solid interfaces, such as stationary objects in a river, the tidal zone, and ponds; the inner surfaces of the mouth and the intestinal tract; the outer surfaces of rocks piled up for bacterial leaching of ore or of rocks stacked in columns for wastewater purification in trickling filters; food particles in the digestive system; and silt particles in rivers or marine environments; (2) liquid–gas interfaces, such as the water surface of a pond and gas bubbles in the ocean; and (3) liquid–liquid interfaces, such as oil droplets in an aqueous solution.

## Liquid–Solid Interfaces

Adhesion to surfaces is an important ecological determinant in many ecosystems. The colonization of a suitable liquid–solid interface may be the prerequisite for exploitation of a habitat. Many organisms that inhabit rushing rivers stick to solid surfaces. In open ecosystems, sessile animals as well as bacteria take advantage of being fixed to a privileged location. In a running river that receives organic waste from upstream, slow-growing bacteria could not compete with fast-growing bacteria if they were not attached to stationary objects at a location where food is still available in concentrations sufficient for growth. Growth in locations of this kind is restricted to those bacteria that can attach themselves to a solid surface. Thick plaits of *Sphaerotilus natans* were found in rivers where wastewater of sugar factories was discharged (Cohn 1881; Demoll and Liebmann 1952; Dondero 1961, 1975; Kolkwitz 1904–1906). A similar habitat for mass development is held by *Leptothrix ochracea* in field drains (Dondero 1975), by *Crenothrix polyspora* in water wells (Völker et al. 1977; Wolfe 1960), and by *Toxothrix trichogenes* in iron springs (Krul et al. 1970; van Veen et al. 1978). The trickling filters of waste-disposal plants resemble the river habitat. In some of these habitats, *Sphaerotilus*, *Caulobacter*, *Asticcacaulis*, *Zoogloea ramigera*, and other stalked, prosthecate, or slime-forming bacteria have been found in addition. In many of these habitats, the bacteria form thick, slimy layers on rocks and pebbles.

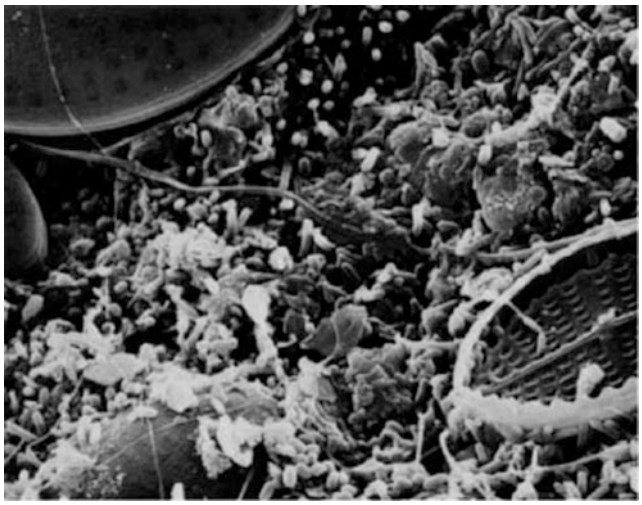


**Fig. 3.1**
**Microflora on the surface of the brown alga *Ascophyllum nodosum*. Scanning electron micrograph (Courtesy of Ralph Mitchell)**

In the marine environment, *Leucothrix mucor* holds a similar position to *Sphaerotilus* in freshwater. *L. mucor* forms sessile filaments and grows as a bacterial epiphyte on seaweed, such as filamentous red and green algae living in habitats where water flows and aeration is good (Kelly and Brock 1969; Raj 1977). The filament is attached to the seaweed by a holdfast (Harold and Stanier 1955) that is part of the sheath (Pringsheim 1957). By scanning electron microscopy (*Fig. 3.1*), bacterial lawns were revealed that consisted of long and short end-attached bacteria, including *Leucothrix mucor* and flexibacteria (Cundell et al. 1977). *Thiothrix nivea* was observed to grow in tufts attached to pebbles in the runoff of a sulfur spring in Seattle, Washington (Bland and Staley 1978).

In rivers and seawater, small, floating particles such as silt, clay, or detritus particles have a remarkable growth-promoting effect on microorganisms. At low-nutrient concentrations, which are suboptimal for efficient growth in homogeneous suspension, the addition of suspended particles favors the growth of bacteria (Heukelekian and Heller 1940) due to adsorption of nutrients to the particle surface. The attachment of bacteria to dense suspensions of silt particles, as is characteristic for certain river waters, has a marked effect on growth and oxygen uptake (Jannasch 1955). The degree of attachment was affected by the concentration of the dissolved organic substrate (Jannasch 1958; Jannasch and Pritchard 1972). Dense growth of bacteria on suspended particles may lead to anoxic microenvironments, as demonstrated by model experiments on denitrification (Jannasch 1960, 1978).

The ability to attach to surfaces may be considered as a means to escape low-nutrient environments. Interfaces play an eminent role in the transport and accumulation of nutrients, such as polysaccharides or proteins. Not only do these nutrients accumulate at solid-liquid interfaces, but they even accumulate at liquid–gas interfaces, and gas bubbles serve as vectors for nutrients (Marshall 1979).

Bacteria are able to stick to solid surfaces in several ways. The outer layers of the bacterial cell envelope (Costerton et al. 1974) evidently play a special role. The cells are either attached by a sheath as in the case of *Sphaerotilus*, *Leptothrix*, and *Leucothrix*; by holdfast substances, which are apparently a gumlike outer layer of the cell envelope and consist of polymers (Umbreit and Pate 1978); or by nonflagellar, filamentous appendages such as fimbriae or pili. The attachment of bacteria to solid surfaces by polymers is apparently widely distributed.

As mentioned above, at high flow rates of a nutrient medium, sessile bacteria are able to exploit the nutritional opportunities of the habitat better than nonattached forms. Cells that do not stick to the surfaces of stationary objects are washed out into another ecosystem; the nutrient exchange is not facilitated by the movement of the medium past the cell. The ability to adhere to surfaces confers a selective advantage on certain metabolic types. Studies on the fine structure of extracellular polysaccharide fibers (Cagle 1975) gave rise to a generalized view of the ability of bacterial cells to adhere to surfaces. The fiber network that extends from the bacterial surface, called bacterial "glycocalyx," mediates adhesion not only to abiotic surfaces but also to other cells, either host or prey.

Adhesion is a way for a bacterium to enter a habitat. The adhesion of the causative agent of caries, *Streptococcus mutans*, to the enamel surface of the tooth, the adhesion of *Vibrio cholerae* to intestinal cells, and other bacterium–host relationships are examples for the potential role of glycocalyx formation in the establishment of infectious diseases (Costerton et al. 1978, 1987). Several unidentified, segmented filamentous bacteria attached to the epithelium of the mucosa of the small bowels of mice have been revealed by scanning electron microscopy (Blumershine and Savage 1978).

For utilization of some solid substrates, a close contact is necessary between the bacterium and its substrate. *Cytophaga* cells adhere closely to cellulose fibers, the rods being aligned with the orientation of the microfibrils. Cellulose degradation by *Cytophaga* and *Sporocytophaga* requires direct contact between the bacteria and the cellulose fibers (Berg et al. 1972; Stanier 1942). The attachment of rumen bacteria to plant particles (Akin 1976; Akin and Amos 1975) is well documented. *Ruminococcus* species were shown to adhere strongly to cotton cellulose fibers and to the cell walls of leaf sections of rye grass, evidently by means of their prominent glycoprotein coats (Latham et al. 1978; Minato and Suto 1978; Patterson et al. 1975). Further examples concern the adherence of starch-digesting bacteria to starch grains, of chitin-hydrolyzing bacteria to chitin, of *Sulfolobus* to sulfur globules (Weiss 1973), and of a thermophilic sulfur oxidizer to sulfide minerals (Golovacheva 1979). Knowledge about the specific mechanisms of attachment of bacteria to solid substrates suggests methods for their enrichment.

Specific interactions between the bacterial cell coat and the plant cell wall apparently precede the invasion of rhizobia into the root hairs of their host plants. Phytohemagglutinins or lectins (Liener 1976) of the plant may be involved in the

recognition process by binding only the corresponding bacteria and not bacteria that infect other legumes (Dazzo et al. 1978; Kato et al. 1979; Marx 1977).

Fimbriation is apparently another means of bacterial attachment to submerged objects (Hirsch and Pankratz 1970). The possession of filamentous, nonflagellar appendages seems to be distributed mainly among members of the Enterobacteriaceae and Pseudomonadaceae but is not restricted to members of these families (Ottow 1975). The ecological importance of fimbriae or pili may consist in their initiating both attachment to solid surfaces as well as contact with other members of the community. The solid surface may be a substrate of low solubility, such as sulfur oxidized by *Sulfolobus* (Brierley 1978; Weiss 1973) or *Thiobacillus* A2 (Korhonen et al. 1978). Attachment to surfaces has not been studied as well as the phenomenon called star formation. The formation of stars, rosettes, or cellular aggregates was originally observed when strains of *Agrobacterium tumefaciens* and *Rhizobium* were studied (Stapp and Bortels 1931; Stapp and Knösel 1954). Since both *Agrobacterium tumefaciens* and rhizobia normally invade plant tissues and must attach to the surface of the plant beforehand, the connection between attachment, important in the natural habitat, and the formation of aggregates as observed in slide cultures under the microscope is obvious. The formation of the frequently very regular rosette-like aggregates in pure cultures of bacteria, such as *Pseudomonas "rhodos"* (Marx and Heumann 1962), *Pseudomonas "echinoides"* (Heumann and Marx 1964; Mayer 1971; Mayer and Schmitt 1971), and *Agrobacterium "luteum"* (Ahrens et al. 1968) may be regarded as a consequence of the presence of polar fimbriae and slime in these bacteria. Similar aggregates are formed on the surface of liquid media (Ahrens et al. 1968).

Aggregation may be either specific, as in the cases of bacterial conjugation, host cell infection, and growth at special surfaces, or may be due to a rather nonspecific process (Fletcher and Loeb 1979), when extracellular polymers such as complex polysaccharides and polyamino acids are excreted or are exposed at the cellular surface under varying physiological conditions, especially during the stationary growth and the death phases and at low-nutrient concentrations. The formation of microbial aggregates is of considerable importance in the soil, in floc formation, in activated sludge treatment, and in industrial biomass production (Harris and Mitchell 1973).

Microbial adhesion has recently grown into a self-contained field of research (see Chap. 11, "Planktonic Versus Sessile Life of Prokaryotes" in Vol. 2). The phenomenon is studied on the physiological (Costerton et al. 1981, 1985; Berkeley et al. 1980), biochemical (Kefford et al. 1982), molecular (Switalski et al. 1989), and biotechnological level (Savage and Fletcher 1985; Characklis and Marshall 1990).

## Liquid–Gas Interfaces

The liquid–gas interface is a unique habitat in many respects. Depending on the ecosystem, this environment harbors a diverse bacterial flora, sometimes called the bacterial neuston. Common to the liquid–air interfaces are (1) direct exposure to the oxygen of the air, (2) accumulation of hydrophobic substances either originating from the air or from the water body, and (3) high light intensities.

The surface film of lakes and ponds formed in summer during calm weather harbors bacteria such as *Pseudomonas*, *Caulobacter*, *Hyphomicrobium*, *Nevskia*, *Flavobacterium*, *Alcaligenes*, and *Micrococcus* (Babenzien 1965, 1967; Hirsch 1974; Hirsch and Pankratz, 1970). Bacteria that contain gas vacuoles may dominate under certain conditions (Walsby 1978).

The surface layer of the ocean is a separate environment (Zaitsev 1971). It has gained special attention because oil pollution has increased and wide areas of the ocean are occasionally covered with petroleum (Bartha and Atlas 1977). A considerable proportion of organic substances accumulating in the marine neuston is released from planktonic organisms; fatty acids, lipids, polysaccharides, hydrocarbons, and proteins have been identified in the surface film (Wangersky 1976). The marine neuston is the habitat of various marine bacteria (Bezdek and Carlucci 1972).

The surface film of water baths in laboratories offers a special habitat for bacteria that either utilize traces of conventional substrates inadvertently added to the water or that utilize volatile or gaseous substrates present in laboratory air (Leifson 1962). The gum formed within several weeks contains *Bacteriogloea*, *Hyphomicrobium*, *Caulobacter*, *Mycobacterium*, and *Nocardia*.

## Gradients as Habitats

In nature, the distribution of nutrients or environmental factors is generally patchy. In many ecosystems, vertical gradients of concentrations of various nutrients exist. The nutrients produced in sediments diffuse into the aqueous layer and, in the absence of mixing by convection or currents, form a concentration gradient. Such gradients are to be expected either in a microscale in sediments, soil, or shallow water bodies or in a macroscale in lakes or oceans. The gradients may either be of substances used as nutrients or of physical factors such as redox potential, temperature, and radiation. Of primary importance for growth and distribution of microorganisms are the concentration gradients of organic acids, hydrogen sulfide, carbon dioxide, and oxygen.

The major amount of organic matter that is decomposed by microorganisms in nature consists of fresh plant litter. Another fraction enters the soil or sediments of aquatic ecosystems after partial degradation by herbivorous animals. Only a comparatively small fraction of the plant tissue is degraded by animals; the major part, cellulose and lignin, is released and becomes the major substrate of the microbial food chain.

Terrestrial and aquatic ecosystems differ greatly with respect to further degradation of the primary biomass and the cellulose-rich detritus derived therefrom. On the land, the solid organic matter stays on or near the surface of the soil and is decomposed

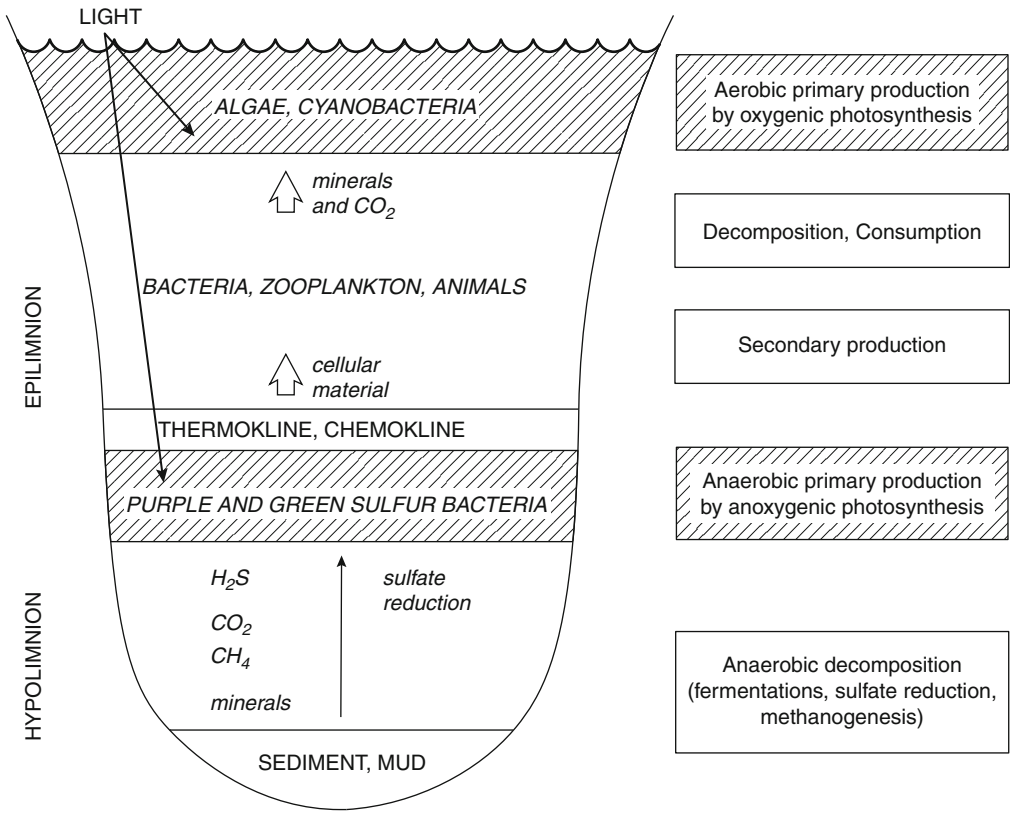



**Fig. 3.2**
**Diagram of production, consumption, and decomposition in an aquatic ecosystem with a chemocline**

mainly aerobically. Only part of the organic matter is transported into the soil by animals as vectors and is degraded by microorganisms either aerobically or anaerobically, depending on aeration and moisture content. Undoubtedly, diffusion processes and concentration gradients play a role in the soil. However, due to the heterogeneous distribution of organic matter, the basic features of anaerobic and aerobic food chains cannot easily be recognized in terrestrial systems.

In contrast, in a stagnant body of water, the solid organic matter produced in the surface layers sinks, either immediately or after passage through a short, animal food chain, to the bottom of the water body. There microbial degradation occurs. Due to its low solubility in water, the oxygen may soon be exhausted in the course of the initial aerobic degradation of organic substances. In highly productive waters and in the absence of convection, the deeper layers become anoxic, which results in anaerobic microbial degradation processes. These processes give rise to the production of various soluble or gaseous substances such as organic acids, hydrogen sulfide, hydrogen, methane, carbon dioxide, and ammonia. These products freely diffuse upward, each forming its own concentration gradient.

The chemical conditions may be further diversified by the liberation of ions from the sediment. These upward gradients are overlayered by the downward gradients of temperature, light, and oxygen. Thus, due to the gradients of various nutrients and other environmental factors, a stagnant water body offers a variety of diverse living conditions for microorganisms. The study of concentration profiles and of the zonation of aquatic ecosystems and the assignment of the microorganisms to the particular conditions of their habitat is a major concern of microbial ecology. Several lakes have been studied as model systems (Clark and Walsby 1978b; Gorlenko et al. 1977; Kuznezow 1959, 1977). The stratified lake will be presented here as an example. The principles derived from these studies can be easily applied to zonations and processes occurring in water-logged soils, tundras, and rice fields. They apply with slight variation also in shallow ponds, sediments, and shallow mats in the runoffs of hot sulfur springs.

## Gradients of a Macroscale: The Stratified Lake

Many lakes are temporarily or permanently stratified (*Figs. 3.2* and *3.3*). Stratification occurs when a dense, either cold or saline (salt-rich) water body is overlayered by a less dense, either warm or salt-free water body. The stratification patterns vary with different climate zones. In temperate climates, normal stratification occurs in freshwater lakes more than 10 m in depth.

The holomictic stratified lake may serve as an example of the stratification and gradient-forming process (Overbeck 1972; Pfennig 1979; Sorokin 1970). After total circulation in winter

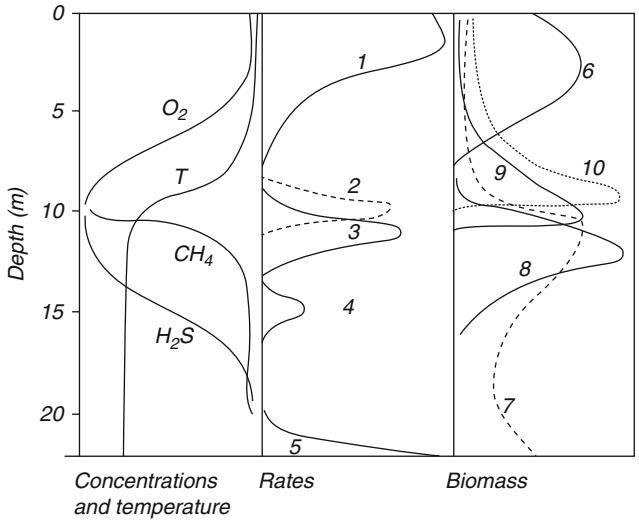



**Fig. 3.3**

**Idealized *vertical* profile of a freshwater lake in the temperate climate zone in summer, showing concentrations, conversion rates, and biomass. The figure is based on drawings and data of Sorokin (1970), Gorlenko, Dubinina, and Kuznezow (1977), and Overbeck (1972). Symbols: *T* temperature, (1–5, conversion rates) *1* $CO_2$ fixation in the light (oxygenic photosynthesis), *2* $CO_2$ fixation in the dark, *3* $CO_2$ fixation in the light (anoxygenic photosynthesis), *4* and *5* sulfate reduction, *6* biomass of algae and cyanobacteria, *7* total bacterial biomass, *8* biomass of phototrophic bacteria, *9* biomass of protozoa, *10* biomass of Copepoda and Cladocera**

time, the warming of the surface layers during the summer yields a less dense upper layer (epilimnion) of circulating, aerated water and a dense, cold, stagnant bottom layer (hypolimnion). The intermediate layer (metalimnion) is characterized by a temperature gradient (the thermocline) and, after oxygen deprivation and anaerobic decomposition of organic matter in the hypolimnion, by a chemical gradient (chemocline) also. In the epilimnion, which is exposed to sunlight, biomass is produced by phototrophic cyanobacteria, diatoms, and green algae; allochthonous matter from the surroundings usually adds to the total organic material. Part of the biomass usually sinks to the hypolimnion and to the bottom of the lake where it is degraded. Degradation is accompanied by oxygen consumption, resulting in a decrease in oxygen concentration and, finally, in anoxic conditions, first in the sediment, then in the bottom layer. Continued anaerobic degradation results in the production of organic fermentation products as well as hydrogen, methane, hydrogen sulfide, and carbon dioxide, which diffuse upward forming concentration gradients. Methane, which is the major carbonaceous substance released from the sediment, is discharged in the form of gas bubbles. Part of the methane is dissolved in the water as it moves upward and is oxidized by methane-utilizing aerobic bacteria. The quick removal of oxygen from the hypolimnion is primarily due to the quick dispersal of methane and the growth of methane-oxidizing bacteria. Finally, the hypolimnion becomes totally anoxic.

As soon as anaerobic conditions have been established in the bottom layer or in the total hypolimnion, the anaerobic conversion processes, which started with the degradation of solid material, mainly cellulose, in the sediment, continue in the water body. Primary soluble fermentation products, hydrogen gas included, are used to reduce sulfate to hydrogen sulfide. The major amount of hydrogen sulfide originates from sulfate reduction in the free water. Simultaneously, nitrate is reduced to nitrite, which transiently accumulates and forms a distinct layer, and then to nitrogen.

The upper part of the epilimnion is the zone of primary production by the oxygenic phototrophic organisms, such as photosynthetic higher plants, algae, diatoms, flagellates, green algae, and cyanobacteria. These primary producers are accompanied by bacteria, protozoa, and metazoa that consume photosynthesized biomass, which results in secondary production and release of cellulosic detritus (Fenchel and Jørgensen 1977). The thermally stratified lake is a very common habitat for cyanobacteria that contain gas vacuoles. *Oscillatoria rubescens*, *O. agardhii*, *Aphanizomenon flos-aquae*, and *Microcystis aeruginosa* form stable populations near the bottom of the epilimnion (Clark and Walsby 1978a, b).

The chemocline and the hypolimnion are the favored habitats of the anaerobic prokaryotes. Provided hydrogen sulfide is present and the light intensity allows, anoxygenic phototrophic bacteria develop underneath the chemocline and form a second layer of primary biomass production (Sorokin 1970). The upper hypolimnion just below the chemocline is the habitat of the purple and green sulfur bacteria, among which the brown forms dominate (*Fig. 3.2*). They produce biomass by anoxygenic photosynthesis from carbon dioxide and hydrogen sulfide. Bacteria that are either buoyant by gas vacuoles or motile by flagellation dominate. The buoyancy of *Lamprocystis*, *Amoebobacter*, *Thiodictyon*, *Thiopedia*, *Pelodictyon*, and *Ancalochloris* is apparently just sufficient to allow them to float in the heavy cold water beneath but insufficient to float in the light warm water above the thermocline. Among the motile purple sulfur bacteria (Chromatiaceae), the large *Chromatium* species (*C. okenii*, *C. weissei*, *C. warmingii*, *C. buderi*) and *Thiospirillum* live in this layer.

Further details on the ecology of photosynthetic bacteria with emphasis on the distribution in stratified lakes were presented in a comprehensive review (Pfennig 1979). The efficiency of biomass production by anoxygenic photosynthesis is remarkable. Part of the biomass that by slight turbulences reaches the upper chemocline is grazed by copepods, cladocera, and protozoa and transported into upper aerobic regions. Photosynthesis by the purple and green sulfur bacteria is accompanied by sulfate production. The sulfate produced is immediately reduced to hydrogen sulfide. As documented by measurements of the sulfate-reducing activity and cell counts of sulfate-reducing bacteria in various depths, the activity of sulfate reduction has two maxima: one in the hypolimnion bottom layers and one below the chemocline (Sorokin 1970). While the reduction power for the first process originates from the sediment, sulfate

reduction below the chemocline seems to be supplied by indigenous solutes released from cells within the region, either by excretion or by decay.

The metalimnion is a layer of high biological activity. Because of its richness in inorganic nutrients as compared to the epilimnion, it is inhabited by a few aerobic photosynthetic prokaryotes that can tolerate hydrogen sulfide and anaerobic conditions. One of them is *Oscillatoria limnetica*, which has been isolated from the sulfide-rich layers of Solar Lake (Eilat, Israel) and studied in detail (Cohen et al. 1975; Padan 1979a, b). It is able to use hydrogen sulfide as an electron donor in a photosystem I-driven reaction and to photoreduce carbon dioxide; hydrogen sulfide is oxidized to elemental sulfur. Once the system has been induced, *O. limnetica* can grow under anaerobic conditions (Oren and Padan 1978; Oren and Shilo 1979). After they return to the oxic zones, the cells switch from anoxygenic to oxygenic photosynthesis. This study of *O. limnetica* explained the predominance of cyanobacteria in sulfide-containing microaerophilic habitats or in habitats of frequently alternating conditions (Padan 1979).

Because of the oxidation of hydrogen sulfide by the purple sulfur bacteria in the light and diffusion of oxygen from the epilimnion, the chemocline moves downward several meters during the daytime. Diurnal vertical fluctuations of the redox discontinuity layer obviously represent a major selective factor for the organisms occupying this habitat.

## Gradients of a Microscale: The Sediments

Among the various types of sediments, only the coastal marine sediment and the lacustrine sediment will be discussed here. In principle, the degradative and biosynthetic processes that occur in sediments are similar to those in stratified lakes. The organic material that supplies the energy for life processes in the sediment is mainly allochthonous. Either leaves or large algae are buried in the sand of coastal marine areas or detritus is incorporated into the mud.

If sulfate is present in excess, as in marine ecosystems, the anaerobic food chain yields mainly hydrogen sulfide and gives rise to the activities of bacteria involved in the sulfur cycle. Habitats of this sort gained early attention by the smell of hydrogen sulfide and the bright red layers and bloom of purple sulfur bacteria and have repeatedly been described (see Bavendamm 1924). There are numerous kinds of sulfureta, most of them estuaries, limans, salt marshes, tidal flats, and swamps.

In typical sulfureta of littoral marine areas, buried organic material is covered by sand, and the water layer is 10–20 cm deep (Fenchel 1969). Hydrogen sulfide production may be high. The sulfide concentration in a few centimeters' depth of these sulfide systems may easily reach 10 mM $H_2S$ (Fenchel and Riedl 1970). The diffusing hydrogen sulfide may give rise to emission to the atmosphere during the night. During the day, the emission of hydrogen sulfide amounts to only 4% of that during the night (Hansen et al. 1978). The diurnal fluctuations of the hydrogen sulfide concentration indicate the significant role of the purple sulfur bacteria in the photic zones of these coastal areas. The bacteria respond to the exhaustion of sulfide and the alternation of the oxygen tension by chemotactic (aerotactic) movements and disappear in the course of illumination into the sediments. The movements parallel the diurnal vertical migrations observed in stratified lakes. Depending on the particular environmental condition, the ecological niche of sulfide oxidation may be occupied by colorless sulfur bacteria such as *Thiovulum*, *Macromonas*, *Achromatium*, and *Beggiatoa* or by phototrophic purple sulfur bacteria.

A characteristic ecosystem, with a very steep gradient from the anoxic hydrogen sulfide-rich zone to the oxic layer, has been described as the "Farbstreifen-Sandwatt" (Hoffmann 1942); the name was coined due to the succession of green, red, and black layers of the sand of marine coastal areas. The phenomenon is restricted to localities with a high level of ground water, sand of grain diameter to provide capillary ascension almost up to the sand surface, sufficient organic material and sulfate in the lower sediment layers, and high light intensities to penetrate the uppermost layers of sand. The green layer contains cyanobacteria exclusively, demonstrating their tolerance to hydrogen sulfide compared to green algae as emphasized by Padan (1979). The red layer underneath is an almost monospecific culture of purple sulfur bacteria, which is in direct contact with the black zone. These observations confirm the ecological significance of the complementary spectral absorption of green plants and purple bacteria (Buder 1919) and are in accordance with measurements showing that long wavelengths penetrate in sand further than short wavelengths (Fenchel and Staarup 1971), in contrast to water (Pfennig 1967).

Multilayered microbial communities in aquatic ecosystems resembling the "Farbstreifen-Sandwatt" have been observed in areas of hot springs and in solar ponds. Various kinds of mats have been described and studied in detail (Cohen and Rosenberg 1989).

A particular environment is apparently required by the sulfide-oxidizing, nonphotosynthetic bacteria such as *Beggiatoa*, *Thiothrix*, *Achromatium*, *Macromonas*, *Thiovulum*, and *Thiospira*. Their habitats appear to be areas where the gradients of both hydrogen sulfide and oxygen are overlapping or, as in running waters, where the hydrogen sulfide-providing sediment is covered by running water containing oxygen.

Due to the instability of hydrogen sulfide in the presence of oxygen, a habitat requiring the simultaneous presence of both compounds will be small and transient. The coexistence of hydrogen sulfide and oxygen has already been convincingly demonstrated by Beijerinck (1895). He introduced a solution of hydrogen sulfide containing a small amount of oxygen into an anaerobic suspension of luminous bacteria—precautions to exclude the access of air were taken. Luminescence occurred, indicating that the hydrogen sulfide solution contained free oxygen and that the affinity of the bacteria for oxygen is high. Hydrogen sulfide and oxygen will be simultaneously available for a long period of time only in areas to which both compounds

are continuously supplied. Observations in nature and when attempting to grow isolates of colorless sulfur-oxidizing bacteria are in agreement with this idea (Bland and Staley 1978; la Riviére 1963, 1965; Strohl and Larkin 1979; Chap. 15, "The Colorless Sulfur Bacteria" in Vol. 3). In a study on the chemolithotrophic nature of *Thiovulum* species (Wirsen and Jannasch 1978), the difficulty of obtaining pure cultures is ascribed to the instability of the oxygen/hydrogen sulfide environment. The individual cells of *Thiovulum* aggregate in "veils" by extruding slime threads, a possible means of stabilizing an oxygen/hydrogen sulfide interface for a period of time (la Riviére 1963).

Careful studies using microelectrodes for the measurement of oxygen and hydrogen sulfide have been carried out on *Beggiatoa* populations at natural oxic/anoxic interfaces of estuarine sediments (Jørgensen 1982; Jørgensen and Revsbech 1983). In *Beggiatoa* "plates" prepared in artificial agar-gelled interfaces or $O_2/H_2S$ gradient cultures, chemoautotrophy was demonstrated (Nelson and Jannasch 1983), and the growth pattern and yield were studied in detail (Nelson et al. 1986). The thickness of these filament plates never reached more than a few mm.

The unusual occurrence of thick mats of *Beggiatoa*-like filaments was observed on the surface of hydrothermal sediments covering hot vents at a depth of 2,000 m in the Guaymas Basin of the Gulf of California (Nelson et al. 1989). These mats were 3-cm thick on the sediments and up to 30-cm thick between stands of vestimentiferan tube worms, the characteristic hosts of symbiotic, sulfide-oxidizing prokaryotes (see below). The unusual mass development of *Beggiatoa* at the hydrothermal vents is speculated to be due to (1) a more efficient flux of $H_2S$ and $O_2$ than by mere diffusion, (2) a continuous supply of an organic growth factor, (3) facultative chemoautotrophy providing an efficient metabolic flexibility in case of intermittent or turbulent vent emissions, and (4) an optimal temperature gradient (Jannasch et al. 1989). Furthermore, the unusually large diameter of the filaments (up to 122 µm) and an "empty" inner space (a liquid vacuole filling more than 80% of the cell, the cytoplasm being distributed only along the outer cell wall) may contribute to their growth and survival in this environment. These allow the cells to grow to an enormous size without diffusional problems, thus providing them with a structural rigidity necessary for exploiting a larger ambient space than smaller cells can without support of a substratum (e.g., sediment). Moreover, an inner low-redox reservoir may enable these organisms to survive in the absence of reduced chemical substrates during flushing by oxygenated ambient seawater for a certain period of time.

The classical filamentous and stalk-forming iron bacteria have to be considered as gradient organisms also. Bacteria such as *Leptothrix ochracea* and related bacteria, as well as *Gallionella ferruginea*, grow in drainage tubes in moist fields, preferably in the narrow zone between the anaerobic bottom layer, which is the source of ferrous iron, and the flowing water, which contains oxygen.

## Horizontal Gradients

Horizontal gradients of environmental factors exist where water of unusual properties and contents is discharged into running water. Gradients are formed in the vicinity of hot springs; sulfur springs; effluents of coal, iron, and salt mines; rivers; ditches; and drainage tubes.

The water discharged from sulfur springs gives rise to a gradient in sulfide concentrations, providing sulfide-rich water and photoanaerobic conditions at the origin and photoaerobic conditions downstream. Hot sulfur springs as they occur in New Zealand, North Island (Castenholz 1976), and Yellowstone National Park (Brock 1978; Castenholz 1977) provide gradients of temperature, sulfide and oxygen concentration, pH value, and secondarily, in the concentration of minerals and organic nutrients; near the spring, the effluent sulfide-rich water of 60–70 °C enables the gliding green bacterium, *Chloroflexus*, to grow and form reddish orange or orange-green mats. Downstream, this green bacterium is accompanied by the cyanobacteria *Oscillatoria*, *Synechococcus*, and, eventually, *Mastigocladus*. The patterns do not vary greatly at different localities (Bauld and Brock 1973; Castenholz 1976, 1977, 1979).

## Possible Mechanisms for Finding or Remaining in the Beneficial Layer of the Gradient

Motility and buoyancy may be involved in keeping the bacteria in horizons of favorable growth conditions. Many motile bacteria manage, by means of their chemotactic response mechanisms, to reach and stay in an area where the concentration of a nutrient or of oxygen is optimal. Bacteria that are motile by flagellation, as well as gliding bacteria, are able to move to and stay in their beneficial environment. Gas vacuoles, occurring exclusively in aquatic microorganisms (Cohen-Bazire et al. 1969), provide buoyancy and may be a means by which bacteria maintain their vertical position (Clark and Walsby 1978a, b).

### Chemotaxis

Chemotaxis is widespread among motile microorganisms (Weibull 1960) and is of ecological importance in many systems (Chet and Mitchell 1976). Usually, the chemotactic response mechanisms result in the accumulation of microorganisms in areas of favorable metabolic conditions and depletion in areas of adverse conditions. However, there is almost no correlation between the properties of a substance to act as an attractant and to serve as an energy source (Adler 1974; Weibull 1960). The effect of an attractant has been shown to increase swimming periods toward the attractant after tumbling, while the swimming periods in the opposite direction are shortened

(Koshland 1974, 1976, 1980, 1981; Repaske and Adler 1981; Adler 1988; Hazelbauer 1988). The role of chemotaxis in the vertical distribution is still a matter of speculation.

### Aerotaxis

The response of bacteria to oxygen may be a dominant mechanism enabling bacteria to find and remain in an environment favorable with respect to oxygen concentration. Vertical movements of clouds of phototrophic bacteria in Winogradsky columns observed during diurnal cycles of light and dark may serve as a model for investigating the response of bacterial populations toward oxygen and substrates.

### Phototaxis

Phototaxis is apparently shared by all anoxygenic phototrophic bacteria that are motile by flagellation. The phototactic response mechanism results in keeping the bacteria in an area of favorable light intensity after they accidentally enter. The response, reversal of flagellar movement, usually occurs when the cells enter an area of lower light intensity. Although phototaxis has been well studied in laboratory cultures (Hustede et al. 1989), investigations relevant to the significance of phototactic behavior in the vertical distribution of phototrophic bacteria in lakes are lacking. The function of photokinesis, the initiation or acceleration of linear velocity by light, has not yet been studied by ecologists (Nultsch 1975).

### Magnetotaxis

Bacteria containing magnetite ($Fe_3O_4$) crystals have been discovered (Blakemore 1975) and isolated (Blakemore et al. 1979; Frankel et al. 1979). It is hypothesized that the downward orientation of the cell within the Earth's inclined magnetic field lines on the northern hemisphere provides favorable growth conditions for these motile and strongly microaerophilic organisms. However, when the studies were repeated in New Zealand and Australia, very similar organisms were found that exhibited a reversed polarity (Blakemore et al. 1980).

The anaerobic formation of magnetite by a marine magnetotactic bacterium (designated strain MV-1) was demonstrated using nitrous oxide as an electron acceptor (Bazylinski et al. 1988). This finding obliterated the notion that the biological production of magnetite can only occur in aerobic top sediments. Some nonmagnetotactic, dissimilatory iron-reducing bacteria were described by Lovley and Phillips (1987) and found also to synthesize extracellular magnetite from hydrous ferric oxide under anaerobic conditions. The formation or "biomineralization" of a ferrimagnetic iron sulfide called greigite ($Fe_3S_4$) has been reported in a multicellular magnetotactic bacterium that has not been isolated but is common in brackish sulfide-rich water and sediments (Mann et al. 1990). These crystals are often aligned in chains and associated with single crystals of nonmagnetic pyrite ($FeS_2$).

### Buoyancy

Assuming that gas vacuoles provide buoyancy to the cells (Walsby 1975, 1977), there may be two ways by which distinct layers are formed. The bacteria may be able to migrate to the favorable depth, or the gas vacuoles may provide neutral buoyancy to guarantee a long residence time to the bacteria at depths supporting their growth. The second alternative has been supported by experimental evidence (Clark and Walsby 1978b).

Gas vacuoles may also serve the distribution of cells and resting stages; this function is exemplified by the gas vacuoles of some clostridia, which are only formed during the transition phase from the vegetative cell to the spore (Duda and Makaer'eva 1977).

An interesting alteration of buoyancy and its significance in the natural habitat has been described in the case of *Metallogenium*. *Metallogenium* has been found in all oligotrophic lakes that contain manganese and also in mesotrophic and eutrophic lakes. Its development occurs during the periods of circulation. The vertical distribution of *Metallogenium* in these lakes is dependent on the concentration of manganese. Manganese is soluble in the reduced state. If oxidized by *Metallogenium*, manganese is precipitated at the cell surface, and the heavy manganese oxides make the cells sink down to the sediment. Under anaerobic conditions, manganese is reduced, the buoyancy is increased, and the cells return to the aerobic layers. Thus, the movement of *Metallogenium* is strictly correlated to the availability of manganese and oxygen.

## Microbial Associations

Under natural conditions, the various types of prokaryotes live in more or less close associations. Interactions between bacteria in a common habitat may be either weak or absent; only species with quite dissimilar nutrient requirements can be expected to show neutralism. The interactions may be strong and may be characterized as mutualism, commensalism, or parasitism. The relationships may be loose or tight, facultative or obligatory. Several examples of microbial associations studied in open systems have been reviewed by Meers (1973).

The most spectacular examples of syntrophic or of mutualistic associations have been discovered in the course of metabolic studies on bacterial cultures presumed to be pure cultures. One example concerns the partners of the culture of *Methanobacterium omelianskii* and illustrates a symbiosis with unidirectional substrate supply of mutual benefit. The other concerns symbiotic associations with bidirectional transfer of small molecules involved in energetic coupling.

Another striking discovery is the symbiotic association between chemolithotrophic sulfide- and thiosulfate-oxidizing prokaryotes and newly described marine invertebrates found

**Table 3.5**

**Examples of interspecies hydrogen transfer by coculturing hydrogen-utilizing (methanogenic) bacteria with various $H_2$-producing bacterial species**

| Substrate | $H_2$-producing species; products of pure culture | $H_2$-utilizing species; products of coculture | Reference |
|---|---|---|---|
| Glucose | ***Ruminococcus albus***; ethanol, acetate, $H_2$, $CO_2$ | ***Vibrio succinogenes***; acetate, succinate | Iannotti et al. (1973) |
| Glucose | ***Selenomonas ruminantium***; lactate | *Methanobacterium ruminantium;* acetate, methane, $CO_2$ | Chen and Wolin (1977) |
| Cellulose | ***Clostridium thermocellum***; ethanol, acetate, $H_2$, $CO_2$ | *Methanobacterium thermoautotrophicum;* acetate, methane, $CO_2$ | Weimer and Zeikus (1977) |
| Cellulose | ***Ruminococcus flavefaciens***; succinate, acetate, formate, $H_2$, $CO_2$ | *Methanobacterium ruminantium;* acetate, methanol, $CO_2$ | Latham and Wolin (1977) |

to live tightly clustered around deep-sea hydrothermal vents (Cavanaugh et al. 1981; Felbeck et al. 1981; Belkin et al. 1986; Distel et al. 1988; Fisher et al. 1989). Furthermore, a new genus of marine blue mussels was found to contain methylotrophic endosymbionts in their gill cells enabling the animal to thrive near methane emissions in the vicinity of "cold seeps" on the seafloor (Childress et al. 1986). A similar association was later described in certain polychaetes collected from marine sediments at the Skagerak in the Baltic Sea (Schmaljohann and Flügel 1987).

## Interspecies Hydrogen Transfer

The concentration of many metabolites in the cell is below measurable levels. The intermediates of reactions, especially those which are catalyzed by multienzyme complexes, scarcely reach measurable concentrations. The intermediates are used as substrates of subsequent enzyme reactions at the same site or in the same compartment where they are produced. The absence of measurable concentrations of a compound does not mean that the compound is not involved in a metabolic reaction.

Similar situations are encountered in many ecosystems where very important intermediates of anaerobic food chains are present at scarcely detectable concentrations. In the rumen, lactate and hydrogen are present only at low concentrations, although they are major products of the predominating anaerobic fermentations; in contrast, acetate, butyrate, propionate, valerate, and formate, which are not subject to further fermentative conversion, may accumulate. In anaerobic ecosystems, only the end produces of anaerobic food chains accumulate or are released into the environment. The concentration of some degradation products, such as hydrogen, acetate, lactate, and ethanol, is low. These compounds are consumed as soon as they are produced.

Metabolic products may be inhibitory to the cells that produce them; subsequent utilization of inhibitory products by commensals, therefore, is useful to the ecosystem. In several cases, the cooperation of two or more organisms is essential, since a substrate would not be degradable at all if the concentration of the product were not kept very low. Interspecies hydrogen transfer is the most significant example of a symbiosis with unidirectional substrate supply from which mutual benefit is drawn.

The principle of interspecies hydrogen transfer became clear when the culture called *Methanobacterium omelianskii* was separated into a strain that produced hydrogen (S-organism) and a strain that oxidized hydrogen (Moh-strain) (Bryant et al. 1967; see also Chap. 2, "Virulence Strategies of Plant Pathogenic Bacteria" in Vol. 3). These strains carry out the following reactions:

$$\text{S-organism: } 2C_2H_5OH + 2H_2O \rightarrow 2CH_3 - COOH + 4H_2$$

$$\text{Moh-strain: } 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

The S-organism grows poorly as a pure culture in media with ethanol or other utilizable alcohols because the accumulated hydrogen inhibits growth. The S-organism lacks the ability to dispose of electrons resulting from the oxidation of ethanol via electron sinks other than hydrogen. For efficient growth of the S-organism, a hydrogen-utilizing bacterium has to be included to remove the hydrogen produced (Reddy et al. 1972a, 1972b). The methanogenic bacteria keep the partial pressure of hydrogen low; for the bovine rumen, a value of about three times $10^{-4}$ atm (two times $10^{-7}$ M) was reported (Hungate 1967). Therefore, both organisms benefit from this symbiotic association. The methanogenic bacterium is continuously supplied with hydrogen, and the hydrogen-producing organism can even degrade and generate energy from substrates such as lactate or ethanol, which, for thermodynamic reasons (Wolin 1976), could not be degraded when hydrogen accumulated in the medium (Wolin and Miller 1982).

As a result of interspecies hydrogen transfer between partners of close symbiotic associations, therefore, special ecological niches can be occupied. The principle has been exemplified by coculturing methanogenic bacteria with various hydrogen-producing bacterial species (*Table 3.5*).

For the fermentative degradation of substrates such as lactate or ethanol, the removal of hydrogen by a hydrogen-utilizing partner is obligatory; without continuous removal of hydrogen, anaerobic growth on these substrates is slight. For the degradation of other substrates, glucose included, the removal of

hydrogen is not obligatory; however, it enables the cell to obtain more energy than would be otherwise possible.

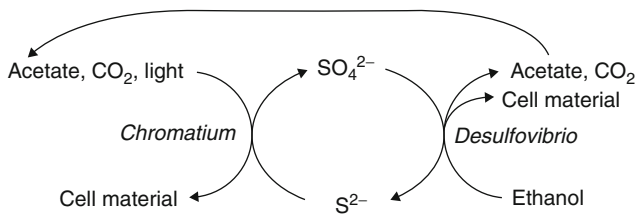
In media that contain little sulfate, *Desulfovibrio vulgaris* grows only modestly on ethanol or lactate producing acetate and hydrogen. Like the S-organism, it lacks the ability to produce a sink for electrons other than protons. In coculture with *Methanobacterium*, ethanol and lactate were actively fermented with the production of acetate and methane (Bryant et al. 1977). Stable methanogenic mixed cultures, which converted glucose to methane, were also obtained by enrichment in the chemostat (Siñeriz and Pirt 1977). Benzoate, which under anaerobic conditions can be utilized by phototrophic bacteria in the light or by chemoorganotrophs through anaerobic respiration, has been shown to be biodegradable even under fermentation conditions (Nottingham and Hungate 1969; Boyd et al. 1983; Sleat and Robinson 1984; Tschech 1989); methane and carbon dioxide are the main fermentation products. Apparently, a microbial consortium of more than two bacteria is involved in methane production from benzoate (Ferry and Wolfe 1976).

The examples discussed above of interspecies substrate transfer concerned the unidirectional transfer of substrates within food chains. In the next section, bidirectional transfer will be discussed.

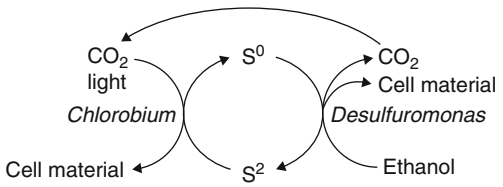
## Bidirectional Transfer of Small Molecules

A bidirectional transfer of small molecules is the basis of some symbiotic associations in which sulfate (or sulfur) and hydrogen sulfide are involved in the energetic coupling of two different strains of bacteria kept in mixed culture.

Sulfate-reducing bacteria such as *Desulfovibrio vulgaris* growing with organic acids as hydrogen donors provide the hydrogen sulfide used for anoxygenic photosynthesis by *Chromatium*, which in turn reoxidizes hydrogen sulfide with the production of sulfate. The occurrence of this light-driven sulfur cycle has been repeatedly described. When sulfate is continuously added to the system, part of the sulfur intracellularly accumulated in the *Chromatium* cells may leave the cycle and be deposited. The deposition of sulfur in certain Cyrenaican (North African) lakes has been attributed mainly to this cycle (Butlin and Postgate 1954), which is as follows:




Although the direct reduction of sulfur was already observed 80 years ago (Beijerinck 1895), a chemoorganotrophic bacterium able to use elemental sulfur as hydrogen acceptor for growth was not known until the 1970s when a bacterium was found capable of oxidizing acetate to carbon dioxide linked to the reduction of sulfur to hydrogen sulfide (Pfennig and Biebl 1976). This bacterium, *Desulfuromonas acetoxidans*, was isolated from a culture called *Chloropseudomonas ethylica*, which had been held as a stable mixed culture for many years. The syntrophic association of the green phototrophic bacterium, *Chlorobium*, and of *D. acetoxidans* occurs because hydrogen sulfide and sulfur permit energetic coupling of the life processes of both partners, as shown below:




In mixed cultures, low concentrations of hydrogen sulfide (7–8 mg/l) are sufficient to allow maximum growth of *Desulfuromonas* strains and of *Chlorobium* or *Prosthecochloris* strains (Biebl and Pfennig 1978). The cell yield of the green bacterium is not limited by the hydrogen sulfide added to the culture; it depends rather on the amount of ethanol, the hydrogen donor.

To understand the function of a species in a habitat doubtless requires studies in mixed culture. Only in mixed culture under conditions of competition is the true actual niche revealed. According to van Niel (1955), Winogradsky "argued convincingly that pure culture studies may reveal characteristics that can express themselves only in the absence of potential competitors." The examples presented demonstrate the necessity for pure-culture studies. Ironically enough, superb examples for the mixed culture concept were noticed when it was discovered that the "pure" cultures were not really pure.

## Specific Aquatic Ecosystems: Marine Environments

Although the borderline between the aquatic and terrestrial habitat of microorganisms is diffuse, certain physical and chemical properties of water characterize the predominantly aqueous environment distinctly with respect to the indigenous microbial population. Inorganic and organic nutrients are available in dissolved and ionic form and are highly mobile and constantly dispersed by diffusion, convection, and currents. These characterizations of nutrient regime, together with the high heat capacity of water, provide the characteristic constancy of the environmental conditions upon which many of the typical aquatic microorganisms depend.

Another typical characteristic of aquatic environments is the variety of interfaces that are of special microbiological importance: the surface film (neuston); the interface between oxygenated and anoxic water or sediment; horizontal layering of water with respect to gradients of light, temperature, and salinity in stagnant waters; and finally the aqueous–solid interface of

submerged surfaces. These interfaces provide specific and often highly selective environmental conditions for the accumulation and growth of microorganisms on the micro- as well as on the macroscale (see above section "Surfaces as Habitats" and Chap. 11, "Planktonic Versus Sessile Life of Prokaryotes" in Vol. 2).

The literature in "aquatic microbiology" largely deals with environments on the macroscale: the qualitative and quantitative assessment of populations in springs, ponds, rivers, lakes, and parts of the ocean. In addition, applied problems led to the investigation of groundwaters, acid mine waters, sewage lagoons, cooling water in pipe systems, etc. Only some of these studies focused specifically on the particular aquatic characteristics.

It was stated earlier that the lack of water-preserving mechanisms makes prokaryotes as a group appear to be typically aquatic organisms. Over and beyond this fact, certain microorganisms are specifically adapted to life at the typical conditions of aquatic environments. An example is *Thiovulum*, a highly motile and large (spherical cells of up to 25-µm diameter) chemolithotrophic organism that oxidizes hydrogen sulfide aerobically (see Chap. 15, "The Colorless Sulfur Bacteria" in Vol. 3). Growth of *Thiovulum* depends on its ability to locate in areas of the short-lived coexistence of hydrogen sulfide and oxygen as spontaneously reacting compounds (Wirsen and Jannasch 1978). The biology of the typically aquatic *Caulobacter* group has been reviewed by Poindexter (1964) and Schmidt (1971). These prokaryotes are uniquely adjusted to metabolize at low-nutrient levels (Poindexter 1979) while largely attached to submerged solid surfaces with the aid of characteristic appendages, the prosthecae.

A large number of typically aquatic microorganisms are microaerophilic, a point of evolutionary interest, and take advantage of the fact that water provides a barrier against a quick replenishment of oxygen, as determined by the rates of dissolution and diffusion. Except for areas of high mixing, as in mountain streams, or of high photosynthesis, as in the surface layer of eutrophic lakes, concentrations of dissolved oxygen in most natural waters are well below saturation values.

The occurrence of a large number of other microorganisms, which can be easily and repeatedly isolated from natural waters, is not necessarily diagnostic for their aquatic specialization but merely for (1) their ability to survive well under these conditions or (2) a high rate of introduction from other habitats. Obvious examples are the seasonal occurrence of typical soil bacteria in streams and rivers or the common existence of enteric bacteria in waters polluted by domestic sewage.

The limited ability of enteric bacteria, as an extreme example, to compete for survival in natural waters indicates another general characteristic of aquatic habitats. The constant dispersion of dissolved nutrients by diffusion and currents has a dilution effect and leads to generally low concentrations of most of the substrates essential for microbial growth. A central theme of aquatic microbiology is the study of the physiology of growth and metabolism in the presence of low-nutrient concentrations (see section "Low-Nutrient Environments" in this chapter).

Relating specific groups of prokaryotes with specific aquatic macrohabitats such as ponds, lagoons, rivers, or parts of the ocean is of limited value. A number of characteristic traits of these ecosystems, however, are of microbiological interest and are dealt with in the following sections.

Seventy-one percent of the globe is covered by seawater, three-quarters of which lies below a depth of 1,000 m. Comparing the mean depth of the ocean to an arbitrarily assumed thickness of the terrestrial biosphere (depth of live soil and the zone of plant and animal life on and above the surface) at 38 m results in an aquatic/terrestrial volume ratio of 99/1. Productivity, on the other hand, is related to surface area and the availability of light and nutrients. It is limited in the sea to the phototrophic surface layer and is estimated, in spite of the above-mentioned volume ratio, in the same order of magnitude as the terrestrial photosynthetic production.

The ambient concentration of dissolved organic carbon in seawater lies in the range of 0.3–1.5 mg/l (Menzel and Ryther 1970). Particulate organic carbon reaches not more than one-tenth to one-fifth of that concentration. Much of this material, especially in deeper waters, is "refractory" to microbial attack ("Low-Nutrient Environments"). Except for a few nutrient-rich areas of high oxygen consumption by microorganisms, offshore waters are aerobic down to the greatest depths. The only attempt to cover the area of marine microbiology was made by ZoBell in 1946. More recent literature reviews deal with particular problems (see below). Initial efforts in reviewing the systematics of the characteristic indigenous bacterial flora, obligately and facultatively aerobic rods, were carried out by Baumann et al. (1972). Luminescent bacteria, largely restricted to the marine environment, are described as belonging to a new genus of the Enterobacteriaceae, the *Beneckea*, and to the genus *Photobacterium* (Baumann and Baumann 1977; Reichelt and Baumann 1973). The former occur as free-living forms and the latter as free-living and quite distinct symbiotic forms living in specific organs of marine fishes and invertebrates (Greenberg et al. 1979; Hastings and Nealson 1977).

## The Deep Sea

The deep sea comprises the largest volume of seawater characterized by the absence of light, by constant temperature around 2–3 °C, by limited input of organic energy sources from the remote surface water, and by considerable hydrostatic pressure. Because of the continuous increase of the last factor with depth, about 1 atm every 10 m, there is no particular depth at which the start of the deep sea can be defined. Oceanographers distinguish between shelf waters of up to 200 m deep, the continental slope area ranging to a depth of about 3,000 m, and the abyssal plains reaching 6,000 m. The deep trenches may reach depths of up to 11,000 m, but the total area of the world's ocean below 6,000 m covers not more than 1.2 %, and the total volume amounts to only 0.01 % (Sverdrup et al. 1942).

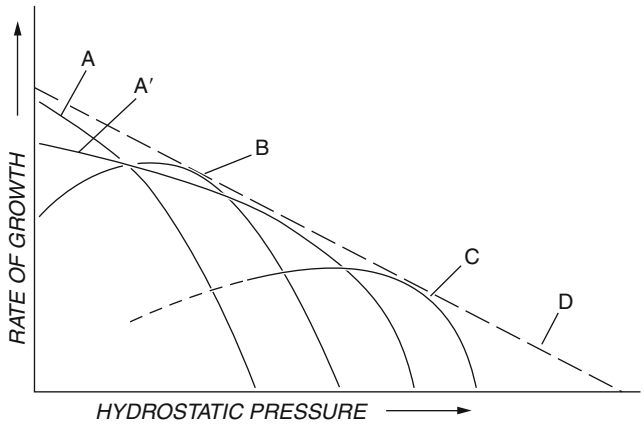



**Fig. 3.4**
**Summarizing scheme of growth responses by marine bacterial isolates to hydrostatic pressure. *A* low barotolerance, *A′* high barotolerance, *B* barophilic, *C* obligate barophilic, *D slope of line* indicates decrease of activity with increasing pressure (Modified from Jannasch and Taylor 1984)**

The remoteness of the deep sea from productive surface waters or from coastal input of organic energy sources causes the sparsity of life in deep waters. Yet a large number of bacteria can be isolated from sediments of almost any depth. The early work in deep-sea microbiology was reviewed by ZoBell (1970) and Morita (1976). Interest in this area was rekindled by experimental work on deep-sea in situ incubation (Jannasch and Wirsen 1973).

The hydrostatic pressure and its possible effect on prokaryote metabolism (Marquis 1976; Marquis and Matsumara 1978) are of special interest. Based on physicochemical considerations, hydrostatic pressure may be assumed to affect metabolic processes only through differential volume changes. Unless dissolution and liberation of gases are involved, in principle, little or no effect can be expected at pressures below 1,000 atm.

A high variability of barotolerance in marine bacteria, mostly documented in terms of growth in various media, has long been known but is not fully understood yet. This is principally also true for "barophilism," a term introduced by ZoBell and Johnson (1949) and today defined as optimal growth response at pressures higher than normal atmospheric pressure. Pure cultures of true barophilic bacteria only first became available at the time Dietz and Yayanos (1978) introduced a technique for their isolation by using pressurized enrichments from decaying deep-sea amphipods in an organic-rich, silica-gel medium. Subsequent isolations from materials collected at depths of 5,800 and 10,500 m yielded organisms that grew optimally at pressures of about 500 and 690 atm (Yayanos et al. 1979, 1981). While some isolates grew at normal atmospheric pressure but at a rate 30-fold lower than at optimum pressure, others did not grow at all after decompression and were called "obligate barophiles." Because of the more striking psychrophilic/barophilic behavior of isolates from greater depths, much of the earlier work concentrated on deep-sea trenches, that is, at depths below 6,000 m, although their seawater volume is only 0.1% of the oceans' total. Thus, the results are more of physiological than of ecological significance.

Since this pioneering work appeared, large numbers of barophilic bacteria have been isolated from various depths (Deming and Colwell 1982; Deming et al. 1981; Yayanos et al. 1982; Jannasch et al. 1982; Jannasch and Wirsen 1982). The data generally indicate a close association between barophilism and psychrophilism, confirming earlier observations (Wirsen and Jannasch 1975) on high barotolerance found primarily, but not exclusively, in psychrophilic isolates. The large variability of barophilism seems to merge gradually with barotolerant growth characteristics.

A direct comparison of all the published data is difficult, since they have been obtained in different growth media. However, the general situation can be schematically depicted as in *Fig. 3.4*, where A and A′ indicate a range of pressure between low and high barotolerance. Barophilic bacteria (B) also exhibit a considerable range of optimal growth pressures and are termed obligate barophiles (C) if they cannot grow at normal atmospheric pressure. The slope indicated by line D is a simplified expression of the overall decrease of microbial activity as a function of increasing pressure with depth. When considering microbial growth in the deep ocean, it should not be forgotten that both the nutrient concentration (mainly the available organic carbon) and the temperature are of predominant importance. It is now technically possible to sample enrich and isolate bacteria from the deep sea in pure culture while maintaining full pressure continuously (Jannasch et al. 1982).

## Hydrothermal Vents

The decreasing cell density observed in the oceans with increasing depth has been explained as the result of the increasing remoteness from the photosynthetically productive surface waters. A striking exception to this general rule was discovered when copious populations of large, sessile invertebrates were found at a depth of 2,700 m clustered around warm-water vents of volcanic origin in the Galapagos Rift area (Ballard 1977). The hydrogen sulfide content of the extruding waters (*Fig. 3.5*) was shown to provide the energy (the electron donor) for the chemosynthetically rather than photosynthetically sustained ecosystem either by free-living or by symbiotic sulfide- and thiosulfate-oxidizing prokaryotes (Jannasch and Wirsen 1979; Grassle 1986; Jannasch 1989). Thus, these deep-sea animal communities are supported by terrestrial rather than solar energy (*Fig. 3.6*), the caveat to this statement being the use of photosynthetically produced oxygen in the pathway for aerobic sulfur oxidation. Although quantitatively of less importance for the production of organic carbon, anaerobic chemosynthesis has also been demonstrated by the isolation of methanogens from various sites at hydrothermal vents (Jones et al. 1989; Huber et al. 1989). Many of the aerobic chemoautotrophic and heterotrophic isolates from the warm and hot hydrothermal vent sites were extremely thermophilic (see above).

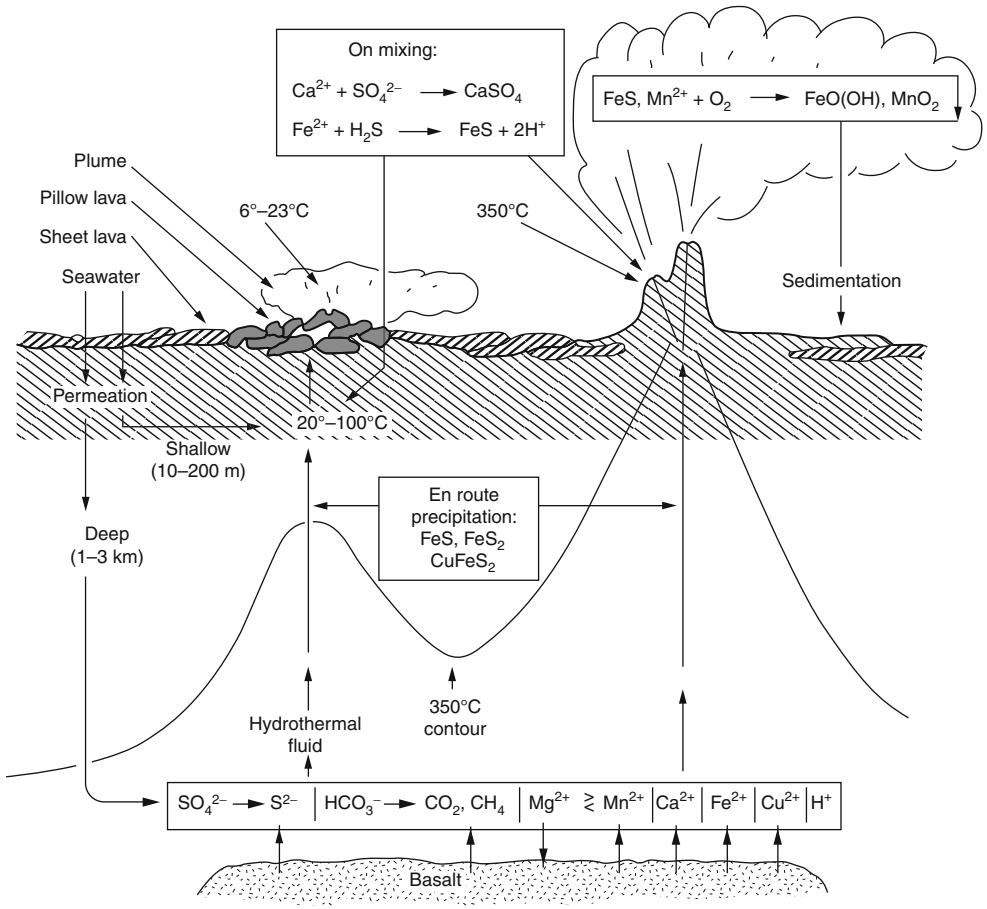



**Fig. 3.5**

**Major geochemical processes occurring within the oceanic crust and on the deep-sea floor. As seawater penetrates several km into the crust, it is heated to 350–400 °C, reacts with basaltic rocks, and leaches various chemical species into solution. The highly reduced "hydrothermal fluid" rises and reaches the seafloor either directly (hot vents) or after mixing with cold, oxygenated seawater before emission (warm vents). On mixing, polymetal sulfides and calcium sulfate (anhydrite) precipitate, either within subsurface lava conduits or as "chimneys" and suspended particulate matter in the "black smokers" (Modified from Jannasch and Mottl 1985)**

## Marine Anoxic Ecosystems

Shallow estuaries are similar to rich freshwater environments, except for frequently and sometimes drastically changing salinities and for the presence of certain characteristic anions and cations. The former is dealt with in the chapters on the habitats of halotolerant and halophilic prokaryotes. Next to chloride, the anion most characteristic for the marine environment is sulfate. In organically rich marine environments, the microbial reduction of sulfate to sulfide initiates the cycling of sulfur compounds with a number of concomitant environmental phenomena: the production of hydrogen sulfide, the precipitation of ferrous (and other heavy metal) sulfide often followed by the formation of a disulfide (e.g., pyrite), the deposition of elementary sulfur, and the rich populations of sulfur-oxidizing prokaryotes at the oxic/anoxic interface. Acidification rarely occurs, due to the high buffer capacity of seawater. Anaerobic marine basins, chemically stabilized by high concentrations of sulfide, range from small estuarine pockets to anoxic water masses on the scale of the Black Sea (see below). The high sodium concentration of seawater has been the key determinant for typically marine bacteria by constituting strict requirements for the cation (McLeod 1968).

The largest anoxic ecosystems in the biosphere are marine. The Black Sea contains no free oxygen from a depth of about 150 m down to the bottom at a 2,000-m depth. In an extensive team effort, the geological, chemical, and biological characteristics have been studied (Degens and Ross 1974). Skopintsev, Karpov, and Vershinina (1959) and Sorokin (1964) have done earlier microbiological work, finding that about 95 % of the sulfide present in the deeper Black Sea water stems from sulfate reduction and that the oxidation of sulfide at the oxic/anoxic interface accounts for the large amounts of dark carbon dioxide fixation (4–6 mg C/$m^3$·day). The auxiliary role of the cycling of sulfur compounds in the turnover of organic and inorganic carbon has been described (Jannasch et al. 1974), and the biomass and total number of microorganisms in the depth of the Black Sea have been measured (Mitskevich 1979).

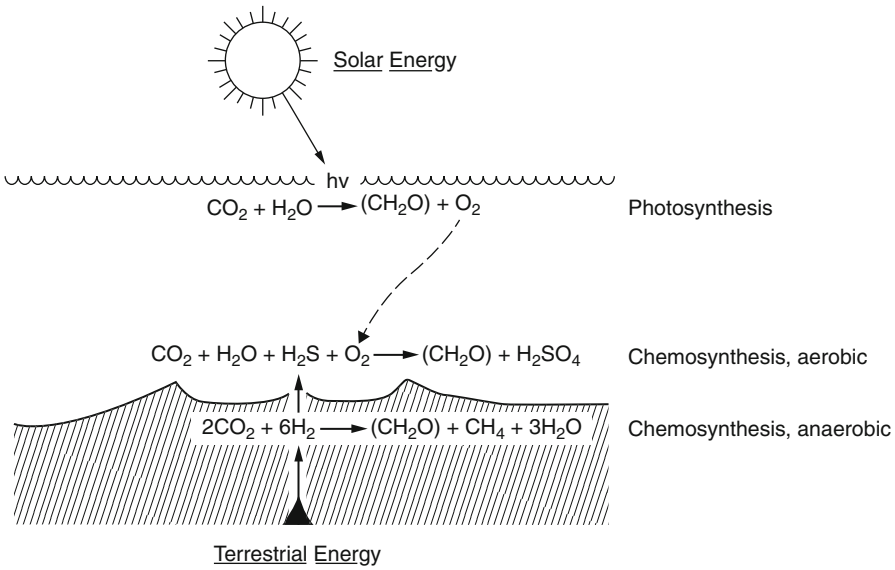



**Fig. 3.6**
**Scheme of the energy supply pathway for photosynthesis and chemosynthesis, showing the role of free oxygen ($O_2$) at deep-sea hydrothermal vents (From Jannasch 1989)**

A series of research cruises on the Black Sea (April–September 1988) has resulted in some unusual oceanographic data. The oxic/anoxic interface, historically at depths between 125 and 175 m in the 2,000-m water column, had risen within the last decade to 80–90 m (*Fig. 3.7*). At the same time, a maximum for bacteriochlorophyll *e* was detected at the same depth where hydrogen sulfide first appears on a downward profile in the water column (Repeta et al. 1989). Such a high concentration of a prokaryotic pigment has never been observed before in the Black Sea, and it exceeded the concentration of the phytoplanktonic chlorophyll *a* in the oxic waters above (*Fig. 3.8*). This first observation of mass bacterial photosynthesis in offshore waters indicates that the rising sulfide level has reached the lower range of the photic zone. Slow-growing isolates from this zone appear to be adapted to low light intensities and are undistinguishable from *Chlorobium phaeobacteroides* (N. Pfennig and H. Cypionka, personal communication). Other organisms isolated from the interface are aerobic, neutrophilic, obligately chemolithotrophic sulfide oxidizers, tentatively placed into the genus *Thiomicrospira*. A number of new sulfide-reducing and methanogenic bacteria were also isolated from the sediment.

The Cariaco Trench off the Venezuelan coast is another permanently anoxic basin of a depth of 1,400 m (Richards 1975; Richards and Vaccaro 1958). Another site is the Orca Basin in the northern Gulf of Mexico, which features an anoxic and saline layer of bottom water at a depth of 2,300 m (Shokes et al. 1977). Trüper (1969) and Watson and Waterbury (1969) investigated anoxic hot brines (56 °C) found at the bottom of the Red Sea and ascribed the absence of readily growing bacteria to toxic concentrations of heavy metals. A unique environment for the discovery of a number of new metabolic types of

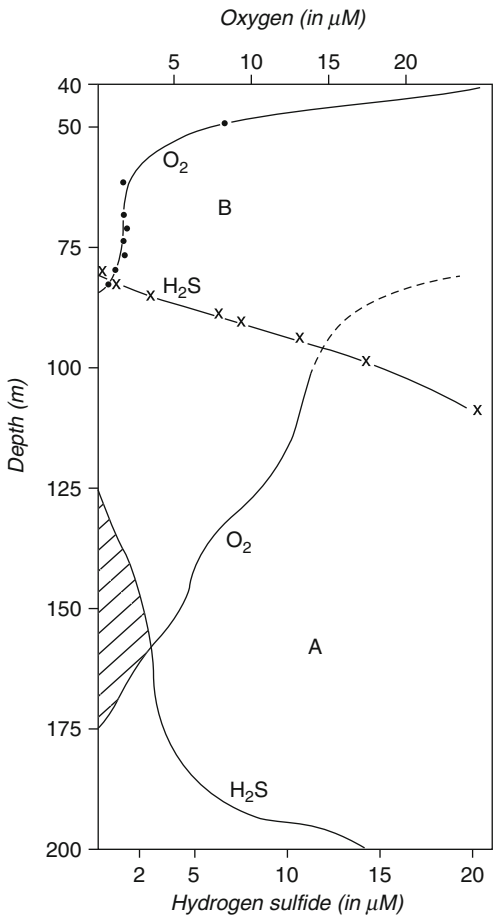



**Fig. 3.7**
**Profiles of dissolved oxygen and hydrogen sulfide in the upper water column of the Black Sea, central western gyre. (*A*, data from Sorokin 1972; *B*, data from Repeta et al. 1989)**

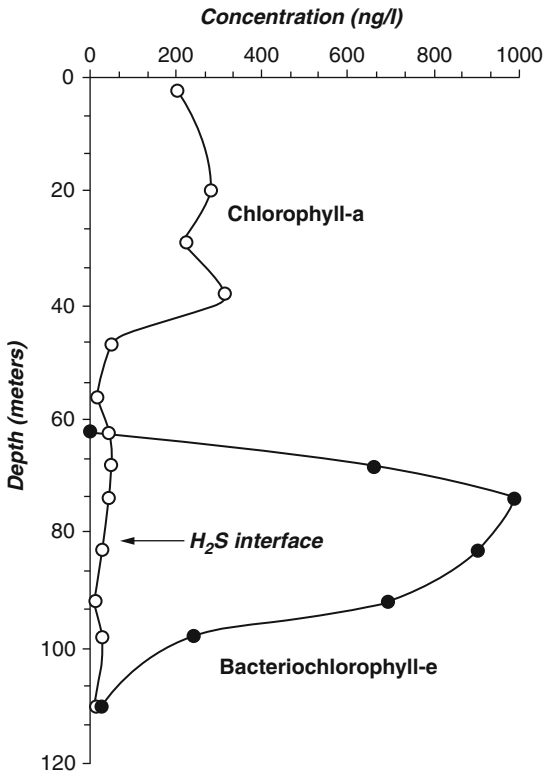



**Fig. 3.8**
**Distribution of phytoplanktonic and bacterial chlorophylls in the upper water column of the Black Sea, central western gyre (Data from Repeta et al. 1989)**

prokaryotes, especially the anaerobically photosynthesizing cyanobacteria, is the Solar Lake near Eilat in the Gulf of Aqaba, which has been described in a series of papers (see Cohen et al. 1977). The relatively low concentration of methane in most of the anoxic marine waters as compared to anoxic freshwaters is in agreement with the competitive behavior of sulfate versus carbon dioxide reduction originally suggested by Cappenberg (1974a, b) and discussed in detail by Rudd and Taylor (1979).

## Eukaryotes as Habitats for Bacteria

Eukaryotes present a multitude of habitats for bacteria. The surfaces, cavities, crevices, and intercellular spaces open to the air, as well as the intestinal tracts, exudates, and excretory substances, offer opportunities for the growth of many bacteria. During evolution, more or less close associations or symbioses between the eukaryotic organisms and bacteria have developed. The symbiotic relationship between the host, the larger partner, and the bacterium is either neutralistic, for example, when bacteria feed on the waste products of the host, or mutualistic. Both of these relationships will be discussed here.

Two kinds of special abilities of the prokaryotes are exploited by the eukaryote and have opened highly specialized habitats: the ability to fix nitrogen and to hydrolyze cellulose.

Nitrogen fixation is exploited by plants. The bacterial symbionts are either held as ectosymbionts in intercellular spaces of leaves, stems, or at the root surface or as endosymbionts in root nodules and rhizothamnia. Animals do not take much advantage of nitrogen-fixing bacteria; nitrogen fixation has only been observed in the hindgut of termites (Benemann 1973; Breznak et al. 1973) and in the gut of humans who eat a carbohydrate-rich diet (Bergensen and Hipsley 1970).

The ability to hydrolyze cellulose is lacking in animals that are higher on the evolutionary scale than the mollusks, with exception of the silverfish, *Lepisma lineata*. Symbiotic relationships between animals and cellulolytic protozoa and bacteria were established because of the abundance of cellulose as food and the general inability of animals to produce cellulolytic enzymes.

Associations of eukaryotic hosts with bacteria have evolutionary aspects. When the plants started to colonize the land and to shape the prerequisites for the evolution of the higher forms of life, the bacteria had already acquired a high degree of biochemical and physiological fitness—presumably the present-day status. Instead of being added to a preexisting system of higher organisms as, for example, plants and animals colonizing the recently emerged island of Surtsey, the bacteria colonized the eukaryotic host during all stages of the host's evolution. The present-day prokaryote–eukaryote relationships must be considered the result of a long selection process. Excluding parasitism, one may hypothesize that in all cases of stable associations, the relationship is of mutual advantage to the partners. The benefit may be as obvious as in the cases of nitrogen fixation in higher plants and of cellulose digestion in animals, or the benefit may just be a protective mechanism of seemingly very little importance, such as preventing harmful bacteria from entering the potential habitat.

## Animal Habitats

Eliminating parasitism from this discussion, only some benign relationships will be considered. In this context, the endosymbiosis of protozoa and bacteria deserves mention. Progress in this area is enormous and has been reviewed in the handbooks edited by Schwemmler and Schenk (1980) and Schenk and Schwemmler (1983). The inner and outer surfaces of animals, the intestinal tract, the skin, and several organs have to be regarded as microbial ecosystems with specific populations of indigenous and nonindigenous bacteria.

### Intestinal Tract

Among the associations of bacteria and animals, the intestinal tract is the ecosystem with the highest population density. The number of bacterial cells within the intestinal tract may even

exceed the number of host cells. With respect to the human gut, this ratio was dramatically described by Savage (1977a): "the normal human organism can be said to be composed of over $10^{14}$ cells, of which only about 10 % are animal cells." The ratio differs from species to species through the animal kingdom.

The intestinal tract is an open ecosystem that resembles a tube receiving food at one end and releasing waste at the other. In omnivores and carnivores, food digestion is accomplished by the animal's own intestinal digestive enzymes without involvement of microbes. There are two modifications of this "straight tube" model. Both types are found especially in herbivores, among the invertebrates as well as the vertebrates. Due to the particular nature of the diet of herbivorous animals, which is rich in celluloses, hemicelluloses, and pectins but is low in protein, the tract has been adapted to accommodate microorganisms. Extensions of the tract function as fermentation vessels and harbor protozoa and bacteria able to convert the plant polymers into microbial cells and degradation products. These fermentation vessels may be located either anteriorly or posteriorly to the areas of the intestinal tract where the gut contents are digested by the enzymes of the animal. In the ruminants, a special compartment of the stomach, the rumen–reticulum, is used as the fermentation vat. Its huge size guarantees a long residence time for the plant material to be degraded by cellulolytic and other microorganisms. Similar vessels destined for foregut fermentation are present in camels, kangaroos, hippopotamuses, and leaf-eating apes. The second modification of the straight tube is represented by extended compartments or blind sacs (ceca) near the end of the intestinal tract. Their function is also that of a fermentation vessel; examples for hindgut fermentation are found among vertebrates (horses, pigs, guinea pigs, rats, rabbits) and insects (termites, wood roaches).

## Human Intestinal Tract

The tract comprises six major areas: mouth, esophagus, stomach, small intestine, cecum, and large intestine. Each may be the habitat of a particular bacterial flora. And these habitats may be subdivided into further categories, such as epithelial (the bacteria grow in association with epithelial surfaces), lumenal (bacteria live free in the lumen), and crystal (bacteria live in crypts). Different areas harbor different bacteria, and each may be the primary habitat for a certain bacterium or a group of bacteria. The habitats are more or less delineated and offer rather constant conditions.

As was recognized very early (see Miller 1890), the mouth, nasopharynx, and throat are inhabited by many aerobic and anaerobic bacteria. *Streptococcus salivarius*, *S. mutans*, *Veillonella alcalescens*, *Treponema dentium*, *Fusobacterium*, *Actinomyces*, lactobacilli, corynebacteria, and cocci belong to the normal flora of the human mouth (Hardie and Bowden 1974). The mouth may be considered as an ecosystem of its own characterized by its anatomical and physiological development, the composition of the saliva, and the succession within the bacterial flora (Russell and Melville 1978). The interaction of the oral streptococci and the mucosal and enamel surfaces deserves special emphasis; special methods have been developed to investigate the specificity of interactions (Rutter and Abbott 1978), which are partially due to the kind and properties of extracellular polysaccharides (Ebisu et al. 1975; Germaine et al. 1974).

The intestinal tract contains climax populations of indigenous as well as allochthonous bacteria, yeasts, and protozoa. Hundreds of different bacteria have been isolated from the intestinal tract of many animals. In many cases, it is difficult to decide whether a certain species is autochthonous or not and which physicochemical niche it fills. Applying modern ecological theory (Alexander 1971) to the ecosystem of the gastrointestinal tract of mammals (Savage 1977a, b), criteria for determining autochthony of microorganisms isolated from the gastrointestinal tract have been developed. "Autochthonous gastrointestinal microorganisms (1) can grow anaerobically, (2) are always found in normal adults, (3) colonize particular areas of the tract, (4) colonize their habitats during succession in infant animals, (5) maintain stable population levels in climax communities in normal adults, and (6) may associate intimately with the mucosal epithelium in the area colonized" (Savage 1977a). These criteria are useful for distinguishing indigenous microorganisms from nonindigenous ones. For detailed information, the excellent reviews on relevant problems and on the present status of knowledge by Savage (1977a, b), Clarke (1977), Bauchop (1977), Costerton et al. (1981a, b, 1987), Costerton and Cheng (1981), Bitton and Marshall (1980), Marshall (1984), and Tannock (1990) should be consulted.

Because of its high acidity, the human stomach is not populated by bacteria. Behind the pylorus, fewer than 10 bacterial cells per milliliter have been counted. From the pylorus via the duodenum and jejunum to the ileum, the number of bacteria increases; up to $10^{11}$ bacteria per gram of feces have been counted; *Escherichia coli* represents less than 1 % of the population. The predominant genus is *Bacteroides*, comprising species such as *B. fragilis*. The Bacteroides are followed by *Fusobacterium*, *Eubacterium*, and *Peptostreptococcus* (Moore and Holdeman 1974), all strictly anaerobic, Gram-negative rods. Other inhabitants belong to the streptococci, lactobacilli, and bifidobacteria (Drasar and Hill 1974; Drasar and Barrow 1985). The dependence of the composition of the flora on the kind of food eaten has not yet been exhaustively studied. The changes caused by the oral application of antibacterial agents like antibiotics and sulfonamides or by special foods such as garlic or cabbage have not been examined with respect to qualitative and quantitative changes of the bacterial flora.

The specific niches of the bacteria in the gastrointestinal tract are not easy to comprehend. The nutritional conditions are certainly optimal for many other bacteria which do not occur there. It is not known whether one or the other special property of intestinal bacteria as discussed by Prins (1977) is responsible for a true autochthony or whether their common occurrence in this habitat is correlated with specific chemical or structural properties.

One could imagine that for lumenal bacteria, high growth rates are required and that bacterial attachment sites and mechanisms favor the colonization of epithelia. The selection of only a few species of bacteria to grow in the gastrointestinal habitats may, among other factors, be due to the tolerance to pH, to lipolytic, peptolytic, and saccharolytic enzymes, to detergent-like bile acids, and to degradation products. Whether certain structural details of the bacterial cell envelope are common to all indigenous inhabitants of the lower intestine is an open question (Costerton et al. 1974, 1981a; Martin 1969).

Basically, in many intestinal tracts, the presence of microorganisms is not necessary. Many animals can grow as gnotobiotes without microorganisms (Coates and Fuller 1977). Studies with such animals have shown that the intestinal microflora confers a kind of resistance to intruding pathogens. While *Vibrio cholerae* and *Shigella dysenteriae* colonize the intestinal tract of germ-free rats easily, they do not colonize the normal gut easily. Stability of the microbial population is a major factor in the gastrointestinal ecosystem.

The use of gnotobiotic animals will help to explore neutralistic, mutualistic, and parasitic relationships of bacteria and their host. However, even simple quantitative and qualitative studies on the populations in intestinal habitats are urgently needed. It was not long ago that *Escherichia coli* was considered the dominant bacterium in the human intestinal tract. With the application to the human gut of methods originally developed for studying the ruminant bacteria, it was found that the number of strict anaerobes exceeded the oxygen-tolerant anaerobes such as *E. coli* by a thousandfold. The routine application of anaerobic techniques to other vertebrate or invertebrate intestinal tracts will certainly result in similar unexpected relationships.

## Rumen and Reticulum

The best-studied ecosystem of foregut fermentation and most probably of all anaerobic ecosystems is the rumen. Rumen symbiosis is an excellent example of a highly developed ecosystem (Hobson 1988). The principles derived from these studies have had profound influence on the study of microbial ecology (Hungate 1966, 1975; Savage 1977a, b).

The main sources of carbohydrates for ruminants are hay, straw, and grass. About 50 % of dried grass consists of fructosans and xylanes, and the rest of the carbohydrate fraction is cellulose. The alimentary tract of ruminants is adapted to this special diet. The first two parts of the bovine stomach, the rumen and the reticulum, serve as a microbial digestive vessel; the total volume is 100–250 l. This ruminoreticulum provides ideal conditions for the growth of many microorganisms. The temperature is constant at 37–39 °C; 100–200 l of saliva are secreted per day; it contains phosphate (10–50 mM), bicarbonate (100–140 mM), and urea (10 mM nitrogen) and is a well-buffered (pH 5.8–7.3) solution. Nutrients are periodically added in the form of well-macerated cellulose-containing fodder; the mixture is mechanically agitated by the contractions of the rumen. The rumen may be considered to be a semicontinuous culture of microorganisms (Hungate 1975).

Between $10^{10}$ and $10^{11}$ bacteria and $10^5$ and $10^6$ protozoa inhabit each milliliter of rumen contents. Most of the bacteria are strict anaerobes and are especially adapted to that habitat. The oxidation–reduction potential amounts to −0.35 V. Because many rumen bacteria are instantly killed by oxygen, special techniques for manipulating nutrient media, inocula, and cultures in the absence of oxygen had to be developed (Holdemann et al. 1977; Hungate 1950). The nitrogen source of most rumen bacteria is ammonia. Many rumen bacteria require a carbon dioxide-rich (10 % $CO_2$) atmosphere (Dehority 1971). *Ruminococcus albus*, *R. flavefaciens*, *Bacteroides succinogenes*, *Butyrivibrio fibrisolvens*, *Eubacterium cellulosolvens*, and *Clostridium lochheadii* are the primary species of cellulose-digesting bacteria of the rumen. The cellobiose and glucose produced from cellulose are fermented by a variety of bacteria which utilize the pectins, starch, fructosans, proteins, and lipids as well. Fermentative degradation of these compounds leads to the accumulation of fatty acids, carbon dioxide, and hydrogen. The latter two gases are combined by *Methanobacterium ruminantium* to give methane.

Besides the bacteria mentioned above, there are many others able to ferment noncellulose carbohydrates or the products of carbohydrate fermentation. These bacteria can only be mentioned here: *Bacteroides amylophilus*, *B. ruminicola*, *Succinimonas amylolytica*, *Selenomonas ruminantium*, *Streptococcus bovis*, *Veillonella alcalescens*, *Lachnospira multiparus*, *Peptostreptococcus elsdenii*, and *Desulfotomaculum ruminis*.

In addition to these bacteria, which have been grown in pure culture, there are some that have been identified microscopically only. Quin's oval and Eadie's oval have been grown in mixed culture (Orpin 1972, 1973). The filamentous bacterium *Oscillospira* has been found mainly in the rumen of sheep. *Lampropedia* is an obligatory aerobe; however, it is found in high numbers in the sheep rumen.

The rumen microflora is in a delicate equilibrium. If, for example, rumen methanogenesis is eliminated by the addition of chloroform to the rumen contents, a series of changes occur: gaseous hydrogen accumulates immediately, and the increased partial pressure of hydrogen results in an increase of the ratio of propionic to acetic acid. These observations indicate that interspecies hydrogen transfer plays an important role in the rumen (Iannotti et al. 1973). Hydrogen is an intermediate in substrate conversions in the rumen (Hungate 1967, 1975).

## Hindgut Fermentation

Although it is not as efficient for the animal as the rumen-type fermentation, hindgut fermentation is widely distributed among herbivorous animals, vertebrates as well as insects. The fermentative organisms and their function in the blind sacs (ceca) and the large intestine of animals have been less well studied than those involved in rumen symbiosis (McBee 1977). The majority

of studies on hindgut fermentations were limited to anatomical–morphological descriptions, and preserved specimens of insects have been used to recognize or describe microorganisms. Recent investigations show that the isolation and cultivation of bacteria so far only recognized in cross sections of cecal mucosa epithelia is possible, although with difficulties (Lee and Phillips 1978). The comprehensive investigations of Buchner (1953) provide an excellent basis and incentive for studies on the axenic culture of protozoa and bacteria present in the insect gut and its appendices. Investigations on the protozoal and bacterial symbionts probably involved in the degradation of plant polymers or having other important functions are urgently needed (Eutick et al. 1978; see Foglesong et al. 1975; McBee 1977). Investigations on the isolation and characterization of heterotrophic bacteria from hindguts of the wood-eating termite, *Reticulitermes flavipes*, revealed the predominance of streptococci and *Bacteroides* species and indicate the existence of a unidirectional food chain from glucose via lactate to propionate and acetate (Schultz and Breznak 1978, 1979).

### Bacteria of the Human Skin

The human skin is a rather homogeneous habitat with respect to temperature. However, moisture conditions vary and create distinctive habitats for a characteristic flora. The available water is the most important environmental factor influencing the size of cutaneous populations. The occlusion of the relatively dry skin of the forearm results in a rapid increase of the bacterial population by a factor of $10^4$ (from an initial colony count of $3 \times 10^3$ to $3.8 \times 10^7$ cells/cm$^2$) by the fourth day of occlusion. The high relative humidity of the axilla results in the survival of dense populations in this region (Marples 1965, 1974, 1976; Noble and Somerville 1974; Woodroffe and Shaw 1974). Many common bacteria, such as the corynebacteria ("diphtheroids"), mycobacteria, micrococci, and streptococci, in addition to yeasts and other fungi, are inhabitants of the human skin. Nutrients are provided by sweat. Fatty acids may act as antibacterial agents and effect a counterselection. The bacteria inhabiting the sweat glands (sebaceous glands) and hair follicles cannot be reached by normal cleaning of the skin and guarantee fast repopulation of the skin after cleaning (Noble and Pitcher 1979; Rosebury 1972).

## Molecular Microbial Ecology

Although the sequencing and hybridization of ribosomal RNAs primarily concern the phylogenetic characteristics of organisms (see Chap. 1, "How We Do, Don't and Should Look at Bacteria and Bacteriology" in Vol. 1 and Woese 1987), first results show that a search for uses of this technique in microbial ecology is well justified (Pace et al. 1986; Giovannoni et al. 1988; DeLong et al. 1988). The advantage of identifying microorganisms within complex natural populations by the use of their individual ribosomal RNA signatures is that the often difficult procedures for isolation, cultivation, and purification become less critical. The disadvantage is that specifically expressed metabolic activities of the phylogenetically identified organisms or their ecological functions in situ or in vitro are not readily apparent from the data. After Torsvick and Goksoyr (1978) pioneered a method for DNA extraction from whole natural populations, hybridization probes were applied to the study of the microbial population in the rumen (Stahl et al. 1988), in soil (Holben et al. 1988), and of communities of marine planktonic microorganisms (Giovannoni et al. 1990), of microbial hot springs (Stahl et al. 1985), and of symbiotic prokaryotes (Distel et al. 1988; Unterman et al. 1989). Much of the present work continues to emphasize methodology (Somerville et al. 1989; Weller and Ward 1989), especially the development of gene probes for individual species or genera and other methods to assess detection and abundance of microbes at specific habitats or within natural populations.

## Outlook

Although the habitat is not specified in the description of a species, it is a major characteristic of a species. Microorganisms have their habitats in various ecosystems and microenvironments. Unfortunately, a discussion of the environments of microorganisms can never be complete. The purpose of the present chapter was to demonstrate the multiplicity of microbial environments with emphasis on the close relationship to the diversity of prokaryotes, their metabolic peculiarities, potential capabilities, and constraints. Obviously, more examples could have been selected, and more will be found in the literature every month. The discoveries of new morphological and metabolic types of prokaryotes, as well as of unsuspected relationships and interactions between microorganisms and their environments, over the last decades can be related, in most cases, to the study of microbial ecosystems which had received no particular attention before. The rumen and its inhabitants have been discussed as one of the most striking examples of this; the illuminated hydrogen sulfide–oxygen interfaces of lakes and lagoons are others. Highly developed symbiotic relationships between bacteria and metazoa in the luminous organs and in the hindguts of mammals and insects as well as between bacteria and protozoa have only been marginally mentioned. The ecosystems consisting of solid matter, such as the various kinds of fertile soils, virgin volcanic soils, and desert soils as well as the rhizospheres of plants, have not been dealt with at all in this chapter.

New artificial microbial ecosystems are arising in increasing numbers in, for instance, the cooling systems of nuclear power plants and the aquatic and terrestrial dumping sites of new, chemically synthesized organic compounds, including herbicides, pesticides, and widely used organic solvents. Special evolutionary pressure is being exerted on prokaryotes by the ever-changing fungicidal and bactericidal environments of hospitals. Microbial populations will also be the first to respond to global environmental changes such as those

following the thinning of the ozone layer and the greenhouse effect. One may also be realistic enough to include here the exposure of this planet's microorganisms to extraterrestrial conditions some of which, for example, certain layers in the atmosphere of Venus, may indeed sustain life of some known chemolithotrophic bacteria. In any case, it can be expected that studies of prokaryotes in relation to their natural environments will remain a most exciting and productive area of microbiology.

## References


Aaronson S (1970) Experimental microbial ecology. Academic, New York

Acher AJ, Juven BJ (1977) Destruction of coliforms in water and sewage by dye-sensitized photooxidation. Appl Environ Microbiol 33:1019–1022

Adler J (1974) Chemoreception and chemotaxis in bacteria. In: Jaenicke L (ed) Biochemistry of sensory functions. Springer, Berlin

Adler J (1988) Chemotaxis: old and new. Botanica Acta 101:93–100

Ahrens R, Moll G, Rheinheimer G (1968) Die Rolle der Fimbrien bei der eigenartigen Sternbildung von *Agrobacterium luteum*. Archiv für Mikrobiologie 63:321–330

Akin DE (1976) Ultrastructure of rumen bacterial attachment to forage cell walls. Appl Environ Microbiol 31:562–568

Akin DE, Amos WE (1975) Rumen bacterial degradation of forage cell walls investigated by electron microscopy. Appl Microbiol 29:692–701

Alexander M (1971) Microbial ecology. Wiley, New YorK

Alexander M (1976) Natural selection and the ecology of microbial adaption in a biosphere. In: Heinrich MR (ed) Extreme environments. Mechanisms of microbial adaption. Academic, New York, pp 3–25

Alexander M (1977) Introduction to soil microbiology, 2nd edn. Wiley, New York

Anwar M, Khan TH, Prebble J, Zagalski PF (1977) Membrane-bound carotenoid in *Micrococcus luteus* protects naphthoquinone from photodynamic action. Nature 270:538–540

Aragno M (1978) Enrichment, isolation and preliminary characterization of a thermophilic, endospore-forming hydrogen bacterium. FEMS Microbiol Lett 3:13–15

Atlas RM, Bartha R (1987) Microbial ecology: fundamentals and applications, 2nd edn. CA The Benjamin/Cummings, Menlo-Park

Babenzien H-D (1965) Über Vorkommen und Kultur von *Nevskia ramosa* Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 1, Suppl 1:111–116

Babenzien H-D (1967) Zur Biologie von Nevskia ramosa. Zeitschrift für Allgemeine Mikrobiologie 7:89–96

Ballard RD (1977) Notes on a major oceanographic find. Oceanus 20:35–44

Barber RT (1968) Dissolved organic carbon from deep waters resists microbial oxidation. Nature 220:274–275

Baross JA, Deming JW (1983) Growth of black smoker bacteria at temperatures of at least 250 °C. Nature 303:423–426

Baross JA, Morita RY (1978) Microbial life at low temperatures: ecological aspects. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, London, pp 9–71

Bartha R, Atlas RM (1977) The microbiology of aquatic oil spills. Adv Appl Microbiol 22:225–266

Bartnicki-Garcia S, Nickerson WJ (1962) Nutrition, growth and morphogenesis of *Mucor rouxii*. J Bacteriol 84:841–858

Bauchop T (1971) Mechanism of hydrogen formation in *Tritrichomonas foetus*. J Gen Microbiol 68:27–33

Bauchop T (1977) Foregut fermentation. In: Clarke RTJ, Bauchop T (eds) Microbial ecology of the gut. Academic, London, pp 223–310

Bauld J, Brock TD (1973) Ecological studies of *Chloroflexus*, a gliding photosynthetic bacterium. Archiv für Mikrobiologie 92:267–284

Baumann P, Baumann L (1977) Biology of the marine enterobacteria: genera Beneckea and Photobacterium. Ann Rev Microbiol 31:39–61

Baumann L, Baumann P, Mandel M, Allen RD (1972) Taxonomy of aerobic marine bacteria. J Bacteriol 110:402–429

Bavendamm W (1924) Die farblosen und roten Schwefelbakterien des Süss-und Salzwassers Pflanzenforschung 2:1–156

Bayley ST, Morton RA (1978) Recent developments in the molecular biology of extremely halophilic bacteria. CRC Crit Rev Microbiol 6:151–205

Bayley ST, Morton RA (1979) Biochemical evolution of halobacteria. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 109–124

Bazylinski DA, Frankel RB, Jannasch HW (1988) Anaerobic magnetite production by a marine, magnetotactic bacterium. Nature 334:518–519

Beijerinck MW (1895) Über *Spirillum desulfuricans* als Ursache von Sulfatreduktion Zentralblatt für Bakteriologie, Parasitenkunde Infektionskrankheiten und Hygiene, Abt. 2, 1:1–9

Beijerinck MW (1921–1940) Verzammelde Geschriften Nijhoff, Den Haag, pp 1–6

Belkin S, Wirsen CO, Jannasch HW (1986a) A new sulfur-reducing, extremely thermophilic eubacterium from a submarine thermal vent. Appl Environ Microbiol 51:1180–1185

Belkin S, Nelson DC, Jannasch HW (1986b) Symbiotic assimilation of $CO_2$ in two hydrothermal vent animals, the mussel *Bathymodiolus thermophilus* and the tube worm *Riftia pachyptila*. Biol Bull 170:110–121

Benemann JR (1973) Nitrogen fixation in termites. Science 181:164–165

Bennett AF (1978) Activity metabolism of the lower vertebrates. Ann Rev Physiol 40:447–469

Berg B, van Hofsten B, Pettersson G (1972) Electron microscopic observations on the degradation of cellulose fibers by *Cellvibrio fulvus* and *Sporocytophaga myxococcoides*. J Appl Bacteriol 35:215–219

Bergensen FJ, Hipsley EH (1970) The presence of $N_2$-fixing bacteria in the intestine of man and animals. J Gen Microbiol 60:61–65

Berkeley RCW, Lynch JM, Melling J, Rutter PR, Vincent B (1980) Microbial adhesion to surfaces. Ellis Horwood, Chichester

Berndt H, Ostwal K-P, Lalucat J, Schumann CH, Mayer F, Schlegel HG (1976) Identification and physiological characterization of the nitrogen fixing bacterium *Corynebacterium autotrophicum* GZ 29. Arch Microbiol 108:17–26

Bezdek HG, Carlucci AF (1972) Surface concentration of marine bacteria. Limnol Oceanogr 17:566–569

Bhuiya ZH, Walker N (1977) Autotrophic nitrifying bacteria in acid tea soils from Bangladesh and Sri Lanka. J Appl Bacteriol 42:253–257

Biebl H, Pfennig N (1978) Growth yield of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. Arch Microbiol 117:9–16

Bitton G, Marshall KC (1980) Adsorption of microorganisms to surfaces. Wiley, New York

Blakemore RP (1975) Magnetotactic bacteria. Science 190:377–379

Blakemore RP, Maratea D, Wolfe RS (1979) Isolation and pure culture of a freshwater magnetic spirillum in chemically defined medium. J Bacteriol 140:720–729

Blakemore RP, Frankel RB, Kalmijn AJ (1980) South-seeking magnetotactic bacteria in the Southern Hemisphere. Nature 286:384–385

Bland JA, Staley JT (1978) Observations on the biology of Thiothrix. Arch Microbiol 117:79–87

Blumershine RV, Savage DC (1978) Filamentous microbes indigenous to the murine small bowel: a scanning electron microscopic study of their morphology and attachment to the epithelium. Microb Ecol 4:95–103

Bokor R (1933) Die Mikrobiologie der Szik-(Salz-oder Alkali-) Böden mit besonderer Berücksichtigung ihrer Fruchtbarmachung. In: Fehäer D (ed) Untersuchungen über die Mikrobiologie des Waldbodens. Springer, Berlin, pp 221–258

Bothe H, De Bruijn FJ, Newton WE (eds) (1988) Nitrogen fixation: hundred years after. Gustav-Fischer-Verlag, Stuttgart

Bousfield IJ, MacKenzie AR (1976) Inactivation of bacteria by freeze-drying. Soc Appl Bacteriol Symp Ser 5:329–344

Boyd SA, Shelton DR, Berry D, Tiedje JM (1983) Anaerobic biodegradation of phenolic compounds in digested sludge. Appl Environ Microbiol 46:50–54

Boyer EV, Ingle MB, Merver GD (1973) *Bacillus alcalophilus* subsp. *halodurans* subsp. nov.: an alkaline-amylase-producing, alkalophilic organism. Int J Syst Bacteriol 23:238–242

Boylen CW (1973) Survival of Arthrobacter crystallopoietes during prolonged periods of extreme desiccation. J Bacteriol 113:33–37

Breznak JA, Brill WJ, Mertins JW, Coppel HC (1973) Nitrogen fixation in termites. Nature 244:577–579

Brierley CL (1977) Thermophilic microorganisms in extraction of metals from ores. In: Underkofler LA (ed) Developments in industrial microbiology. Proceedings of the 33rd general meeting of the society for industrial microbiology, vol 18. Washington American Institute of Biological Sciences, Washington, DC, pp 273–284

Brierley CL (1978a) Bacterial leaching. CRC Crit Rev Microbiol 6:207–262

Brierley JA (1978b) Thermophilic iron-oxidizing bacteria found in copper leaching dumps. Appl Environ Microbiol 36:523–525

Brierley CL, Brierley JA (1973) A chemoautotrophic and thermophilic microorganism isolated from an acid hot spring. Can J Microbiol 19:183–188

Brierley JA, Lockwood SJ (1977) The occurrence of thermophilic iron-oxidizing bacteria in a copper leaching system. FEMS Microbiol Lett 2:163–165

Briston J, Courtois D, Denis F (1974) Microbiological study of a hypersaline lake in French Somaliland. Appl Microbiol 27:819–822

Brock TD (1967) Life at high temperatures. Science 158:1012–1019

Brock TD (1969) Microbial growth under extreme conditions. Soc Gen Microbiol Symp 19:15–41

Brock TD (1970) High temperature systems. Ann Rev Ecol Syst 1:191–220

Brock TD (1978) Thermophilic microorganisms and life at high temperatures. Springer, New York

Brock TD (1979) Ecology of saline lakes. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 39–47

Brock TD (ed) (1986) Thermophiles: general, molecular and applied microbiology. Wiley, New York

Brock TD (1987) The study of microorganisms in situ: progress and problems. In: Fletscher M, Gray TRG, Jones JG (eds) Ecology of microbial communities. Cambridge University Press, Cambridge, pp 1–17

Brock TD, Boylen KL (1973) Presence of thermophilic bacteria in laundry and domestic hot-water heaters. Appl Microbiol 25:72–76

Brock TD, Freeze H (1969) *Thermus aquaticus* gen. n. and sp. n., a non-sporulating extreme thermophile. J Bacteriol 98:289–297

Brock TD, Brock ML, Bott TL, Edwards MR (1971) Microbial life at 90 °C: The sulfur bacteria of Boulder Spring. J Bacteriol 107:303–314

Brock TD, Brock KM, Belly RT, Weiss RL (1972) Sulfolobus: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Archiv für Mikrobiologie 84:54–68

Brown AD (1978) Microbial water stress. Bacteriol Rev 40:803–846

Bryant MP, Wolin EA, Wolin MJ, Wolfe RS (1967) *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Archiv für Mikrobiologie 59:20–31

Bryant MP, Campbell LL, Reddy CA, Crabill MR (1977) Growth of *Desulfovibrio* in lactate or ethanol media low in sulfate in association with $H_2$-utilizing methanogenic bacteria. Appl Environ Microbiol 33:1162–1169

Buchner P (1953) Endosymbiose der Tiere mit pflanzlichen Mikroorganismen. Birkhäuser Verlag, Basel

Buder J (1919) Zur Biologie des Bacteriopurpurins und der Purpurbakterien. Jahrbücher der Wissenschaftlichen Botanik 58:525–628

Burggraf S, Jannasch HW, Nicolaus B, Stetter KO (1990) *Archaeoglobus profundus* sp. nov., represents a new species of within the sulfate-reducing archaebacteria. Syst Appl Microbiol 10:24–28

Butlin KR, Postgate JR (1954) The microbiological formation of sulphur in Cyrenaican lakes. In: Cloudsley-Thompson JL (ed) Biology of deserts. Institute of Biology, London, pp 112–122

Cagle GD (1975) Fine structure and distribution of extracellular polymer surrounding selected aerobic bacteria. Can J Microbiol 21:395–408

Cappenberg TH-E (1974a) Interrelations between sulfate-reducing and methane-producing bacteria in bottom deposits of a fresh-water lake I. Field of observations. Antonie van Leeuwenhoek J Microbiol Serol 40:285–295

Cappenberg TH-E (1974b) Interrelations between sulfate-reducing and methane-producing bacteria in bottom deposits of a fresh-water lake. II. Inhibition experiments. Antonie van Leeuwenhoek J Microbiol Serol 40:297–306

Castenholz RW (1969) Thermophilic blue-green algae and the thermal environment. Bacteriol Rev 33:476–504

Castenholz RW (1976) The effect of sulfide on the blue-green algae of hot springs. I. New Zealand and Iceland. J Phycol 12:54–68

Castenholz RW (1977) The effect of sulfide on the blue-green algae of hot springs. II. Yellowstone National Park. Microb Ecol 3:79–105

Castenholz RW (1979) Evolution and ecology of thermophilic microorganisms. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 373–392

Cavanaugh CM, Gardiner SL, Jones MLS, Jannasch HW, Waterbury JB (1981) Prokaryotic cells in the hydrothermal vent tube worm. Riftia pachyptila: possible chemoautotrophic symbionts. Science 213:340–342

Characklis WG, Marshall KC (1990) Biofilms. Wiley Interscience, New York

Chen M, Wolin MJ (1977) Influence of $CH_4$ production by *Methanobacterium ruminantium* on the fermentation of glucose and lactate by *Selenomonas ruminantium*. Appl Environ Microbiol 34:756–759

Chet I, Mitchell R (1976) Ecological aspects of microbial chemotactic behavior. Ann Rev Microbiol 30:221–239

Childress JJ, Fisher CR, Brook JM, Kennicutt MC II, Bridigare R, Anderson AE (1986) A methanotrophic marine molluscan (Bivalvia, Mytilidae) symbiosis: mussels fueled by gas. Science 233:1306–1308

Chislett ME, Kushner DJ (1961) A strain of Bacillus circulans capable of growing under highly alkaline conditions. J Gen Microbiol 24:187–190

Clark FE (1967) Bacteria in soil. In: Burges A, Raw F (eds) Soil biology. Academic, London, pp 15–49

Clark AE, Walsby AE (1978a) The occurrence of gas-vacuolate bacteria in lakes. Arch Microbiol 118:223–228

Clark AE, Walsby AE (1978b) The development and vertical distribution of populations of gas-vacuolate bacteria in a eutrophic, monomictic lake. Arch Microbiol 118:229–233

Clarke RTJ (1977) The gut and its micro-organisms. In: Clarke RTJ, Bauchop T (eds) Microbial ecology of the gut. Academic, London, pp 35–71

Coates ME, Fuller R (1977) The gnotobiotic animal in the study of gut microbiology. In: Clark RTJ, Bauchop T (eds) Microbial ecology of the gut. Academic, London, pp 311–346

Cohen Y, Rosenberg E (1989) Microbial mats: physiological ecology of benthic microbial communities. American Society for Microbiology, Washington, DC

Cohen Y, Padan E, Shilo M (1975) Facultative anoxygenic photosynthesis in the cyanobacterium *Oscillatoria limnetica*. J Bacteriol 123:855–861

Cohen Y, Krumbein WE, Goldberg M, Shilo M (1977) Solar lake (Sinai). I. Physical and chemical limnology. Limnol Oceanogr 22:597–608

Cohen-Bazire G, Stainer RY (1958) Inhibition of carotenoid synthesis in photosynthetic bacteria. Nature 181:250–252

Cohen-Bazire G, Kunisawa R, Pfennig N (1969) Comparative study of the structure of gas vacuoles. J Bacteriol 100:1049–1061

Cohn F (1881) Gutachten über die Abwässer verschiedener Zuckerfabriken im Winter 1881 Quoted from Kolkwitz 1906

Colmer AR, Temple KL, Hinkle ME (1950) An iron-oxidizing bacterium from the drainage of some bituminous coal mines. J Bacteriol 59:317–328

Cornax R, Morinigo MA, Romero P, Borrego JJ (1990) Survival of pathogenic microorganisms in seawater. Curr Microbiology 220:293–298

Costerton JW, Cheng KJ (1981) The Bacterial glycocalyx in nature and disease. Ann Rev Microbiol 35:299–324

Costerton JW, Ingram JM, Cheng K-J (1974) Structure and function of the cell envelope of Gram-negative bacteria. Bacteriol Rev 38:87–110

Costerton JW, Geesey GG, Cheng K-J (1978) How bacteria stick. Sci Am 238:86–95

Costerton JW, Irvin RT, Cheng KJ (1981) The role of bacterial surface structures in pathogenesis. Crit Rev Microbiol 8:303–338

Costerton JW, Marrie TJ, Cheng KJ (1985) Phenomena of bacterial adhesion. In: Savage DC, Fletcher M (eds) Bacterium adhesion. Plenum, New York, pp 3–43

Costerton JW, Cheng K-J, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ (1987) Bacterial biofilms in nature and disease. Ann Rev Microbiol 41:435–464

Cross T (1968) Thermophilic actinomycetes. J Appl Bacteriol 31:36–53

Csonka LN (1989) Physiological and genetic responses of bacteria to osmotic stress. Microbiol Rev 53:121–147

Cundell AM, Sleeter TD, Mitchell R (1977) Microbial populations associated with the surface of the brown alga *Ascophyllum nodosum*. Microb Ecol 4:81–91
Dazzo FB, Yanke WE, Brill WJ (1978) Trifoliin: a RHIZOBIUM recognition protein from white clover. Biochim Biophys Acta 539:276–286
De Bont JAM, Mulder EG (1974) Nitrogen fixation and co-oxidization of ethylene by a methane-utilizing bacterium. J Gen Microbiol 83:113–121
Degens ET, Ross DA (1974) The Black Sea: geology, chemistry and biology Memoir 20. American Association of Petroleum, Tulsa
Dehority BA (1971) Carbon dioxide requirement of various species of rumen bacteria. J Bacteriol 105:70–76
DeLong EF, Wickham GS, Pace NR (1988) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243:1360–1363
Deming JW, Colwell RR (1982) Barophilic bacteria associated with digestive tracts of abyssal holothurians. Appl Environ Microbiol 44:1222–1230
Deming JW, Tabor PS, Colwell RR (1981) Barophilic growth from intestinal tracts of deep-sea invertebrates. Microb Ecol 7:85–94
Demoll R, Liebmann H (1952) Über die Verteilung von Sphaerotilus natans im Fluss. Schweizerische Zeitschrift für Hydrologie 14:289–297
Dietz AS, Yayanos AA (1978) Silica gel for isolating and studying bacteria under hydrostatic pressure. Appl Environ Microbiol 36:966–968
Distel DL, Lane DJ, Olsen GJ, Giovannoni SJ, Pace B, Pace NR, Stahl DA, Felbeck H (1988) Sulfur-oxidizing bacterial endosymbionts: analysis of phylogeny and specificity by 16S rRNA sequences. J Bacteriol 170:2506–2510
Dondero NC (1961) *Sphaerotilus*, its nature and economic significance. Adv Appl Microbiol 3:77–107
Dondero NC (1975) The Sphaerotilus-Leptothrix group. Ann Rev Microbiol 29:407–465
Drasar BS, Barrow PA (1985) Intestinal microbiology (Aspects of Microbiology 10). van Nostrand Reinholf, England
Drasar BS, Hill MJ (1974) Human intestinal flora. Academic, London
Duckworth RB (1975) Water relations in foods. Proceedings of an international symposium in Glasgow, Sept 1974. Academic, London
Duda VI, Makaer'eva DE (1977) Morphogenesis and function of gas caps on spores of anaerobic bacteria of the genus *Clostridium*. Mikrobiologiya 46:689–694, In Russian, with English summary
Dugan PR, MacMillan CB, Pfister RM (1970) Aerobic heterotrophic bacteria indigenous to pH 2.8 acid mine water: microscopic examination of acid streamers. J Bacteriol 101:973–981
Dundas IED (1977) Physiology of Halobacteriaceae. Adv Microb Physiol 15:85–120
Dundas ID, Larsen H (1962) The physiological role of the carotenoid pigments of *Halobacterium salinarium*. Archiv für Mikrobiologie 44:233–239
Ebisu S, Kato K, Kotani S, Misaki A (1975) Structural differences in fructans elaborated by *Streptococcus mutans* and *S. salivarius*. J Biochem 78:879–887
Ellwood DC, Hedger JN, Latham MH, Lynch JM, Slater JH (1980) Contemporary microbial ecology. Academic, London
Ensign JC, Wolfe RS (1964) Nutritional control of morphogenesis in *Arthrobacter crystallopoietes*. J Bacteriol 87:924–932
Eutick ML, O'Brien RW, Slaytor M (1978) Bacteria from the gut of Australian termites. Appl Environ Microbiol 35:823–828
Fairbairn D (1970) Biochemical adaptation and loss of genetic capacity in helminth parasites. Biol Rev 45:29–72
Felbeck H, Childress JJ, Somero GN (1981) Calvin-Benson cycle and sulphide oxidation enzymes in -animals from sulphiderich habitats. Nature 293:291–293
Fenchel TM (1969) The ecology of marine microbenthos. IV. Structure and function of the benthic ecosystem. Ophelia 6:1–182
Fenchel TM, Jørgensen BB (1977) Detritus food chains in aquatic ecosystems: the role of bacteria. Adv Microb Ecol 1:1–58
Fenchel TM, Riedl RJ (1970) The sulfide system: a new biotic community underneath the oxidized layer of marine sand bottoms. Mar Biol 7:255–268
Fenchel TM, Staarup BJ (1971) Vertical distribution of photosynthetic pigments and the penetration of light in marine sediments. Oikos 22:172–182
Ferry JG, Wolfe RS (1976) Anaerobic degradation of benzoate to methane by a microbial consortium. Arch Microbiol 107:33–40
Fiala G, Stetter KO, Jannasch HW, Langworthy TA, Madon J (1986) *Staphylothermus marinus* sp. nov. represents a novel genus of extremely thermophilic submarine heterotrophic archaebacteria growing up to 98 °C. Syst Appl Microbiol 8:106–113
Fisher CR, Childress JJ, Minnich E (1989) Autotrophic carbon fixation by the chemoautotrophic symbionts of *Riftia pachyptila*. Biol Bull 177:372–385
Fletcher M, Loeb GI (1979) Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Appl Environ Microbiol 37:67–72
Fletcher M, Marshall KC (1982) Are solid surfaces of ecological significance to aquatic bacteria? In: Marshall KC (ed) Advances in microbial ecology. Plenum, New York
Fliermans CB, Brock TD (1972) Ecology of sulfur-oxidizing bacteria in hot acid soils. J Bacteriol 111:343–350
Focht DD, Verstraete W (1977) Biochemical ecology of nitrification and denitrification. Adv Microb Ecol 1:135–214
Foglesong MA, Walker DH Jr, Puffer JS, Markovetz AJ (1975) Ultrastructural morphology of some prokaryotic microorganisms associated with the hindgut of cockroaches. J Bacteriol 123:336–345
Foster JW (1949) Chemical activities of fungi. Academic, New York
Frankel RB, Blakemore RP, Wolfe RS (1979) Magnetite in freshwater magnetotactic bacteria. Science 203:1355–1356
Fridovich I (1974) Superoxide dismutases. Adv Enzymol 41:35–97
Fridovich I (1975) Oxygen: boon and bane. Am Sci 63:54–59
Fridovich I (1976) Oxygen radicals, hydrogen peroxide, and oxygen toxicity. In: Pryor WA (ed) Free radicals in biology, vol 1. Academic, New York, pp 239–277
Gerber NN (1975) Prodigiosin-like pigments. CRC Crit Rev Microbiol 3:469–485
Germaine GR, Chludzinski AM, Schachtele CF (1974) Streptococcus mutans dextransucrase: requirement for primer dextran. J Bacteriol 120:287–294
Gillespy TG, Thorpe RH (1968) Occurrence and significance of thermophiles in canned foods. J Appl Bacteriol 31:59–65
Giovannoni SJ, DeLong EF, Olsen GJ, Pace NR (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J Bacteriol 170:720–726
Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345(6270):60–63
Golovacheva RS (1976) Thermophilic nitrifying bacteria from hot springs. Mikrobiologiya 45:377–379, In Russian, with English summary
Golovacheva RS (1979) Attachment of *Sulfobacillus thermosulfidooxidans* cells to the surface of sulfide minerals. Mikrobiologiya 48:528–533, In Russian, with English summary
Golovacheva RS, Karavaiko GI (1978) *Sulfobacillus*, a new genus of thermophilic sporeforming bacteria. Mikrobiologiya 47:815–822, In Russian, with English summary
Gorini L (1960) Antagonism between substrate and repressor in controlling the formation of a biosynthetic enzyme. Proc Nat Acad Sci USA 46:682–690
Gorlenko WM, Dubinina GA, Kuznezow SJ (1977) Ecology of aquatic microorganisms. Nauka, Moscow
Goto E, Kodama T, Minoda Y (1977) Isolation and culture conditions of thermophilic hydrogen bacteria. Agric Biol Chem 41:685–690
Gottlieb SF (1971) Effect of hyperbaric oxygen on microorganisms. Ann Rev Microbiol 25:111–152
Grant WD, Mills AA, Schofield AK (1979) An alkalophilic species of Ectothiorhodospira from a Kenyan soda lake. J Gen Microbiol 110:137–142
Grassle JF (1986) The ecology of deep-sea hydrothermal vent communities. Adv Mar Biol Ecol 23:301–362
Greenberg EP, Hastings JW, Ulitzur S (1979) Induction of luciferase synthesis in Beneckea harveyi by other marine bacteria. Arch Microbiol 120:87–91
Griffin DM (1981) Water and microbial stress. Adv Microb Ecol 5:91–136
Griffin DM, Luard EJ (1979) Water stress and microbial ecology. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 49–63
Gromet-Elhanan Z (1977) Electron transport and photophosphorylation in photosynthetic bacteria. In: Trebst A, Avron M (eds) Encyclopaedia of plant physiology, vol 5. Springer, Berlin, pp 637–662
Gunner HB, Alexander M (1964) Anaerobic growth of *Fusarium oxysporum*. J Bacteriol 87:1309–1316

Hansen MH, Ingvorsen K, Jørgensen BB (1978) Mechanisms of hydrogen sulfide release from coastal marine sediments to the atmosphere. Limnol Oceanogr 23:68–76

Harder W, Veldkamp H (1968) Physiology of an obligate psychrophilic marine Pseudomonas species. J Appl Bacteriol 31:12–33

Harder W, Veldkamp H (1971) Competition of marine psychrophilic bacteria at low temperatures. Antonie van Leeuwenhoek J Microbiol Serol 37:51–63

Hardie JM, Bowden GH (1974) The normal microbial flora of the mouth. In: Skinner FA, Carr JG (eds) The normal microbial flora of man. Academic, London, pp 47–83

Harold R, Stanier RY (1955) The genera Leucothrix and Thiothrix. Bacteriol Rev 19:49–58

Harris RH, Mitchell R (1973) The role of polymers in microbial aggregation. Ann Rev Microbiol 27:27–50

Hassan HM, Fridovich I (1979) Superoxide dismutase and its role for survival in the presence of oxygen. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 179–193

Hastings JW, Nealson KH (1977) Bacterial bioluminescence. Ann Rev Microbiol 31:549–595

Hattori T, Ishida Y, Maruyama Y, Morita RY, Uchida A (1989) Recent advances in microbial ecology. In: Proceedings of the 5th international symposium on microbial ecology, Japan Science Society Press, Tokyo

Hazelbauer GL (1988) The bacterial chemosensory system. Can J Microbiol 34:466–474

Heinen W (1974) Proceedings of the first European workshop on microbial adaptation to extreme environments. Biosystems 6:57–80

Held AA (1970) Nutrition and fermentative energy metabolism of the water mold *Aqualinderella fermentans*. Mycologia 62:339–358

Held AA, Emerson R, Fuller MS, Gleason FH (1969) Blastocladia and Aqualinderella: fermentative water molds with high carbon dioxide optima. Science 165:706–708

Henrici AT, Johnson DE (1935) Studies of freshwater bacteria. II. Stalked bacteria, a new order of Schizomycetes. J Bacteriol 30:61–86

Herdman M, Janvier M, Waterbury JB, Rippka R, Stanier RY (1979) Deoxyribonucleic acid base composition of cyanobacteria. J Gen Microbiol 111:63–71

Heukelekian H, Heller A (1940) Relation between food concentration and surface for bacterial growth. J Bacteriol 40:547–558

Heumann W, Marx R (1964) *Feinstruktur und Funktion der Fimbrien bei dem sternbildenden Bakterium Pseudomonas echinoides*. Archiv für Mikrobiologie 47:325–337

Hirsch P (1974) Budding bacteria. Ann Rev Microbiol 28:392–444

Hirsch P (1979) Life under conditions of low nutrient concentrations. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 357–372

Hirsch P, Pankratz St H (1970) Study of bacterial populations in natural environments by use of submerged electron microscope grids. Zeitschrift für Allgemeine Mikrobiologie 10:589–605

Hobson PN (1988) The rumen microbial ecosystem. Elsevier Science, London

Hochachka PW, Mustafa T (1972) Invertebrate facultative anaerobiosis. Science 178:1056–1060

Hochachka PW, Somero GN (1973) Strategies of biochemical adaptation. WB Saunders, London

Hoffmann C (1942) Beiträge zur Vegetation des FarbstreifenSandwattes. Kieler Meeresforschungen 4:85–108

Holben WE, Jansson J, Chelm B, Tiedje T (1988) DNA probe method for detection of specific microorganisms in the soil bacterial community. Appl Environ Microbiol 54:703–711

Holdemann LV, Cato EP, Moore WEC (1977) Anaerobe laboratory manual, 4th edn. Virginia Polytechnic Institute and State University, Blacksburg

Horikoshi K, Akiba T (1982) Alkalophilic microorganisms. A new microbial world. Japan Scientific Societies Press/Springer, Berlin/New York

Huber R, Langworthy TA, König H, Thomm M, Woese CR, Sleytr UB, Stetter KO (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, Huber G, Segerer A, Seger J, Stetter KO (1987a) Aerobic and anaerobic extremely thermophilic autotrophs. In: van Verseveld HW, Duine JA (eds) Proceedings of the 5th international symposium. Martinus Nijhoff, Dordrecht, pp 44–51

Huber R, Kristjansson J, Stetter KO (1987b) *Pyrobaculum* gen. nov., a new genus of neutrophilic, rod-shaped archaebacteria from continental solfataras growing optimally at 100 °C. Arch Microbiol 149:95–101

Huber R, Kurr M, Jannasch HW, Stetter KO (1989) A novel group of abyssal methanogenic archaebacteria (*Methanopyrus*) growing at 110 °C. Nature 342:833–834

Huber R, Stoffers P, Cheminee JL, Richnow HH, Stetter KO (1990) Hyperthermophilic archaebacteria within the crater and open-sea plume of erupting Macdonald Seamount. Nature 345:179–181

Hughes MN, Poole RK (1989) Metals and microorganisms. Chapman and Hall, London

Hungate RE (1950) The anaerobic mesophilic cellulolytic bacteria. Bacteriol Rev 14:1–49

Hungate RE (1962) Ecology of bacteria. In: Gunsalus JC, Stanier RY (eds) The bacteria, vol IV, The physiology of growth. Academic, New York, pp 95–119

Hungate RE (1966) The rumen and its microbes. Academic, New York

Hungate RE (1967) Hydrogen as an intermediate in the rumen fermentation. Archiv für Mikrobiologie 59:158–164

Hungate RE (1975) The rumen microbial ecosystem. Ann Rev Ecol Syst 6:39–66

Hussain HM (1973) Ökologische Untersuchungen über die Bedeutung thermophiler Mikroorganismen für die Selbsterhitzung von Heu. Zeitschrift für Allgemeine Mikrobiologie 13:323–334

Hustede E, Liebergesell M, Schlegel HG (1989) The photophobic response of various sulfur and nonsulfur purple bacteria. Photochem Photobiol 50:809–815

Iannotti EL, Kafkewit D, Wolin MJ, Bryant MP (1973) Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes*: changes caused by interspecies transfer of $H_2$. J Bacteriol 114:1231–1240

Imhoff JF, Trüper HG (1977) *Ectothiorhodospira halochloris* sp. nov., a new extremely halophilic bacterium containing bacteriochlorophyll b. Arch Microbiol 114:115–121

Inniss WE (1975) Interaction of temperature and psychrophilic microorganisms. Ann Rev Microbiol 29:445–465

Inniss WE, Ingraham JL (1978) Microbial life at low temperatures: mechanisms and molecular aspects. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, London, pp 73–104

Jaggar J (1983) Physiological effects of near-ultraviolet radiation on bacteria. Photochem Photobiol Rev 7:1–75

Jannasch HW (1955) Zur Ökologie der zymogenen planktischen Bakterienflora natürlicher Gewässer. Archiv für Mikrobiologie 23:146–180

Jannasch HW (1957) Die bakterielle Rotfärbung der Salzseen des Wadi Natrun (Ägypten). Archiv für Hydrobiologie 53:425–433

Jannasch HW (1958) Studies of planktonic bacteria by means of a direct membrane filter method. J Gen Microbiol 18:609–620

Jannasch HW (1960) Versuche über Denitrifikation und die Verfügbarketi des Sauerstoffes in Wasser und Schlamm. Archiv für Hydrobiologie 56:335–369

Jannasch HW (1967) Enrichment of aquatic bacteria in continuous culture. Archiv für Mikrobiologie 59:165–173

Jannasch HW (1977) Growth kinetics of aquatic bacteria. Soc Appl Bacteriol Symp Ser 6:55–68

Jannasch HW (1978) Microorganisms and their aquatic habitat. In: Krumbein WE (ed) Environmental biogeochemistry and geomicrobiology, vol 1. Ann Arbor Scientific, Ann Arbor, pp 17–24

Jannasch HW (1979) Microbial ecology of aquatic low-nutrient habitats. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 243–260

Jannasch HW (1984) Microbial processes at deep sea hydrothermal vents. In: Rona PA, Bostrom K, Laubier L, Smith KL (eds) Hydrothermal processes at seafloor spreading centers. Plenum, New York, pp 677–709

Jannasch HW (1989) Chemosynthetically sustained ecosystems in the deep sea. In: Schlegel HG, Bowien B (eds) Autotrophic bacteria. Springer, Berlin, pp 147–166

Jannasch HW, Mateles RI (1974) Experimental bacterial ecology studies in continuous culture. Adv Microb Physiol 11:165–212

Jannasch HW, Pritchard PH (1972) The role of inert particulate matter in the activity of aquatic microorganisms. In: Melchiorri-Santolinie U, Hopton JW (ed) Detritus and its role in aquatic ecosystems. Memorie dell'Istituto Italiano di Idrobiologia Dott Marco de Marchi Pallanza Italy Suppl 29:289–308

Jannasch HW, Taylor CD (1984) Deep-sea microbiology. Ann Rev Microbiol 37:487–514

Jannasch HW, Wirsen CO (1973) Deep-sea microorganisms: in situ response to nutrient enrichment. Science 180:641–643

Jannasch HW, Wirsen CO (1977) Retrieval of concentrated and undecompressed microbial populations from the deep sea. Appl Environ Microbiol 33:642–646

Jannasch HW, Wirsen CO (1979) Chemosynthetic primary production at East Pacific sea floor spreading centers. Bioscience 29:592–598

Jannasch HW, Wirsen CO (1981) Morphological survey of microbial mats near deep sea thermal vents. Appl Environ Microbiol 41:528–538

Jannasch HW, Wirsen CO (1982) Microbial activities in undecompressed and decompressed deep-seawater samples. Appl Environ Microbiol 43:1116–1124

Jannasch HW, Trüper HG, Tuttle JH (1974) The microbial sulfur cycle in the Black Sea. Dergens ET, Ross DA (ed) The Black Sea: its geology, chemistry and biology, Memoir 20. American Association of Petroleum, Tulsa

Jannasch HW, Wirsen CO, Taylor CD (1976) Undecompressed microbial populations from the deep sea. Appl Environ Microbiol 32:360–367

Jannasch HW, Wirsen CO, Taylor CD (1982) Deep-sea bacteria: isolation in the absence of decompression. Science 216:1315–1317

Jannasch HW, Huber R, Belkin S, Stetter KO (1988) *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus *Thermotoga*. Arch Microbiol 150:103–104

Jannasch HW, Nelson DC, Wirsen CO (1989) Massive natural occurrence of unusually large bacteria (*Beggiatoa* sp.) at a hydrothermal deep-sea vent site. Nature 342:834–836

Jones OTG (1977) Electron transport and ATP synthesis in the photosynthetic bacteria. In: Haddock BA, Hamilton WA (eds) Microbial energetics. Cambridge University Press, Cambridge, pp 151–183

Jones WJ, Leigh JA, Mayer F, Woese CR, Wolfe RS (1983) *Methanococcus jannaschii*, sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch Microbiol 163:154–261

Jones WJ, Stugard CE, Jannasch HW (1989) Comparison of thermophilic methanogens from submarine hydrothermal vents. Arch Microbiol 151:314–318

Jørgensen BB (1982) Ecology of the bacteria of the sulfur cycle with special reference to anoxic-oxic interface environments. Phil Tran R Soc Lond Biol Sci 298:543–561

Jørgensen BB, Revsbech NP (1983) Colorless sulfur bacteria, *Beggiatoa* spp. and *Thiovulum* spp., in $O_2$ and $H_2S$ microgradients. Appl Environ Microbiol 45:1261–1270

Jørgensen BB, Zawacki LX, Jannasch HW (1990) Thermophilic bacterial sulfate reduction in deep-sea sediments at the Guaymas Basin hydrothermal vent site (Gulf of California). Deep-Sea Res 37:695–710

Kato G, Maruyama Y, Nakamura M (1979) Role of lectins and lipopolysaccharides in the recognition process of specific legume-Rhizobium symbiosis. Agric Biol Chem 43:1085–1092

Kefford B, Kjelleberg S, Marshall KC (1982) Bacterial scavenging: utilization of fatty acids localized at a solid-liquid interface. Arch Microbiol 133:257–260

Kelly MT, Brock TD (1969) Physiological ecology of Leucothrix mucor. J Gen Microbiol 59:153–162

Kjelleberg S, Hermansson M, Mardén P, Jones GW (1987) The transient phase between growth and non growth of heterotrophic bacteria, with emphasis on the marine environment. Annu Rev Microbiol 41:25–49

Kluyver AJ, Donker HJL (1925) The unity of the chemistry of the fermentative sugar dissimilation processes of microbes. Proc Roy Acad Amsterdam 28:297–313

Kluyver AJ, Donker HJL (1926) Die Einheit in der Biochemie Chemie der Zelle und Gewebe 13:134–190

Koch AL (1979) Microbial growth in low concentrations of nutrients. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 261–279

Kolkwitz R (1904–1906) Mykologie und Reinigung der städtischen und der Zuckerfabriksabwässer 391. In: Lafar F (ed) Handbuch der technischen Mykologie, vol 3. Jena Gustav, Fischer Verlag

Korhonen TK, Nurmiaho E-L, Tuovinen OH (1978) Fimbriation in *Thiobacillus* A2. FEMS Microbiol Lett 3:195–198

Koshland DE Jr (1974) The chemotactic response in bacteria. In: Jaenicke L (ed) Biochemistry of sensory functions. Springer, Berlin, pp 133–160

Koshland DE Jr (1976) Bacterial chemotaxis as a simple model for a sensory system. Trends Biochem Sci 1:1–3

Koshland DE (1980) Bacterial chemotaxis as a model behavioral system. Raven, New York

Koshland DE (1981) Biochemistry of sensing and adaptation in a simple bacterial system. Ann Rev Biochem 50:765–782

Krinsky NI (1979) Carotenoid pigments: multiple mechanisms for coping with the stress of photosensitized oxidations. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 163–177

Krul JM, Hirsch P, Staley JT (1970) *Toxothrix trichogenes* (Chol.) Beger et Bringmann: the organism and its biology. Antonie van Leeuwenhoek J Microbiol Serol 36:409–420

Krulwich TA, Guffanti AA (1983) Physiology of acidophilic and alkalophilic bacteria. Adv Microb Physiol 24:173–214

Kuenen JG, Boonstra HG, Schröder J, Veldkamp H (1977) Competition for inorganic substrates among chemoorganotrophic and chemolithotrophic bacteria. Microb Ecol 3:119–130

Kushner DJ (1971) Life in extreme environments. In: Buvet R, Ponnamperuma C (eds) Chemical evolution and origin of life. North-Holland, Amsterdam, pp 485–491

Kushner DJ (1978) Life in high salt and solute concentrations: halophilic bacteria. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, London, pp 317–368

Kuznezow SI (1959) Die Rolle der Mikroorganismen im Stoffkreislauf der Seen. Berlin VEB Deutscher Verlag der Wissenschaften

Kuznezow SI (1977) Trends in the development of ecological microbiology. In: Droop MR, Jannasch HW (eds) Advances in aquatic microbiology. Academic, London, pp 1–48

la Riviére JWM (1963) Cultivation and properties of *Thiovulum majus* Hinze. In: Oppenheimer CH (ed) Marine microbiology. Charles C Thomas, Springfield, pp 61–72

la Riviére JWM (1965) Enrichment of colorless sulfur bacteria Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 1, Suppl 1:17–27

Langworthy TA (1978) Microbial life in extreme pH values. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, London, pp 279–315

Lanyi JK (1979) Physical-chemical aspects of salt-dependence in Halobacteria. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 93–107

Lapage SP, Shelton JE, Mitchell TG, MacKenzie AR (1970) Culture collections and the preservation of bacteria. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 3A. Academic, London

Larsen H (1967) Biochemical aspects of extreme halophilism. Adv Microb Physiol 1:97–132

Larsen H (1971) Halophilism, microbial. In: McGraw-Hill encyclopedia of science and technology, 3rd edn. McGraw-Hill, New York

Larsen H (1973) The halobacteria's confusion to biology. The fourth A.J. Kluyver memorial lecture delivered before the Netherlands Society for Microbiology, April 1972 at the Delft University of Technology. Antonie van Leeuwenhoek J Microbiol Serol 39:383–396

Larsen H (1986) Halophilic and halotolerant microorganisms—an overview and historical perspective. FEMS Microbiol Rev 39:3–7

Latham MJ, Wolin MJ (1977) Fermentation of cellulose by *Ruminococcus flavefaciens* in the presence and absence of *Methanobacterium ruminantium*. Appl Environ Microbiol 34:297–301

Latham MJ, Brooker BE, Pettipher GL, Harris PJ (1978) *Ruminococcus flavefaciens* cell coat and adhesion to cotton cellulose and to cell walls in leaves of perennial ryegrass (*Lolium perenne*). Appl Environ Microbiol 35:156–165

Le Roux NW, Wakerley DS, Hunt SD (1977) Thermophilic Thiobacillus-type bacteria from Icelandic thermal areas. J Gen Microbiol 100:197–201

Leathen WW, Braley SA Sr, McIntyre LD (1953) The role of bacteria in the formation of acid from certain sulfuritic constituents associated with bituminous coal. II. Ferrous iron oxidizing bacteria. Appl Microbiol 1:65–68

Lee A, Phillips M (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

Leifson E (1962) The bacterial flora of distilled and stored water. I. General observations, techniques and ecology. Int Bull Bacteriol Nomen Taxon 12:133–153

Levi P, Linkletter A (1989) Metals, microorganisms and biotechnology. In: Hughes MN, Poole RK (eds) Metals and microorganisms. Chapman and Hall, London

Liener IE (1976) Phytohemagglutinins (phytolectins). Ann Rev Plant Physiol 27:291–319

Loesche WJ (1969) Oxygen sensitivity of various anaerobic bacteria. Appl Microbiol 18:723–727

Lovley DR, Phillips EJP (1987) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl Environ Microbiol 54:1472–1480

Madigan MT, Brock TD (1977) Adaptation by hot springs phototrophs to reduced light intensities. Arch Microbiol 113:111–120

Mandel M, Leadbetter ER, Pfennig N, Trüper HG (1971) Deoxyribonucleic acid base compositions of phototrophic bacteria. Int J Syst Bacteriol 21:222–230

Mann S, Sparks NH, Frankel RB, Bazylinski DA, Jannasch HW (1990) Biomineralization of ferrimagnetic greigite (FeS) and iron pyrite (FeS) in a magnetotactic bacterium. Nature 343:258–261

Marchlewitz B, Schwartz W (1961) Untersuchungen über die Mikroben-Assoziation saurer Grubenwässer. Zeitschrift für Allgemeine Mikrobiologie 1:100–114

Marples MJ (1965) The ecology of the human skin. Thomas Charles C, Springfield

Marples MJ (1974) The normal microbial flora of the skin. In: Skinner FA, Carr JG (eds) The normal microbial flora of man. Academic, London, pp 7–12

Marples MJ (1976) Life on the human skin. Sci Am 220:108–115

Marquis RE (1976) High-pressure microbial physiology. In: Rose AH, Tempest DW (eds) Advances in microbial physiology, vol 14. Academic, London, pp 159–241

Marquis RE, Matsumara P (1978) Microbial life under pressure. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, London, pp 105–158

Marshall KC (1976) Interfaces in microbial ecology. Harvard University Press, Cambridge/London

Marshall KC (1979) Growth at interfaces. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim

Marshall KC (ed) (1984) Microbial adhesion and aggregation. Dahlem workshop reports, Life Science, vol 31. Springer, New York

Martin SM (1964) Conservation of microorganisms. Ann Rev Microbiol 18:1–16

Martin HH (1969) Die Struktur der Zellwand bei Gram-negativen. Bakterien Arzneimittel-Forschung 19:266–272

Marx JL (1977) Looking at lectins: do they function in recognition processes? Science 196:1429–1430

Marx R, Heumann W (1962) Uber Geisselfeinstrukturen und Fimbrien bei zwei Psuedomonas-Stämmen. Archiv für Mikrobiologie 43:245–254

Matin A (1979) Microbial regulatory mechanisms at low nutrient concentrations as studies in chemostat. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 323–339

Matin A, Veldkamp H (1978) Physiological basis of the selective advantage of a *Spirillum* sp. in a carbon-limited environment. J Gen Microbiol 105:187–197

Matin A, Veldhuis C, Stegemann V, Veenhuis M (1979) Selective advantage of a *Spirillum* sp. in a carbon-limited environment. Accumulation of poly-β-hydroxybutyric acid and its role in starvation. J Gen Microbiol 112:349–355

Matin A, Auger EA, Blum PH, Schultz JE (1989) Genetic basis of starvation survival in nondifferentiating bacteria. Ann Rev Microbiol 43:293–316

Matthews MM, Sistrom WR (1959) Function of carotenoid pigments in non-photosynthetic bacteria. Nature 184:1892–1893

Mayer F (1971) Elektronenmikroskopische Untersuchung der Fimbrienkontraktion bei dem sternbildenden Bodenbakterium *Pseudomonas echinoides*. Archiv für Mikrobiologie 76:166–173

Mayer F, Schmitt R (1971) Elektronenmikroskopische, diffraktometrische und disc-elektrophoretische Untersuchungen an Fimbrien des sternbildenden Bodenbakteriums *Pseudomonas echinoides* und einer nicht-sternbildenden. Mutante Archiv für Mikrobiologie 79:311–326

Mazanec K, Kocur M, Martinec T (1965) Electron microscopy of ultrathin sections of *Sporosarcina ureae*. J Bacteriol 90:808–816

McBee RH (1977) Fermentation in the hindgut. In: Clarke RTJ, Bauchop T (eds) Microbial ecology of the gut. Academic, London, pp 185–222

McLeod RA (1968) On the role of inorganic ions in the physiology of marine bacteria. Adv Microbiol Sea 1:95

Meers JL (1973) Growth of bacteria in mixed cultures. CRC Crit Rev Microbiol 2:139–184

Megusar F, Gantar M (eds) (1986) Perspectives in microbial ecology. Slovene Society for Microbiology Ljubljana, Yugoslavia

Menzel DW, Ryther JH (1970) Distribution and cycling of organic matter in the oceans. In: Hood DW (ed) Organic matter in natural waters. Alaska Institute of Marine-Sciences, Alaska

Millar WN (1973) Heterotrophic bacterial population in acid coal mine water: *Flavobacterium acidurans*, sp. n. Int J Syst Bacteriol 23:142–150

Miller WD (1890) The micro-organisms of the human mouth. Basel Karger, Philadelphia, unaltered reprint from original work

Miller RE, Simons LA (1962) Survival of bacteria after twenty-one years in the dried state. J Bacteriol 84:1111–1114

Minato H, Suto T (1978) Technique for fractionation of bacteria in rumen microbial ecosystem. II. Attachment of bacteria isolated from bovine rumen to cellulose powder in vitro and elution of bacteria attached therefrom. J Gen Appl Microbiol 24:1–16

Mitskevich IN (1979) The total number of biomass of microorganisms in deep waters of the Black Sea (In Russian, with English summary). Mikrobiologiya 48:552–557

Moore WEC, Holdeman LV (1974) Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl Microbiol 27:961–979

Morita RY (1975) Psychrophilic bacteria. Bacteriol Rev 39:144–167

Morita RY (1976) Survival of bacteria in cold and moderate hydrostatic pressure environments with special reference to psychrophilic and barophilic bacteria. In: Gray TGR, Postgate JR (eds) The survival of vegetative microbes. Cambridge University Press, Cambridge, pp 279–298

Morris JG (1975) The physiology of obligate anaerobiosis. Adv Microb Physiol 12:169–246

Morris JG (1976) Fifth Stenhouse-Williams Memorial Lecture—oxygen and the obligate anaerobe. J Appl Bacteriol 40:229–244

Morris JG (1978) The biochemistry of anaerobiosis. Biochem Soc Trans 6:353–356

Morris JG (1979) Nature of oxygen toxicity in anaerobic microorganisms. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 149–162

Morris JG, O'Brien RW (1971) Oxygen and clostridia: a review. In: Barker AN, Gould GW, Wolf J (eds) Spore research 1971. Academic, London, pp 1–37

Mossel DAA (1975) Water and micro-organisms in foods—a synthesis. In: Duckworth RB (ed) Water relations of foods. Academic, London, pp 347–361

Mossel DAA, Ingram M (1955) The physiology of the microbial spoilage of foods. J Appl Bacteriol 18:232–268

Mountfort DO, Asher RA (1983) Role of catabolic regulatory mechanism in control of carbohydrate utilization by the rumen anaerobic fungus *Neocallimastix frontalis*. Appl Environ Microbiol 46:1331–1338

Mulder EG, Brotonegoro S (1974) Free-living heterotrophic nitrogen-fixing bacteria. In: Quispel A (ed) The biology of nitrogen fixation. North-Holland, Amsterdam, pp 37–85

Müller M (1975) Biochemistry of protozoan microbodies: peroxisomes, glycerophosphate oxidase bodies, hydrogenosomes. Annu Rev Microbiol 29:467–483

Müller-Neuglück M, Engel H (1961) Photoinaktivierung von Nitrobacter winogradskyi Buch. Archiv für Mikrobiologie 39:130–138

Nasim A, James AP (1978) Life under conditions of high irradiation. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, London, pp 409–439

Neijssel OM, Hueting S, Crabbendam KJ, Tempest DW (1975) Dual pathways of glycerol assimilation in *Klebsiella aerogenes* NCIB 418. Their regulation and possible functional significance. Arch Microbiol 104:83–87

Nelson DC, Jannasch HW (1983) Chemoautotrophic growth of a marine *Beggiatoa* in sulfide-gradient cultures. Arch Microbiol 136:262–269

Nelson DC, Jørgensen BB, Revsbech NP (1986) Growth pattern and yield of a chemoautotrophic *Beggiatoa* sp. in oxygen-sulfide microgradients. Appl Environ Microbiol 53:225–233

Nelson DC, Wirsen CO, Jannasch HW (1989) Characterization of large, autotrophic *Beggiatoa* spp. abundant at hydrothermal vents of the Guaymas Basin. Appl Environ Microbiol 55:2909–2917

Noble WC, Pitcher DG (1979) Microbial ecology of the human skin. Adv Microb Ecol 2:245–289

Noble WC, Somerville DA (1974) Microbiology of human skin. Saunders, London

Nottingham PM, Hungate RE (1969) Methanogenic fermentation of benzoate. J Bacteriol 98:1170–1172

Nultsch W (1975) Phototaxis and photokinesis. In: Carlile MJ (ed) Primitive sensory and communication systems: the taxes and tropisms of microorganisms and cells. Academic, London, pp 29–90

O'Brien RW, Morris JG (1971) Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. J Gen Microbiol 68:307–318

Odum EP (1977) Ecology: the link between the natural and the social sciences, 2nd edn. Holt, Rinehart & Winston, London

Ohta K, Kiyomiya A, Koyama N, Nosoh Y (1975) The basis of the alkalophilic property of a species of bacillus. J Gen Microbiol 86:259–266

Okon Y, Albrecht SL, Burris RH (1976) Factors affecting growth and nitrogen fixation of Spirillum lipoferum. J Bacteriol 127:1248–1254

Oren A, Padan E (1978) Induction of anaerobic, photoautotrophic growth in the cyanobacterium *Oscillatoria limnetica*. J Bacteriol 133:558–563

Oren A, Shilo M (1979) Anaerobic heterotrophic dark metabolism in the cyanobacterium *Oscillatoria limnetica*: sulfur respiration and lactate fermentation. Arch Microbiol 122:77–84

Orpin CG (1972) The culture in vitro of the rumen bacterium Quin's oval. J GenMicrobiol 73:523–530

Orpin CG (1973) The intracellular polysaccharide of the rumen bacterium Eadie's oval. Archiv für Mikrobiologie 90:247–254

Orpin CG, Joblin KN (1988) The rumen anaerobic fungi. In: Hobson PN (ed) The rumen microbial ecosystem. Elsevier Science, London

Ottow JCG (1975) Ecology, physiology, and genetics of fimbriae and pili. Annu Rev Microbiol 29:79–108

Overbeck J (1972) Zur Struktur und Funktion des aquatischen Ökosystems Berichte der Deutschen Botanischen Gesellschaft 85:553–579

Pace NR, Stahl D, Lane DJ, Olsen GJ (1986) The analysis of natural microbial populations by ribosomal RNA sequences. Adv Microbiol Ecol 9:1–55

Padan E (1979a) Facultative anoxygenic photosynthesis in cyanobacteria. Ann Rev Plant Physiol 30:27–40

Padan E (1979b) Impact of facultatively anaerobic photoautotrophic metabolism on ecology of cyanobacteria (blue-green algae). Adv Microb Ecol 3:1–48

Pask-Hughes RA, Williams RAD (1975) Extremely thermophilic Gram-negative bacteria from hot tap water. J Gen Microbiol 88:321–328

Pask-Hughes RA, Williams RAD (1977) Yellow-pigmented strains of *Thermus* spp. from Icelandic hot springs. J Gen Microbiol 102:375–383

Patterson H, Irvin R, Costerton JW, Cheng K-J (1975) Ultrastructure and adhesion properties of *Ruminococcus albus*. J Bacteriol 122:278–287

Pfennig N (1961) Eine vollsynthetische Nährlösung zur selektiven. Anreicherung einiger Schwefelpurpurbakterien Naturwissenschaften 48:136

Pfennig N (1965) Anreicherungskulturen für rote und grüne Schwefelbakterien Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 1, Suppl 1:179–189, 503–504

Pfennig N (1967) Photosynthetic bacteria. Ann Rev Microbiol 21:286–324

Pfennig N (1979) General physiology and ecology of photosynthetic bacteria. In: Sistrom WR, Clayton R (eds) Photosynthetic bacteria. Plenum, New York, pp 3–18

Pfennig N, Biebl H (1976) *Desulfuromonas acetoxidans* gen. nov. and sp. nov., a new anaerobic, sulfur-reducing, acetate-oxidizing bacterium. Arch Microbiol 110:3–12

Pfennig N, Cohen-Bazire G (1967) Some properties of the green bacterium *Pelodictyon clathratiforme*. Arch Mikrobiol 59:226–236

Pierson BK, Castenholz RW (1974) A phototrophic gliding filamentous bacterium of hot springs. *Chloroflexus aurantiacus*, gen. and sp. nov. Arch Microbiol 100:5–24

Poindexter JS (1964) Biological properties and classification of the Caulobacter group. Bacteriol Rev 28:231–295

Poindexter JS (1979) Morphological adaptation to low nutrient concentrations. In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 341–356

Prebble J, Huda S (1977) The photosensitivity of the malate oxidase system of a pigmented strain and a carotenoidless mutant of *Sarcina lutea* (*Micrococcus luteus*). Arch Microbiol 113:39–42

Pringsheim EG (1957) Observations on *Leucothrix mucor* and *Leucothrix cohaercus* nov. sp. with a survey of colorless filamentous organisms. Bacteriol Rev 21:69–81

Prins RA (1977) Biochemical activities of gut microorganisms. In: Clarke RTJ, Bauchop T (eds) Microbial ecology of the gut. Academic, London, pp 73–183

Raj HD (1977) Leucothrix. CRC Crit Rev Microbiol 5:271–301

Ramaley RF, Hixson J (1970) Isolation of a nonpigmented, thermophilic bacterium similar to *Thermus aquaticus*. J Bacteriol 103:527–528

Reddy CA, Bryant MP, Wolin MJ (1972a) Characteristics of S organism isolated from *Methanobacillus omelianskii*. J Bacteriol 109:539–545

Reddy CA, Bryant MP, Wolin MJ (1972b) Ferredoxin-independent conversion of acetaldehyde to acetate and $H_2$ in extracts of S organism. J Bacteriol 110:133–138

Reichelt JL, Baumann P (1973) Taxonomy of the marine, luminous bacteria. Arch für Mikrobiol 94:283–330

Repaske DR, Adler J (1981) Change in intracellular pH of escherichia coli mediates the chemotactic response to certain attractants and repellents. J Bacteriol 145:1196–1208

Repeta DJ, Simpson DJ, Jørgensen BB, Jannasch HW (1989) Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea. Nature 342:69–72

Richards FR (1975) The Cariaco basin (Trench). Oceanogr Marine Biol Ann Rev 13:11–67

Richards FA, Vaccaro RF (1958) The Cariaco Trench, an anaerobic basin in the Caribbean Sea. Deep-Sea Res 3:214–228

Rittenberg SC (1979) *Bdellovibrio*: a model of biological interactions in nutrient impoverished environments? In: Shilo M (ed) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, pp 305–322

Robinson JB, Salonius PO, Chase FE (1965) A note on the differential response of *Arthrobacter* spp. and *Pseudomonas* spp. to drying in soil. Can J Microbiol 11:746–748

Rose AH (1968) Physiology of microorganisms at low temperatures. J Appl Bacteriol 31:1–11

Rosebury T (1972) Der Reinlichkeitstick. Hoffmann & Campe Verlag, Hamburg

Rudd JWM, Taylor CD (1980) Methane cycling in aquatic environments. Adv Aquat Microbiol 2:77–150

Rupela OP, Tauro P (1973) Isolation and characterization of Thiobacillus from alkali soils. Soil Biol Biochem 5:891–897

Russell C, Melville TH (1978) A review: bacteria in the human mouth. J Appl Bacteriol 44:163–181

Rutter PR, Abbott A (1978) A study of the interaction between oral streptococci and hard surfaces. J Gen Microbiol 105:219–226

Sadoff HL (1973) Comparative aspects of morphogenesis in three prokaryotic genera. Ann Rev Microbiol 27:133–153

Sadoff HL (1975) Encystment and germination in *Azotobacter vinelandii*. Bacteriol Rev 39:516–539

Savage DC (1977a) Microbial ecology of the gastrointestinal tract. Ann Rev Microbiol 31:107–133
Savage DC (1977b) Interactions between the host and its microbes. In: Clarke RTJ, Bauchop T (eds) Microbial ecology of the gut. Academic, London
Savage DC, Fletcher M (1985) Bacterial adhesion. Plenum, New York
Scarr MP (1968) Thermophiles in sugar. J Appl Bacteriol 31:66–74
Schenk A, Aragno M (1979) *Bacillus schlegelii*, a new species of thermophilic, facultatively chemolithoautotrophic bacterium oxidizing molecular hydrogen. J Gen Microbiol 115:333–341
Schenk EA, Schwemmler W (1983) Endocytobiology II. Walter de Gruyter, Berlin
Schlegel HG (ed) (1965) Anreicherungskultur und Mutantenauslese Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 1 Orig., Suppl. 1:179–189
Schlegel HG, Bowien B (1989) Autotrophic bacteria. Science Tech/Springer, Madison/New York
Schlegel HG, Jannasch HW (1967) Enrichment cultures. Ann Rev Microbiol 21:49–70
Schlegel HG, Pfennig N (1961) Die Anreicherungskultur einiger Schwefelpurpurbakterien. Archiv für Mikrobiologie 38:1–39
Schmaljohann R, Flügel HJ (1987) Methane-oxidizing bacteria in Pogonophora. Sarsia 72:91–98
Schmidt JM (1971) Prosthecate bacteria. Ann Rev Microbiol 25:93–110
Schmidt-Lorenz W (1967) Behavior of microorganisms at low temperatures. Bulletin de l'Institut International du Froid 1–59
Schnaitman C, Lundgren DG (1965) Organic compounds in the spent medium of *Ferrobacillus ferrooxidans*. Can J Microbiol 11:23–27
Schön GH, Engel H (1962) Der Einfluss des Lichtes auf Nitrosomonas europaea. Win Archiv für Mikrobiologie 42:415–428
Schroff G, Schöttler U (1977) Anaerobic reduction of fumarate in the body wall musculature of *Arenicola marina* (Polychaeta). J Comp Physiol 116:325–336
Schultz JE, Breznak JA (1978) Heterotrophic bacteria present in hindguts of wood-eating termites [*Reticulitermes flavipes* (Kollar)]. Appl Environ Microbiol 35:930–936
Schultz JE, Breznak JA (1979) Cross-feeding of lactate between *Streptococcus lactis* and *Bacteroides* sp. isolated from termite hindguts. Appl Environ Microbiol 37:1206–1210
Schweinfurth G, Lewin L (1898) Beiträge zur Topographie und Geochemie des ägyptischen Natron-thals. Zeitschrift für die Gesamte Erdkunde 33:1–25
Schwemmler W, Schenk EA (1980) Endocytobiology. Walter de Gruyter, Berlin
Shilo M (ed) (1979) Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim
Shokes RF, Trabant PK, Presley BJ, Reid DF (1977) Anoxic, hypersaline basin in the northern. Gulf of Mexico Sci 196:1443–1446
Siñeriz F, Pirt SJ (1977) Methane production from glucose by a mixed culture of bacteria in the chemostat: the role of Citrobacter. J Gen Microbiol 101:57–64
Singer CE, Ames BN (1970) Sunlight ultraviolet and bacterial DNA base ratios. Science 170:822–826
Skopintsev BA, Karpov AV, Vershinina OA (1959) Study of the dynamics of some sulfur compounds in the Black Sea under experimental conditions. Soviet Oceanogr Ser 4:55–72, English translation
Sleat R, Robinson JP (1984) The bacteriology of anaerobic degradation of aromatic compounds. J Appl Bacteriol 57:381–394
Smith DW (1978) Water relations of microorganisms in nature. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, London, pp 369–380
Sneath PHA (1962) Longevity of micro-organisms. Nature 195:643–646
Somerville CC, Knight IT, Straube WL, Colwell RR (1989) Simple, rapid method for direct isolation of nucleic acids from aquatic environments. Appl Environ Microbiol 55:548–554
Sorokin YI (1964) On the primary production and bacterial activities in the Black Sea. Journal du Conseil Conseil International pour l'Exploration de la Mer 29:41–60
Sorokin YI (1970) Interrelations between sulphur and carbon turnover in meromictic lakes. Archiv für Hydrobiologie 66:391–446
Sorokin YI (1972) The bacterial population and process of sulfide oxidation in the Black Sea. J Cons Int Explor Mer 34:423–454
Souza KA, Deal PH (1977) Characterization of a novel extremely alkaline bacterium. J Gen Microbiol 101:103–109
Souza KA, Deal PH, Mack HM, Turnbill CE (1974) Growth and reproduction of microorganisms under extremely alkaline conditions. Appl Microbiol 28:1066–1068
Stahl DA, Lane DJ, Olsen GJ, Pace NR (1985) Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. Appl Environ Microbiol 49:1379–1384
Stahl DA, Flesher B, Mansfield HR, Montgomery L (1988) Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl Environ Microbiol 54:1079–1084
Stanier RY (1942) The cytophaga group: a contribution to the biology of Mycobacteria. Bacteriol Rev 6:143–196
Stapp C, Bortels H (1931) Der Pflanzenkrebs und sein Erreger *Pseudomonas tumefaciens*. II. Mitteilung: Über den Lebenskreislauf von *Pseudomonas tumefaciens* Zeitschrift für Parasitenkunde 4:101–125
Stapp C, Knösel D (1954) Zur Genetik sternbildender Bakterien Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abt. 2. 108:244–259
Steinbüchel A (1986) Anaerobic pyruvate metabolism of *Trichomonas foetus* and *Trichomonas vaginalis* hydrogenosomes. Mole Biochem Parasitol 20:57–65
Stetter KO (1986) Diversity of extremely thermophilic archaebacteria. In: Brock TD (ed) Thermophiles, general, molecular and applied microbiology. Wiley, New York, pp 39–74
Stetter KO (1989) Extremely thermophilic chemolithoautotrophic archaebacteria. In: Schlegel HG, Bowien B (eds) Autotrophic bacteria. Science Tech, Madison, pp 167–176
Stetter KO, Zillig W (1985) *Thermoplasma* and the thermophilic sulfur-dependent archaebacteria. In: Wolfe RS, Woese CR (eds) The bacteria, vol 8. Academic, New York, pp 85–170
Stetter KO, König H, Stackebrandt E (1983) *Pyrodictium* gen. nov., a new genus of submarine disc-shaped sulphur reducing archaebacteria growing optimally at 105 °C. Syst Appl Microbiol 4:535–551
Stetter KO, Fiala G, Huber G, Huber R, Segerer A (1990) Hyperthermophilic microorganisms. FEMS Microbiol Rev 75:117–124
Stockhausen F (1907) Okologie, "Anhäufungen" nach Beijerinck. Institut für Gärungsgewerbe, Berlin
Strange RE (1976) Microbiol response to mild stress. Meadowfield Press, Durham
Strength WJ, Isani B, Linn DM, Williams FD, Vandermolen GE, Laughon BE, Krieg NR (1976) Isolation and characterization of *Aquaspirillum fascilus* sp. nov., a rod-shaped, nitrogen-fixing bacterium having unusual flagella. Int J Syst Bacteriol 26:253–268
Strohl WR, Larkin JM (1979) Enumeration, isolation, and characterization of *Beggiatoa* from freshwater sediments. Appl Environ Microbiol 36:755–770
Stumm CK, Zwart KB (1986) Symbiosis of protozoa with hydrogen-utilizing methanogens. Microbiol Sci 3:100–105
Sverdrup HW, Johnson MW, Fleming RH (1942) The oceans. Prentice-Hall, London
Swart-Füchtbauer H, Rippel-Baldes A (1951) Die baktericide Wirkung des Sonnenlichtes. Archiv für Mikrobiologie 16:358–362
Switalski L, Hook M, Beachey EH (1989) Molecular mechanisms of microbial adhesion. Springer, New York
Tannock GW (1990) The Microecology of lactobacilli inhabiting the gastrointestinal tract. Adv Microb Ecol 11:147–171
Tansey MR, Brock TD (1978) Microbial life at high temperatures: ecological aspects. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, London, pp 159–216
Tempest DW, Neijssel OM (1976) Microbial adaptation of low-nutrient environments. In: Dean ACR, Ellwood DC, Evans CGT, Melling J (eds) Continuous culture 6: applications and new fields. Ellis Horwood, Chichester, pp 283–296
Tempest DW, Neijssel OM (1979) Eco-physiological aspects of microbial growth in aerobic nutrient-limited environments. Adv Microb Ecol 2:105–153
Tempest DW, Meers JL, Brown CM (1970) Synthesis of glutamate in *Aerobacter aerogenes* by a hitherto unknown route. Biochem J 117:405–407
Tempest DW, Meers JL, Brown CM (1973) Glutamate synthetase (Gogat): a key enzyme in the assimilation of ammonia by prokaryotic organisms. In:

Prusiner S, Stadtman ER (eds) The enzymes of glutamine metabolism. Academic, New York, pp 167–182
Thiele HH (1968) Die Verwertung einfacher organischer Substrate durch Thiorhodaceae. Archiv für Mikrobiologie 60:124–138
Tindall BJ, Trüper HG (1986) Ecophysiology of the aerobic halophilic archaebacteria. Syst Appl Microbiol 7:202–212
Torma AE (1977) The role of *Thiobacillus ferrooxidans* in hydrometallurgical processes. Adv Biochem Engin 6:1–37
Torsvik VL, Goksoyr J (1978) Determination of bacterial DNA in soil. Soil Biol Biochem 10:7–12
Trüper HG (1969) Bacterial sulfate reduction in the Red Sea hot brines. In: Degens ET, Ross DA (eds) Hot brines and recent heavy metal deposits in the Red Sea. Springer, New York, pp 262–271
Trüper HG (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
Tschech A (1989) Der anaerobe Abbau von aromatischen Verbindungen Forum. Mikrobiologie 12:251–264
Tuovinen OH, Kelly DP (1972) Biology of Thiobacillus ferrooxidans in relation to the microbiological leaching of sulphide ores. Zeitschrift für Allgemeine Mikrobiologie 12:311–346
Tuttle JH, Randles CI, Dugan PR (1968) Activity of microorganisms in acid mine water. I. Influence of acid water on aerobic heterotrophs of a normal stream. J Bacteriol 95:1495–1503
Uesugi I, Yajima M (1978) Oxygen and "strictly anaerobic" intestinal bacteria. I. Effects of dissolved oxygen on growth. Zeitschrift für Allgemeine Mikrobiologie 18:287–295
Umbreit TH, Pate JL (1978) Characterization of the holdfast region of wild-type cells of holdfast mutants of *Asticcacaulis biprosthecum*. Arch Microbiol 118:157–168
Unterman PM, Baumann P, McLean DL (1989) Pea aphid symbiont relationships established by analysis of 16S rRNAs. J Bacteriol 171:2970–2974
van Gemerden H (1974) Coexistence of organisms competing for the same substrate: an example among the purple sulfur bacteria. Microb Ecol 1:104–119
van Niel CB (1932) On the morphology and physiology of the purple and green sulphur bacteria. Archiv für Mikrobiologie 3:1–112
van Niel CB (1936) On the metabolism of the Thiorhodaceae. Archiv für Mikrobiologie 7:323–358
van Niel CB (1955) The microbe as a whole. In: Waksman SA (ed) Perspectives and horizons in microbiology. Rutgers University Press, New Brunswick, pp 3–12
van Veen WL, Mulder EG, Deinema MH (1978) The Sphaerotilus-Leptothrix group of bacteria. Microbiol Rev 42:329–356
Vedder A (1934) *Bacillus alcalophilus* sp. nov., benevens enkle ervaringen met sterk alcalische voedingsbodems. Antonie van Leeuwenhoek J Microbiol Serol 1:141–147
Veldkamp H (1970) Enrichment cultures of prokaryotic organisms. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 3A. Academic, London, pp 305–361
Veldkamp H (1976) Continuous culture in microbial physiology and ecology patterns of progress. Meadowfield Press, Durham
Veldkamp H, Jannasch HW (1972) Mixed culture studies with the chemostat. J Appl Chem Biotechnol 22:105–123
Veldkamp H, van den Berg G, Zevenhuizen LPTM (1963) Glutamic acid production by *Arthrobacter globiformis*. Antonie van Leeuwenhoek J Microbiol Serol 29:35–51
Voelz H, Dworkin M (1962) Fine structure of *Myxococcus xanthus* during morphogenesis. J Bacteriol 84:943–952
Völker H, Schweisfurth R, Hirsch P (1977) Morphology and ultrastructure of *Crenothrix polyspora* Cohn. J Bacteriol 131:306–313
Walsby AE (1970) The gas vesicles of aquatic prokaryotes Regulations between structure and function in the prokaryotic cell. In: Society for General Microbiology Symposium, vol 28. Cambridge University Press, London, New York, Melbourne, pp 327–357
Walsby AE (1975) Gas vesicles. Ann Rev Plant Physiol 26:427–439
Walsby AE (1977) The gas vacuoles of blue-green algae. Sci Am 237:90–97
Wangersky PJ (1976) The surface film as a physical environment. Ann Rev Ecol Syst 7:161–176
Watson SW, Waterbury JB (1969) The sterile hot brines of the Red Sea. In: Degens ET, Ross DA (eds) Hot brines and recent heavy metal deposit in the Red Sea. Springer, New York, pp 272–281
Weibull C (1960) Movement. In: Gunsalus IC, Stanier RY (eds) The bacteria, vol 1, Structure. Academic, New York, pp 153–205
Weimer PJ, Zeikus JG (1977) Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. Appl Environ Microbiol 33:289–297
Weiner RM, Devine RA, Powell DM, Dagasan L, Moore RL (1985) *Hyphomonas oceanitis* spec. nov., *H. hirschiana* spec. nov. and *H. jannaschiana* spec. nov. Int J System Bact 35:237–243
Weiss RL (1973) Attachment of bacteria to sulphur in extreme environments. J Gen Microbiol 77:501–507
Weller R, Ward DM (1989) Selective recovery of 16 S rRNA sequences from natural microbial communities in the form of cDNA. Appl Environ Microbiol 55:1818–1822
Whittaker RH, Levin SA, Root RB (1973) Niche, habitat and ecotope. Am Nat 107:321–338
Whittenbury R, Davies SL, Davey JF (1970) Exospores and cysts formed by methane-utilizing bacteria. J Gen Microbiol 61:219–226
Wiegel J, Schlegel HG (1976) Enrichment and isolation of nitrogen fixing hydrogen bacteria. Arch Microbiol 107:139–142
Wiegel J, Wilke D, Baumgarten J, Opitz R, Schlegel HG (1978) Transfer of the nitrogen fixing hydrogen bacterium *Corynebacterium autotrophicum* (Baumgarten et al.) to *Xanthobacter* gen. nov. Int J Syst Bacteriol 28:573–581
Wiley WR, Stokes JL (1963) Effect of pH and ammonium ions on the permeability of *Bacillus pasteurii*. J Bacteriol 86:1152–1156
Williams AG (1986) Rumen holotrich ciliate protozoa. Microbiol Rev 50:25–49
Windberger E, Huber R, Trincone A, Fricke H, Stetter KO (1989) *Thermotoga thermarum* sp. nov. and *Thermotoga neapolitana* occurring in African continental sulfataric springs. Arch Microbiol 151:506–512
Winfrey MR, Zeikus JG (1977) Effect of sulfate on carbon and electron flow during microbial methanogenesis in fresh-water sediments. Appl Environ Microbiol 33:275–281
Winogradksy SN (1926) Études sur la microbiologie du sol. Sur les microbes fixateurs d'azote. Annales de l'Institut Pasteur 40:455–520
Winogradsky SN (1925) Etudes sur la microbiologie du sol. I. Sur la m;aaethode. Annales de l'Institut Pasteur 39:299–354
Winogradsky SN (1947) Principles de la Microbiologie Ecologique. Antonie van Leeuwenhoek J Microbiol Serol 12:5–15
Winogradsky SN (1949) Microbiologie du sol: problèmes et méthodes. Masson et Cie, Paris
Wirsen CO, Jannasch HW (1975) Activity of marine psychrophilic bacteria at elevated hydrostatic pressures and low temperatures. Mar Biol 31:201–209
Wirsen CO, Jannasch HW (1978) Physiological and morphological observations on Thiovulum sp. J Bacteriol 136:765–774
Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271
Wolfe RS (1960) Observations and studies of *Crenothrix polyspora*. J Am Water Works Assoc 52:915–918
Wolin MJ (1976) Interactions between $H_2$-producing and methane-producing species. In: Schlegel HG, Gottschalk G, Pfennig N (eds) Microbial production and utilization of gases. Goltze, Göttingen, pp 141–150
Wolin MJ, Miller TL (1982) Interspecies hydrogen transfer: 15 years later. ASM-News 48:561–565
Woodroffe RCS, Shaw DA (1974) Natural control and ecology of microbial populations on skin and hair. In: Skinner FA, Carr JG (eds) The normal microbial flora of man. Academic, London, pp 13–34
Yayanos AA, Dietz AS (1983) Death of a hadal deep-sea bacterium after decompression. Science 220:497–498, Washington
Yayanos AA, Dietz AS, Van Boxtel R (1979) Isolation of a deep-sea barophilic bacterium and some of its growth characteristics. Science 205:808–810, Washington
Yayanos AA, Dietz AS, Van Boxtel R (1981) Obligately barophilic bacterium from the Marianas Trench. Proc Nat Acad Sci USA 78:5212–5215

Yayanos AA, Dietz AS, Van Boxtel R (1982) Dependence of reproduction rate on pressure as a hallmark of deep-sea bacteria. Appl Environ Microbiol 44:1356–1361

Zaitsev Yu-P (1971) Vinogradov KA (ed) Marine neustonology. Keter Press, Jerusalem

Zebe E (1977) Anaerober Stoffwechsel bei wirbellosen Tieren Vorträge der Rheinisch-Westfälischen Akademie der Wiseenschaften

Zeikus JG, Wolfe RS (1972) *Methanobacterium thermoautotrophicus* sp. n., an anaerobic, autotrophic, extreme thermophile. J Bacteriol 109:707–713

Zhao H, Wood AG, Widdel F, Bryant MP (1988) An extremely thermophilic *Methanococcus* from a deep-sea hydrothermal vent and its plasmids. Arch Microbiol 150:178–183

Zillig W, Stetter KO, Schäfer W, Janekovic D, Wunderl S, Holz I, Palm P (1981) Thermoproteales: a novel type of extremely thermoacidophilic anaerobic archaebacteria isolated from Icelandic solfataras Zlb. Bakt. Hyg., I. Abt. Orig. C 2:205–227

Zillig W, Schnabel R, Tu J, Stetter KO (1982) The phylogeny of archaebacteria, including novel anaerobic thermoacidophiles in the light of RNA polymerase structure. Naturwiss 69:197–204

ZoBell CE (1946) Marine microbiology, a monograph on hydrobacteriology. Chronica Botanica, Waltham

ZoBell CE (1970) Pressure effects of morphology and life processes. In: Zimmermann A (ed) High pressure effects on cellular processes. Academic, London, pp 85–130

ZoBell CE, Johnson FH (1949) The influence of hydrostatic pressure on the growth and viability of terrestrial and marine bacteria. J Bacteriol 57:179–189

ZoBell CE, Morita RY (1957) Barophilic bacteria in some deep sea sediments. J Bacteriol 73:563–568

# 4 Origin of Life: RNA World Versus Autocatalytic Anabolist

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## Introduction

The deep past of the Earth is unobservable. Therefore, the problem of the origin of life can only be approached by a theory. Such theories are scientific to the extent that they explain known facts of biology, predict unknown facts of chemistry, agree with unproblematic geohistoric theories, and are compatible with universal laws of physics and chemistry. These requirements follow from Popper's methodology of science (Popper 1959). Only two theories on the origin of life appear to be detailed enough for an evaluation along these lines:

1. The *RNA world* theory (Gesteland et al. 1999; Cech 2011; Szostak 2011) postulates an origin with a population of RNA-like molecules that replicated in a cold prebiotic broth of activated nucleotides, and served at the same time as catalysts (ribozymes) for replication and for metabolic transformations.
2. The *autocatalytic anabolist* theory (Wächtershäuser 1992, 2006, 2010) posits a hot origin by transition metal-catalyzed carbon fixation that evolved by organic products bonding as catalyst-promoting ligands to metal centers (also termed FeS world in view of the dominance of Fe and S).

These two theories are here compared with regard to their relative empirical merit. Selected references are cited for reasons of being very relevant, very recent, or very forgotten.

## Sources for Nutrients and Energy

Nutrients required by the *anabolist* theory must have always been available as volcanic-hydrothermal fluids ($CO_2$, CO, COS, $CH_3SH$, HCN, $P_4O_{10}$, $H_2S$, $H_2$, $N_2$, $NH_3$) with chemical potentials due to quenching to low temperatures of, say 100–160 °C. They show multiple interrelated functions by contact to crustal transition metal minerals, notably by the reaction $FeS + H_2S \rightarrow FeS_2 + 2H^+ + 2e^-$ ($E^{\circ\prime} = -620$ mV) as electron source for metabolic reactions and $N_2$-fixation (Dörr et al. 2003).

The *RNA world* theory postulates a "prebiotic broth" with activated nucleotides formed from preaccumulated organic compounds. Experiments typically follow a modular scheme: nucleosides made from bases and ribose with subsequent activation. Besides requiring mutually incompatible reaction conditions (Shapiro 1986), they typically result in intractable mixtures (Schwartz 2008), if they are chemically feasible at all. For solving some of these problems an alternative synthesis of pyrimidine nucleotides departs from an aqueous, phosphate-buffered solution of cyanamide with sequential additions of pure glycolaldehyde, pure glyceraldehyde, and pure cyanoacetylene (Powner et al. 2009)—reactants that pose more problems than they solve. In view of these problems it has been suggested that the first reproducing "informational" molecules were proteins, with thioesters as energy source for polycondensation (De Duve 1991). This idea of a "protein world" has never matured to a theory.

## Induction, Reproduction, Evolution

Both approaches assume a molecular autocatalytic system that forms de novo in a certain chemical context (induction), and subsequently promotes its own synthesis (reproduction) with modifications (evolution). For the original *RNA world* theory induction means de novo formation of a population of oligo- or polynucleotides in a prebiotic broth. There is experimental support for nucleotides that are activated by nonprebiotic imidazole-phosphate groups in combination with clay catalysis (Prakash et al. 2009). A spontaneous onset of replication and evolution is too improbable to be expected under such experimental conditions, the required strand separation by temporary elevation of temperature (Kuhn 1972) adding to the problem. For such polycondensations liquid water is a problem, notably at elevated temperatures. Experiments with preestablished RNAs

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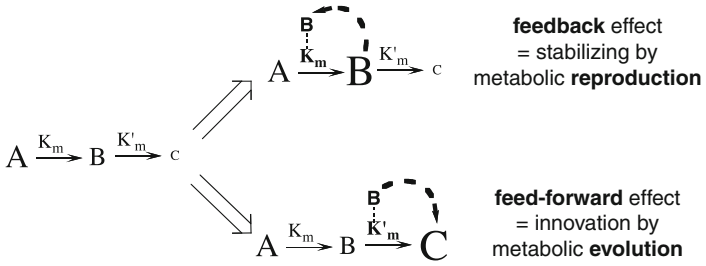


**Fig. 4.1**
**Product ligand effects (*A*: starting material; *B, C*: organic products; $K_m$, $K'_m$: transition metal catalysts; *dashed arrow*: ligand effect; *double arrow*: evolutionary change)**

and nucleotides in the absence of a sophisticated catalyst (mainly due to Orgel and his school) have provided something like a conclusive falsification. As a consequence the experimental program shifted to in vitro evolution experiments with the aim to create catalytic RNA (a ribozyme) that could serve as catalyst for its own replication from nucleoside triphosphates. Experiments in this direction were successful, but the demonstrated requirement of long ribozymes of at least 165 nucleotides renders their emergence in a prebiotic broth extremely improbable (Johnston et al. 2001). From there the research program shifted to RNA replication inside lipid vesicles (Szostak et al. 2001). For an origin in a prebiotic broth this move compounded the problems further by requiring a high content of lipids, while in another sense it leads us to the realization that the genetic machinery must have originated inside a cellular entity.

According to the *anabolist* theory reproduction and evolution are induced by the reductive formation of low-molecular-weight organic compounds from volcanic C1-compounds by transition metal catalysis. For these redox reactions liquid water is a benefit rather than a detriment. Key to understanding the possibility of reproduction and evolution at this simple molecular level is the recognition that some of the produced organic compounds have functional groups, by which they may become ligands of transition metal centers, increasing their catalytic activity. There are two effects of an organic product B (*Fig. 4.1*): Feedback effect on catalyst for producing the same organic product B (metabolic reproduction); or feed-forward effect on catalyst for producing another organic product C (metabolic evolution). New ligands improve catalysts, which then elicit new metabolic reactions with new products that become further new ligands and so forth. In this manner the metabolism evolves by terminal extension or lateral branching of its pathways, by closing reaction cycles, by recruiting new nutrients or new catalytic metals, or by conquering new environments. By these effects the metabolism self-expands at an accelerating pace, resulting in an avalanche breakthrough.

Demonstrated induction reactions comprise formation of RSH from $CO_2$/FeS/$H_2S$ (Heinen and Lauwers 1996); RSH and RCOOH from CO/NiS/$H_2S$ (Loison et al. 2010); COS, $CH_3SH$, and $CH_3COSCH_3$ from CO/NiS/$H_2S$ via COS (Huber and Wächtershäuser 1997); $CH_3$-CO-COOH from CO/FeS/$C_9H_{19}SH$ at 200 MPa and 250 °C (Cody et al. 2000), from $Co_2(CO)_8$/$CH_3SH$/CO (Huber et al. 2012), or from $Ni(OH)_2$/(CO,$H_2$)/CN (Huber et al. 2012); peptides from α-amino acids by activation with CO/$H_2S$ via COS (Huber et al. 2003); and α-hydroxy/amino acids (or derivatives) from HCN/$Ni(OH)_2$/(CO, $H_2$) (Huber et al. 2012). Phenyl-acetate and phenyl-lactate (via phenyl-pyruvate) form by reaction of benzyl mercaptan with HCN/CO/$Ni(OH)_2$. Yield of the latter reaction increases by addition of Gly or Ala, which demonstrates the chemical possibility of a feed-forward effect (Huber et al. 2012).

## Evolutionary Connex with Extant Genetic Machinery

The *RNA world* theory assumes a phase of life, whereby the catalytic biopolymers consisted exclusively of ribozymes. Hence the name "RNA world." With the appearance of the ur-ribosome began the translation of RNA sequences into protein sequences. This gave rise to an RNA-protein world with a step-by-step replacement of ribozymes by enzymes (enzymatic take-over). Much later, RNA as replicating informational molecule was replaced by DNA, with ribonucleotide reductases appearing first as ribozymes to be replaced later by enzymes.

By the *anabolist* theory life began with a *direct* mechanism of evolution, organic products, e.g., peptides, feeding directly as ligands into metallocatalysts. Originally, the components of nucleic acids (ribose, bases, nucleosides, nucleotides) earned their keep also as ligands; and so did oligonucleotides giving rise to catalytic metalloribonucleo particles. The earliest oligonucleotide sequences were not hereditary. Nevertheless base pairing would have started soon, first as structural determinant and later as a new kind of catalysis by sequence complementation. Now information transfer and its fidelity began to become important, ushering in a massively parallel evolution of the incipient genetic machinery: from sequence copying to replication and from peptide formation to translation, drawing in more and more amino acids, and generating ever longer coded peptide/protein ligands. DNA as stable information storage molecule may well have followed transient catalytic RNA on its heels. In this way an *indirect* mechanism of evolution appeared, with accidental sequence mutations being translated into modified proteins for metalloenzymes, followed by selection: life's great randomizer invented by life itself.

Much of the early history of life is recorded in the structures of the ribosome (Harish and Caetano-Anollés 2012). By generating mutually compatible rooted phylogenetic trees directly from rRNA substructures and from ribosomal protein domain structures in accordance with cladistic principles it was possible to establish a timeline of ribosomal evolution, correlating substructures with ages. This revealed a gradual coevolution of ribosomal RNAs and ribosomal proteins with the ribosomal center for peptide bond formation having less antiquity than the ribonucleoprotein ratchet of the small subunit. The authors suggest that these oldest regions of the ribosome are "relics of an ancient ribonucleoprotein world," which corresponds nicely with the structural complementarity of mutual scaffolding of RNAs and peptides before the dawn of the genetic machinery (Carter and Kraut 1974). These findings contradict the central thesis of the *RNA world* theory that ribozymes "invented" peptide bond formation, while in accordance with the *anabolist* theory RNA is predated by metallopeptides.

## Evolutionary Connex with Extant Cellular Structures

A multicomponent genetic machinery could not become established prior to cellularization. For the *RNA world* theory this means a nutritional paradox: RNA replication requires ionic or hydrophilic nutrients, for which a lipid membrane is impermeable. A lipid vesicle that is competent for containment would cause starvation and a nonstarving, leaky vesicle would be ineffective as means for containment.

According the *anabolist* theory the metabolism evolves to the point where it generates lipids intrinsically. These are inert and accumulate on the mineral surfaces with the effect of surface lipophilization and reduced activity of $H_2O$ and $H_3O^+$. Thereby all kinds of condensation reactions are protected from the hydrolytic effect of liquid water (collective feed-forward effect) including the formation of lipids by condensation reactions (collective feedback effect). Further accumulation will eventually lead first to mineral-supported bilayer membranes and later to semi-cellular structures bounded partly by the mineral base and partly by a self-supporting lipid membrane. At this stage the surface metabolism coexists with a membrane metabolism and a cytosol metabolism and the genetic machinery can begin to come into the world of life. Ultimately autonomous lipid vesicles are formed with mineral inclusions. A nutritional paradox does not arise, since bilayer lipid membranes are permeable for the small, neutral inorganic nutrients.

In view of their low water solubility and strong bonding to transition metal centers relatively short alkyl mercaptans would have been effective initial lipids, soon to be replaced by long-chain fatty acids. All extant forms of lipid biosynthesis may go back to a common precursor (*Figs. 4.2*, *4.3*). However, primordial condensation of acetyl-thioester (AT) requires conversion to an anionic intermediate ($^-$AT) by deprotonation in α-position. This in turn requires a strong base, which cannot exist under aqueous conditions. Alternatively, the anionic

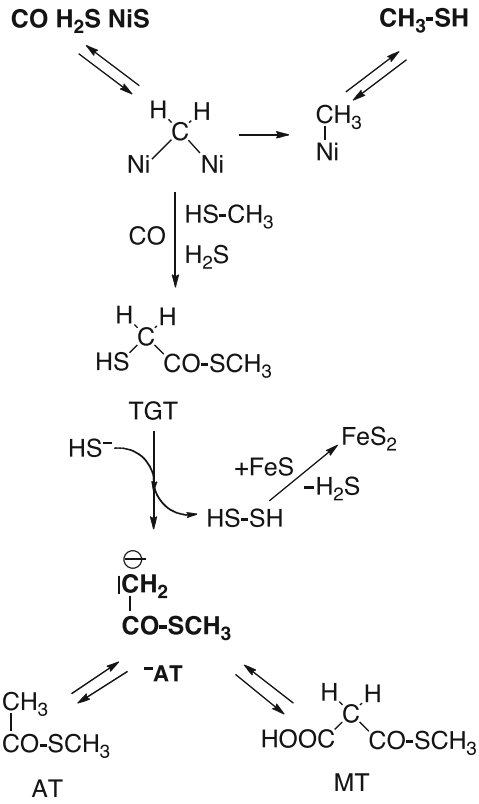


**Fig. 4.2**
**Formation of deprotonated anionic acetyl-thioester ($^-$AT)**

intermediate ($^-$AT) may form by a redox reaction with $FeS/H_2S$ involving a nucleophilic attack of a sulfhydryl anion on the α-mercapto group of thioglycolyl thioester (TGT) (reductive elimination of HSSH en route to pyrite). Desulfurization of thioglycolic acid by $FeS/H_2S$ (Blöchl et al. 1992) and conversion of thioglycolic acid to acetyl-amide by $FeS/H_2S$ (Keller et al. 1994) support this proposal. TGT might form reductively from CO and $NiS/H_2S$. The anionic intermediate ($^-$AT) may in turn become reversibly carboxylated to form malonyl-thioester (MT).

For lipid synthesis (*Fig. 4.3*) the anionic intermediate ($^-$AT) would engage in a subsequent nucleophilic attack on the thioester group of acetyl-thioester (AT) to generate 3-keto-butyryl-thioester (3KBT) en route to fatty acid lipids by the chain length arithmetic 2 + 2 + 2 + 2 + 2 + 2 = 12 (pathway A); or on the thioester group of malonyl-thioester (MT) to generate 3-keto-glutaryl-thioester (3KGT), 3-hydroxy-glutaryl-thioester (3HGT), glutaconyl-thioester (GT) and its decarboxylation product, an anionic form of butenyl-thioester ($^-$BT) en route to fatty acid lipids by the chain length arithmetic 4 + 4 + 4 = 12 (pathway B); or on the keto group of 3-keto-butyryl-thioester (3KBT) to generate 3-hydroxy-3-methyl-glutaryl-thioester (3H3MGT) and 3-methyl-glutaconyl-thioester (3MGT) and its decarboxylation product, an anionic form of 3-methyl-butenyl-thioester ($^-$3MBT) en route to isoprenoid acid lipids by the chain length arithmetic 4 + 4 + 4 = 12 (pathway C). Pathways B and C are favored, because they require fewer steps than pathway A.

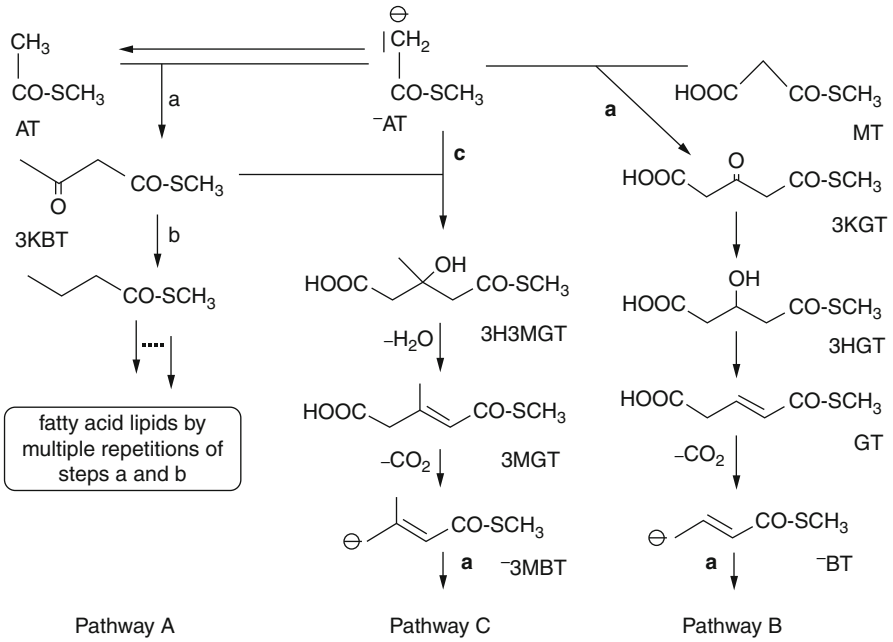


**Fig. 4.3**
**Family of anionic species for lipid synthesis (a = condensation with thioester group; b = elimination of oxo group; c = condensation with keto group)**

They are vinylogs of pathway A. Pathway B generates unsaturated straight-chain fatty acids and pathway C generates unsaturated branched isoprenoid acids. All three carbanionic species may co-react.

A crucial consequence of the formation of closed cellular structures is the automatic formation of an ion ($H^+$) gradient across the membrane in conjunction with the appearance of electron transport chains and a new kind of energy source: chemiosmosis. It would at first have operated by coupling exergonic and endergonic redox reactions while coupling with membrane-bound ATPases would have appeared later.

## Evolutionary Connex with Extant Metabolism

Extant metabolisms are highly integrated with multifunctional catalysts and tightly interconnected pathways and cycles. By the *anabolist* theory integration must have been at work from the start. If a ligand effect promotes one pathway at the expense of all others, the overall metabolism is weakened. This generates a selective force for multi-catalytic ligand effects. Also, the less integrated periphery of the metabolism would evolve faster than its center.

If a new pathway branches from a preestablished metabolism and a product of said new pathway feeds back into its own synthesis, we speak of an egotistic or parasitic catalyst. It may be seen as the earliest form of a virus (virulyst). A dramatically different situation arises, if a product of a branch pathway exhibits a "dual feedback" effect: an "egotistic feedback" effect directly promoting its own synthesis *and* a compensatory "altruistic feedback" effect promoting the preexisting metabolism (vitalizer effect). It may persist by virtue of its egotistic feedback component even though the chemical conditions for its de novo synthesis may have vanished. Therefore, with every new dual feedback, the metabolism switches into a new, relatively stable expanded state. This constitutes a stabilizing memory effect. Extant cellular organisms are replete with vitalizers, e.g., DNA, ribosomes, translocases, coenzymes.

Autotrophic metabolisms have a particular need for integration, since carbon flows from volcanic C1-compounds into organic carbon skeletons of increasing size (C2 → C3 → C4). In an expanding metabolism the traffic through these early steps increases drastically and quickly causes a bottleneck, notably in case of limitations of nutrients, transition metals, reductants, and ligand feedback. This metabolic burden is today relieved by a special kind of stabilizing feedback: The reductive citric acid cycle (RCC), which is really a thioester cycle. It is autocatalytic, doubling with every turn, and potentially goes back to a very early primitive version, perhaps with thio acids instead of thioesters and $FeS/H_2S$ as reductant/catalyst (Wächtershäuser 1990). In view of its complex arithmetic (2 + 1 + 1 + 1 + 1 → 6 → 4 + 2), a simpler version (2 + 1 + 1 → 4 → 2 + 2) has been suggested, with cleavage at the malyl-thioester(acid) level (Wächtershäuser 1998a). Other extant autocatalytic $CO_2$-fixation pathways (Fuchs 2011) share with the RCC acetyl-thioester and succinyl-thioester as common pivot points and promise deep insights into early metabolic evolution. In extant metabolisms a protein cycle is operative with needed proteins being synthesized by the ribosome while unneeded proteins are

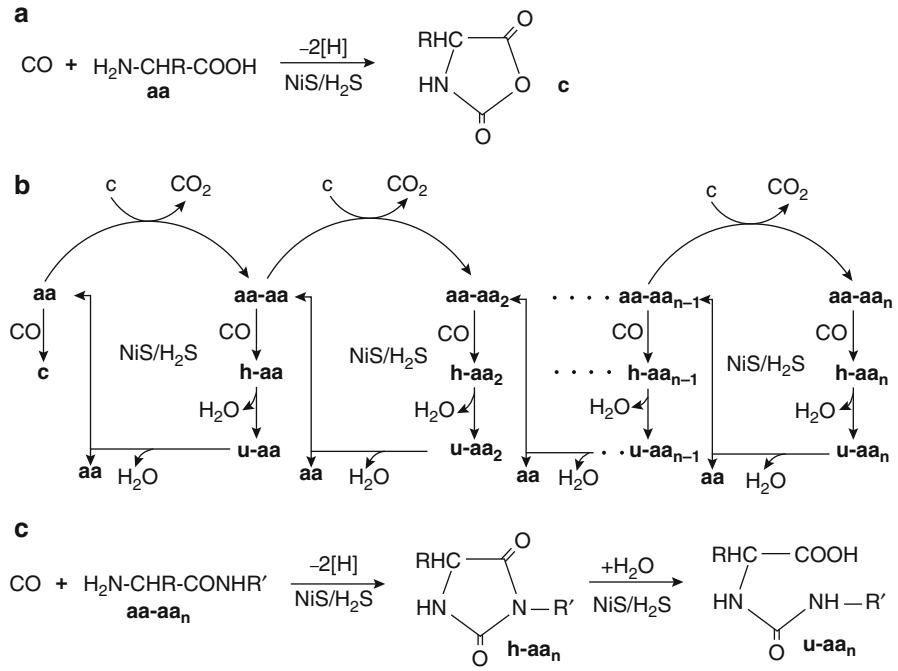


**Fig. 4.4**

**Peptide cycle: (a) activating reaction; (b) concatenated cycles; (c) degradative reactions (aa = amino acid unit; h = hydantoin ring; u = urea group; NHR′ = -(NH-CHR-CO)$_n$-OH)**

degraded by the proteasome. Early peptides are in an analogous situation. They are synthesized and degraded by the same catalytic system, NiS/CO/$H_2S$, thus forming a concatenation of peptide cycles (Fig. 4.4) and a dynamic peptide library, which is self-selecting since peptides are not only stabilized by binding to pyrite surfaces (Schreiner et al. 2011) or other catalytic transition metal species but also promote their own synthesis.

Homochirality unites biochemistry and sets it apart from the abiotic world. How did this occur? Extant RNA is homochiral, yet the *RNA world* theory assumes a prebiotic broth of racemic or prochiral nucleotides, which means that any broth-born RNA would have been heterochiral and fallen stillborn from the copying process. By the *anabolist* theory the racemic state of organic products is a benefit, because it broadens the space of feedback possibilities. Homochirality is a later "invention" of life, perhaps forced in complementary fashion for L-amino acids and D-ribose by RNA-peptide complexation (Carter and Kraut 1974). By the influence of chiral pyrite crystals enantioselectivity may be predetermined and not a frozen accident (Wächtershäuser 1992).

## Temperature, Phylogeny, and Time Frame of Life

The discovery of hyperthermophilic Bacteria and Archaea has made room for the previously unthinkable possibility that the last universal common ancestor (LUCA) and by extension the origin of life may have been hyperthermophiles that evolved down the temperature scale with polyphyletic appearance of mesophiles (Woese 1987).

The *RNA world* theory is committed to a mesophilic origin of life and makes the implicit assumption of symmetry between thermally upward and downward evolution. Temperature is all-pervasive, profoundly influencing all structures and processes. A thermally upward adaptation could only occur for one (the weakest) trait at a time, and by tiny increments. This has the consequence that thermally upward evolution of whole organisms is extremely slow (probabilistically forbidden). Thermally downward evolution, by contrast, proceeds by opportunistic losses of thermal traits, e.g., losses of CysS-metal bonds as cross-linkers: evolution by degeneration rather than adaptation. Let us look at three main consequences: (1) Species that appear at successively higher branch points in the tree of life will have successively fewer thermal stabilizers. (2) Organisms with lower thermal status will harbor some traits of a higher thermal status (thermal relics). (3) A recently acquired low thermal status may be readily reversed (thermal atavism). This explains a striking fact concerning ancient proteins with metal-binding pairs of CXXC signatures. Some of these have the same CXXC signatures in all homologues, while others (e.g., aminoacyl synthetases and ribosomal proteins) show on average a decrease (but not an increase!) of frequency of these signatures in the direction of higher branch points. In the course of thermally downward evolution such thermal traits may readily deteriorate one C at a time, while in the course of thermally upward evolution the functionally required introduction of whole signatures (e.g., CXXC—CXXC)

into the right places by covariation would be highly improbable. The earliest peptides would have bonded to transition metals (Woese 1972), and longer ones to extended mineral surfaces, and self-supporting folding structures would first have been acquired by local cross-linking to metals and much later by a multitude of distributed weak group interactions.

Informational RNAs cannot fold well at high temperatures, which means that the *RNA theory* requires a mesophilic or psychrophilic origin (Moulton et al. 2000). The *anabolist* theory allows for an origin of RNA at high temperature. It merely assumes that the earliest RNAs were bonded to surfaces and only those with a proper sequence and high G + C content lifted off as well-folded, self-supporting structures stabilized by peptides. Later, with the formation of longer ribosomal proteins by the incipient genetic machinery and of enzymes for stabilizing RNAs by secondary modifications, the restriction of the RNA sequence space became more and more relaxed. By comparative analysis of RNA sequences (from which highly variable positions were eliminated) it was possible to reconstruct ancestral RNAs, with very high G + C contents for LUCA and also for the common ancestors of all bacteria, and all archaea, thus strongly supporting a hyperthermophilic origin of life (Di Giulio 2003).

The oldest microfossils with remains of cell walls of uniform thickness, carbon content with $^{13}C$-depletion and pyrite deposits have been dated to 3.4 billion years ago (Wacey et al. 2011), more than a billion years after the origin of the Solar System (4.567 billion years ago). It appears from careful analysis of Hadean crystals of extremely stable $ZrSiO_4$ (zircon) found preserved as detrital particles in much younger sedimentary host rocks, that as early as ~4.4 billion years ago the Earth had cooled down sufficiently to bear an ocean of liquid water (Wilde et al. 2001; Mojzsis et al. 2001), which must have been hot. For the *anabolist* theory this finding makes room for an early Hadean origin of life, at a time when volcanic-hydrothermal sites must have been rampant, thus providing enough time for biochemical/cellular evolution. The theory is compatible with and even requires a hot origin for kinetic reasons (Stockbridge et al. 2010). Hadean life could have survived intense bombardment with very large impactors simply by protection inside rocks thrown into orbit around the Earth (Cockel 2006). An *RNA world* origin would have had to wait until the Earth cooled down and heavy bombardment had subsided, i.e., until after 3.8 billion years ago. Thus, by the empirical facts of geology an *anabolist* origin had plenty of time while the *RNA world* theory is squeezed for time.

Attempts to employ comparative analysis of polymer sequences for reconstructing LUCA (as clue for the origin of life) are hampered by lateral gene transfer, notably of genes concerning the metabolism (Woese 1998). The situation may be remedied by comparing bacterial and archaeal genomes for the presence of more or less extended conserved *clusters* of genes for multicomponent information processing systems. Lateral transfer of a cluster of several genes encoding components of such systems is expected to lead to functionally impaired or corrupted hybrid systems. Therefore, lateral transfer of such gene clusters is statistically forbidden. Now, by overlapping alignment of gene clusters of different organisms, it was possible to reconstruct a hypothetical segment of the genome of LUCA (Wächtershäuser 1998b). It comprises genes for transcription, translation, and protein translocation. This means that LUCA had already a cellular lipid membrane, an organized DNA genome, RNA, genetic code, modern sets of bases and amino acids, and an extended metabolism for supporting all that.

The above results support the proposal that organisms at the dawn of LUCA were "pre-cells" in the sense of being modern in almost every respect, except for an intense lateral transfer of metabolic genes, but not of genes for the genetic machinery (Kandler 1998). Owing to the primitivity of its enzymes LUCA must have generated its chiral phosphoglycerol lipids as racemates. Racemic lipids are able to form stable membranes with a patchwork of chirally segregated lipid domains and with cellular structures undergoing frequent fusions and fissions as in rapidly growing *Thermococcus coalescens* (Kuwabara et al. 2005), thereby generating a first subset of pre-cells with a predominance of one lipid enantiomer and a second subset of pre-cells with a predominance of the other lipid enantiomer. Between these two subsets fusions would have been less probable than within each subset. They would have served as placeholders for the later independent emergence of the phylogenetic domains of the Bacteria and Archaea by the appearance of enantioselective enzymes for lipid synthesis—origin of speciation preordained in the universal laws of physical chemistry. From this perspective the Eukarya are seen as the result of an endosymbiosis between a population of bacterial host cells and a population of pre-cells as guests, that later turned into the cell nucleus (Wächtershäuser 1992, 2003, 2006, 2010).

## Explanatory Power and Explanatory Fallacies

*Explanatory power*: The *anabolist* theory is based on the heuristic of biochemical retrodiction (common precursor functions for disparate successor functions), which generates automatically a backward convergence toward the pioneer organism of life and conversely explanatory power for numerous traits: concentric pattern of metabolism; FeS-proteins; metalloenzymes; organic ligands; inorganic ligands; organic sulfhydryl groups with catalytic or redox functions; CO-fixation; multifunctionality of catalysts. Extant amino acid activation by ATP and synthetases may be seen as reversal of presumptive primordial formation of ATP by energy transfer from amino acids activated by COS or $CO/H_2S$. Catalytic enzyme surfaces are seen as functional successors of catalytic mineral surfaces. Bioorganic carboxylate, thiolate, or phosphate groups are explained as derived from mineral bonding mediators. Finally, extant digital sequence information is explained by a takeover from primordial analog information with ribosomes operating as digital-to-analogue converters.

*Explanatory fallacies*: The theory of primordial RNA has no explanatory power, because the *explanans* is more complex than the *explanandum*. The proposal of ribozymes as sole biocatalysts

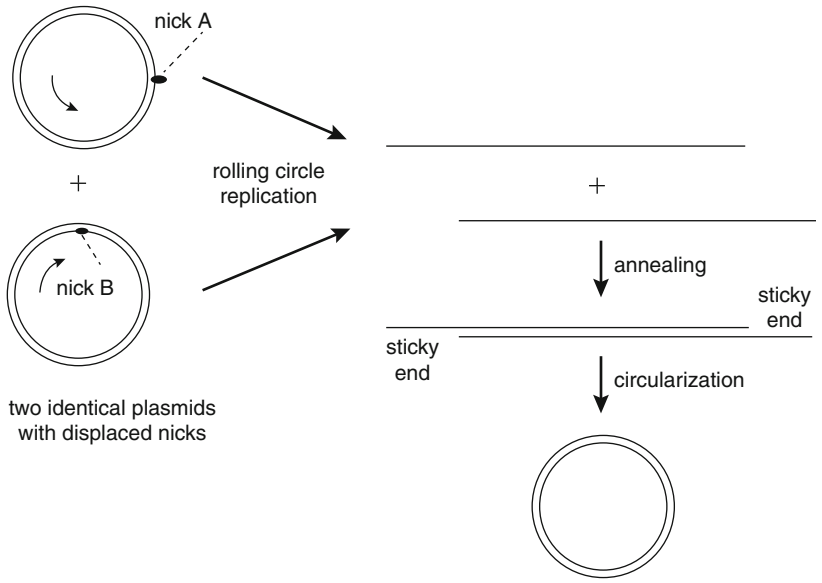


**Fig. 4.5**
**Hypothetical rolling circle replication of multiple LUCA DNA plasmids with nicks in both strands (takeover from rolling circle transcription by expression of ribonucleotide reductase)**

lost its explanatory power, with the discovery that the peptidyl transfer center is predated by other nucleoprotein regions of the ribosome. The proposal of RNA as hereditary polymer is explanatory for conversion of ribonucleotides to deoxyribonucleotides and for RNA in telomerases, but not for RNA primers since DNA primers work well in Archaea (Chemnitz Galal et al. 2012), and not for viral RNA genomes since viruses lack cytosol. Nucleotidyl rests of coenzymes are better explained as activation for covalent bonding to enzymes (Wächtershäuser 1992), rather than as relics of "handles" for RNA bonding. Finally, the nonrelation between archaeal and bacterial DNA replicases is better explained by rolling circle replication of LUCA DNA (Fig. 4.5) than by a LUCA RNA genome.

An imaginative proposal postulates a primordial seafloor mound that grew like a chemical garden by repeated precipitation and bursting of an FeS membrane at the boundary between hot, alkaline, $H_2S$-laden vent water, and cold, acidic, $Fe^{2+}$-laden ocean water, thereby acquiring an interior open-cell structure. Early evolution in its entirety supposedly happened inside this mound within the time frame of its growth: initial carbon fixation (oceanic $CO_2$ + vent $H_2$) at the bottom, later RNA catalysis and replication in the middle, LUCA at the top, with Bacteria and Archaea breaking loose and entering the ocean—formation of ATP by chemiosmosis across the top membrane serving as continuous source of fuel (Martin and Russell 2003). The proposal fails because (a) RNA cannot exist under the hot, alkaline interior conditions, (b) an experimental FeS layer "behaved more like a permeable reactive barrier than a membrane" (Rickard and Luther 2007) and thus cannot support chemiosmosis, and (c) experimental FeS cells, the main support of this proposal, turned out to be a freeze-drying artifact (Wächtershäuser 2006).

## Conclusion and Outlook

The *RNA world* theory posits the origin of life as highly improbable one-time event that is knowable only in principle. By contrast, the *anabolist* theory assumes an origin of life as rapid, chemically predetermined, singular, and directional process: a universal chemical law of life's origin. Fundamentally different experimental programs transpire from the two theories. Within the *RNA world* theory experiments have the role of rationalizing partial aspects of the theory. The *anabolist* theory by contrast has the ultimate experimental goal to recreate the pioneer organism in vitro and to watch it evolve.

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## References

Blöchl E, Keller M, Wächtershäuser G, Stetter KO (1992) Reactions depending on iron sulfide and linking geochemistry with biochemistry. Proc Natl Acad Sci USA 89:8117–8120

Carter CW, Kraut J (1974) A proposed model for interaction of polypeptides with RNA. Proc Natl Acad Sci USA 72:283–287

Cech TR (ed) (2011) The RNA worlds in context. Cold Spring Harb Perspect Biol. doi:10.1101/cshperspect.a006742

Chemnitz Galal W, Pan M, Kelman Z, Hurwitz J (2012) Characterization of the DNA Primase complex isolated from the archaeon, *Thermococcus kodakarensis*. J Biol Chem 287(20):16209–16219

Cockel CS (2006) The origin and emergence of life under impact bombardment. Phil Trans R Soc B 361:1845–1875

Cody GD, Boctor NZ, Filley TR, Hazen RM, Scott JH, Sharma A, Yoder HS Jr (2000) Primordial carbonylated iron-sulfur compounds and the synthesis of pyruvate. Science 289:1337–1340

De Duve C (1991) Blueprint for a cell: the nature and origin of life. Neil Patterson, Burlington

Di Giulio M (2003) The universal ancestor and the ancestor of bacteria were hyperthermophiles. J Mol Evol 57:721–730

Dörr M, Käßbohrer J, Grunert R, Kreisel G, Brand WA, Werner RA, Geilmann H, Apfel C, Robl C, Weigand W (2003) A possible prebiotic formation of ammonia from dinitrogen on iron-sulfide surfaces. Angew Chem Int Ed 42:1540–1543

Gesteland RF, Cech TR, Atkins JF (eds) (1999) The RNA world: the nature of modern RNA suggests a prebiotic RNA. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Fuchs G (2011) Alternative pathways of carbon dioxide fixation: insights into the early evolution of life? Annu Rev Microbiol 65:631–658

Harish A, Caetano-Anollés G (2012) Ribosomal history reveals origin of modern protein synthesis. PLoS One 7:e32776

Heinen W, Lauwers AM (1996) Organic sulfur compounds resulting from the interaction of iron sulfide, hydrogen sulfide and carbon dioxide in an anaerobic aqueous environment. Orig Life Evol Biosph 26:131–150

Huber C, Wächtershäuser G (1997) Activated acetic acid by carbon fixation on (Fe, Ni)S under primordial conditions. Science 276:245–247

Huber C, Eisenreich W, Hecht S, Wächtershäuser G (2003) A possible primordial peptide cycle. Science 301:938–940

Huber C, Kraus F, Hanzlik M, Eisenreich W, Wächtershäuser G (2012) Elements of metabolic evolution. Chem Eur J 18:2063–2080

Johnston WK, Unrau PJ, Lawrence MS, Glasner ME, Bartel DP (2001) RNA-catalyzed RNA-polymerization: accurate and general RNA-templated primer extension. Science 292:1319–1325

Kandler O (1998) The early diversification of life and the origin of the three domains: a proposal. In: Wiegel J, Adams MWW (eds) Thermophiles: the keys to molecular evolution and the origin of life. Taylor & Francis, London, pp 19–31

Keller M, Blöchl E, Wächtershäuser G, Stetter KO (1994) Formation of amide bonds without condensation agent and implications for the origin of life. Nature 368:836–838

Kuhn H (1972) Selbstorganisation molekularer systeme und die evolution des genetischen apparats. Angew Chem 84:838–862

Kuwabara T, Minaba M, Ogi N, Kammekura M (2005) *Thermococcus coalescens* sp. nov., a cell-fusing hyperthermophilic archaeon from Suiyo Seamount. Int J Syst Microbiol 55:2507–2514

Loison A, Dubant S, Adam P, Albrecht P (2010) Elucidation of an iterative process of carbon-carbon bond formation of prebiotic significance. Astrobiology 10:973–988

Martin W, Russell MJ (2003) On the origin of cells: an hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautotrophic prokaryotes, and from prokaryotes to nucleated cells. Phil Trans R Soc B 358:27–85

Mojzsis SJ, Harrison TM, Pidgeon RT (2001) Oxygen-isotope evidence from ancient zircons for liquid water at the Earth's surface 4.300 Myr ago. Nature 409:178–181

Moulton V, Gardner PP, Pointon RF, Creamer LK, Jameson GB, Penny D (2000) RNA folding argues against a hot-start origin of life. J Molec Evol 51:416–421

Powner MW, Gerland B, Sutherland JD (2009) Synthesis of activated pyrimidine ribonucleotides in prebiotically plausible conditions. Nature 459:239–242

Popper KR (1959) The logic of scientific discovery. Hutchinson, London

Prakash CJ, Aldersley MF, Delano JW, Ferris JP (2009) Mechanism of montmorillonite catalysis in the formation of RNA oligomers. J Am Chem Soc 131:13369–13374

Rickard D, Luther GW III (2007) Chemistry of iron sulfides. Chem Rev 107: 514–562

Schreiner E, Nair NN, Wittekindt C, Marx D (2011) Peptide synthesis in aqueous environments: the role of extreme conditions and pyrite mineral surfaces on formation and hydrolysis of peptides. J Am Chem Soc 133:8216–8226

Schwartz AW (2008) Intractable mixtures and the origin of life. In: Herdewijn P, Kisakürek MV (eds) Origin of life: chemical approach. Verlag Helvetica Chimica Acta, Zürich, pp 175–183

Shapiro R (1986) Origins: a sceptic's guide to the creation of life on Earth. Summit Books, New York

Stockbridge RB, Lewis CA Jr, Wolfenden R (2010) Impact of temperature on the time required for the establishment of primordial biochemistry, and for the evolution of enzymes. Proc Natl Acad Sci 107:22102–22105

Szostak JW (2011) An optimal degree of physical and chemical heterogeneity for the origin of life? Phil Trans R Soc B 366:2894–2901

Szostak JW, Bartel DP, Luisi PL (2001) Synthesizing life. Nature 409:387–390

Wacey D, Kilburn MR, Saunders M, Cliff J, Brasier MD (2011) Microfossils of sulphur-metabolizing cells in 3.4-billion-year-old rocks of Western Australia. Nature Geosci 4:698–702

Wächtershäuser G (1990) Evolution of the first metabolic cycles. Proc Natl Acad Sci USA 87:200–204

Wächtershäuser G (1992) Groundworks for an evolutionary biochemistry: the iron-sulfur world. Prog Biophys Mol Biol 58:85–201

Wächtershäuser G (1998a) The case for a hyperthermophilic, chemolithoautotrophic origin of life in an iron-sulfur world. In: Wiegel J, Adams MWW (eds) Thermophiles: the keyes to molecular evolution and the origin of life? Taylor & Francis, London, pp 47–57

Wächtershäuser G (1998b) Towards a reconstruction of ancestral genomes by gene cluster alignment. Syst Appl Microbiol 21:473–477

Wächtershäuser G (2003) From pre-cells to eukarya — a tale of two lipids. Mol Microbiol 47:13–22

Wächtershäuser G (2006) From volcanic origins of chemoautotrophic origin of life to bacteria, archaea and eukarya. Phil Trans R Soc B Lond 361: 1787–1808

Wächtershäuser G (2010) Chemoautotrophic origin of life: the iron-sulfur world hypothesis. In: Barton LL, Mandl M, Loy A (eds) Geomicrobiology: molecular and environmental perspective. Springer, Dordrecht, pp 1–35

Wilde SA, Valley JW, Peck WH, Graham CM (2001) Evidence from detrital zircons for the existence of continental crust and oceans on the Earth 4.4 Gyr ago. Nature 409:175–178

Woese CR (1972) The emergence of genetic organization. In: Ponnamperuma C (ed) Exobiology. North-Holland, Amsterdam, pp 301–341

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271

Woese CR (1998) The universal ancestor. Proc Natl Acad Sci USA 95:6854–6859

# 5 Morphological and Physiological Diversity

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## Introduction

Over 5,000 species and nearly 1,000 genera of isolated prokaryotes were tabulated as of 1999 (Garrity and Holt 2000). Moreover, studies examining 16S ribosomal DNA in natural populations have provided convincing evidence that these cultured organisms are just the "tip of the iceberg" with several entire phyla/divisions having no or few cultured representatives (Hugenholtz et al. 1998). Prokaryotic diversity is an immensely valuable resource—not only as a source of an almost infinite variety of metabolic capabilities, enzymes, and genes, but also as a veritable cornucopia of strategies for dealing with the world. If what we wish to understand is not only how an organism operates but also how what it does enables it to deal with an extremely variable and occasionally hostile environment, then the study of microbial diversity truly holds the answers.

Much attention has focused on understanding the properties of a relatively few organisms (such as *Escherichia coli* and some of its close relatives) in ever greater depth and detail that is unmatched in any other cellular organism. This understanding has tended to serve as a paradigm for understanding bacteria in general, and in a larger sense, all organisms. Its ultimate expression is attributed to Jacques Monod:SprS "What was true for *Escherichia coli* would be true for the elephant" (Judson 1979), although he was actually referring to an "old axiom" of A. J. Kluyver on the unity in biochemistry: "From elephant to butyric acid bacterium—it is all the same" (Singleton 2000). In any event, we have come to understand that though some fundamental strategies are indeed universal, each organism has evolved its own combination of tactics to arrive at the solution to its peculiar problems. It is thus appropriate, indeed often even useful, to continue the pursuit of the unusual microbe, so eloquently referred to by Ralph Wolfe in the foreword to this treatise.

Among the many rewards of this pursuit has been the recent recognition that, in addition to the diversity among the familiar and commonly recognized bacteria, there is yet a higher level of diversity. The existence of the Archaea has led to the recognition of a group of prokaryotes that not only does not share many of the properties considered fundamental to bacteria (Stanier and van Niel 1962) but also has some properties, most notably transcription, more closely resembling those of eukaryotes (Reeve et al. 1997).

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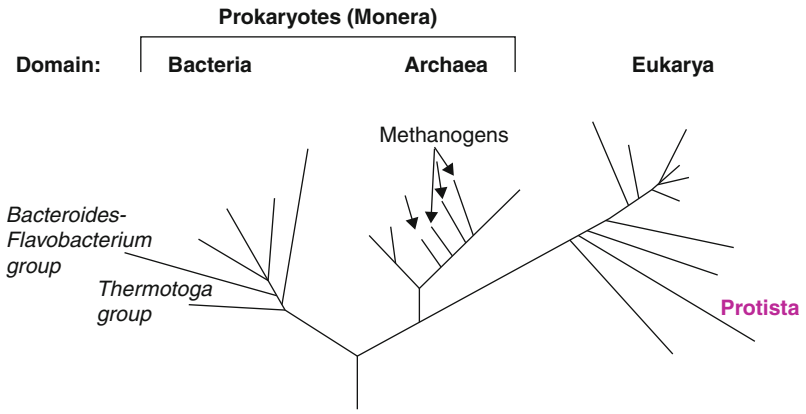


**Fig. 5.1**
**Phylogenetic tree based on 16S rRNA. The three domains are shown, as well as the five classically defined kingdoms (From Woese et al. 1990)**

To get a sense of the evolutionary diversity of prokaryotes, one need only examine the universal 16S rDNA tree (*Fig. 5.1*) to conclude that about two thirds of evolutionary diversity is prokaryotic, and indeed most of eukaryotic evolutionary diversity is also microbial, mainly consisting of protists (Sogin and Silberman 1998). Some "macrobiologists," notably the eminent evolutionary biologist Ernst Mayr (1998), insist that the phenotypic diversity of prokaryotes pales before that of eukaryotes, which includes "jellyfish, butterflies, dinosaurs, hummingbirds, yeasts, giant kelp, and giant sequoias." Woese in his reply (Woese 1998) justly states that Mayr is comparing apples and oranges, and that the human visual system has evolved to discern differences in plants and animals as a matter of survival. Whereas, as we will demonstrate presently, there is ample morphological variation in prokaryotes; their specialty is metabolic variation and adaptation to habitats and niches. Can eukaryotes use sulfur or a pesticide as their lunch, fix nitrogen, or grow in boiling water? Clearly not. Indeed, some of Mayr's naturalist colleagues at Harvard now appreciate the importance of prokaryotes. Stephen Jay Gould (1996) states that "on any reasonable or fair criterion, bacteria are—and always have been—the dominant form of life on earth," and E. O. Wilson speculates at the end of his autobiography (Wilson 1995) that were he to start over again in the twenty-first century, he would become a microbial ecologist.

It is not our intention in this chapter to construct a bestiary. Nor is it our goal to provide an exhaustive description of prokaryotic diversity; in effect, that is the entire content of this book. Our choice of examples of diversity has been selective, and this chapter shall be limited to using a variety of prokaryotes to illustrate the diversity of structural, physiological, and metabolic strategies used by bacteria to adapt to the world.

## Diversity in Cell Size

The conventional wisdom that prokaryotic cell diameters are typically near 1 μm, whereas eukaryotic cells are 10 μm or larger is generally true, but significant exceptions exist. Members of the Bacteria can show enormous diversity in cell size. For example, one of us works with a disk-shaped organism, *Dehalococcoides ethenogenes*, with a diameter of 0.4–0.5 μm and a height of 0.1–0.2 μm so that its volume is near 0.024 $\mu m^3$, about one twentieth that of *E. coli*. On the other hand, *Epulopiscium fishelsoni*, found in the gastrointestinal tracts of certain fish, are cigar-shaped cells with a diameter as great as 80 μm and lengths up to 600 μm (Angert et al. 1993). The volume of these cells is near 2 million $\mu m^3$, about eight orders of magnitude larger than of *D. ethenogenes*. Another example of prokaryotic gigantism, *Thiomargarita*, is a sphere (nearly a millimeter in diameter) composed of a thin layer of cytoplasm around a large internal vacuole in which nitrate, concentrated from the surrounding water, can reach concentrations as high as 0.8 M (Schulz et al. 1999). This nitrate-filled vacuole allows the organisms to oxidize sulfide under anaerobic conditions using the nitrate as a respiratory electron acceptor. Curiously, no examples of archaeal cells more than a few μm in diameter are known (*Fig. 5.2*).

An advantage of existing as micrometer-sized cells is a greater surface-to-volume ratio. For example, if one compares spherical cells with 1-μm and 10-μm diameters, it requires 1,000 of the smaller cells (ca. 0.5 $\mu m^3$) to equal the larger cell (ca. 500 $\mu m^3$) in volume. Moreover, the smaller cells have 10 times greater total surface area (ca. 3,140 $\mu m^2$) than the larger one (ca. 314 $\mu m^2$), allowing them to present more surface to the environment for uptake of scarce nutrients. The smaller size apparently obviates the need for the organellar compartmentation and internal membrane systems generally found in eukaryotes because molecules can traverse the distances in the prokaryotic cell more easily by diffusion. Indeed, the cells of *Epulopiscium* contain an extensive internal membrane system (Clements and Bullivant 1991), indicating that their large size necessitates such a system.

## Diversity in Cell Shape

The controversy between the pleomorphists and the monomorphists led to a view of bacterial morphology first

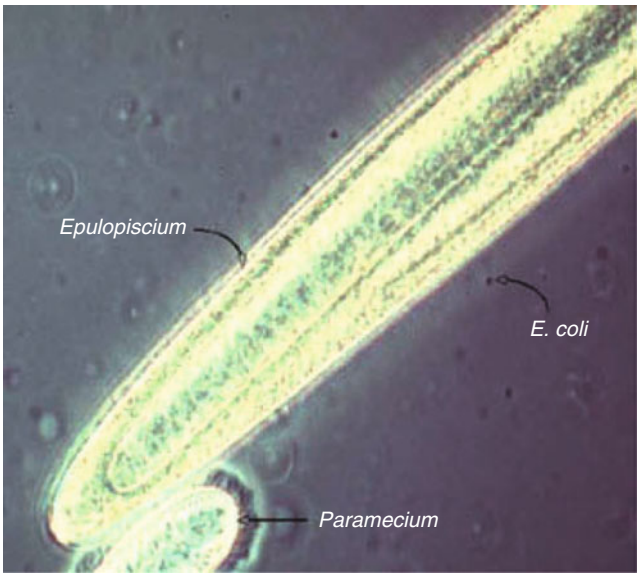


**Fig. 5.2**
**Comparison of *Epulopiscium* with cells of the protist *Paramecium* and *E. coli* (Photo courtesy of E. Angert)**

propounded by Ferdinand Cohn (1875) and hammered into a dogma by the subsequent work of Robert Koch. The classical textbook view of bacteria described them as spherical or cylindrical, the latter category divided between straight and curved rods. This view, based on the observations made on laboratory-grown cultures cultivated under conditions designed to result in a maximum growth rate and morphological uniformity, came to represent a picture of the "true" morphology of bacteria. It obscured the spectrum of enormous morphological diversity manifested by the bacteria. It also obscured the fact that that diversity was often the result of life cycles that represented, for bacteria, survival strategies in addition to those of maximizing growth rates.

The shape of a cell is not a trivial or casual aspect of its adaptation to its environment but is instead a strategic consequence of this adaptation. Nor is the shape of a cell limited in any fundamental way to a rod, coccus, or spiral. Ferdinand Cohn in 1875 divided bacteria into these three forms in one of the first systematic attempts to classify bacteria. It is now quite clear that the variety of cell shapes and groupings far exceeds those offered in early views. There are bacteria that are amorphous, ovoid, square, stellate, filamentous, or stalked (*Fig. 5.3*). They may be grouped as pairs, clumps, chains, rosettes, cuboid packets, flat squares, networks, mycelia, or fruiting bodies (*Figs. 5.4*, *5.5*, *5.6*).

What are the possible functions of cell shape and grouping? Certainly one of the most obvious relates to the surface-volume (S/V) ratio of a cell referred to above in the context of cell size. The sphere has the lowest possible S/V ratio, and as a cell becomes longer and thinner, its S/V ratio increases. One can imagine that a cell such as the oligotrophic *Caulobacter*, whose lifestyle involves the scavenging of extremely low concentrations of nutrients, will benefit from a rod shape, and from the consequent increase in surface area available for nutrient uptake. It has also been suggested (Pate and Ordal 1965) that the characteristic stalks of *Caulobacter* (and perhaps also *Asticcacaulis*) function to increase the surface area of the cell yet further. Conversely, one may suggest that organisms that characteristically find themselves under conditions of ample nutrient levels would not be disadvantaged by low S/V ratios and may even find spherical shapes advantageous for other reasons. For example, one could imagine that an organism whose cell surface was subject to assault by antibodies or other host-defense factors might evolve a coccal shape that would minimize the amount of accessible surface.

It is conceivable that the shape of a cell may also be related to optimal hydrodynamic properties. It may not be a coincidence that motile organisms are rarely spherical; the number of flagellated cocci is quite small. Gliding bacteria are generally rod shaped (*Fig. 5.6*) although an interesting exception to this is found in *Isosphaera pallida*, a budding, chain-forming, gliding coccus (*Fig. 5.4*). In addition, the spiral shape of organisms such as spirochetes may be causally related to the ability of the organism to screw its way through a relatively highly viscous medium. In fact, it has been shown that the motility of spirochetes is enhanced at higher than normal viscosities (Canale-Parola 1978).

It was appropriate at one time to view any departure from the rod-coccus-spiral morphology as a reflection of abnormal conditions. It is now quite clear that there are a variety of cell morphologies, each of which is uniquely appropriate for the ecological niche of that cell.

## Diversity in Cell Grouping

It is also interesting to think about the functions of cell groupings. Is it possible that the characteristic clustering of the staphylococci further reduces the amount of accessible cell surface? And what is the conceivable function of the striking tendency of *Thiopedia* and *Lampropedia* (the latter a chemotrophic aerobe and the former a phototrophic anaerobe) to grow as sheets of cells (*Fig. 5.3*)?

One instance where a characteristic grouping has been rationalized is in the myxobacteria. These organisms undergo a complex life cycle and feed on insoluble macromolecular debris by excreting a complex of hydrolytic enzymes. They are thus at the mercy of diffusion both of their enzymes away from the cell and of the hydrolyzed nutrients back to the cell. However, the complex life cycle of these organisms which causes them to periodically collect into densely packed fruiting bodies, as well as to form swarming, gliding masses of cells (*Fig. 5.6b*), guarantees a high local concentration of periodically excreted enzymes. This has been shown to optimize their feeding (Rosenberg et al. 1977) and has been referred to as a "microbial wolf-pack effect" (Dworkin 1973).

Though the collection of resting cells compressed in the fruiting body represents the potential for releasing a high-density population once germination occurs, one can only

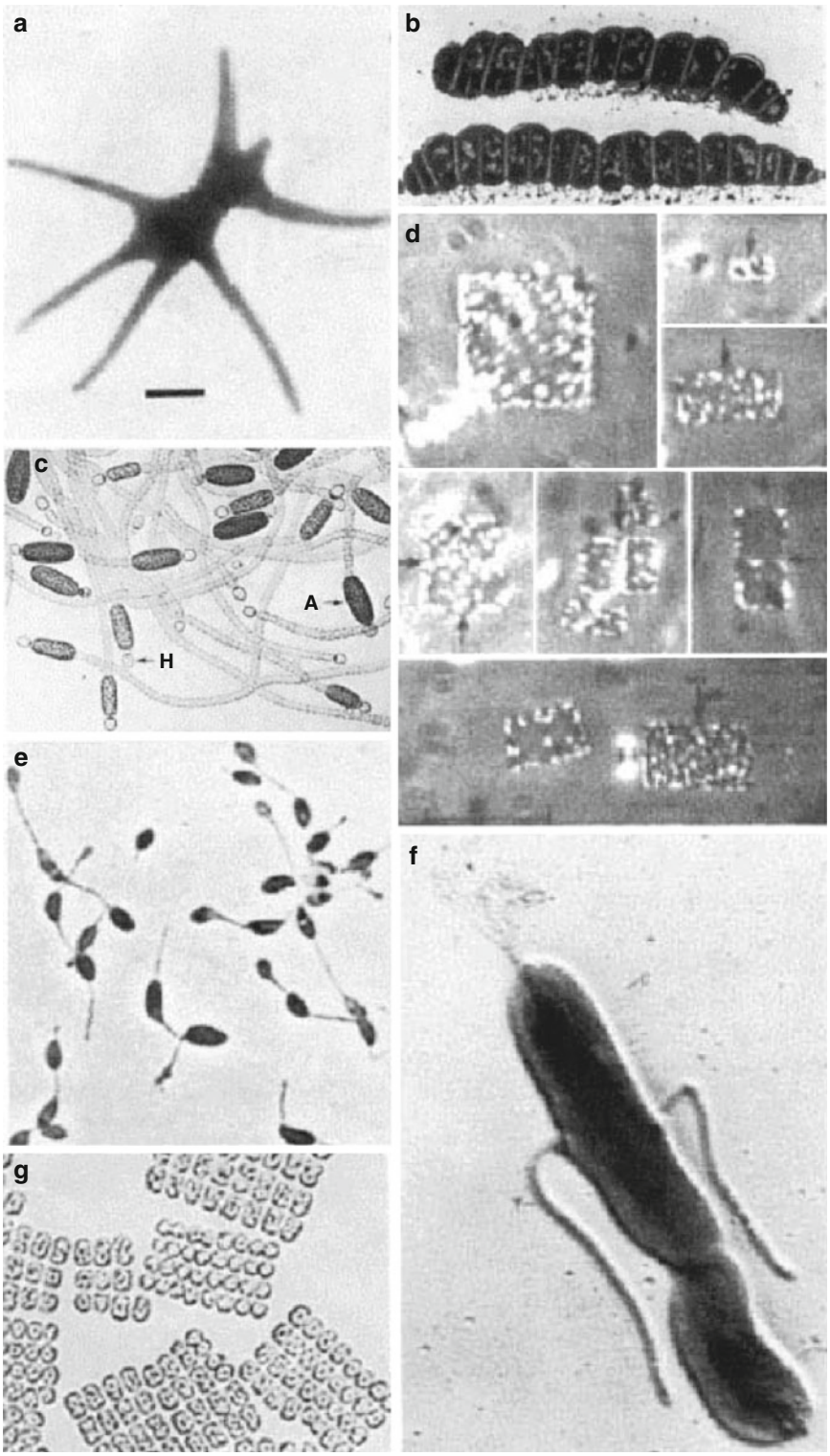


**Fig. 5.3**
**A variety of cell shapes found among prokaryotes. (a) *Prosthecomicrobium*. (b) *Simonsiella*. (c) *Cylindrospermum*. (d) Square bacterium. (e) *Rhodomicrobium*. (f) *Asticcacaulis*. (g) *Thiopedia rosea* (a from Staley 1968; b from Pangborn et al. 1977; c from Stanier et al. 1981; d from Walsby 1980, with permission; f from Pate and Ordal 1965, with permission; e courtesy of P. Hirsch; and g courtesy of N. Pfennig)**

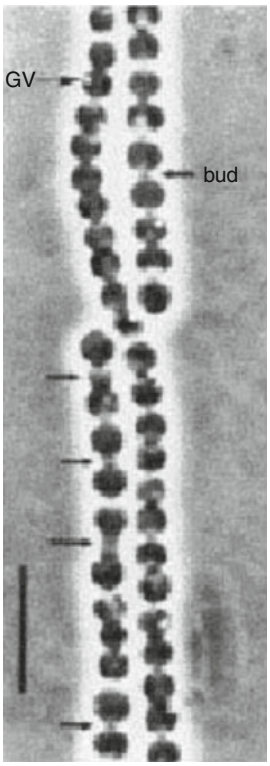


**Fig. 5.4**
***Isosphaera pallida*** **(From Giovannoni et al. 1987)** ***Arrows*** **denote new cells growing as buds forming between cells, and gv denotes gas vesicles**

speculate as to the function of fruiting body complexity. It is feasible, however, that each germinated cluster of cells has an optimal size, and that the number of cells in each of the sporangiole packages arrayed on the fruiting body determines this size.

There are other groupings that are familiar, for example, chain formation by cocci or by rods (Fig. 5.7a, b), clusters (e.g., *Stomatococcus*, Fig. 5.7c), sheathed cells (e.g., *Sphaerotilus*, Fig. 5.7e), mycelium formation (e.g., *Streptomyces*, Fig. 5.7f), rosette formation (e.g., *Caulobacter*, Fig. 5.7d), cuboidal packets (e.g., *Sarcina*, Fig. 5.7g), and networks (e.g., *Rhodomicrobium*, Fig. 5.3). These groupings are probably not casual or accidental properties of the cells but are likely functional and should be viewed as other manifestations of the diversity of prokaryotic adaptation to the environment. Understanding how these various groupings contribute to a cell's fitness lingers as one of the challenges of structure-function studies.

## The Cell Surface

The interface between a bacterial cell and its surroundings is where the cell will first have to deal with a variable environment. It is thus the part of the bacterial cell where one might expect to find the greatest diversity. It is the cell surface that determines the nature of attachment to a substrate or to another cell. The cell surface also determines what gets into and out of the cell and the nature of that transport process.

## Diversity in Capsules

Microbiologists have always had a fondness for working with organisms that grow in liquid in a nicely dispersed state. This has tended to obscure the fact that, in nature, most bacteria are not growing in a dispersed state but are clinging tenaciously to a substratum as a clump of cells or in a biofilm. Many organisms are surrounded by a more or less amorphous layer of slime, generally designated as the glycocalyx.

Furthermore, as was pointed out originally by Whittenbury and Dow (1977) and later emphasized by Costerton et al. (1986), the physiological properties of planktonic bacteria growing in a dispersed state are substantially different from those of the same cells when they are in their sessile state, embedded in a matrix of glycocalyces as pure- or mixed-culture biofilms (Costerton et al. 1999).

The principal component of the glycocalyx may be either polypeptide, as is the case with a number of species of *Bacillus*, or polysaccharide, characteristic of both Gram-positive and Gram-negative organisms. These layers have a variety of functions depending on the organism and the nature of the layer. They serve in some cases to facilitate attachment to surfaces, either cellular or otherwise, and the nature of the capsule must, in some way, determine the kind of substratum to which the cell can attach.

The capsule has been shown to interfere with the function of the phagocytic cells of the immune system and may form a barrier to phage adsorption. Cook and Colvin (1980) have shown that the cellulosic fibers produced by *Acetobacter* serve to maintain these highly aerobic organisms at the liquid-air interface. Finally, it has been suggested that the hygroscopic quality of the glycocalyx may help a cell to resist desiccation. Based on such a variety of functions that a glycocalyx may play, it is no surprise that the composition of this structure varies so widely.

Another aspect of capsular diversity relates to the role of a capsule in interfering with the ability of phagocytic cells to engulf their bacterial prey. Certainly the ability of the pneumococcus to manifest a variety of capsular polysaccharide types plays an important role in the evasion of host defenses by the bacterium; the generation of diversity of the cell surface is a general strategy for compromising host-defense mechanisms.

## Cell Wall Diversity

In Stanier and van Niel's classic description of the prokaryotic cell (Stanier and van Niel 1962), one of the few positive characteristics of prokaryotes (as opposed to lack of nucleus, mitochondria, etc.) was the presence of a muramic acid-containing peptidoglycan cell wall. Indeed, because this structure is not present in eukaryotes, its synthesis is a primary target of several

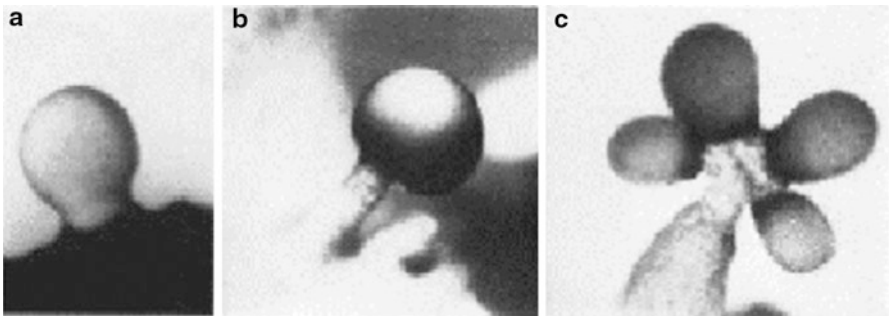


**Fig. 5.5**
**Fruiting bodies of myxobacteria. (a) *Myxococcus fulvus*. (b) *Myxococcus stipitatus*. (c) *Stigmatella aurantiaca* (a and b from Reichenbach and Dworkin 1981b; c courtesy of H. Reichenbach)**

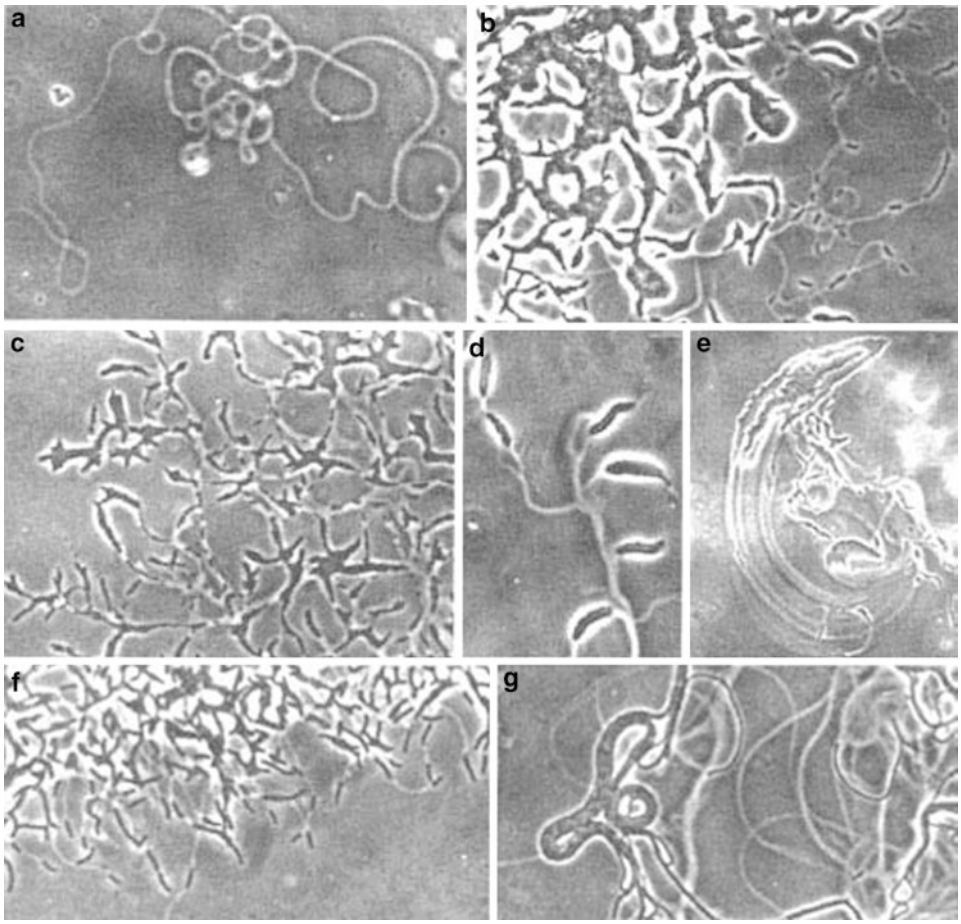


**Fig. 5.6**
**Gliding bacteria. (a) *Nannocystis exedens*. (b) *Myxococcus fulvus*. (c) *Cytophaga* sp. (d) *Flexibacter elegans*. (e) *Vitreoscilla stercoraria*. (f) *Lysobacter* sp. (g) *Herpetosiphon giganteus* (From Reichenbach and Dworkin 1981a)**

important antibiotics, such as the penicillin/cephalosporin group. At that time, cell walls were divided into Gram-positive, with its thick peptidoglycan layer, and Gram-negative, with a thin peptidoglycan layer and outer membrane (Beveridge 1995). Stanier and van Niel were aware of mycoplasmas and similar organisms lacking a cell wall. It is now clear that though nearly all members of the Bacteria do indeed have a muramic acid-containing cell wall, many have protein cell walls, such as planktomycetes and chlamydia (Weisburg et al. 1986). The Gram-negative cell wall seems to be the default phenotype in the Bacteria, being found in most branches including the deeply branching Aquificae and Thermotogae. Moreover, even some

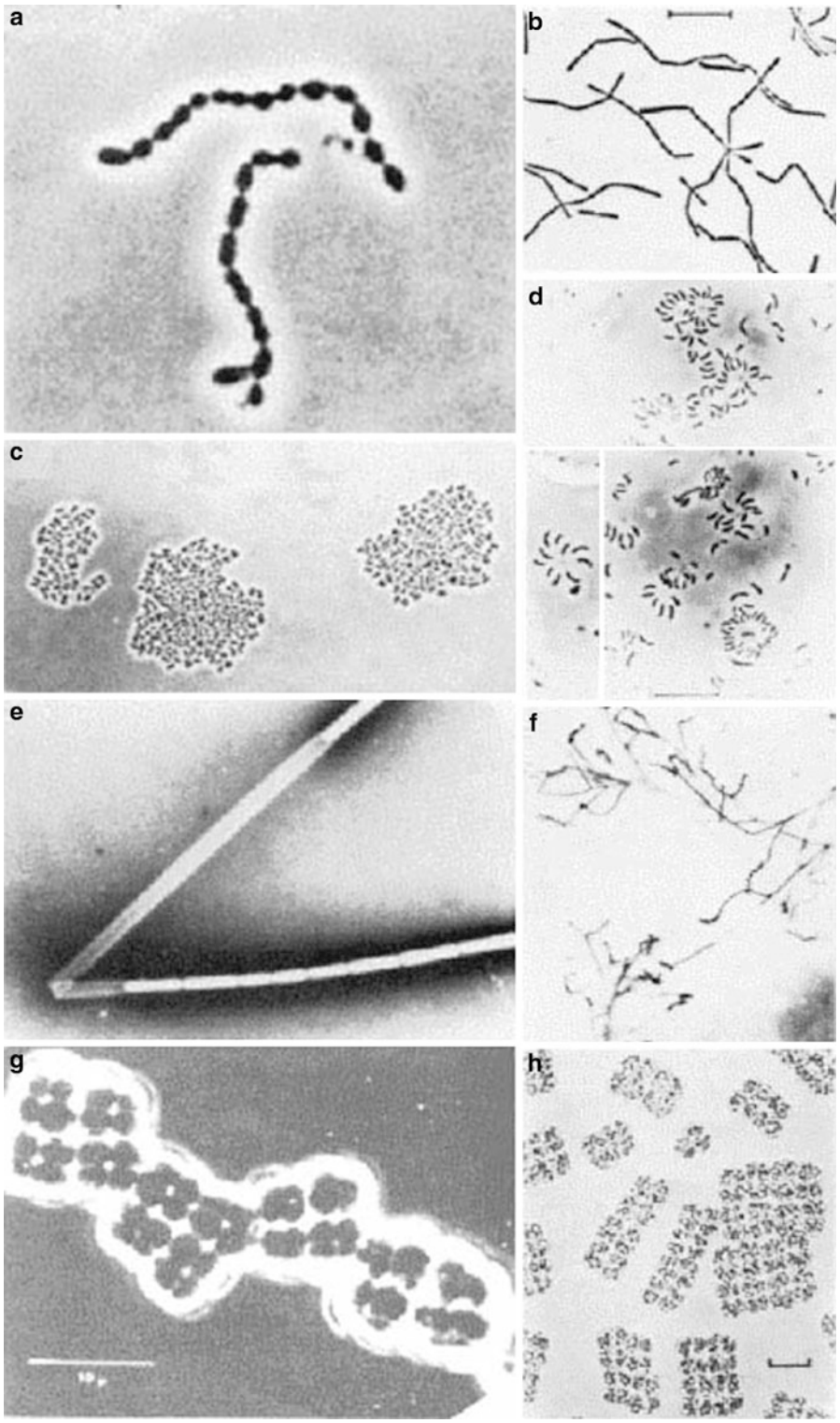


**Fig. 5.7**

**A variety of cell arrangements among the prokaryotes. (a) Phase-contrast micrograph of chains of *Streptococcus lactis*. (b) Chain of rod-shaped cells of *Lactobacillus gasseri*. (c) Irregular cluster of *Stomatococcus mucilaginosus*. (d) Rosette of *Caulobacter*. (e) Motile rods within a sheath, *Sphaerotilus natans*. (f) Branched filament, *Streptomyces* sp. (g) Three-dimensional packet of cocci, *Sarcina maxima*. (h) Two-dimensional packet of cocci, *Amoebobacter pedioformis* (a courtesy of T. D. Brock; b from Kandler and Weiss 1986; c from Schleifer 1986; d from Poindexter 1964; e from Stokes 1954; f from Kutzner 1981; g from Holt and Canale-Parola 1967; and h from Eichler and Pfennig 1986)**

deep branches of the Firmacutes contain organisms (such as *Sporomusa* and *Desulfotomaculum*) with Gram-negative cell wall structures. On the other hand, there are those who propose that the Gram-positive cell wall is the default structure (Gupta 1998).

Arguably, the default cell wall architecture in the Archaea is the protein S-layer, a paracrystalline two-dimensional array of protein subunits (Sleytr et al. 1993). S-layers also are found outside the cell wall in many bacteria. In the genera *Methanosarcina* and *Halococcus*, there is a thick fibrous polysaccharide layer outside the S-layer, apparently serving as a corset to prevent lysis at relatively low osmolarities (Kandler 1994; Sowers et al. 1993). In two methanogenic orders, the Methanobacteriales and Methanothermales, the cell walls consist of a thick peptidoglycan layer called "pseudomurein" which causes them to stain Gram-positive. Pseudomurein is similar to eubacterial murein but differs in a number of interesting aspects. It contains *N*-acetyl talosamineuronic acid instead of *N*-acetyl muramic acid, the carbohydrate that, along with *N*-acetyl glucosamine, represents the carbohydrate backbone of murein. The amino acids in the peptide chain of the archaebacteria are all L-isomers and the glycosidic linkage is α-1,3 rather than −1,4.

## Motility

Motility by multicellular eukaryotes serves a number of different functions. It enables an organism to escape from a predator or some other threat, it facilitates movement toward a potential mate, it allows developmental movement, and perhaps most importantly, it allows an organism to seek out and move toward a supply of food.

In the case of prokaryotes, it seems unlikely that cells can perceive the presence of predators such as protozoa, leukocytes, slime molds, phage, or *Bdellovibrio* sufficiently in advance to allow them to exercise an avoidance strategy. Bacteria, however, can detect gradients of repellent chemicals and respond by moving away (see below).

With regard to mating, it does not seem that motility to facilitate mating interactions occurs among the bacteria. In those cases where a mating pheromone has, in fact, been demonstrated (Dunny et al. 1978), the participating organisms are nonmotile and depend on random motion to effect contact. The function of the pheromone is to prepare the organisms for the events involved in adhesion and gene transfer. Prokaryotic movement to accomplish a developmental event seems to be limited to the myxobacteria, where it allows the cells to maintain a high population density and to move into aggregation centers as a prelude to fruiting body formation (Spormann 1999).

The most common function of motility among the prokaryotes seems to be to allow the organisms to position themselves optimally in their microenvironment, for example, to move along a concentration gradient toward a food source or away from a repellant (see Chap. 15, "Bacterial Behavior" in Vol. 2). The notion of a concentration gradient has implicit in it the condition that the substance in question is soluble rather than particulate, and that the milieu is thus an aqueous one. This serves to rationalize the observation that all the bacteria for which chemotaxis has been demonstrated are motile by means of flagella, with which they swim through an aqueous milieu. In fact, it is reasonable to suggest that the only conceivable function of swimming motility in bacteria is to allow the cell to travel along a perceived concentration gradient. The importance for chemotaxis and motility to the hunt for food is underlined by the finding that many naphthalene-degrading *Pseudomonas* strains possess plasmids which carry both the necessary biodegradation genes and genes encoding chemotaxis toward naphthalene (Grimm and Harwood 1997), thereby forming a biodegradative package. Similarly, prokaryotes can position themselves in a gradient of light, a phenomenon called "phototaxis."

### Swimming Motility

In all cases where they have been examined carefully, flagella in the Bacteria are fundamentally similar. They are usually composed of one, and in some cases two or three, species of a self-assembling protein, generically designated as "flagellin." This is attached, via a hook-shaped adaptor, to a motor-like basal body whereby the flagellar filament is inserted into the cell membrane. The flagellin subunits are translocated down the center of the flagellar shaft to the distal growth point where flagella are synthesized. An interesting adaptive use of this system is type III secretion found in pathogens, in which an assembly with protein subunits homologous to those in the flagellar filament is used to inject toxic proteins directly into eukaryotic host cells (Macnab 1999). Diversity is manifested by the number and arrangement of the flagella around the cell. This varies from the polarly flagellated cell with a single flagellum (*Fig. 5.8*) to the profusely and peritrichously flagellated cells (*Fig. 5.11*). In between, there are cells with bipolar flagellation (*Fig. 5.9*) or with polar tufts of flagella (*Fig. 5.10*).

A most unusual style of flagellation is found among the spirochetes. Rather than being freely turning propellers, the flagella are enshrouded by an outer cell envelope. It has been suggested (Berg 1976) that their motion within this outer sheath generates a torque that, in combination with the helical structure of the cell, propels the cell in a screw-like fashion. It has been shown that, for certain of the spirochetes (Canale-Parola 1978), flagellar motility is enhanced in a high-viscosity medium. This peculiar variation on flagellar organization may have evolved in response to high-viscosity environments that would hamper or prevent ordinary flagellar motility, allowing the spirochetes to move through viscous media like a corkscrew through a cork. Interestingly, spirochete motility seems to have at least three modes: swimming forward, swimming backward, and flexing (Fosnaugh and Greenberg 1988).

Only flagellated prokaryotes move, in effect, via a rotating propeller principle. In this sense it is completely unlike the movement of conventional eukaryotic flagella, whose action is

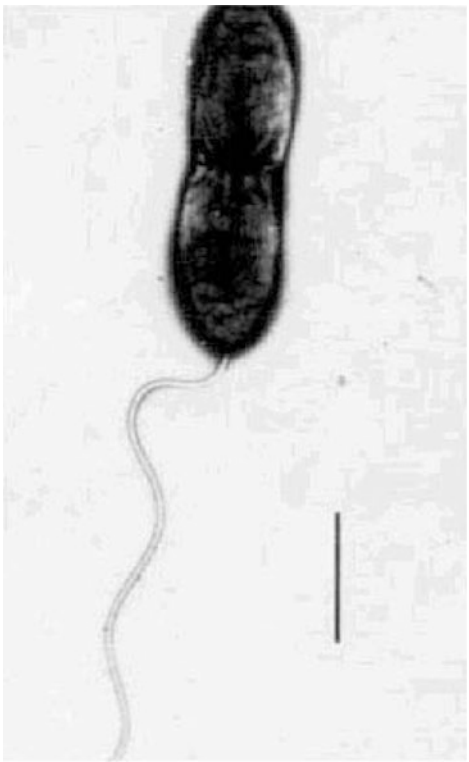

Fig. 5.8
Polarly flagellated cell of *Pseudomonas andropogonis*. Bar = 0.2 μm (From Palleroni 1984)

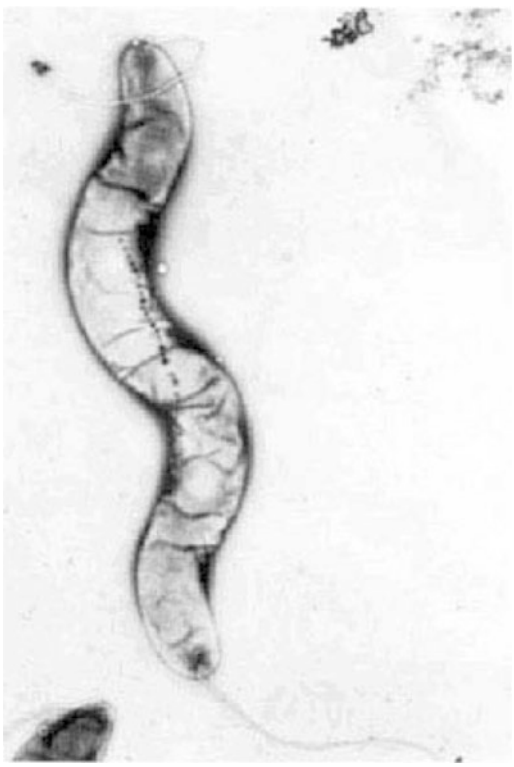

Fig. 5.9
Bipolarly flagellated cell of a magnetotactic spirillum (From Balkwill et al. 1980)

Fig. 5.10
Flagellar fascicle of *Spirillum volutans* (From Kreig 1984)

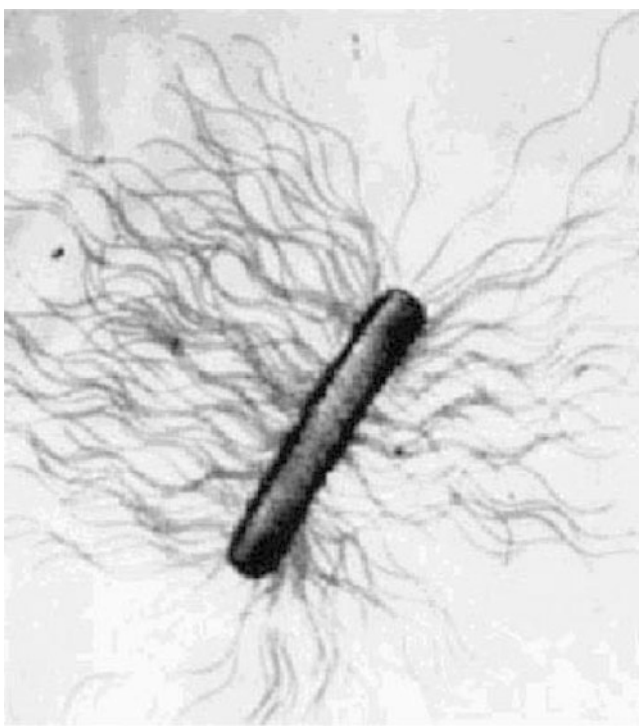

Fig. 5.11
Peritrichously flagellated swarmer cell of *Proteus mirabilis* (From Hoeniger 1965)

based instead on the sinusoidal beating of the flagellum within a single plane. One might ask either how the bacterium manages to operate an intracellular, rotating structure or, on the other hand, why there are no fish with propellers. The answer may lie in two features of the biophysics of movement. First, the efficiency of converting input energy to thrust is about 96–98 % for an oscillating, flexible foil (such as the eukaryotic flagellum; Katz and Weihs 1979); this is substantially higher than what can be obtained with a propeller-type mechanism. Second, the bacterium does not have this option; the tremendous viscous drag exerted on a small cell would damp out the oscillations of a sinusoidally beating flagellum. A screw-like motion is its solution to the need to move through an aqueous but (from the viewpoint of the bacterium) tar-like milieu.

### Swarming Motility

Certain species of *Proteus* and of *Vibrio* carry out a kind of motility called "swarming" (Williams 1978) that is an alternative to their normal, swimming motility. Under certain conditions, usually the perception of a solid surface or of an increased viscosity (in the case of *Vibrio*), the cell morphology and the manner of flagellation change. The cells become longer and, in the case of *Proteus*, filamentous, and the sparsely or polarly flagellated cells become profusely peritrichous (*Fig. 5.11*). In *Proteus*, when the cells are placed on an agar surface under the appropriate conditions, the swarming phase is periodic and alternates with a growth phase, during which shorter, less extensively flagellated swimming cells are formed (Matsuyama et al. 2000).

### Twitching Motility

Certain of the Gram-negative cocci and coccobacilli, such as *Neisseria*, *Moraxella* and *Acinetobacter*, manifest a peculiar type of movement known as "twitching" (Henrichsen 1983). The cells exhibit jerking or jumping movements of a few micrometers in any direction. It now appears that retraction of type IV pili is responsible for this motility (Merz et al. 2000).

### Gliding Motility

There are, however, alternative mechanisms for motility in prokaryotes. An entire group of bacteria, colloquially termed "the gliding bacteria," move by an undefined mechanism. These organisms are able to glide across solid surfaces such as agar and occasionally glass or plastic. The mechanism or mechanisms of such motility are not understood. The cells do not inch, wiggle, or sidle. There seem to be no organelles of locomotion, although various authors have, on occasion, seen structures that they have interpreted as locomotory organelles (Lünsdorf and Reichenbach 1989; Pate and Chang 1979). Various mechanisms for gliding motility have been proposed (Burchard 1984). They range from moving tracks (Lapidus and Berg 1982), rotating organelles (Pate and Chang 1979), polarized excretion of surfactants (Keller et al. 1983), and contractile fibers (Burchard 1984). No one of these proposals is supported by other than those who have suggested them, and it now appears that there may be more than one mechanism of gliding (Spormann 1999).

The gliding bacteria, though customarily grouped together, have in common only the facts that they move by gliding and that they all have Gram-negative cell wall architecture. The motility mechanisms seem to be different, and the organisms cross the entire spectrum of physiological types.

While there is no hard evidence, it seems appropriate that those organisms whose nutrition depends on the extracellular solubilization of particulate material would glide rather than swim to gain access to their substrates. Though this seems to be the case for the myxobacteria, it is not clearly the case for the cyanobacteria or other gliding phototrophs.

### Motility in Archaea

Some archaea show a flagellar-based motility, though gliding motility has never been detected in the Archaea. Whereas the archaeal flagellar assembly superficially resembles those of the Bacteria, it lacks a hook region, and, interestingly, the amino acid sequences of the subunits of the flagellar shaft resemble those of type IV pili in bacteria, rather than those of flagellins (Jarrell et al. 1996). Moreover, whereas bacterial flagellins are translocated outside the cell via a sec-independent transport system involving transport through the interior of the flagellum shaft, the flagellar subunits in the Archaea are transported using a standard sec-dependent system (Jarrell et al. 1996). Homologues of methyl-accepting chemotaxis proteins and the CheA-CheY two-component signal transduction system (Rudolph and Oesterhelt 1996) have been found in the haloarchaea. Indeed, the haloarchaea have special sensory rhodopsins that, instead of pumping ions as do bacteriorhodopsin and halorhodopsin, couple with the methyl-accepting chemotaxis proteins to form a phototaxis system (Perazzona and Spudich 1999).

## Cell Division Strategies

Bacterial multiplication is usually thought of as being synonymous with binary transverse fission, the division strategy used by most of the commonly studied bacteria. This process has been intensively investigated, and, though many of the regulatory processes of division remain unknown, the descriptive details of the process are clearly defined (Ingraham et al. 1983). There is, however, a variety of other division strategies found among the bacteria, once one strays from the old laboratory standbys. Thus, division by budding among such organisms as *Hyphomicrobium* and *Rhodomicrobium* (*Fig. 5.3e*), by mycelial extension and subsequent fragmentation among the filamentous

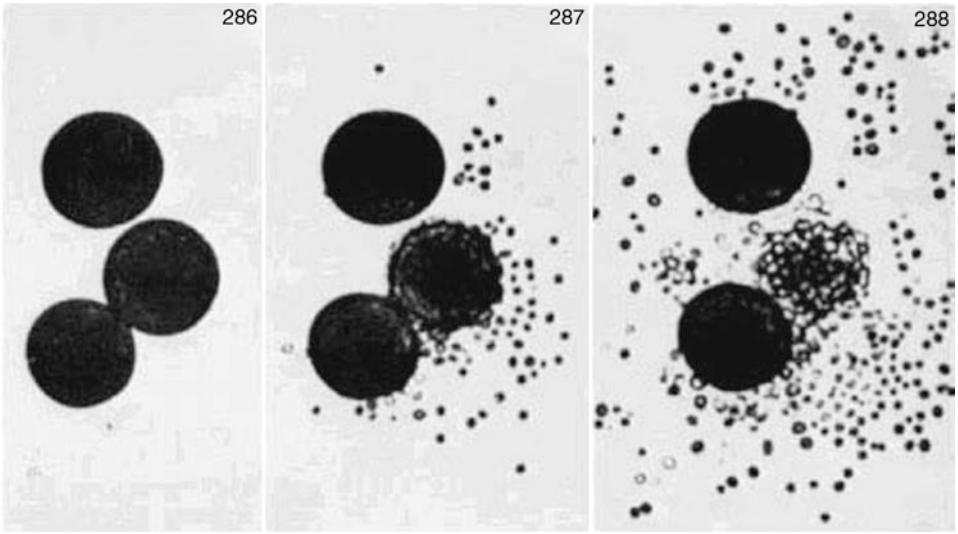


**Fig. 5.12**
**Multiple fission and release of baeocytes from *Dermocarpa*. The numbers represent the hours that have elapsed during the growth of the initial, small baeocyte (From Stanier et al. 1981)**

actinomycetes (*Fig. 5.7f*), and by multiple fission among the pleurocapsalean cyanobacteria (*Fig. 5.12*) has been described among the bacteria.

While it does seem a useful and interesting exercise to wonder what particular advantages are associated with the different strategies for cell division, this is not a subject that has received a great deal of attention from microbiologists. Nevertheless, it does seem obvious that these different mechanisms of cell division must be functionally related to the larger aspects of the biology of the cell. Thus, for example, mycelial extension followed by fragmentation seems a reasonable way to alternate filamentous and single-celled modes. And the unequal division of the stalked cell of *Caulobacter* (*Fig. 5.13*) leading to the generation of a flagellated swarmer cell must certainly reflect the division of the organism's activity between a sessile, stalked stem cell and a motile cell that may expand the territory occupied by that clone. Certainly, other speculations are possible to rationalize other modes of prokaryotic cell division.

## Developmental Diversity

Chapter 16, "Prokaryotic Life Cycles" in Vol. 2 presents a more extensive discussion of the traditional view (and a more detailed description) of development among bacteria. The purpose of this section is to point out that development represents an additional set of strategies for diversity.

Development implies a set of alternative states. These alternatives may be expressed as a function of time, in which case we think of the alternative states as parts of a life cycle; on the other hand, the heterogeneity may be spatial, with different parts of the cell differentiated so as to fulfill specific functions. An

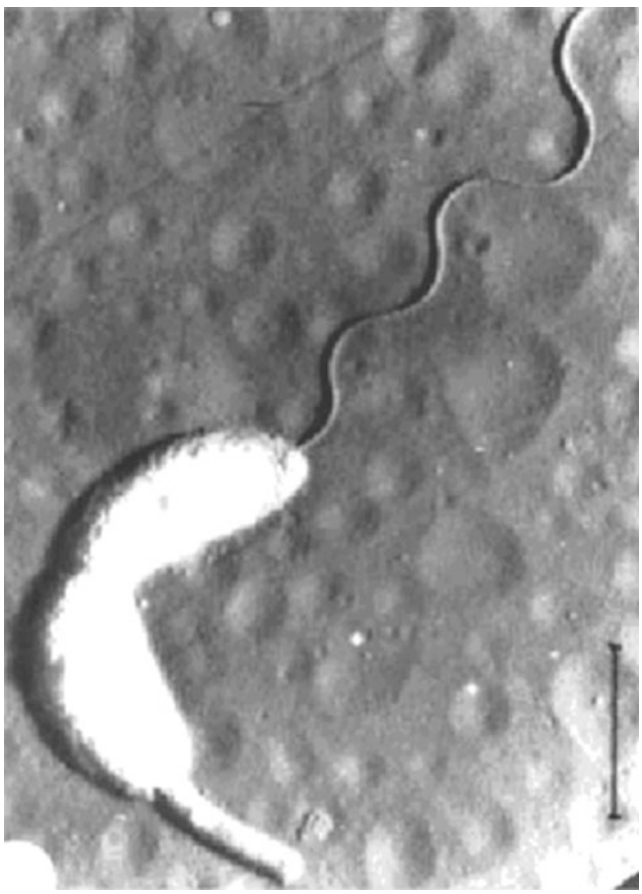

**Fig. 5.13**
**Generation of swarmer cell from stalked cell of *Caulobacter crescentus* (From Poindexter 1964)**

example of the latter would be the stalked cell of *Caulobacter* (Fig. 5.13). In the case of either temporal or spatial alternatives, prokaryotic development generates considerably greater diversity than was afforded by the traditional nineteenth-century view of bacteria as rods, cocci, or spirilla.

The generation of diversity among bacteria by developmental morphogenesis or by differentiation expands the kinds of niches that the bacteria can occupy. As an example, the ability of *Caulobacter* to form both a swarmer cell and a stalked cell allows it to separate the processes of growth and reproduction from dispersal. As an oligotroph, that is, an organism designed to feed on relatively low concentrations of nutrients, its ability to seek out, detect, and move toward such low concentrations must be optimized. That is the presumptive function of the swarmer cell. Once such a site has been discovered, it is the function of the stalked cell to attach to a surface and begin the process of feeding, growth, and reproduction. This sort of alternation between a sessile, feeding and reproductive stage and a nongrowing, swarming stage is also characteristic of organisms such as the actinoplanes, in which a mycelial phase alternates with a motile zoospore-like cellular stage. This type of developmental strategy facilitates the colonization of sites that might otherwise be inaccessible or inappropriate.

The characteristic swarm of the myxobacteria is another developmental strategy used by a bacterium to optimize its feeding. The myxobacteria feed by excreting a battery of potent hydrolytic enzymes that degrade proteins, peptidoglycan, polysaccharides, lipids, and nucleic acids. The cells are thus at the mercy of the process of diffusion of enzymes away from the cell and of low-molecular-weight products of hydrolysis toward the cell. The myxobacteria optimize this process by traveling as a swarm—a high population density of cells (Fig. 5.14). The rate of cell growth on such substrates increases as a function of the cell density—a reflection of the fact that the cells are feeding cooperatively (Rosenberg et al. 1977). This sort of microbial "wolf-pack effect" has been suggested to rationalize the overall life cycle of the myxobacteria (Dworkin 1973).

The ability of organisms to convert from a vegetative, growing stage to a resting stage allows them to persist in environments that might periodically become inhospitable to normal, prokaryotic life. As was pointed out earlier, the ability to grow in an extreme environment is invariably at the expense of the ability to grow in the so-called normal environment. However, morphogenesis to a resistant, metabolically quiescent resting cell allows the organism to survive in the absence of growth until conditions are once again suitable for growth. A remarkable feature of this adaptation is that the state of almost total quiescence is juxtaposed with the ability to respond to the reappearance of conditions suitable for growth by germinating in an almost hair-trigger fashion.

One particular type of spore is referred to as an "endospore" and is found among the Gram-positive bacteria. This resting cell is formed within the vegetative cell and is usually released as a free spore as the process of sporulation is completed. Endosporulation occurs widely across traditional taxonomic lines and is found among the genera Bacillus, *Clostridium*, *Sporolactobacillus*, *Sporosarcina*, and *Thermoactinomyces* (Fig. 5.15). In all of these organisms, the essential features of the spore and of the process of sporulation are quite similar. Another type of sporulation among the Gram-positive bacteria is found among the actinomycetes. Actinospores include the nonmotile conidial spores of *Streptomyces* (Fig. 5.16), the sporangiospores of *Actinoplanes* (Fig. 5.17), and the endospores of *Thermoactinomyces* (Fig. 5.18), which, despite their traditional taxonomic distance from the Bacillaceae, share many of the properties of *Bacillus* endospores. In this context, it is interesting that molecular techniques have revealed a moderate but distinct phylogenetic relationship between *Bacillus stearothermophilus* and *Thermoactinomyces vulgaris* (Stackebrandt et al. 1987).

**Fig. 5.14**
**Swarm edge of *Stigmatella erecta* at low magnification. Bar = 110 μm (From Reichenbach 1984)**

The exospores of Gram-negative bacteria are, in general, less resistant to environmental extremes than are endospores. They are, however, metabolically quiescent and substantially more resistant to desiccation, physical breakage, and environmental extremes than the corresponding vegetative cells. Among the Gram-negative bacteria, resistant resting cells that have been fairly well characterized are found among the myxobacteria, as myxospores; among the cyanobacteria, as akinetes; and among *Azotobacter* and related genera, as azotocysts. Less well characterized resting cells have been described for the photosynthetic bacterium *Rhodomicrobium* (Dow and Whittenbury 1979) and some of the methylotrophic bacteria (Whittenbury et al. 1970).

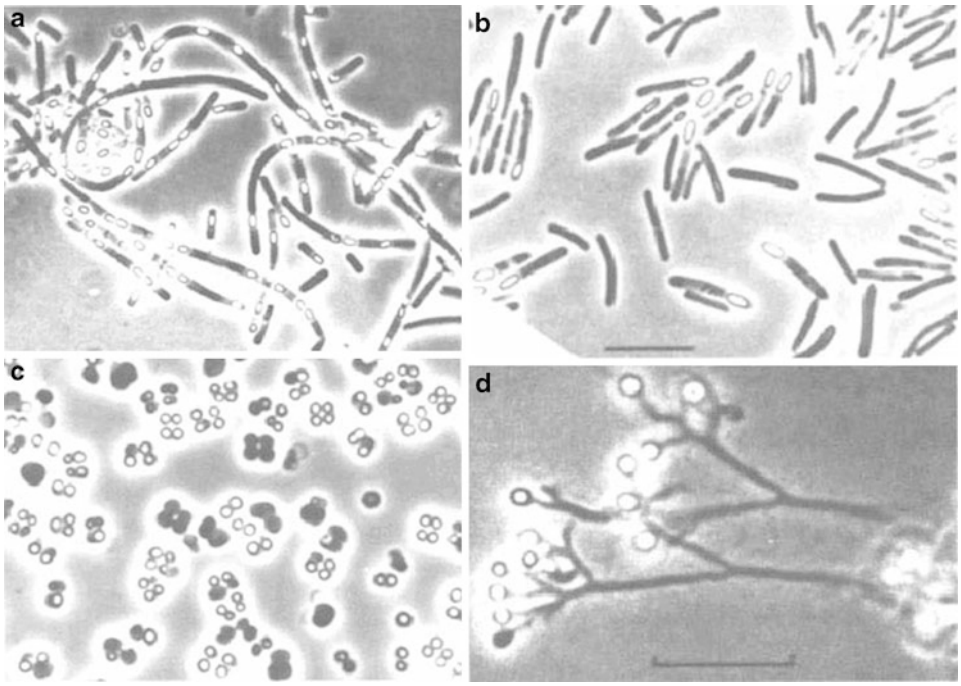


**Fig. 5.15**
**Endospores of (a) *Bacillus fastidiosus*, (b) *Clostridium butyricum*, (c) *Sarcina ureae*, and (d) *Thermoactinomyces dichotomicus* (a courtesy of S. C. Holt; b from Gottschalk et al. 1981; c from Sneath 1986; and d from Cross 1981)**

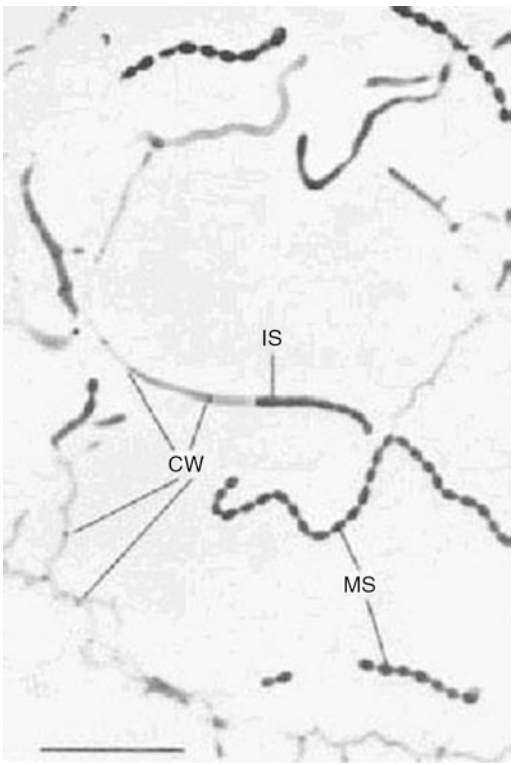


**Fig. 5.16**
**Phase-contrast micrographs of vegetative hyphae and conidiospores of *Streptomyces coelicolor*. CW cross walls, IS immature spores, and MS mature spores. Bar = 10 μm (From Chater and Hopwood 1973)**

Myxobacterial resting cells are contained in the characteristic fruiting bodies formed by the myxobacteria (Fig. 5.5), and their shapes vary from the round, resistant, optically refractile cells formed by the genus Myxococcus (Fig. 5.19a) to the slightly shortened, oval rods formed by *Stigmatella* (Fig. 5.19b). The myxospore of *Myxococcus xanthus* is formed by the shortening and rounding up of the entire rod-shaped vegetative cell and is finally enclosed by a multilayered spore coat/capsule (White 1984). The *Myxococcus xanthus* myxospore is the only myxobacterial resting cell that has been well characterized both structurally and biochemically.

The cyanobacteria form a variety of cell types that have been considered to be spores or resting cells. These include the hormocysts of *Westiella*, the exospores of *Chamaesiphon*, the endospores or baeocytes of the Pleurocapsales (Fig. 5.12), and the akinetes of *Anabaena* and other genera. The cyanobacteria, in general, have not been characterized from a physiological or biochemical point of view with the same intensity that many of the eubacteria have. Thus, information about the properties of cyanobacterial resting cells is quite sparse. Most of the available information centers on the akinetes of *Anabaena*, and even in that case, the difficulty of obtaining large populations of relatively pure akinetes has limited the available information (Nichols and Adams 1982). While the akinetes of *Anabaena* are more resistant to extreme low temperature and desiccation than the corresponding vegetative cells, their respiratory rate is twice that of the vegetative cells. Figure 9.20 illustrates the akinetes of *Anabaena*.

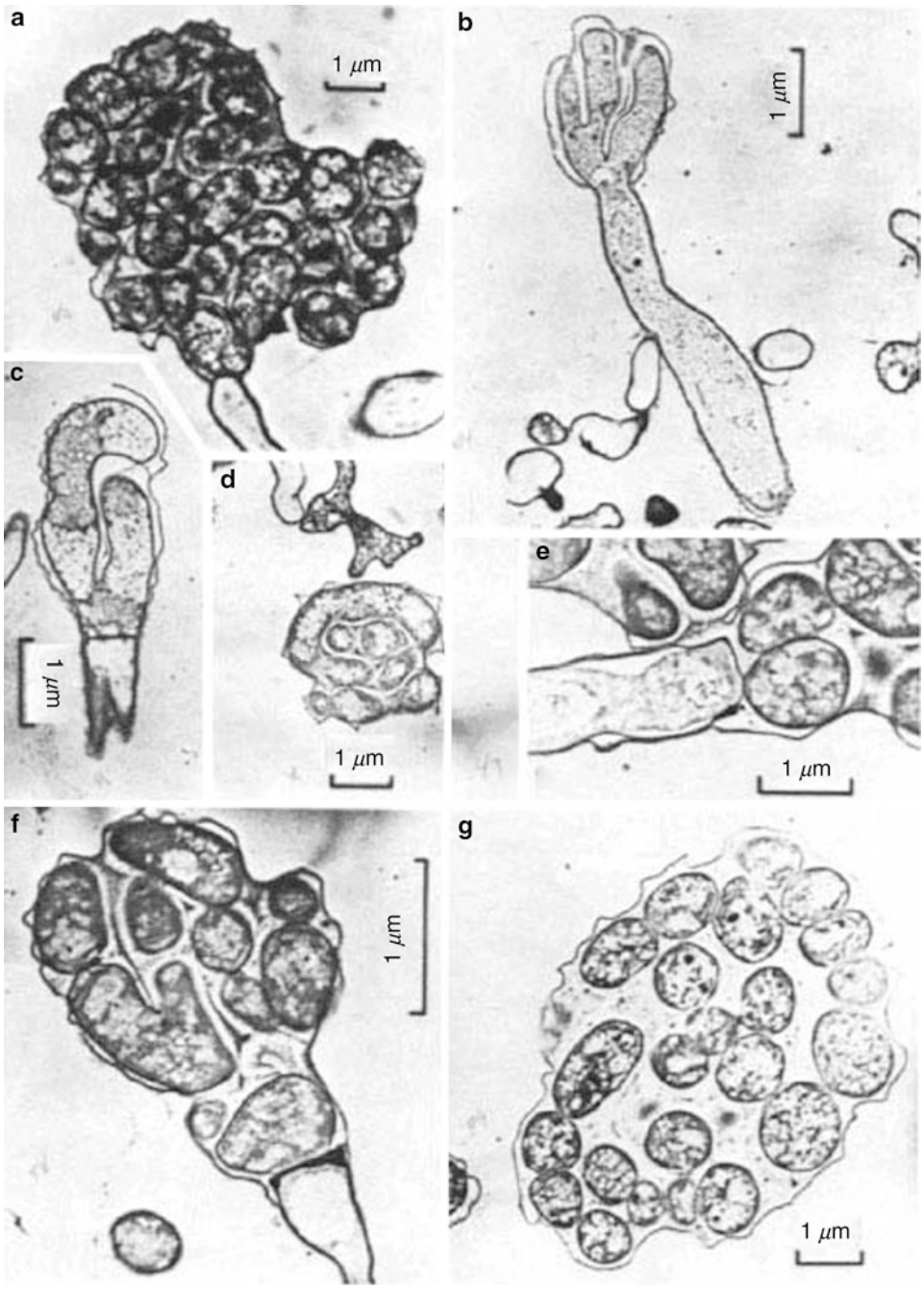


**Fig. 5.17**
**Electron micrographs of thin sections of sporangiospores and sporangia of *Actinoplanes*. (a) Mature sporangium, 4 days old. (b, c, and d) Immature sporangium, 2 days old. (e, f), and (g) Mature sporangium, 4 days old (From Lechevalier and Holbert 1965)**

Cells of the Gram-negative, nitrogen-fixing genus *Azotobacter* are able to convert to cysts when the cells have exhausted the nutrients of the growth medium (Sadoff 1976). The resting cells also can be induced by placing the cells in a medium containing hydroxybutyrate as the carbon source. *Azotobacter* cysts (*Fig. 5.21*) are superficially similar to myxospores, in that they are only slightly more resistant to temperature extremes than the corresponding vegetative cells but are considerably more resistant to desiccation and seem to be truly metabolically quiescent. (See Sudo and Dworkin (1973) for an extensive, comparative survey of prokaryotic resting cell properties.)

Another interesting type of life cycle is shown by intracellular pathogens of the chlamydia group. Cells of the chlamydia must be able to carry out three very different kinds of processes. Because they are not transmitted by any sort of a vector, but may exist free in the environment, they must be able to persist in

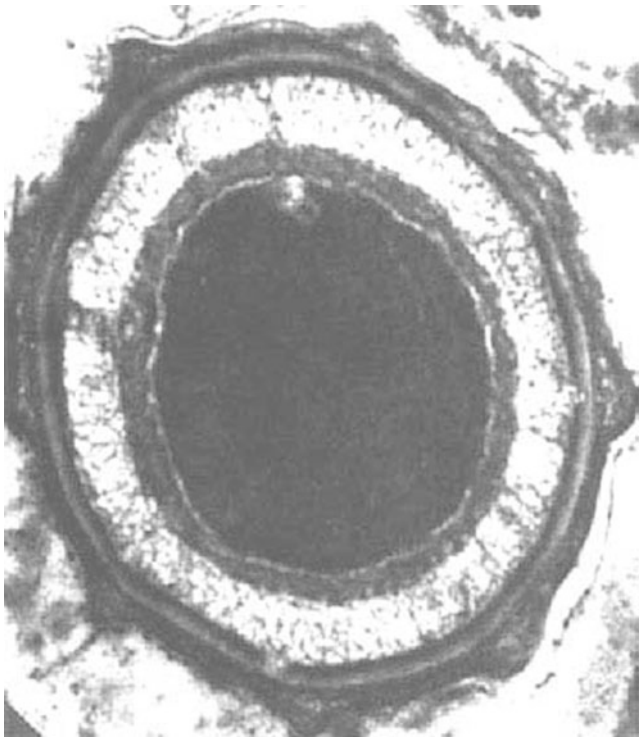

**Fig. 5.18**
**Electron micrograph of a thin section of a spore of *Thermoactinomyces sacchari* (From Lacy 1971)**

a nutrient-free, desiccated environment, subject to the normal variations of environmental conditions. Second, they must be able, from this state, to infect a specific host and to enter the host cell. Third, they must then be able to grow and reproduce intracellularly. Chlamydia accomplishes these processes by alternating between two states—the elementary body (a small, dense, resistant, and nongrowing cell) and the reticulate body (the vegetative form of the cell that can grow and reproduce).

Readers interested in a more detailed description of the various groups of prokaryotes that undergo development are referred to the excellent recent monograph on the subject (Brun and Shimkets 2000).

## Metabolic Diversity

Catabolism is the part of metabolism involved in conservation of energy that can be used for biosynthesis and other cellular functions. Energy can be conserved from chemical reactions (chemotrophy) or from light (phototrophy). Catabolic diversity in prokaryotes greatly exceeds that in eukaryotes. There are many modes of metabolism, including anaerobic respiration, or lithotrophy, which eukaryotes cannot perform; even in those that the two cell types share, such as fermentation or photosynthesis, the eukaryotes are greatly outstripped by the prokaryotes in terms of substrates utilized and metabolic modes. Majorie Stephenson (1949) expressed this concept presciently in the introduction to the first edition of her textbook on bacterial metabolism (also in the third edition), written well before the modern era of molecular biochemistry or bacterial genetics. In it she states:

"Bacteria may be tentatively regarded as biochemical experimenters; owing to their small size and rapid growth, variations must arise very much more frequently than in more differentiated forms of life, and they can in addition afford to occupy more precarious positions in the natural economy than larger organisms with more exacting requirements."

In terms of anabolism, all organisms share essentially the same pathways for biosynthesis of protein, nucleic acids, carbohydrates, and lipids. Prokaryotes can biosynthesize certain compounds like vitamin $B_{12}$ or certain antibiotics not found in the eukaryotes, but one must also give due credit to the biosynthetic capabilities of the fungi, and especially those of the plants that biosynthesize an incredible diversity of chemical compounds including hydrocarbons, aromatic compounds, heterocyclics, and alkaloids. Indeed, the formidable biodegradative abilities of soil microorganisms can be partially attributed to the selective pressures of diverse plant compounds in their environment serving as potential growth substrates.

Two crucial aspects of anabolism are the fixation of carbon and nitrogen, processes that are essential for primary production of biomass on earth. It has long been known that fixation of nitrogen is a solely prokaryotic process. Moreover, if one considers chloroplasts to be descended from cyanobacteria, which is clearly the case (Moreira et al. 2000), then essentially all fixation of carbon on earth is also prokaryotic.

In the following sections, we will give an overview of various metabolic modes found in prokaryotes, discussing catabolic diversity and the fixation of carbon and nitrogen.

## Anaerobic Fermentation

Fermentation can be defined as the utilization of an organic compound in the absence of external electron acceptors, including oxygen. Microbes are unique in their ability to exploit anaerobic environments, and Louis Pasteur's insight into "La vie sans l'air" opened up a whole new area of metabolism. Among the microbes, the prokaryotes have most extensively exploited this modus vivendi. As is the case with almost all aspects of biological diversity, there are few clear-cut categories but rather a spectrum of differences that diminish as one learns more about the property. Thus, the spectrum of relations to oxygen includes those organisms that cannot use oxygen as a terminal electron acceptor and are in fact damaged by exposure to oxygen, those that are likewise obligately anaerobic but are indifferent to the presence of oxygen, those facultative organisms that have the option of metabolizing either aerobically or anaerobically, and finally those organisms that are obliged to use oxygen as a terminal electron acceptor.

Eukaryotes can ferment a few common carbohydrates such as starch, cellulose, glucose, or sucrose to a limited number of products: lactate or ethanol and $CO_2$, or, in the case of hydrogenosome-containing anaerobic protists, to ethanol,

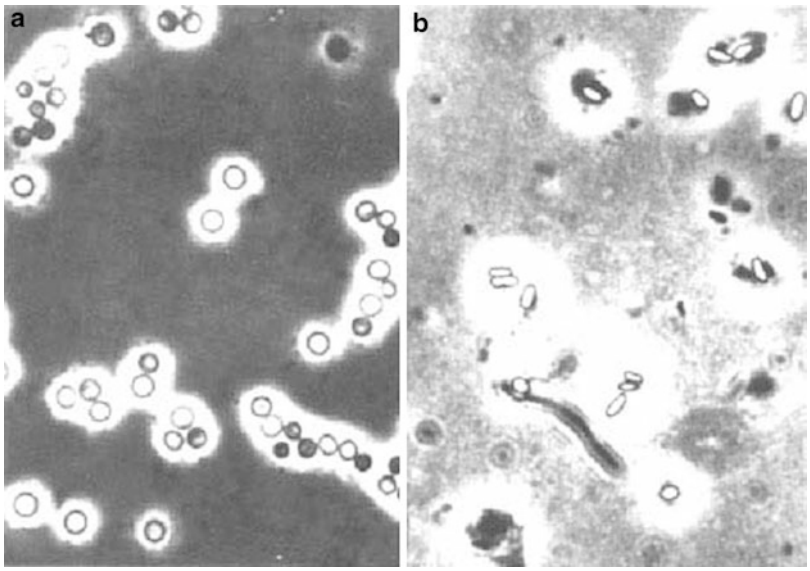


**Fig. 5.19**
**Myxospores of myxobacteria. (a) *Myxococcus xanthus*. (b) *Stigmatella aurantiaca* (Courtesy of H. Reichenbach)**

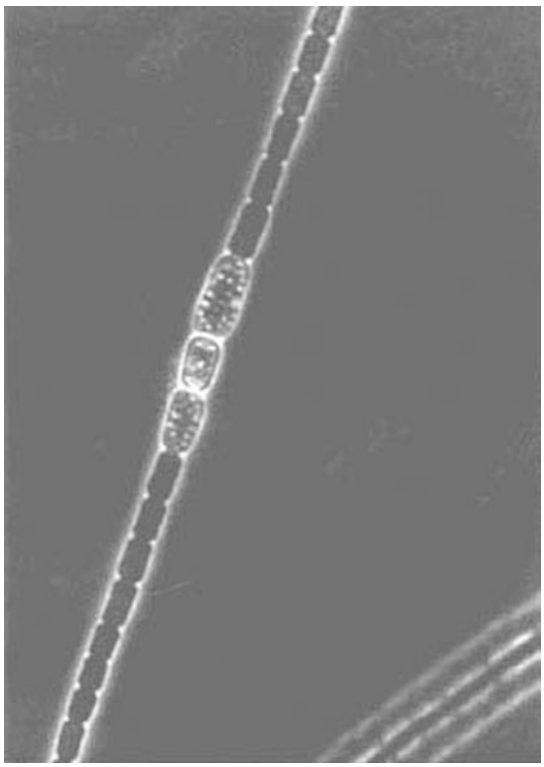

**Fig. 5.20**
**Akinetes of *Anabaena*, positioned on both sides of a heterocyst (From Carr 1979)**

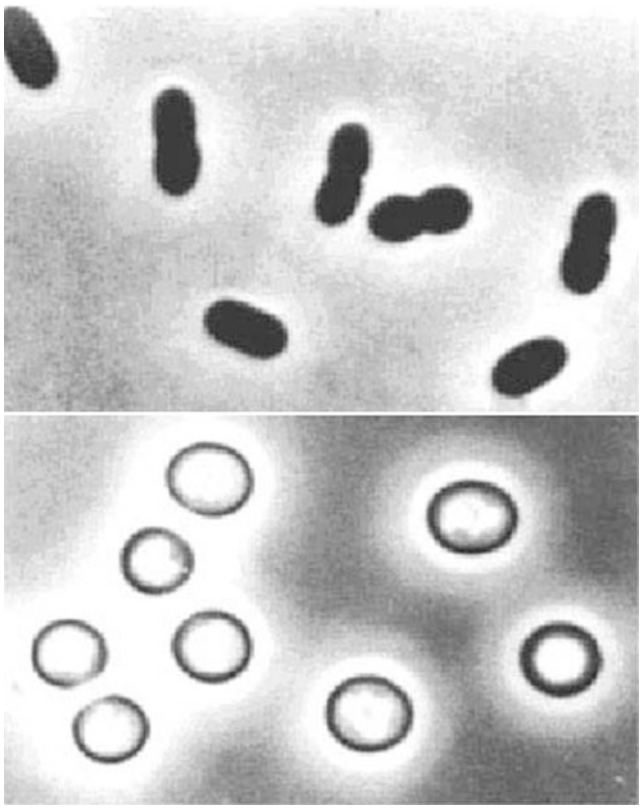

**Fig. 5.21**
**Vegetative cells (*top*) and azotocysts (*bottom*) of *Azotobacter vinelandii* (Courtesy of H. Sadoff)**

acetate, and $CO_2$ and $H_2$. The fermentative abilities of prokaryotes are more extensive.

Carbohydrates often are the main organic substrate available for fermentation. The canonical pathway for utilization of carbohydrates is the Embden-Meyerhof-Parnas (EMP) pathway found in eukaryotes and many prokaryotes. The Entner-Doudoroff (ED) pathway involves oxidation of glucose-6-phosphate to glucuronic acid-6-phosphate and leads to the

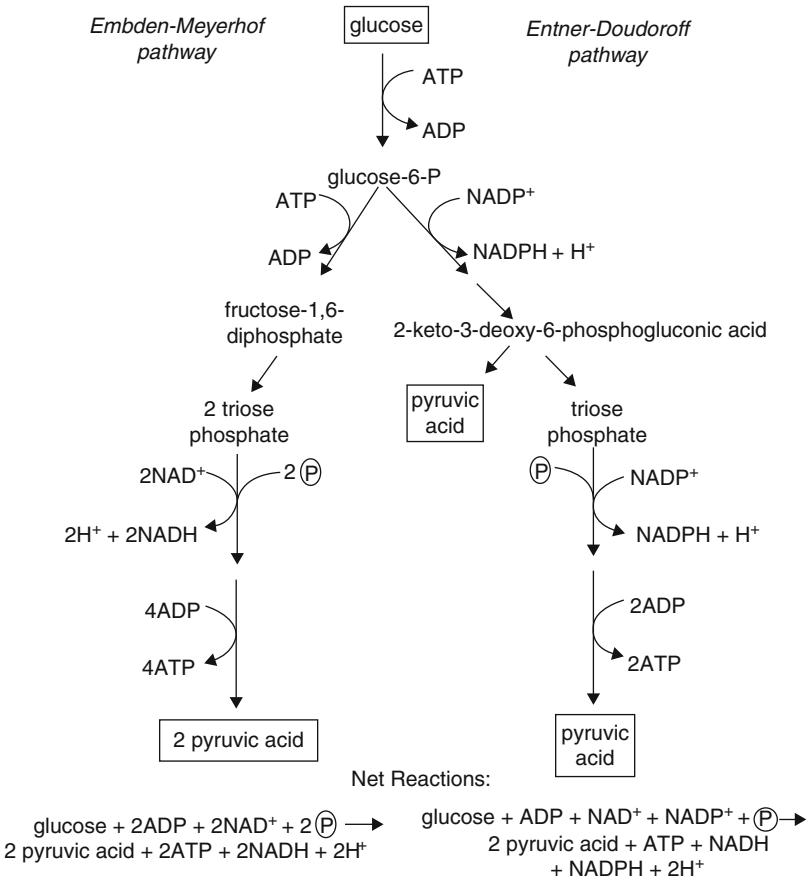


**Fig. 5.22**
**The Embden-Meyerhof and Entner-Doudoroff pathways (Adapted from Stanier et al. 1979)**

conservation of only a single ATP per hexose via substrate-level phosphorylation (Conway 1992, *Fig. 5.22*). The ED pathway is common in the Proteobacteria, especially aerobes, but can be found in some clostridia and bacilli and even some eukaryotic microbes such as *Entamoeba histolytica* and *Penicillium notatum* (Conway 1992). Most of the organisms that use the ED pathway are aerobes, but the Proteobacterium *Zymomonas mobilis* uses it as a primary fermentative pathway for hexoses. *Escherichia coli* possesses both the EMP and the ED pathways and apparently uses the latter when dining on uronic acids in the gut (Peekhaus and Conway 1998). Other glycolytic pathways in the Bacteria include that used by heterolactic Gram-positive bacteria (also leading to the conservation of a single ATP per hexose), the pathway found in bifidobacteria, and the pentose phosphate pathway, which is generally used for biosynthesis of five carbon sugars for nucleic acids (Gottschalk 1986).

When sugar-fermenting Archaea were examined for glycolytic pathways, they were shown to have interesting modifications of both the EMP and the ED pathways (Kengen et al. 1996; Selig et al. 1997). For example, the hyperthermophile Pyrococcus furiosus, which ferments hexoses to acetate, hydrogen, $CO_2$, and alanine, was found to utilize a modified EMP pathway in which ADP rather than ATP serves as the phosphoryl donor for hexokinase and phosphofructokinase (*Fig. 5.23*). Moreover, instead of the standard glyceraldehyde 3-phosphate dehydrogenase, which uses $NAD^+$ as an electron acceptor and produces 1,3-diphosphoglycerate, *Pyrococcus* and certain other Archaea possess a novel tungsten-containing glyceraldehyde 3-phosphate/ferredoxin oxidoreductase (Adams 1993) that produces 3-phosphoglycerate. Thus, the net high-energy phosphodiester bonds conserved from hexose to pyruvate by this pathway are zero. The ATP from acetyl-CoA produced from pyruvate, however, is conserved by a novel ADP-dependent acetyl-CoA synthetase (previously described acetyl-CoA synthetases cleave ATP to AMP and pyrophosphate in the acetyl-CoA synthesis direction).

In contrast, *Thermotoga maritima*, a phylogenetically deep-branching hyperthermophilic fermentative bacterium, converts hexoses to pyruvate using the conventional EMP pathway and conserves ATP from acetyl-CoA using phosphotransacetylase and acetate kinase, as is found in mesophilic bacteria. The advantage of the glyceraldehyde 3-phosphate/ferredoxin oxidoreductase over the $NAD^+$-utilizing dehydrogenase is unclear, but one possibility is that ferredoxin is a very strong electron donor capable of reducing protons to $H_2$. In contrast, $H_2$ production from NADH is thermodynamically unfavorable at $H_2$ partial pressures above $10^{-3}$ atm (Wolin and Miller 1982). Therefore, the modified EMP pathway is less likely to be inhibited under conditions of high $H_2$ partial pressures. Indeed, growth of

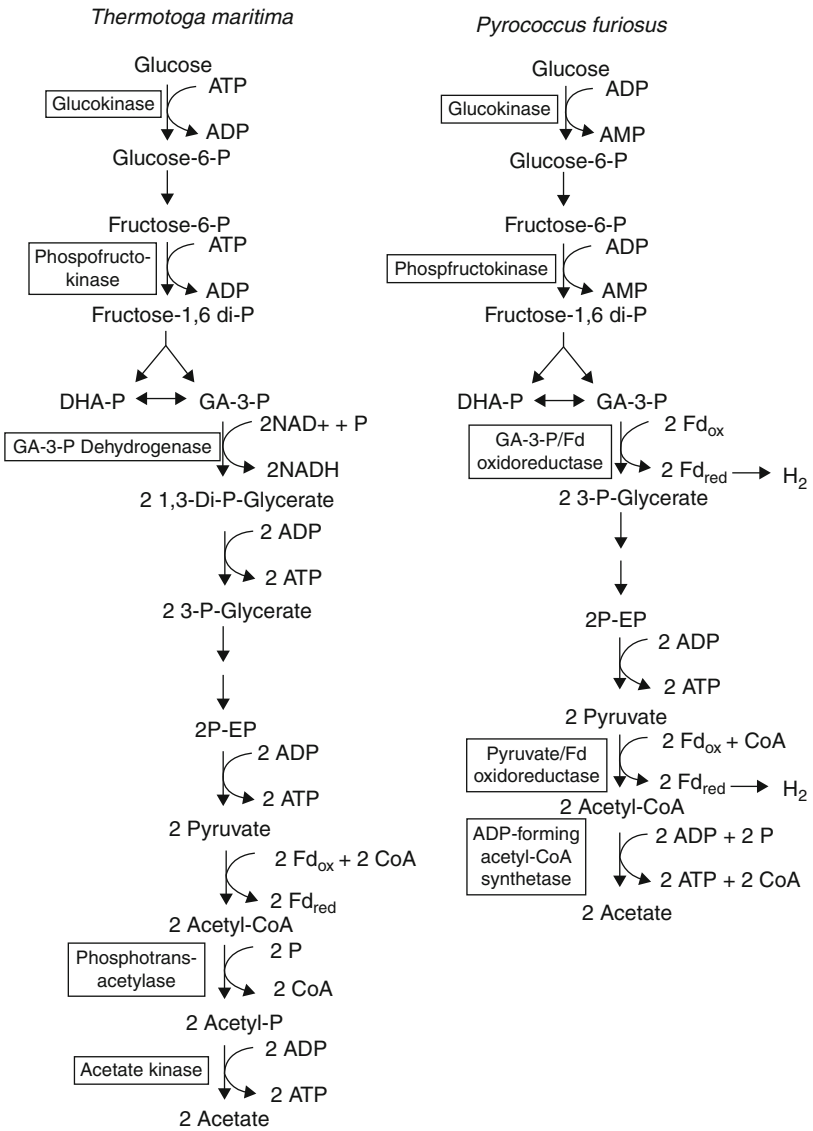


**Fig. 5.23**
**Comparison of glycolytic pathways in *Thermotoga maritima* and *Pyrococcus furiosus*. *P* phosphate, *DHA* dihydroxyacetone, *GA* glyceraldehyde, *P-EP* phosphoenolpyruvate, *Fd* ferredoxin, and *CoA* coenzyme A**

*T. maritima* on sugars becomes inhibited unless an electron acceptor such as elemental sulfur or a hydrogen-utilizing methanogen is added to remove $H_2$.

Archaea possess other variants on the EMP pathway (Menendez et al. 1999). In *Desulfurococcus*, ATP is used in both hexokinase and phosphofructokinase as in the classic EMP pathway, whereas in *Thermoproteus*, ATP is used by hexokinase and pyrophosphate is used by a phosphofructokinase, as has been found in some eubacteria and eukaryotes. All EMP pathways studied in the Archaea thus far utilize glyceraldehyde 3-phosphate/ferredoxin oxidoreductase instead of the $NAD^+$-utilizing dehydrogenase. A modification of the ED pathway in certain Archaea (including *Sulfolobus*, *Thermoplasma*, and *Halobacterium*) has been described in which glucose is not phosphorylated before oxidation to gluconic acid, and phosphorylation occurs at a later step in the ED pathway (Conway 1992).

Besides carbohydrates, amino acids, derived from the hydrolysis of proteins by proteases, are fermented by a variety of prokaryotes, particularly the clostridia, either singly or in pairs, as in the Stickland reaction (Gottschalk 1986). The products of amino acid fermentation, amines (including putrescine and cadaverine), branched-chain fatty acids, and sulfide and mercaptans from sulfur-containing amino acids, essentially define the word putrid. Our great olfactory sensitivity to these compounds no doubt has played a selective role in protecting us from eating spoiled food potentially containing botulin and other toxins. The purines and pyrimidines derived from nucleic acids are also readily fermented by prokaryotes.

Originally it was thought that only a small number of sugars and amino acids were fermented by prokaryotes. As anaerobic culture techniques have improved, an astounding diversity of compounds has been found to serve as substrates for fermentation, including aromatic compounds and even saturated alkane hydrocarbons (Zengler et al. 1999). Many of these fermentations take place in the presence of a hydrogen-consuming anaerobe such as a methanogen. The removal of hydrogen (or acetate in some cases) allows the fermentation to be energetically favorable. This syntrophic interaction is called "interspecies hydrogen transfer" (Schink 1997).

Indeed, our concepts of fermentation have had to change over the past few decades. It was generally considered that fermentations involved substrate-level phosphorylation. However, consider the example of *Propionigenium modestum*, which grows by decarboxylating succinate to propionate by the following reaction:


There is no net redox reaction, only a decarboxylation. Readers might rightly guess that the pathway is not a simple decarboxylation, but rather involves coenzyme A derivatives and includes a rearrangement to methylmalonyl-CoA. However, the substrate-level phosphorylations in this pathway provide a net ATP yield of zero. Instead, it was shown that membrane-bound methylmalonyl-CoA decarboxylase pumps sodium ions with each decarboxylation it carries out. A sodium-dependent version of an $F_1F_0$-ATPase can then conserve ATP from the sodium gradient across the cell membrane. Thus, in the absence of electron transport, a chemiosmotic potential is generated in this organism, as well as in others carrying out similar decarboxylations (Dimroth and Schink 1998).

## Anaerobic Respiration

For aerobic respiration, eukaryotes are dependent upon mitochondria, clearly derived from endosymbiotic prokaryotes in the α-subphylum of the Proteobacteria (Gray et al. 1999). If the mitochondria were once capable of using electron acceptors other than oxygen, they no longer are, nor have the eukaryotes evolved other mechanisms to use alternate electron acceptors. Thus, anaerobic respiration is solely the domain of prokaryotes. Moreover, prokaryotes can utilize a large range of electron acceptors other than oxygen (*Table 5.1*).

Nitrate and nitrite are produced aerobically from ammonia by nitrifiers (see below) and can be used as electron acceptors by diverse members of both the Bacteria and the Archaea (Zumft 1997). Nitrate is initially reduced to nitrite, and nitrite can be reduced either to $N_2$ and $N_2O$ gas via the denitrification pathway or to ammonia. The former process is more common as a respiratory process, whereas the latter is often used to assimilate nitrate and nitrite but can be used as a respiratory process by *E. coli*, for example (Stewart 1994). The energetics of using nitrate and nitrate compounds as electron acceptors is comparable to that of using $O_2$, and most of the organisms reducing nitrogen oxides are facultative aerobes.

In the final decade of the twentieth century, recognition of the importance of Fe(III) as an electron acceptor in anaerobic habitats increased. Whereas at low pH, Fe(III) mainly exists as the free ion and the Fe(III)/Fe(II) oxidation-reduction potential is near +0.77v, at circumneutral pH values, Fe(III) exists primarily as hydroxide precipitates, which causes the oxidation/reduction potential to be between +0.2 and −0.2v (Widdel et al. 1993). Amorphous ferric hydroxide precipitates are much more bioavailable than are more crystalline ones. Most known Fe(III) reducers are members of the Proteobacteria, but even certain deep-branching thermophilic Bacteria and Archaea can reduce Fe(III) (Vargas et al. 1998), suggesting a role for this process early in life's history on earth. Also, certain purple photosynthetic bacteria oxidize Fe(II) to Fe(III) (Widdel et al. 1993), making for a photosynthetic producer-consumer cycle analogous to those for oxygen or sulfur.

Sulfur compounds can serve as electron acceptors, and it is apparent from *Table 5.1* that the amount of energy available from their reduction is considerably less than that of the preceding compounds. Sulfate is an abundant form of sulfur, especially in seawater, where its concentration is near 28 mM. Two genera, *Desulfovibrio* and *Desulfotomaculum*, were essentially the only ones known until better anaerobic techniques were applied, revealing an enormous diversity of organisms. Most sulfate reducers belong to the δ- and ε-subphyla of the Proteobacteria or to the Firmicutes (Gram-positive bacteria) in the Bacteria and to the genus *Archaeoglobus* in the Archaea. Sulfate reducers and other organisms can generally utilize sulfite or thiosulfate, and these two compounds can be used even in the absence of organic compounds in an inorganic "fermentation" to sulfide and sulfate (Bak and Pfennig 1987).

Elemental sulfur also is used as an electron acceptor by diverse anaerobes, either as a respiratory electron acceptor leading to energy conservation by an electron transport chain or simply as a way to recycle NADH to $NAD^+$ for certain fermentative organisms such as *Thermotoga*. Elemental sulfur is reduced to sulfide by many hyperthermophiles growing under conditions of "fire and brimstone," and sulfur reduction is a good candidate for the first respiratory process on earth.

When other electron acceptors in anaerobic habitats are absent or depleted, the acceptor remaining is carbon dioxide, which can be used by either methanogens or acetogens. Methanogens represent the predominant phenotype in the Euryarchaeota phylum in the Archaea, demonstrating an enormous phylogenetic and morphological diversity. Methanogens use only a small number of simple substrates (the most complex is acetic acid) and an intricate pathway for reduction of one-carbon units to methane (DiMarco et al. 1990). This pathway was thought to contain many unique enzymes and cofactors, but a large portion of the pathway is now known to be used to oxidize formaldehyde to $CO_2$ in aerobic methylotrophic bacteria

**Table 5.1**
**Electron acceptor utilization for respiration**

| Reactants | Products | $\Delta G^{\circ\prime}/H_2$ (kJ) | Representative organisms |
|---|---|---|---|
| $O_2 + 2\ H_2$ | $2\ H_2O$ | −237 | *Homo sapiens* |
| | | | *Escherichia coli* |
| | | | *Sulfolobus acidocaldarius* |
| $NO_3^- + H_2$ | $NO_2^- + H_2O$ | −163 | *Escherichia coli* |
| | | | *Pyrobaculum aerophilum* |
| $NO_2^- + 2\ H^+ + 3\ H_2$ | $NH_4^+ + 2\ H_2O$ | −145 | *Escherichia coli* |
| $2\ NO_2^- + 2\ H^+ + 3\ H_2$ | $N_2 + 4\ H_2O$ | −265 | *Pseudomans stuzeri* |
| | | | *Pyrobaculum aerophilum* |
| $2\ Fe(OH)_3 + 2\ HCO_3^- + 2\ H^+ + H_2$ | $2\ FeCO_3 + 6\ H_2$ | −118 | *Geobacter metallireducens* |
| | | | *Shewanella putrefaciens* |
| $SO_4^{2-} + H^+ + 4\ H_2$ | $HS^- + 4\ H_2O$ | −38 | *Desulfovibrio desulfuricans* |
| | | | *Archaeoglobus fulgidus* |
| $S + H_2$ | $HS^- + H^+$ | −28 | *Desulfuromonas acetoxidans* |
| | | | *Pyrodictium brockii* |
| $HCO_3^- + 4\ H_2 + H^+$ | $CH_4 + 3\ H_2O$ | −34 | *Methanococcus jannaschii* |
| | | | *Methanospirillum hungatei* |
| $2\ HCO_3^- + 4\ H_2 + H^+$ | $CH_3COO^- + 4\ H_2O$ | −26 | *Acetobacterium woodii* |
| $Fumarate^{2-} + H_2$ | $Succinate^{2-}$ | −86 | *Escherichia coli* |
| $(CH_3)_2SO + H_2$ | $(CH_3)_2\ S + H_2O$ | −124 | *Escherichia coli* |
| | | | *Rhodobacter capsulatus* |
| R–Cl | R–H | −170 | *Desulfomonile tiedjei* |
| | | | *Dehalococcoides ethenogenes* |
| $ClO_4^- + 4\ H_2$ | $Cl^- + 4\ H_2O$ | −268 | *Ideonella dechloratans* |

[a]$H_2$ is used as a model electron donor for the sake of comparisons of the reactions, and its use does not imply that the representative organisms use $H_2$. Most $\Delta G^{\circ\prime}$ values are taken from Thauer et al. 1977

(Chistoserdova et al. 1998). Moreover, coenzyme M, the immediate precursor of methane in methanogens, has been found in an aerobic alkane-oxidizing *Xanthobacter* species (Allen et al. 1999). Thus, there is indeed unity in biochemistry (Singleton 2000).

Acetogens are anaerobic eubacteria generally in the Firmicutes capable of reducing two $CO_2$ moieties to the methyl and acetyl groups of acetyl-CoA (see section "Autotrophic $CO_2$ Fixation" in this chapter) by a pathway utilizing an enzyme complex sometimes called the "carbon monoxide dehydrogenase/acetyl-coenzyme A synthetase complex" (Ragsdale 1997). A variant on this enzyme complex is used by methanogenic Archaea to fix carbon dioxide for autotrophic growth and by acetate-utilizing methanogens to split acetyl-coenzyme A. Unlike the methanogens, the acetogens are metabolically versatile, using a wide variety of substrates for acetogenesis.

In most anaerobic habitats, the amount of electron donor is limiting, and prokaryotes capable of anaerobic respiration are in fierce competition for those electrons. It has long been known that the outcome of this competition correlates with the free energy available from the reactions, so that denitrifiers could outcompete sulfate reducers, which, in turn, outcompete methanogens, which generally outcompete acetogens. This hierarchy of competitive exclusion is best explained by a threshold model based on thermodynamic principles, so that if the substrate is $H_2$, sulfate reducers are capable of utilizing that $H_2$ at concentrations below that at which methanogens can conserve energy from it (Cord-Ruwisch et al. 1988).

Besides these biogeochemically important electron acceptors, a wide diversity of others can be utilized, and we will touch on only a few. It is generally considered that electron acceptors in anaerobic respiration are inorganic, but, for example, *E. coli* and many other organisms capable of anaerobic growth can use fumarate or dimethyl sulfoxide (Weiner et al. 1988) as respiratory electron acceptors. Indeed, during its "fermentation" of glucose, *E. coli* produces succinate, which is the product of the respiratory reduction of fumarate.

Besides its importance to the biogeochemical cycles of carbon, nitrogen, and sulfur, anaerobic respiration plays an important role in biodegradation of pollutants. One important set of reactions is that involving reductive dechlorination of chlorinated organics, which are among the most important of pollutants. The most highly chlorinated organics are often resistant to aerobic attack, but there is considerable energy available for conservation if they can use the chlorinated organic compound

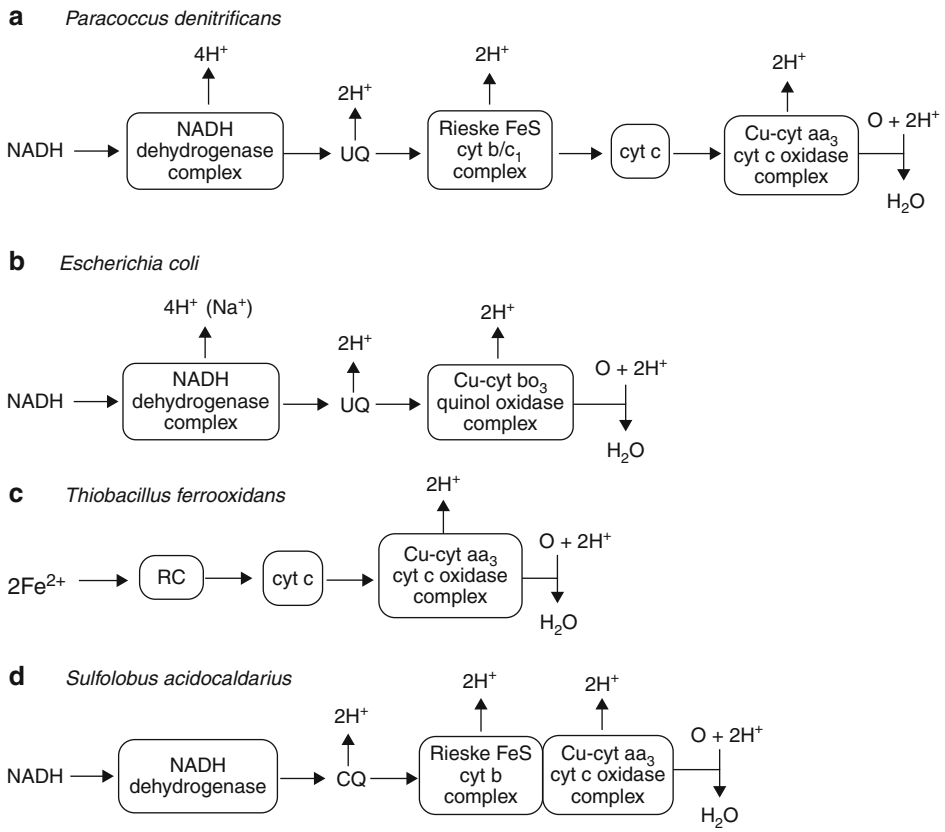


**Fig. 5.24**

**Electron transport in various prokaryotic aerobes: (a) *Paracoccus denitrificans*, (b) *Escherichia coli*, (c) *Thiobacillus ferrooxidans*, and (d) *Sulfolobus acidocaldarius*. *UQ* ubiquinone, *cyt* cytochrome, *RC* rusticyanin, and *CQ* caldariellaquinone**

as a respiratory electron acceptor (Mohn and Tiedje 1992), a process sometimes called "dehalorespiration." It was first demonstrated that 3-chlorobenzoate could serve as an electron acceptor for energy conservation via reductive dechlorination for an organism called *Desulfomonile tiedjei* (DeWeerd et al. 1990; Suflita et al. 1982), a member of the δ-Proteobacteria. Since then, organisms have been shown to conserve energy by reductive dechlorination of chlorophenols and chlorinated ethenes (Holliger et al. 1998; Maymó-Gatell et al. 1997; Mohn and Tiedje 1992), the latter including the solvents tetrachloroethene and trichloroethene, which are particularly pervasive groundwater pollutants. Curiously, although evidence for anaerobic breakdown of less chlorinated methanes is ample (Mägli et al. 1996; Messmer et al. 1993), respiratory utilization of chloroform and carbon tetrachloride has not been described.

Other environmental applications utilizing anaerobic respiration involve reduction of various metals besides Fe(III) (Lovley and Coates 2000). For example, *Desulfovibrio desulfuricans* can reduce U(VI) to U(IV), which is a precipitate, and allows immobilization of radioactive wastes (Lovley and Phillips 1992). Another novel reaction with environmental potential is the respiratory reduction of perchlorate, which is part of rocket propellant mixtures and has contaminated several groundwater aquifer sites, to chloride (Coates et al. 1999; Malmquist et al. 1994).

## Aerobic Metabolism

In aerobic eukaryotes, respiration is carried out by mitochondria, which are now known to be descended from the α-Proteobacteria (Gray et al. 1999). Thus, all respiration on earth is prokaryotic. In essentially all aerobic organisms, electrons travel down an electron transport chain from the organic compound to oxygen in a manner such that protons, or sometimes sodium ions, are pumped out of the cell or mitochondrion, leading to development of an electric potential that can be converted to ATP by membrane-associated ATPases (Saraste 1999).

Diversity of prokaryotic electron transport chains can be considerable. For example, the α-Proteobacterium *Paracoccus denitrificans* utilizes a complex transport chain very closely resembling that in mitochondria consisting of three large membrane-bound enzyme complexes (*Fig. 5.24a*). The first is an NADH/ubiquinone oxidoreductase complex (NADH dehydrogenase or complex I) containing a bound flavin and several iron/sulfur centers. The second is a ubiquinol/cytochrome

*c*-oxidoreductase complex (cytochrome $bc_1$ complex or complex III), which contains heme groups, and the high potential Rieske iron/sulfur center carries out a Q cycle (Saraste 1999), thereby conserving energy from ubiquinone transport. Cytochrome c is reduced in the periplasm and transfers its electrons to a copper- and heme-containing cytochrome c oxidase (complex IV), which passes the electrons from a periplasmic cytochrome *c* to oxygen. Protons are pumped out of the cell by each of the complexes, and by the asymmetric reduction of ubiquinone on the inside of the cell membrane and its oxidation of the outside (the Q loop) and by the consumption of protons inside the cell by the reduction of oxygen to water. It should be mentioned that *P. denitrificans* actually has several different terminal oxidases that it regulates in response to growth conditions (De Gier et al. 1994).

*Escherichia coli* has a simpler electron transport chain that lacks complex III, so that electrons are passed directly from the quinol to an oxidase complex (Fig. 5.24b). Thus, it does not pump as many protons per oxygen as does *P. denitrificans*. Moreover, under low oxygen conditions, *E. coli* induces more of a quinol oxidase complex containing hemes *b* and *d*. This complex does not pump protons at all, but has a lower $K_m$ for oxygen, which is a useful trade-off.

*Thiobacillus ferrooxidans* is an acidophile which transports electrons from Fe(II) to oxygen, and inasmuch as the Fe(II)/Fe(III) couple is near +0.77 v, Fe(II) cannot reduce NADH (−0.32 v) or quinones (ca. 0.0 v). Therefore, *T. ferrooxidans* has a truncated electron transport chain in which electrons flow from Fe(II) to a small periplasmic copper-containing protein called "rusticyanin," then to cytochrome c, and finally to a typical oxidase (Fig. 5.24c). Thus, *T. ferrooxidans* conserves less energy per electron than do organisms using reactions in which NADH is the electron donor.

As an example of an archaeal electron transport chain, the thermoacidophile *Sulfolobus acidocaldarius* can use elemental sulfur or organic compounds as electron donors for its aerobic growth. When growing on organic compounds, it transports electrons from NADH to oxygen using an electron transport chain that is similar to those in eubacteria, but with some interesting differences (Fig. 5.24d). Its NADH dehydrogenase complex is relatively small and does not appear to pump protons; it uses a sulfur-containing quinone called "calderiellaquinone," and the electrons then go through a supercomplex equivalent to the two found in *Paracoccus*, but lacking cytochrome *c* (Schäfer et al. 1996). The main subunit of the terminal oxidase complex shows genetic relatedness to other copper-heme oxidases, which form a gene family (Garcia-Horsman et al. 1994) that also includes nitric oxide reductase from denitrifiers, suggesting that the different reductases had a common origin.

The energetics of aerobic respiration is so energetically favorable that the oxidation of essentially any organic compound is thermodynamically feasible, and the only limitation for an organism to utilize a particular organic compound is devising an energy-conserving breakdown pathway. Typically, microorganisms can break down any compound made by nature, as well as many nonnatural synthetic compounds (xenobiotics). There are exceptions to this doctrine of microbial infallibility (Alexander 1981) including some polymers and some smaller molecules that do not resemble natural substrates, leading to persistence of some toxic chemicals in the environment. Nevertheless, a novel compound existing in an environment at a reasonably high concentration may eventually select for organisms capable of using it. For example, *Burkholderia cepacia* strain AC1100 has, apparently by mutation and genetic exchange, developed a pathway to utilize the herbicide 2,4,5-T, originally considered nondegradable, as a growth substrate (Haugland et al. 1990; Huebner et al. 1998).

## Lithotrophy and Methanotrophy

Another metabolic capability found uniquely in prokaryotes is lithotrophy, the ability to use inorganic electron donors for energy conservation. Their ability to oxidize inorganic nitrogen and sulfur compounds makes prokaryotic lithotrophs important components of the biogeochemical cycles of these elements. Both aerobes and anaerobes can oxidize inorganic substrates, such as hydrogen oxidation by methanogens, but we will confine our discussion mainly to aerobes. Because they use inorganic substrates for energy conservation, lithotrophs are often autotrophs, that is, they fix carbon dioxide, but some can incorporate organic carbon. Methanotrophy, the ability to utilize methane as an electron donor, has only been found in certain aerobic members of the Proteobacteria.

The nitrifiers, bacteria that oxidize ammonia and nitrite to nitrate, were originally characterized in the classic studies of Winogradsky. One set of organisms, sometimes called "nitrosofiers," oxidizes ammonia to nitrite, followed by oxidation of nitrite to nitrate. The reason that oxidation of ammonia to nitrate requires two separate microbial groups is not understood. Most extant nitrifiers are members of the Proteobacteria, although the nitrite oxidizer *Nitrospira* is in a distinct phylum, and all can fix $CO_2$ by the Calvin cycle. Because organic matter generally inhibits nitrofiers, Winogradsky used silica gel plates rather than agar to isolate them.

The aerobic oxidation of ammonia begins with oxidation to hydroxylamine by ammonia monooxygenase, making that process obligately aerobic. However, an anaerobic oxidation process, called the "anammox reaction," has been found in which ammonia is the electron donor and nitrite is the electron acceptor (Table 5.2). The organism responsible for this reaction has not been isolated, but strong molecular biological evidence shows that the predominant organism in the enrichment culture is a member of the phylum Planctomycetes (Strous et al. 1999).

The "colorless" sulfur bacteria are so named to differentiate them from the purple sulfur and green sulfur photosynthetic bacteria. They oxidize reduced sulfur compounds, often the product of sulfate reducers in anaerobic zones, so they are often found at anaerobic/aerobic interfaces. Typically they can oxidize sulfide, elemental sulfur (to which they can sometimes be attached or which they store in cellular vacuoles), or

**Table 5.2**
**Reactions carried out by aerobic lithotrophs and methanotrophs**

| Reaction | $\Delta G^{\circ\prime}/2e^-$ (kJ) | Organism type | Example species |
|---|---|---|---|
| $NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + 2\ H_2O$ | −137 | Nitrosofying bacteria | *Nitrosomonas europaea* |
| $NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$ | −76 | Nitrifying bacteria | *Nitrobacter winogradskyi* |
| $NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$ | −238 | Anammox organisms | *Brocadia anammoxidans* |
| $S^\circ + 1.5\ O_2 \rightarrow SO_4^{2-} + 2\ H^+$ | −196 | "Colorless" sulfur bacteria | *Thiobacillus thiooxidans* |
| | | | *Sulfolobus acidocaldarius* |
| $2\ Fe^{2+} + 2\ H^+ + 0.5\ O_2 \rightarrow 2\ Fe^{3+} + H_2O$ | −66[a] | Iron bacteria | *Thiobacillus ferrooxidans* |
| | | | *Sulfolobus acidocaldarius* |
| $2\ FeS_2 + 7.5\ O_2 + 7\ H_2O \rightarrow 2\ Fe(OH)_3 + 8\ H^+ + 4\ SO_4^{2-}$ | −164[a] | Iron bacteria | *Thiobacillus ferrooxidans* |
| | | | *Metallosphaera sedula* |
| $H_2 + 0.5\ O_2 \rightarrow H_2O$ | −237 | Hydrogen bacteria | *Ralstonia eutropha* |
| $CH_4 + 2\ O_2 \rightarrow HCO_3^- + H^+ + H_2O$ | −203 | Methanotrophs | *Methylococcus capsulatus* |

Abbreviation: *Anammox* Anaerobic ammonium oxidation
[a]Values for pH = 2

thiosulfate. Because their main metabolic product is sulfuric acid, some of the sulfur oxidizers are acidophiles. Many of the Gram-negative rods, both acidophilic and neutrophilic, which carry out this reaction, were named "*Thiobacillus*." However, these organisms are scattered throughout the Proteobacteria and many will require new generic assignments. There are also some filamentous sulfur-oxidizing bacteria such as *Beggiatoa* and *Thiothrix*, which are also in the proteobacteria (although *Beggiatoa*, because of its close morphological resemblance to the filamentous cyanobacterium *Oscillatoria*, was once considered by some to be a colorless cyanobacterium). There are also archaeal sulfur oxidizers, such as *Sulfolobus*.

Some sulfur oxidizers can use nitrate as an electron acceptor. One interesting example is the large (ca. 50 μm in diameter) gliding filaments called *Thioploca*, which form large mats in upwelling zones. When these filaments are in the aerobic zone, they concentrate nitrate from seawater, where its concentration is near 25 mM, into intracellular vacuoles, where the nitrate concentrations can reach the remarkably high concentration of 5 M. They can then glide down into the anaerobic parts of the mat, where they can then use the nitrate for oxidation of sulfide (Fossing et al. 1995). An even larger organism, up to 1 mm in diameter, is the spherical organism, called "*Thiomargarita*" (sulfur-pearl), which consists of a thin film of cytoplasm around a large nitrate-filled vesicle. These organisms are uniquely poised to exploit the anaerobic/aerobic interface by using an electron acceptor that allows them to use sulfide in anaerobic layers where aerobic metabolism is excluded.

Ferrous iron spontaneously oxidizes at pH 7, which has hampered studies on neutrophilic iron oxidizers. Several organisms, such as the sheathed bacterium *Leptothrix* and the stalked bacterium *Gallionella*, are known to precipitate iron and manganese oxides, but it is still unclear what role they play in their metabolism. More recently, Emerson and Moyer (1997) used agar gradient cultures in which reduced iron diffused from an agar plug at the bottom while oxygen diffused from the top. Microorganisms grew as a band at the iron/oxygen interface in these cultures, from which neutrophilic iron oxidizers were isolated.

At low pH, ferrous iron is stable in the presence of oxygen, and *Thiobacillus ferrooxidans* and other iron-oxidizing organisms such as *Leptospirillum ferrooxidans* can be readily isolated from acidophilic environments. Acidophilic Archaea such as *Sulfolobus acidocaldarius* and the newly described mesophile *Ferroplasma acidarmanus* (Edwards et al. 2000) can also oxidize ferrous iron. Most organisms that can oxidize ferrous iron and sulfur compounds play a role in the leaching of metal sulfide minerals such as pyrite ($FeS_2$). The exposure of pyrite minerals to oxygen during coal mining leads to their oxidation to sulfuric acid, resulting in acid mine drainage. On the other hand, this ability has been taken advantage of to recover metals such as copper from low-grade sulfide ores. Thus, the iron-oxidizing prokaryotes can have positive or negative economic effects.

The ability to oxidize hydrogen (which only requires the ability to link a hydrogenase with an electron transport chain) is widespread in the Bacteria and Archaea. Because of labile iron/sulfur centers in hydrogenases, often hydrogen oxidation occurs under microaerophilic conditions in aerobes. Some hydrogen bacteria can grow as autotrophs, but many will incorporate organic carbon when available.

Methanotrophy, the ability to oxidize methane, has only been found in two clusters in the Proteobacteria. Methylotrophy, the ability to use single-carbon compounds such as methanol, is more widespread, occurring in many bacteria and archaea, as well as in certain yeasts. The first step in aerobic methanotrophs is the hydroxylation of methane to methanol by methane monooxygenase, making the process obligatorily aerobic. However, there is considerable evidence that methane may be oxidized under anaerobic conditions (Boetius et al. 2000), but the organisms involved have thus far proved elusive. It was long believed that the intermediates after methanol in the

**Table 5.3**
**Properties of photosynthetic bacteria and chloroplasts**

| | Phylogenetic group | Reaction center chlorophyll | Accessory pigments | Electron donors | Primary electron acceptor | $CO_2$ fixation pathway |
|---|---|---|---|---|---|---|
| Purple nonsulfur bacteria | α and β Proteobacteria | bcl *a* or *b* | chl *a* or *b* | Organic compounds, $H_2$, $Fe^{2+}$, $H_2S$ (at low concentration) | Q | Calvin cycle |
| Purple sulfur bacteria | γ Proteobacteria | bcl *a* | chl *a* or *b* | $H_2S$, $H_2$, some organic compounds, etc. | Q | Calvin cycle |
| Heliobacteria | Firmacutes | bcl *g* | — | Organic compounds | Fd | Calvin cycle |
| Green sulfur bacteria | Chlorobi | bcl *a* | bcl *c,d,e* (chlorosomes) | $H_2S$, $S_2O_3^{2-}$, $H_2$ | Fd | Reverse TCA cycle |
| Green nonsulfur bacteria | Chloroflexi | bcl *a* | bcl *c,d,e* (chlorosomes) | Organic compounds, $H_2$, $H_2S$ | Q | Hydroxypropionate cycle |
| Cyanobacteria | Cyanobacteria | chl *a* | Phycobilins or chl *b* | $H_2O$ | Q (PSI)<br>Fd (PSII) | Calvin cycle |
| Chloroplasts | Cyanobacteria | chl *a* | Phycobilins or chl *b* | $H_2O$ | Q (PSI)<br>Fd (PSII) | Calvin cycle |

Abbreviations: *Q* Quinone, *Fd* Ferredoxin, *TCA* Tricarboxylic acid, *PS* Photosystem

pathway of methane oxidation were free one-carbon compounds such as formaldehyde and formate (Dworkin and Foster 1956), but it has recently been shown that the pathway in many methanotrophs and methylotrophs closely resembles that in methanogenic Archaea in which intermediates are bound to tetrahydromethanopterin and methanofuran and involves which enzymes homologous to those in methanogens (Chistoserdova et al. 1998).

Eukaryotes are unable to utilize inorganic energy sources or methane, and therefore, some living in habitats in which these energy sources are abundant, such as anaerobic/aerobic sediment interfaces or near undersea spreading centers, have entered symbioses with lithotrophs or methanotrophs. One of the best known examples is the giant tube worm *Riftia* living near undersea spreading centers and using intracellular bacteria that oxidize sulfide and fix $CO_2$ via the Calvin cycle (Robinson et al. 1998). A wide variety of marine invertebrates, especially clams and mussels, have either sulfide-oxidizing or methane-oxidizing symbionts, sometimes both (Distel and Cavanaugh 1994). Thus, the eukaryotic hosts have used the prokaryotes to augment their metabolic capabilities, much as eukaryotic hosts did when entering symbioses with the ancestors of the mitochondria and chloroplasts or with symbiotic bacteria providing needed nutrients (Moran and Baumann 2000).

## Phototrophy

Phototrophy, the ability to use light as an energy source, is yet another invention of prokaryotes. Photosynthesis in eukaryotes is carried out in chloroplasts, conclusively shown to be derived from cyanobacteria and probably the result of a single endosymbiotic event (Moreira et al. 2000). There are two general classes of phototrophy in prokaryotes. The first is one based on the now inappropriately named "bacteriorhodopsin" (it should be "archaerhodopsin") found in halophilic Archaea. Bacteriorhodopsin is a polypeptide with a retinal prosthetic group, which allows it to pump protons, thereby generating a protonmotive force. This system seems to be supplemental to normal heterotrophic growth in these organisms, although its actual ecophysiological role is unclear. Besides bacteriorhodopsin, the halobacteria can possess halorhodopsin, a light-driven chloride pump, and two types of sensory rhodopsins used in phototaxis (Spudich 1993). Recently, a DNA fragment cloned from ocean water was found to have, besides a 16S rDNA gene, which showed it to be from a proteobacterium, a gene encoding rhodopsin-like protein (Beja et al. 2000). When expressed in *E. coli* and provided with retinal, this protein pumped protons, and it has been given the name "proteorhodopsin."

The major type of phototrophy is that based on chlorophylls. Among the prokaryotes, there is considerable diversity of pigments, photosystems, and electron donors, all adapted to a variety of ecological niches. The photosynthetic bacteria can be placed into five groups, the purple bacteria (traditionally divided into the purple sulfur and nonsulfur bacteria, based on their use of reduced sulfur or organic compounds as the preferred electron donor), the heliobacteria, the green sulfur bacteria, the green nonsulfur bacteria, and the cyanobacteria (*Table 5.3*). Phylogenetic analysis of the 16S rDNA of the photosynthetic bacteria (Stackebrandt et al. 1996) shows that these groups occupy different phyla.

The purple bacteria are found in three subphyla of the diverse phylum Proteobacteria, which includes many typical "Gram-negatives" such as *E. coli*, *Pseudomonas*, and *Rhizobium*.

The purple nonsulfur bacteria are spread within the α- and β-subphyla, and the purple sulfur bacteria are associated with the γ-subphylum. This distribution strongly suggests that the ancestor of these groups was itself a "purple bacterium" and that the current nonphotosynthetic organisms in the Proteobacteria, like *E. coli*, evolved from photosynthetic ancestors.

Similarly, it was a surprise when the heliobacteria, a group physiologically similar to the purple nonsulfur bacteria, were found to be members of the Gram-positive phylum Firmacutes, although in this case, only a single small branch within the phylum contains photosynthetic organisms. The green sulfur bacteria form essentially their own separate phylum, the Chlorobia. According to 16S rDNA phylogeny, the green nonsulfur bacteria, despite similarities of their photopigments, are not closely related to the green sulfur bacteria and are the founding members of the phylum Chloroflexus. Interestingly, the phylogeny of genes for chlorophyll synthesis in the green sulfur and nonsulfur bacteria indicates a relatively close affinity of the two groups, suggesting horizontal gene transfer of the photosynthesis genes (Xiong et al. 2000). Finally, the cyanobacteria also form a separate phylum, which also includes chloroplasts.

The cyanobacteria and chloroplasts contain chlorophyll *a* (chl *a*) as their primary photosynthetic pigment in their reaction centers, which absorbs light maximally at 680–700 nm, depending on whether it is in photosystem I or II. The other photosynthetic bacteria possess only a single photosystem, most containing mainly bacteriochlorophyll a (bcl *a*), which absorbs maximally in the infrared at 800–870 nm. Exceptions include certain purple nonsulfur bacteria which utilize bcl *b*, which absorbs maximally in the far infrared at 1,050 nm, and the heliobacteria, which utilize the interesting pigment bcl g, which absorbs maximally at 790 nm and breaks down to chl *a* upon exposure to oxygen.

All phototrophs contain carotenoids (which make the purple bacteria "purple" and green bacteria green) as accessory pigments to widen the spectrum of light used. In the purple bacteria, the structure of the antenna complex in the membrane, which feeds excitation energy into the reaction center, forms a set of concentric circles around the reaction center (Cogdell et al. 1999). In the green sulfur and nonsulfur bacteria, light energy is transmitted to the reaction center in the cell membrane by the chlorosome (a macromolecular complex containing protein and bacteriochlorophylls *c*, *d*, and *e*), which is associated with the inside face of the cell membrane. In most cyanobacteria, the main accessory pigments that transmit energy to photosystem II are the phycobilins (tetrapyrrole-containing proteins, phycocyanin being the one that confers the blue part of the blue-green color of most members of this group). Chloroplasts can contain either phycobilins, as in the case of the red algae, or chl *b*, as found in plants and green algae, as the main accessory pigment. Finding cyanobacteria with chl *b* suggested that they were the ancestors of the green plant chloroplasts; however, phylogenetic analyses have demonstrated that these "prochlorophytes" are simply distinct strains of cyanobacteria with no direct relationship with chloroplasts. The genes for chl *b* synthesis in cyanobacteria and chloroplasts appear to share a common evolutionary origin (Tomitani et al. 1999), but whether that is due to the ancestor of the cyanobacteria and chloroplasts containing chl *b* or horizontal gene transfer has yet to be determined.

Van Niel's insight that the photosynthetic reduction of $CO_2$ in green plants was a consequence of [H] released by the photolysis of water (van Niel 1949) was a stroke of genius and led to a unifying equation of photosynthesis: $CO_2 + 2\ H_2A \rightarrow CH_2O\ H_2O + A$, where A could be O, S, an organic constituent, or even nothing in the case of $H_2$. It also rationalized the diversity among the photosynthetic bacteria, as it became clear that water in the cyanobacteria reduced sulfur in the green and purple sulfur bacteria, and organic molecules in the purple nonsulfur bacteria all served the same purpose—that of providing reducing power for biosynthesis. And each of these sources of reducing power made it possible for that particular group of photosynthetic prokaryotes to exploit a particular ecological niche: water in the case of the cyanobacteria, areas rich in reduced sulfur for the green and purple sulfur bacteria, and areas with organic substrates for the purple and green nonsulfur bacteria.

Photosynthetic pigments have adapted further. All of the chlorophyll molecules have the same basic ground plan—a substituted Mg-tetraporphyrin. However, the nature of the substitutions and of the sequence of conjugated double bonds generates a series of absorption spectra that span from 680 nm for the major absorption peak of chlorophyll *a* in the cyanobacteria to 1,035 nm for the major absorption maximum for bacteriochlorophyll *b* in certain purple bacteria. Thus, the photosynthetic prokaryotes are able to span a broad range of photic zones. The green and purple bacteria usually occupy shallow, aquatic areas, underlying the oxygenic cyanobacteria that filter out the relatively short, visible wavelengths of light and transmit the longer, near-infrared wavelengths. This orientation is consistent with the fact that photosynthesis in the green and purple bacteria is an anoxygenic process and their photosynthesis is obligately anaerobic.

## Autotrophic $CO_2$ Fixation

Carbon dioxide is an abundant and available source of carbon, but it must be reduced to cellular organic carbon at approximately the level of $CH_2O$, a process that requires reducing power and usually requires energy. Organisms that use $CO_2$ as their primary carbon source are autotrophs and serve as primary producers in ecosystems. Autotrophic carbon dioxide fixation in eukaryotes is represented solely by the Calvin cycle, though among the prokaryotes, there are several distinct pathways of autotrophic $CO_2$ fixation: (1) the Calvin cycle, (2) the reverse TCA cycle, (3) acetogenesis, and (4) the hydroxypropionate pathway.

The Calvin cycle, which was discovered initially in the green algae, is also found among many of the photolithotrophic and chemolithotrophic Bacteria, where it serves as the major mechanism for carbon assimilation (*Fig. 5.25*). The first step of $CO_2$

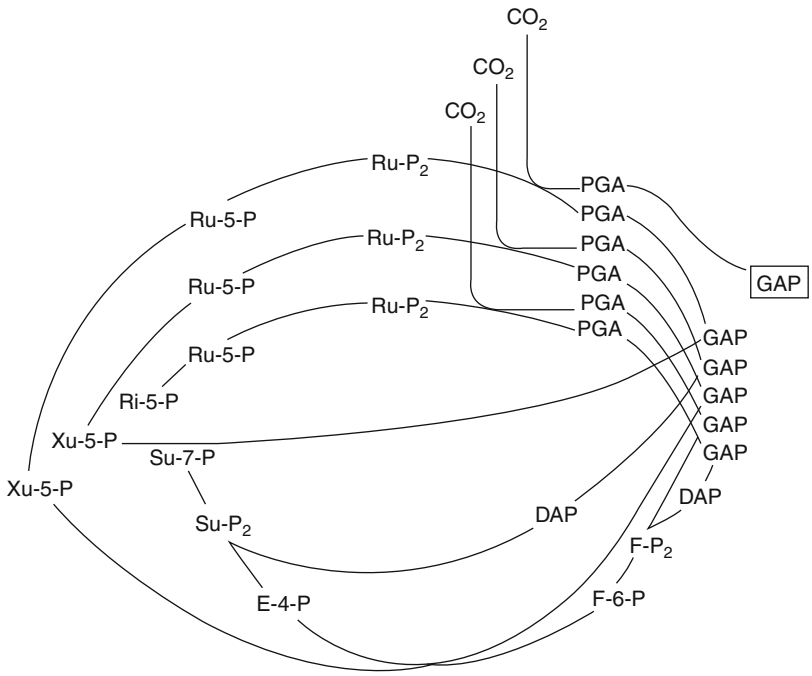


**Fig. 5.25**
**The Calvin cycle. Abbreviations: *Ru-P2* ribulose-1,5-bisphosphatem *PGA* 3-phosphoglycerate, *GAP* glyceraldehyde 3-phosphate, *DAP* dihydroxyacetone phosphate, *F-P2* fructose-1,6-bisphosphate, *F-6-P* fructose-6-phosphate, *E-4-P* erythrose-4-phosphate, *Su-P2* sedoheptulose-1,7-bisphosphate, *Su-7-P* sedoheptulose-7-phosphate, *Xu-5-P* xylulose-5-phosphate, *Ri-5-P* ribose-5-phosphate, *Ru-5-P* ribulose-5-phosphate (Adapted from Gottschalk 1986)**

fixation is the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) reaction, resulting in the conversion of one molecule of ribulose-1,5-bisphosphate and $CO_2$ to two molecules of 3-phosphoglyceric acid. These are then reduced to glyceraldehyde 3-phosphate, followed by several rearrangements to regenerate ribulose-1,5-bisphosphate. Rubisco from the Bacteria has been classified into two types. Type I is found in plants, cyanobacteria, and several other prokaryotes and has a subunit structure consisting of eight large and eight small subunits. Type II Rubisco is found in certain purple nonsulfur bacteria and a few other organisms and has been found to be a dimer of large subunits. The phylogenetic tree for Rubisco shows many branching orders considerably different from that of the 16S rRNA tree, and this has been considered strong evidence for rampant genetic transfer of this gene (Delwiche and Palmer 1996). Moreover, genes resembling those encoding Rubisco have been found in archaeal genomes, and it has been shown that the corresponding proteins have Rubisco activity (Maeda et al. 1999; Watson et al. 1999). These Rubisco homologues form yet another phylogenetic cluster, and the metabolic role of these enzymes in their host organisms has yet to be determined because most evidence indicates that $CO_2$ in methanogenic archaea is fixed via the acetogenic pathway (see below).

The Chlorobiaceae, known colloquially as the "green sulfur bacteria," were shown not to use the Calvin cycle for $CO_2$ fixation. Instead, they run the tricarboxylic acid (TCA) cycle in the reverse, reductive direction, using it to fix $CO_2$ eventually into acetyl-CoA and pyruvate, rather than in the conventional oxidative direction (*Fig. 5.26*). Most, but not all, of the enzymes will catalyze the reactions in the reverse direction. Thus, the Chlorobiaceae have replaced succinic dehydrogenase with fumarate reductase, substituted an α-ketoglutarate dehydrogenase-ferredoxin oxidoreductase for the conventional α-ketoglutarate dehydrogenase complex, and replaced the irreversible citrate synthase with an ATP-citrate lyase. Since its discovery in the Chlorobiaceae, the reductive TCA cycle has also been found in Desulfobacter hydrogenophilus in the δ-subphylum of the Proteobacteria; in members of the Aquificae, a deeply branching hyperthermophilic phylum in the Bacteria; and in anaerobic members of the Crenarchaeota. It was thought to be present in some aerobic Crenarchaeota, but recent results indicate that a different pathway functions in those organisms (see below).

As mentioned previously in the discussion of anaerobic respiration, acetogenic bacteria can convert $H_2$ and $CO_2$ into acetate, the equivalent of fixing $CO_2$ into organic matter. Indeed, many acetogens can grow in mineral medium using $CO_2$ as a carbon source and are therefore autotrophs. The pathway, shown in *Fig. 5.27*, leads to the fixation of two moles of carbon into acetate. The methyl group of acetate is synthesized by reducing one-carbon units to methyl-tetrahydrofolate. This methyl group is transferred to a corrinoid-containing iron-nickel-sulfur enzyme complex called "carbon monoxide dehydrogenase" or "acetate synthetase/decarbonylase." The methyl

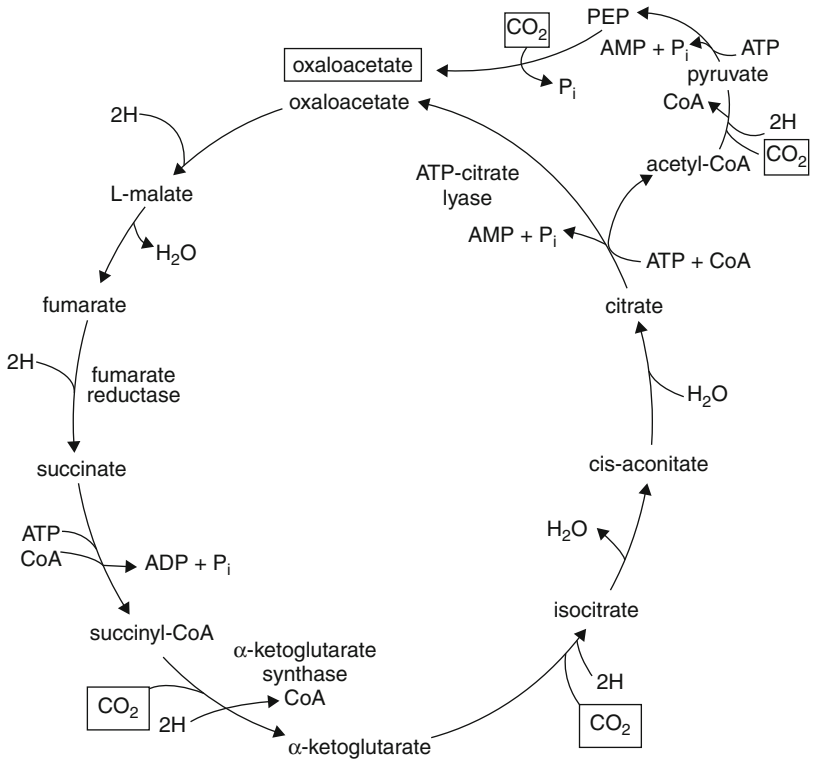


**Fig. 5.26**
**The reductive or reversed tricarboxylic acid cycle (Adapted from Gottschalk 1986)**

group is transferred to the cobalt of a corrinoid, and $CO_2$ is reduced to the equivalent of carbon monoxide. These are then assembled into an enzyme-bound acetyl group, which is released as acetyl-coenzyme A (CoA), which can be conserved as ATP in catabolism or can be used for biosynthesis. This pathway costs a cell only one ATP per two carbons fixed to acetyl-CoA. This pathway is also found in certain sulfate reducers in the δ-subphylum of the Proteobacteria (Menendez et al. 1999). A variation of this pathway using methanopterin derivatives rather than folates is found in autotrophic methanogenic Archaea as well as in the sulfate-reducing Archaeoglobus (Menendez et al. 1999). Thus, the acetogenic pathway is responsible for much of the $CO_2$ fixation occurring in anoxic habitats.

Finally, the pathway for $CO_2$ fixation in the green nonsulfur photosynthetic bacterium *Chloroflexus aurantiacus* was not clear until it was demonstrated (Strauss and Fuchs 1993) that a novel pathway based on carboxylation of acetyl-CoA and propionyl-CoA (*Fig. 5.28*), sometimes called the "3-hydroxypropionate pathway," is responsible for $CO_2$ fixation to glyoxalate in these organisms. The key enzymes of this pathway are acetyl-CoA carboxylase and propionyl-CoA carboxylase. This pathway has also been found in aerobic lithotrophic Crenarchaeota such as *Sulfolobus* (Menendez et al. 1999). The distribution of these four autotrophic pathways is summarized in *Table 5.4*.

## Nitrogen Fixation

Fixation of $N_2$ is carried out solely by prokaryotes. Until the twentieth century, when a large amount of nitrogen was fixed anthropogenically by the Born-Haber process, essentially all nitrogen found in eukaryotes was originally fixed by prokaryotes and transferred to them either indirectly through the food chain or directly in symbiosis, as in the case of the symbioses between legumes and rhizobia found in their root nodules. Nitrogen fixation is found in a variety of physiological types of prokaryotes, occurring in aerobes, microaerophiles, anaerobes, phototrophs, and free-living as well as symbiotic bacteria. Phylogenetically it is widespread in the Bacteria, including members of the phyla Proteobacteria, Firmacutes, Actinobacteria, Chlorobia, and Cyanobacteria. In the Archaea, nitrogen fixation has only been found thus far in the methanogens (Lobo and Zinder 1992).

The enzyme that is responsible for the process, nitrogenase, is a complex of two metalloprotein components. One component usually contains an iron/sulfur/molybdenum cluster that is considered the active site for dinitrogen reduction, as well as an unusual iron/sulfur cluster called the "P cluster" (Dean et al. 1993). The second component is sometimes called the "Fe protein" and contains a single 4Fe-4S cluster per homodimer and transfers electrons to the first component, accompanied by hydrolysis of approximately two ATPs to ADPs per electron transferred. Because this process is so energetically expensive, it is not surprising that most free-living organisms regulate expression of nitrogenase genes (Berger et al. 1994) and activity

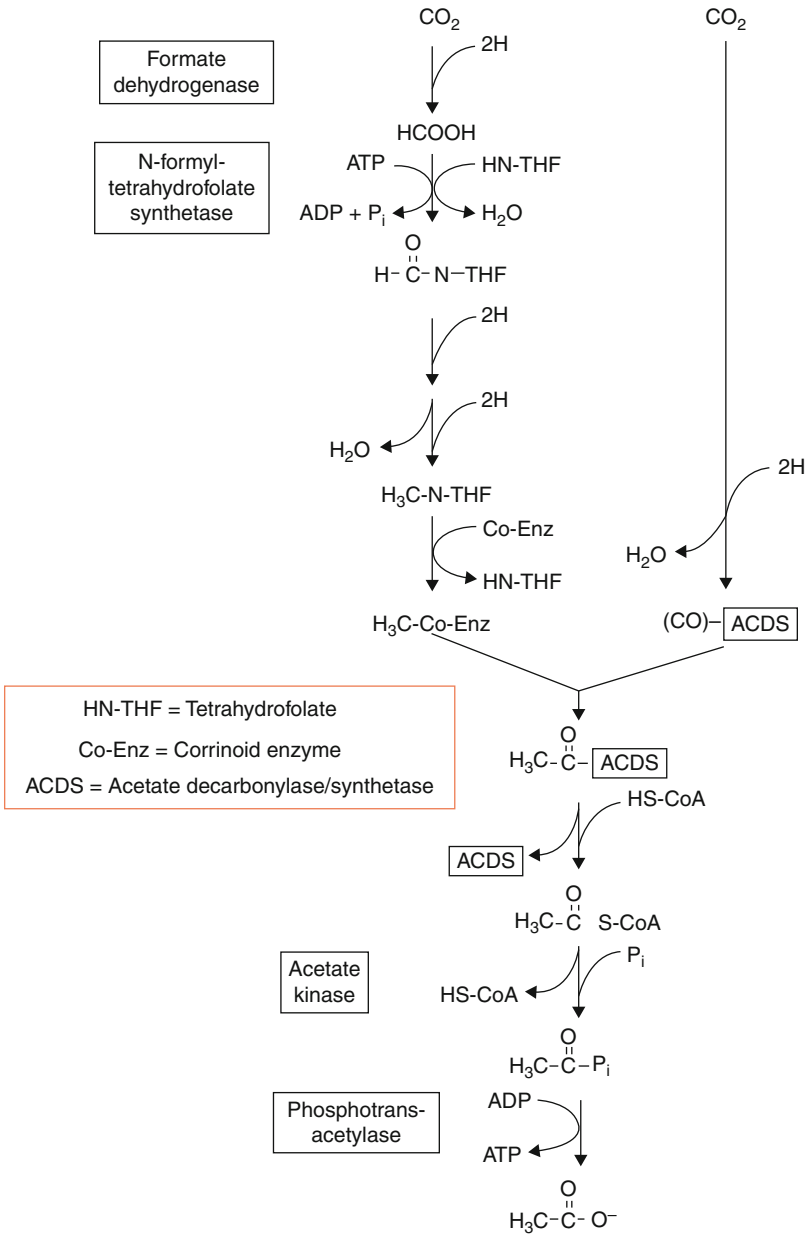


**Fig. 5.27**
**The acetogenic pathway**

(Ludden and Roberts 1988). Alternative nitrogenases exist in which molybdenum (Mo) is replaced by vanadium (V) or in which no metal other than iron has been found (Bishop and Premakumar 1992). The nitrogenase protein components contain many highly conserved amino acid sequence motifs involved in cofactor binding and subunit interactions. Interestingly, these proteins are homologous to proteins involved in reductive steps of chlorophyll *a* biosynthesis (Fujita and Bauer 2000), providing a link between the processes of nitrogen fixation and photosynthesis.

Both protein components of nitrogenase are extremely sensitive to oxygen, and the bacteria fixing nitrogen aerobically have evolved a variety of strategies to protect the nitrogenase from oxygen poisoning. Among the members of the genus *Azotobacter*, there are three mechanisms for nitrogenase protection. These are respiratory protection, conformational protection, and oxygen regulation of nitrogenase synthesis (Kennedy and Toukdarian 1987). Respiratory protection occurs because *Azotobacter* can consume oxygen much faster than its rate of entry into the cell. These unusually high rates of respiration thus result in maintaining the nitrogenase in an essentially anoxic environment. Indeed, limiting *Azotobacter* respiration increases their sensitivity to oxygen during nitrogen fixation. Conformational protection is a result of the ability of *Azotobacter* to synthesize another FeS protein that enters into an association with the nitrogenase complex and protects it from $O_2$ inactivation

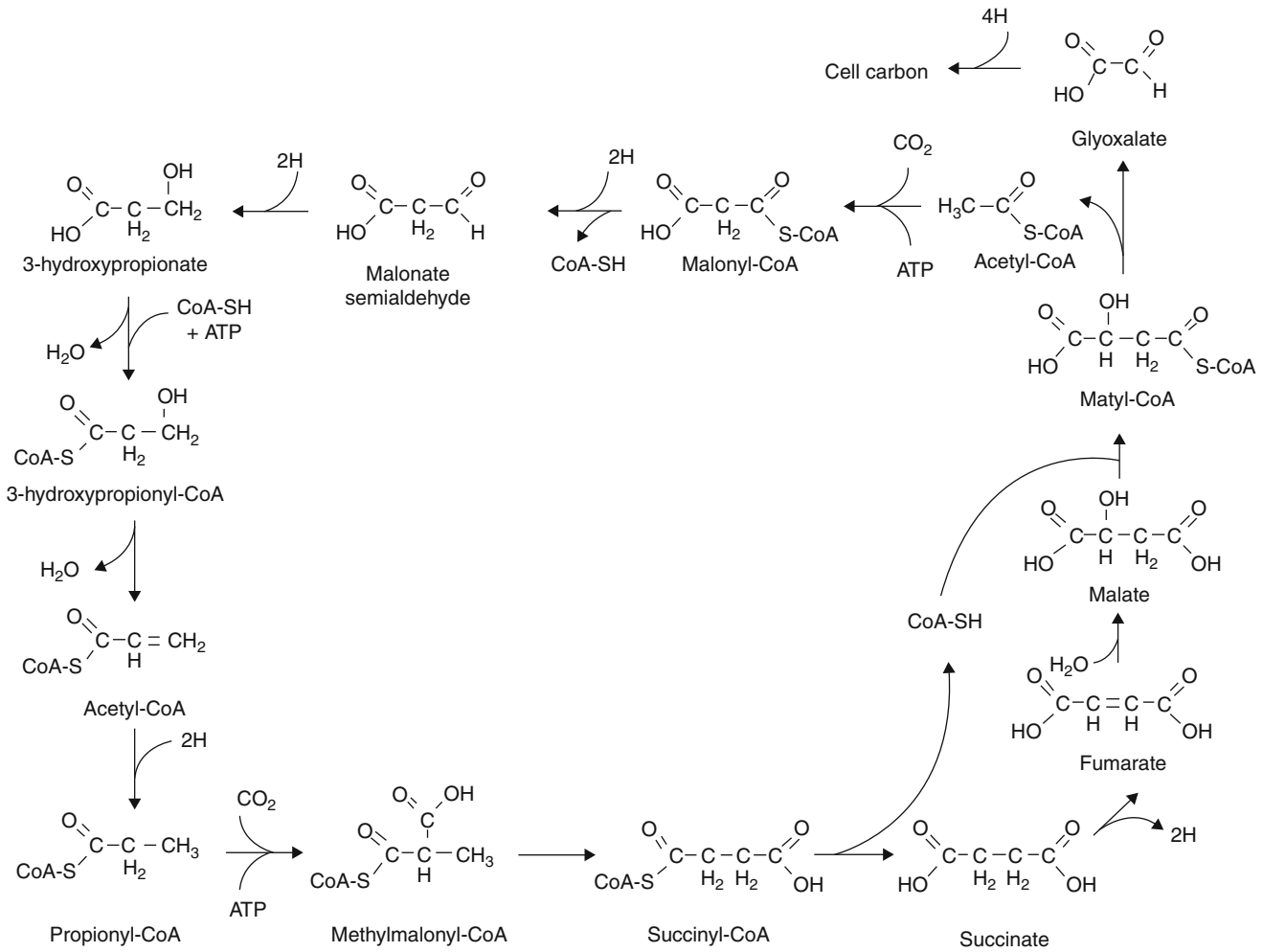


**Fig. 5.28**
**The 3-hydroxypropionate cycle (From Menendez et al. 1999)**

(Moshiri et al. 1994). During this association, the complex is unable to manifest any nitrogenase activity.

Many organisms are not as adept as Azotobacter at protecting their nitrogenase from $O_2$ and fix nitrogen only under microaerophilic conditions, even though they otherwise are not microaerophiles. One example of this is the rhizobia (Spaink 2000). They were not shown to be able to fix nitrogen until 1975, nearly a century after their isolation by Beijerinck. When in the plant root nodule, the rhizobia are protected by the heme protein leghemoglobin, which has an extremely high affinity for $O_2$ and transports the $O_2$ necessary for *Rhizobium* growth, simultaneously preventing the access of the $O_2$ to the nitrogenase (Dilworth and Appleby 1979).

The nitrogen-fixing cyanobacteria are presented with an even greater challenge than other aerobes because $O_2$ is one of the main products of their photosynthetic metabolism. Anabaena and other related filamentous, nitrogen-fixing cyanobacteria solve the problem of $O_2$ poisoning of nitrogenase by segregating the nitrogen-fixing enzymes in a specialized cell called "the heterocyst." The heterocyst insulates the nitrogenase from $O_2$ in two ways. First, it lacks photosystem II and thus does not generate any $O_2$; photosystem I is still operative and continues to generate ATP by photophosphorylation. Second, it is surrounded by a laminated structure consisting of a series of unique glycolipids that seem to act as a physical barrier to prevent $O_2$ from penetrating into the cell. Thus, the cell separates its nitrogenase both from endogenous as well as exogenous $O_2$. The heterocyst can feed the fixed, reduced nitrogen products to the adjoining vegetative cells, from which it receives the reducing power necessary to convert dinitrogen to amino acids. To add to the elegance of the solution, the heterocysts are interspersed along the filament, spaced so as to provide an optimum supply of fixed nitrogen to the growing and dividing vegetative cells. A peptide signal, similar to those used for quorum sensing by Gram-positive bacteria, is used to regulate this spacing (Yoon and Golden 1998). The actinomycete *Frankia*, which fixes nitrogen in nodules in alder trees and several shrubs, utilizes a similar solution, forming specialized cell aggregates called "vesicles" (Benson and Silvester 1993).

Some of the nonheterocystous cyanobacteria have solved the problem of nitrogen fixation and photosynthetic $O_2$ evolution by separating the two processes in time rather than in space.

**Table 5.4**
**$CO_2$ fixation pathways found in prokaryotes**

| Pathway | Representative organisms | Phylum |
|---|---|---|
| Calvin cycle | Plant chloroplasts | Cyanobacteria |
| | *Anabaena cylindrical* | Cyanobacteria |
| | *Rhodobacter sphaeroides* | Proteobacteria |
| | *Thiobacillus ferrooxidans* | Proteobacteria |
| | *Methanococcus jannaschii*[a] | Euryarchaeota |
| Reductive TCA cycle | *Chlorobium limicola* | Chlorobi |
| | *Desulfobacter hydrogenophilus* | Proteobacteria |
| | *Aquifex pyrophilus* | Aquificae |
| | *Thermoproteus neutrophilus* | Crenarchaeota |
| Reductive acetyl-CoA pathway | *Clostridium thermoaceticum* | Firmacutes |
| | *Desulfobacterium autotrophicum* | Proteobacteria |
| | *Methanococcus jannaschii*[b] | Euryarchaeota |
| | *Ferroglobus placidus* | Euryarchaeota |
| Hydroxypropionate cycle | *Chloroflexus aurantiacus* | Chloroflexi |
| | *Sulfolobus metallicus* | Crenarchaeota |

Data from Menendez et al. (1999)
Abbreviations: *TCA* Tricarboxylic acid, and *CoA* Coenzyme A
[a]The function of Rubisco found in methanogens and other Euryarchaeota are not presently known
[b]Pathway uses methanofuran and tetrahydromethanopterin instead of tetrahydrofolate

Thus, nitrogenase is synthesized, and nitrogen fixation takes place in the dark. During the photoperiod, the nitrogenase formed during the previous dark period is presumably destroyed (Stal and Krumbein 1985). Indeed, this was the first evidence for a biological clock in prokaryotes (Johnson and Golden 1999). It is still not clear, however, how the filamentous nonheterocystous colonial cyanobacterium *Trichodesmium* fixes nitrogen during photosynthesis (Capone et al. 1997).

Finally, it should be mentioned that a thermophilic actinomycete utilizes a nitrogenase enzyme complex in which a modified Mo-Fe protein is coupled to a carbon monoxide dehydrogenase (Ribbe et al. 1997). This system is much more $O_2$ resistant than the standard nitrogenases, and it is not clear why similar systems have not been found in other aerobes. In summary, the variety of mechanisms devised by the prokaryotes for protecting nitrogenase from $O_2$ poisoning is an impressive example of the strategic versatility of the prokaryotes.

## Adaptation to Environmental Extremes

The prokaryotes not only can tolerate the broadest spectrum of environmental extremes of any group of organisms, but some of their optimum conditions for growth would roast, freeze, acidify, or shrivel up most other organisms. Thus, *Pyrolobus fumarii* can grow up to 113 °C and survive 1 h of autoclaving at 121 °C (Blochl et al. 1997), *Halobacterium salinarum* grows optimally at 5.2 M salt (Kushner 1985), *Ferroplasma* grows at pH 0 (Edwards et al. 2000), and a wide variety of organisms can grow at temperatures below 0 °C (Morita 1975). It should be pointed out that the organisms specifically referred to above are Archaea. While the ability to thrive under extreme conditions is not an exclusive property of the Archaea, they seem to be able to withstand high temperatures, at least partially attributable to their unusual ether-linked lipids.

In most cases, the mechanisms whereby the organisms are able to thrive in an extreme environment have reduced or eliminated the ability of that organism to tolerate the normal or common environment. In other words, the adaptation to the extreme environment has not extended a particular organism's milieu but rather replaced one optimum with another. Thus, the ability to tolerate, indeed the requirement for, a high salt concentration renders *Halobacterium salinarum* fragile in a normal osmotic milieu, *Pyrolobus* cannot grow at below 80 °C, and *Ferroplasma* cannot grow at a neutral pH.

The ability to exploit a wide variety of ecological niches and to grow optimally at environmental extremes that are intolerable for higher, more complex organisms is a result of the intense physiological and metabolic specialization among the prokaryotes. In a sense, then, the ability of a complex, multicellular organism to deal with its environment is a compromise among the various specialized and differentiated cells; the extremes that such an organism can tolerate are the lowest common denominator of the properties of its individual cells. That is the price paid for the operational complexity of the multicellular organism. On the other hand, the small size, structural simplicity, and unicellular nature of the prokaryote are admirably suited to permit adaptation to a wide variety of environmental extremes, with each adaptive response narrowly focused on dealing optimally with that particular extreme.

## Conclusion

It is appropriate that we end this chapter with a quotation from the late mentor of one of us (M.D.), Professor Jackson W. Foster (Foster 1964):

> The source of the microbiologist's strength, and at the same time, his refuge, is the infinitely large numbers and varieties of microbes known or presumed to be extant. Compounded with those that can be modified artificially, this pool represents an infinitely versatile catalyst. Because, figuratively speaking, it catalyzes innumerable reactions and transformations with virtually any material occurring in nature, the microbe has to be regarded as a prime, natural resource of all men regardless of national boundaries.... As a means of emphasizing. . . the diverse, mysterious, and gratifying potential of the microbe, I paraphrase a trenchant saying made famous many years ago by the influential American magazine, the *Ladies Home Journal*. Whereas they were concerned with women, and I with microbes, the small

perversion I make does no injustice to either – "Never underestimate the power of the Microbe."

## References


Adams MWW (1993) Enzymes and proteins from organisms that grow near and above 100 degrees C. Annu Rev Microbiol 47:627–648

Alexander M (1981) Biodegradation of chemicals of environmental concern. Science 211:132–138

Allen JR, Clark DD, Krum JG, Ensign SA (1999) A role for coenzyme M (2-mercaptoethanesulfonic acid) in a bacterial pathway of aliphatic epoxide carboxylation. Proc Natl Acad Sci USA 96:8432–8437

Angert ER, Clements KD, Pace NR (1993) The largest bacterium. Nature 362:239–241

Bak F, Pfennig N (1987) Chemolithotrophic growth of *Desulfovibrio sulfodismutans* sp. nov. by disproportionation of inorganic sulfur compounds. Arch Microbiol 147:184–189

Balkwill DL, Maratea D, Blakemore RP (1980) Ultrastructure of a magnetotactic spirillum. J Bacteriol 141:1399–1408

Beja O, Aravind L, Koonin EV, Suzuki MT, Hadd A, Nguyen LP, Jovanovich SB, Gates CM, Feldman RA, Spudich JL, Spudich EN, DeLong EF (2000) Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. Science 289:1902–1906

Benson DR, Silvester WB (1993) Biology of Frankia strains, actinomycete symbionts of actinorhizal plants. Microbiol Rev 57:293–319

Berg HC (1976) How spirochaetes may move. J Theor Biol 56:269–273

Berger DK, Narberhaus F, Kustu S (1994) The isolated catalytic domain of NIFA, bacterial enhancer-binding protein, activates transcription in vitro: activation is inhibited by NIFL. Proc Natl Acad Sci USA 91:103–107

Beveridge TJ (1995) The periplasmic space and periplasm in gram-positive and Gram-negative bacteria. ASM News 61:125–130

Bishop PE, Premakumar R (1992) Alternative nitrogen fixation systems. In: Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation. Chapman and Hall, New York, pp 736–762

Blochl E, Rachel R, Burggraf S, Hafenbradl D, Jannasch HW, Stetter KO (1997) *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113 degrees C. Extremophiles 1:14–21

Boetius A, Ravenschlag K, Schubert CJ, Rickert D, Widdel F, Gieseke A, Amann R, Jorgensen BB, Witte U, Pfannkuche O (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature 407:623–626

Brun YV, Shimkets LJ (2000) In: Brun YV, Shimkets LJ (eds) Prokaryotic development. ASM Press, Washington, DC, pp 1–500

Burchard RP (1984) Gliding motility and taxes. In: Rosenberg E (ed) Myxobacteria: development and cell interactions. Springer, New York, pp 139–161

Canale-Parola E (1978) Motility and chemotaxis in spirochaetes. Annu Rev Microbiol 32:69–99

Capone DG, Zehr JP, Paerl HW, Bergman B, Carpenter EJ (1997) Trichodesmium, a globally significant marine cyanobacterium. Science 276:1221–1229

Carr NG (1979) Differentiation in filamentous cyanobacteria. In: Parish JH (ed) Developmental biology of prokaryotes. University of California Press, Berkeley, pp 167–201

Chater KE, Hopwood DA (1973) Differentiation in actinomycetes. In: Ashworth JM, Smith JE (eds) Microbial differentiation. Cambridge University Press, London, pp 143–160

Chistoserdova L, Vorholt JA, Thauer RK, Lidstrom ME (1998) C1 transfer enzymes and coenzymes linking methylotrophic bacteria and methanogenic Archaea. Science 281:99–102

Clements KD, Bullivant S (1991) An unusual symbiont from the gut of surgeonfishes may be the largest known prokaryote. J Bacteriol 173:5359–5362

Coates JD, Michaelidou U, Bruce RA, O'Connor SM, Crespi JN, Achenbach LA (1999) Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. Appl Environ Microbiol 65:5234–5241

Cogdell RJ, Isaacs NW, Howard TD, McLuskey K, Fraser NJ, Prince SM (1999) How photosynthetic bacteria harvest solar energy. J Bacteriol 181:3869–3879

Cohn F (1875) Untersuchungen über Bacterien. Beiträge zur Biologie der Pflanzen 1:127–222

Conway T (1992) The Entner-Doudoroff pathway: history, physiology and molecular biology. FEMS Microbiol Rev 9:1–27

Cook KE, Colvin JR (1980) Evidence for a beneficial influence of cellulose production on growth of *Acetobacter xylinum* in liquid medium. Curr Microbiol 3:203–205

Cord-Ruwisch R, Steitz H-J, Conrad R (1988) The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Arch Microbiol 149:350–357

Costerton JW, Nickel JC, Ladd TI (1986) Suitable methods for the comparative study of free-living and surface-associated bacterial populations. In: Poindexter JS, Leadbetter EL (eds) Bacteria in nature, vol 2. Plenum Press, New York, pp 49–84

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Cross T (1981) The monosporic actinomycetes. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds) The prokaryotes. Springer, Berlin, pp 2102–2091, www.prokaryotes.com

De Gier JW, Lubben M, Reijnders WN, Tipker CA, Slotboom DJ, van Spanning RJ, Stouthamer AH, van der Oost J (1994) The terminal oxidases of *Paracoccus denitrificans*. Mol Microbiol 13:183–196

Dean DR, Bolin JT, Zheng LM (1993) Nitrogenase metalloclusters—structures, organization, and synthesis. J Bacteriol 175:6737–6744

Delwiche CF, Palmer JD (1996) Rampant horizontal transfer and duplication of rubisco genes in eubacteria and plastids. Mol Biol Evol 13:873–882

DeWeerd KA, Mandelco L, Tanner RS, Woese CR, Suflita JM (1990) *Desulfomonile tiedjei* gen. nov. and sp. nov., a novel anaerobic dehalogenating, sulfate-reducing bacterium. Arch Microbiol 154:23–30

Dilworth MJ, Appleby CA (1979) Leghemogiobin and Rhizohium hemoproteins. In: Hardy RWF, Bottomly F, Burns RC (eds) A treatise on dinitrogen fixation. Sec I, II. Wiley, New York, pp 691–764

DiMarco AA, Bobik TA, Wolfe RS (1990) Unusual coenzymes of methanogenesis. Annu Rev Biochem 59:355–394

Dimroth P, Schink B (1998) Energy conservation in the decarboxylation of dicarboxylic acids by fermenting bacteria. Arch Microbiol 170:69–77

Distel DL, Cavanaugh CM (1994) Independent phylogenetic origins of methanotrophic and chemoautotrophic bacterial endosymbioses in marine bivalves. J Bacteriol 176:1932–1938

Dow CS, Whittenbury R (1979) Prosthecate bacteria. In: Parish JH (ed) Developmental biology of prokaryotes. University of California Press, Berkeley

Dunny CM, Brown BL, Clewell DB (1978) Induced cell aggregation and mating in Streptococcus faecalis: evidence for a bacterial sex hormone. Proc Nat Acad Sci USA 75:3479–3483

Dworkin M (1973) Cell-cell interactions in the myxobacteria. In: Ashworth JM, Smith JE (eds) Microbial differentiation. Cambridge University Press, London, pp 125–142

Dworkin M, Foster JW (1956) Studies on *Pseudomonas methanica* (Sohngen) nov. comb. J Bacteriol 72:646–659

Edwards KJ, Bond PL, Gihring TM, Banfield JF (2000) An archaeal iron-oxidizing extreme acidophile important in acid mine drainage. Science 287:1796–1799

Eichler B, Pfennig N (1986) Characterization of a new platelet-forming purple sulfur bacterium. *Amoebobacter pedioformis* sp. nov. Arch Microbiol 146:295–300

Emerson D, Moyer C (1997) Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. Appl Environ Microbiol 63:4784–4792

Fosnaugh K, Greenberg EP (1988) Motility and chemotaxis of Spirochaeta aurantia: computer-assisted motion analysis. J Bacteriol 170:1768–1774

Fossing H, Gallardo VA, Jorgensen BB, Huttel M, Nielsen LP, Schulz H, Canfield DE, Forster S, Glud RN, Gundersen JK, Kuver J, Ramsing NB, Teske A,

Thamdrup B, Ulloa O (1995) Concentration and transport of nitrate by the mat-forming sulphur bacterium *Thioploca*. Nature 374:713–715

Foster JW (1964) Speculative discourse on where and how microbiological science as such may advance with application. In: Starr MP (ed) Global impacts of applied microbiology. Wiley, New York, pp 61–73

Fujita Y, Bauer CE (2000) Reconstitution of light-independent protochlorophyllide reductase from purified bchl and BchN-BchB subunits: in vitro confirmation of nitrogenase-like features of a bacteriochlorophyll biosynthesis enzyme. J Biol Chem 275:23583–23588

Garrity GM, Holt JG (2001) A road map to the manual. In: Garrity GA, Boone DR, Castenholz RW (eds) Bergey's manual of systematic bacteriology, vol 1, 2nd edn. Springer, New York, pp 119–166, http://www.cme.msu.edu//bergeys

Giovannoni SJ, Schabatach E, Castenholz R (1987) *Isosphaera pallida*, gen. and comb. nov., a gliding, budding eubacterium from hot springs. Arch Microbiol 147:276–284

Gottschalk G (1986) Bacterial metabolism. Springer, New York, pp 1–380

Gottschalk G, Andreeson JR, Hippe H (1981) The genus *Clostridium* (nonmedical aspects). In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds) The prokaryotes. Springer, Berlin, pp 1176–1803, http://www.prokaryotes.com

Gould SJ (1996) The power of the modal bacter, or why the tail can't wag the dog. Full House Three Rivers Press, New York, pp 167–216

Gray MW, Burger G, Lang BF (1999) Mitochondrial evolution. Science 283:1476–1481

Grimm AC, Harwood CS (1997) Chemotaxis of *Pseudomonas* spp. to the polyaromatic hydrocarbon naphthalene. Appl Environ Microbiol 63:4111–4115

Gupta RS (1998) Protein phylogenies and signature sequences: a reappraisal of evolutionary relationships among archaebacteria, eubacteria, and eukaryotes. Microbiol Mol Biol Rev 62:1435–1491

Haugland RA, Schlemm DJ, Lyons RP, Sferra PR, Chakrabarty AM (1990) Degradation of the chlorinated phenoxyacetate herbicides 2,4-dichlorophenoxyacetic acid and 2,4,5, thrichlorophenoxyacetic acid in pure and mixed bacterial cultures. Appl Environ Microbiol 56:1357–1362

Henrichsen J (1983) Twitching motility. Annu Rev Microbiol 37:81–93

Hoeniger JFM (1965) Development of flagella by Proteus mirabilis. J Gen Microbiol 40:29–42

Holliger C, Wohlfarth G, Diekert G (1998) Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol Rev 22:383–398

Holt SC, Canale-Parola E (1967) Fine structure of *Sarcina maxima* and *Sarcina ventriculi*. J Bacteriol 93:399–410

Huebner A, Danganan CE, Xun L, Chakrabarty AM (1998) Genes for 2,4,5-trichlorophenoxyacetic acid metabolism in *Burkholderia cepacia* AC1100: characterization of the tftC and tftD genes and locations of the tft operons on multiple replicons. Appl Environ Microbiol 64:2086–2093

Hugenholtz P, Goebel BM, Pace NR (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 180:4765–4774

Ingraham JL, Maaløe O, Neidhardt FC (1983) Growth of the bacterial cell. Sinauer Associates, Sunderland

Jarrell KF, Bayley DP, Kostyukova AS (1996) The archaeal flagellum: a unique motility structure. J Bacteriol 178:5057–5064

Johnson CH, Golden SS (1999) Circadian programs in cyanobacteria: adaptiveness and mechanism. Annu Rev Microbiol 53:389–409

Judson HF (1979) Afterward: always the same impasse. In: The eighth day of creation. Simon and Schuster, New York, pp 605–616

Kandler O (1994) Cell wall biochemistry and three-domain concept of life. Syst Appl Microbiol 16:501–509

Kandler O, Weiss N (1986) Regular, non-sporing Gram-positive rods. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 2. Williams and Wilkins, Baltimore, pp 1208–1260, http://www.cme.msu.edu//bergeys

Katz J, Weihs D (1979) Large amplitude on steady motion of a flexible slender propulsor. J Fluid Mech 90:713–723

Keller KH, Grady M, Dworkin M (1983) Surface tension gradients: feasible model for gliding motility of *Myxococcus xanthus*. J Bacteriol 155:1358–1366

Kengen SWM, Stams AJM, de Vos WM (1996) Sugar metabolism in hyperthermophiles. FEMS Microbiol Rev 18:119–137

Kennedy C, Toukdarian A (1987) Genetics of azotobacters: applications to nitrogen fixation and related aspects of metabolism. Annu Rev Microbiol 41:227–258

Kreig N (1984) Aerobic, microaerophilic, motile, helical/vibroid Gram-negative bacteria. In: Krieg NR, Holt JG (eds) Bergey's manual of determinative bacteriology, vol 1. Williams and Wilkins, Baltimore, pp 71–124, http://www.cme.msu.edu//bergeys

Kutzner HJ (1981) The family Streptomycetaceae. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds) The prokaryotes. Springer, Berlin, pp 2028–2090

Lacy J (1971) *Thermoactinomyces sacchari* sp. nov., a thermophilic actinomycete causing bagassosis. J Gen Microbiol 66:327–338

Lapidus IR, Berg HC (1982) Gliding motility of *Cytophaga* strain U67. J Bacteriol 151:384–398

Lechevalier H, Holbert PE (1965) Electron microscopic observations of the sporangial structure of a strain of Actinoplanes. J Bacteriol 89:217–222

Lobo AL, Zinder SH (1992) Nitrogen fixation by methanogenic bacteria. In: Evans H, Stacey G, Burris R (eds) Biological nitrogen fixation. Routledge, Chapman and Hall, New York, pp 191–211

Lovley DR, Coates JD (2000) Novel forms of anaerobic respiration of environmental relevance. Curr Opin Microbiol 3:252–256

Lovley DR, Phillips EJ (1992) Reduction of uranium by *Desulfovibrio desulfuricans*. Appl Environ Microbiol 58:850–856

Ludden PW, Roberts EA (1988) Regulation of nitrogenase activity by reversible ADP-ribosylation of dinitrogenase reductase. In: Boethe H, de Bruijn FJ, Newton WE (eds) Nitrogen fixation: hundred years after. Gustav Fischer Verlag, Stuttgart, pp 157–162

Lünsdorf H, Reichenbach H (1989) Ultrastructural details of the apparatus of gliding motility of Myxococcus fulvus (Myxobacterales). J Gen Microbiol 135:1633–1641

Macnab RM (1999) The bacterial flagellum: reversible rotary propellor and type III export apparatus. J Bacteriol 181:7149–7153

Maeda N, Kitano K, Fukui T, Ezaki S, Atomi H, Miki K, Imanaka T (1999) Ribulose bisphosphate carboxylase/oxygenase from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1 is composed solely of large subunits and forms a pentagonal structure. J Mol Biol 293:57–66

Mägli A, Wendt M, Leisinger T (1996) Isolation and characterization of *Dehlobacterium formicoaceticum* gen. nov. sp. nov., a strictly anaerobic bacterium utilizing dichloromethane as a source of carbon and energy. Arch Microbiol 166:101–108

Malmquist A, Welander T, Moore E, Ternstrom A, Molin G, Stentrom IM (1994) *Edeonella dechloratans*, gen. nov., sp. nov., a new bacterium capable of growing anaerobically with chlorate as an electron acceptor. Syst Appl Microbiol 17:58–64

Matsuyama T, Takagi Y, Nakagawa Y, Itoh H, Wakita J, Matsushita M (2000) Dynamic aspects of the structured cell population in a swarming colony of *Proteus mirabilis*. J Bacteriol 182:385–393

Maymó-Gatell X, Chien YT, Gossett JM, Zinder SH (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science 276:1568–1571

Mayr E (1998) Two empires or three? Proc Natl Acad Sci USA 95:9720–9723

Menendez C, Bauer Z, Huber H, Gad'on N, Stetter KO, Fuchs G (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

Merz AJ, So M, Sheetz MP (2000) Pilus retraction powers bacterial twitching motility. Nature 407:98–102

Messmer M, Wohlfarth G, Diekert G (1993) Methyl chloride metabolism of the strictly anaerobic, methyl chloride-utilizing homoacetogen strain MC. Arch Microbiol 160:383–387

Mohn WW, Tiedje JM (1992) Microbial reductive dehalogenation. Microbiol Rev 56:482–507

Moran NA, Baumann P (2000) Bacterial endosymbionts in animals. Curr Opin Microbiol 3:270–275
Moreira D, Le Guyader H, Phillippe H (2000) The origin of red algae and the evolution of chloroplasts. Nature 405:69–72
Morita RY (1975) Psychrophilic bacteria. Bacteriol Rev 39:144–167
Moshiri F, Kim JW, Fu C, Maier RJ (1994) The FeSII protein of *Azotobacter vinelandii* is not essential for aerobic nitrogen fixation, but confers significant protection to oxygen-mediated inactivation of nitrogenase in vitro and in vivo. Mol Microbiol 14:101–114
Nichols JM, Adams DG (1982) Akinetes. In: Carr NG, Whitton BA (eds) The biology of cyanobacteria. University of California Press, Berkeley, pp 387–412
Palleroni NJ (1984) Gram-negative aerobic rods and cocci. In: Krieg NR, Holt JG (eds) Bergey's manual of determinative bacteriology, vol 1. Williams and Wilkins, Baltimore, pp 140–406, http://www.cme.msu.edu//bergeys
Pangborn J, Kuhn DA, Woods JR (1977) Dorsal-ventral differentiation in Simonsiella and other aspects of its morphology and ultrastructure. Arch Microbiol 113:197–204
Pate JL, Chang LYE (1979) Evidence that gliding motility in prokaryotic cells is driven by rotary assemblies in the cell envelope. Curr Microbiol 2:257–262
Pate JL, Ordal EJ (1965) The fine structure of two unusual stalked bacteria. J Cell Biol 27:133–150
Peekhaus N, Conway T (1998) What's for dinner?: Entner-Doudoroff metabolism in escherichia coli. J Bacteriol 180:3495–3502
Perazzona B, Spudich JL (1999) Identification of methylation sites and effects of phototaxis stimuli on transducer methylation in *Halobacterium salinarum*. J Bacteriol 181:5676–5683
Poindexter JS (1964) Biological properties and classification of the Caulobacter group. Bacteriol Rev 28:231–295
Ragsdale SW (1997) The eastern and western branches of the Wood/Ljungdahl pathway: how the east and west were won. Biofactors 6:3–11
Reeve JN, Sandman K, Daniels CJ (1997) Archaeal histones, nucleosomes, and transcription initiation. Cell 89:999–1002
Reichenbach H (1984) Myxobacteria: a most peculiar group of social prokaryotes. In: Rosenberg E (ed) Myxobacteria: development and cell interactions. Springer, Berlin, pp 1–50
Reichenbach H, Dworkin M (1981a) Introduction to the gliding bacteria. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds) The prokaryotes. Springer, Berlin, pp 316–327, http://www.prokaryotes.com
Reichenbach H, Dworkin M (1981b) The order myxobacterales. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds) The prokaryotes. Springer, Berlin, pp 328–355, http://www.prokaryotes.com
Ribbe M, Gadkari D, Meyer O (1997) $N_2$ fixation by *Streptomyces thermoautotrophicus* involves a molybdenum-dinitrogenase and a manganese-superoxide oxidoreductase that couple $N_2$ reduction to the oxidation of superoxide produced from $O_2$ by a molybdenum-CO dehydrogenase. J Biol Chem 272:26627–26633
Robinson JJ, Stein JL, Cavanaugh CM (1998) Cloning and sequencing of a form II ribulose-1,5-biphosphate carboxylase/oxygenase from the bacterial symbiont of the hydrothermal vent tubeworm Riftia pachyptila. J Bacteriol 180:1596–1599
Rosenberg E, Keller KH, Dworkin M (1977) Cell density-dependent growth of *Myxococcus xanthus* on casein. J Bacteriol 129:770–779
Rudolph J, Oesterhelt D (1996) Deletion analysis of the che operon in the archaeon *Halobacterium salinarium*. J Mol Biol 258:548–554
Sadoff HL (1976) Encystment and germination in *Azotobacter vinelandii*. Bacteriol Rev 39:516–539
Saraste M (1999) Oxidative phosphorylation at the fin de siecle. Science 283:1488–1493
Schäfer G, Purschke W, Schmidt CL (1996) On the origin of respiration: electron transport proteins from archaea to man. FEMS Microbiol Rev 18:173–188
Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Rev 61:262–280
Schleifer KH (1986) Gram-positive cocci. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 2. Williams and Wilkins, Baltimore, pp 999–1002, http://www.cme.msu.edu//bergeys
Schulz HN, Brinkhoff T, Ferdelman TG, Marine MH, Teske A, Jorgensen BB (1999) Dense populations of a giant sulfur bacterium in Namibian shelf sediments. Science 284:493–495
Selig M, Xavier KB, Santos H, Schonheit P (1997) Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium Thermotoga. Arch Microbiol 167:217–232
Singleton R (2000) Historical roots. ASM News 66:510–511
Sleytr UB, Messner P, Pum D, Sara M (1993) Crystalline bacterial cell surface layers. Mol Microbiol 10:911–916
Sneath PHA (1986) Endospore forming Gram-positive rods and cocci. In: Krieg NR, Holt JG, Krieg NR, Holt JG (eds) Bergey's manual of determinative bacteriology, vol 2. Williams and Wilkins, Baltimore, pp 1104–1207, http://www.cme.msu.edu//bergeys
Sogin ML, Silberman JD (1998) Evolution of the protists and protistan parasites from the perspective of molecular systematics. Int J Parasitol 28:11–20
Sowers KR, Boone JE, Gunsalus RP (1993) Disaggregation of *Methanosarcina* spp. and growth as single cells at elevated osmolarity. Appl Environ Microbiol 59:3832–3839
Spaink HP (2000) Root nodulation and infection factors produced by rhizobial bacteria. Annu Rev Microbiol 54:257–288
Spormann AM (1999) Gliding motility in bacteria: insights from studies of *Myxococcus xanthus*. Microbiol Mol Biol Rev 63:621–641
Spudich JL (1993) Color sensing in the archaea—a eukaryotic-like receptor coupled to a prokaryotic transducer. J Bacteriol 175:7755–7761
Stackebrandt E, Ludwig W, Weizenegger M, Dorn S, McGill TJ, Fox GE, Woese CW, Schubert W, Schleifer KH (1987) Comparative 16S rRNA oligonucleotide analyses and murein types of round-spore-forming bacilli and non-spore-forming relatives. J Gen Microbiol 133:2523–2529
Stackebrandt E, Rainey FA, Ward-Rainey N (1996) Anoxygenic phototrophy across the phylogenetic spectrum: current understanding and future perspectives. Arch Microbiol 166:211–223
Stal LJ, Krumbein WE (1985) Nitrogenase activity in the non-heterocystous cyanobacterium *Oscillatoria* sp. grown under alternating light–dark cycles. Arch Microbiol 143:67–71
Staley JT (1968) Prosthecomicrobium and Ancalomicrobium: new prosthecate freshwater bacteria. J Bacteriol 95:1921–1942
Stanier RY, van Niel CB (1962) The concept of a bacterium. Arch Mikrobiol 42:17–35
Stanier RY, Adelberg EA, Ingraham JL, Wheelis ML (1979) Microbial metabolism: the generation of ATP Introduction to the microbial World. Prentice-Hall, New York, pp 154–186
Stanier RY, Pfennig N, Trüper HG (1981) Introduction to the phototrophic prokaryotes. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds) The prokaryotes. Springer, Berlin, pp 197–211, http://www.prokaryotes.com
Stephenson M (1949) Bacterial metabolism, 3rd edn. Longmans, Green, London
Stewart V (1994) Regulation of nitrate and nitrite reductase synthesis in enterobacteria. Antonie van Leeuwenhoek 66:37–45
Stokes J (1954) Studies on the filamentous sheathed iron bacterium Sphaerotilus natans. J Bacteriol 67:278–291
Strauss G, Fuchs G (1993) Enzymes of a novel autotrophic $CO_2$ fixation pathway in the phototrophic bacterium *Chloroflexus aurantiacus*, the 3-hydroxypropionate cycle. Eur J Biochem 215:633–643
Strous M, Fuerst JA, Kramer EH, Logemann S, Muyzer G, van de Pas-Schoonen KT, Webb R, Kuenen JG, Jetten MS (1999) Missing lithotroph identified as new planctomycete. Nature 400:446–449
Sudo SZ, Dworkin M (1973) Comparative biology of prokaryotic resting cells. Adv Microbiol Physiol 9:153–224
Suflita JM, Horowitz A, Shelton DR, Tiedje JM (1982) Dehalogenation: a novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science 218:1115–1117
Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180
Tomitani A, Okada K, Miyashita H, Matthijs HC, Ohno T, Tanaka A (1999) Chlorophyll b and phycobilins in the common ancestor of cyanobacteria and chloroplasts. Nature 400:159–162

van Niel CB (1949) The comparative biochemistry of photosynthesis. In: Franck J, Loomis WE (eds) Photosynthesis in plants, vol 400. Iowa State College Press, Ames, pp 437–495

Vargas M, Kashefi K, Blunt-Harris EL, Lovley DR (1998) Microbiological evidence for Fe(III) reduction on early Earth. Nature 395:65–67

Walsby AE (1980) A square bacterium. Nature 283:69–71

Watson GM, Yu JP, Tabita FR (1999) Unusual ribulose 1,5-bisphosphate carboxylase/oxygenase of anoxic *Archaea.* J Bacteriol 181:1569–1575

Weiner JH, MacIsaac DP, Bishop RE, Bilous PT (1988) Purification and properties of *Escherichia coli* dimethyl sulfoxide reductase, an iron-sulfur molybdoenzyme with broad substrate specificity. J Bacteriol 170:1505–1510

Weisburg WG, Hatch TP, Woese CR (1986) Eubacterial origin of chlamydiae. J Bacteriol 167:570–574

White D (1984) Structure and function of myxobacteria cells and fruiting bodies. In: Rosenberg E (ed) Myxobacteria: development and cell interactions. Springer, Berlin, pp 51–67

Whittenbury R, Dow CS (1977) Morphogenesis and differentiation in *Rhodomicrobium vannielii* and other budding and prosthecatebacteria. Bacteriol Rev 41:754–808

Whittenbury R, Davies SL, Davy JF (1970) Exospores and cysts formed by methane-utilizing bacteria. J Gen Microbiol 61:219–226

Widdel F, Schnell S, Heising S, Ehrenreich A, Assmus B, Schink B (1993) Ferrous iron oxidation by anoxygenic phtotrophic bacteria. Nature 362:834–836

Williams FD (1978) Nature of the swarming phenomenon in Proteus. Annu Rev Microbiol 32:101–122

Wilson EO (1995) Naturalist. Warner Books, New York, pp 101–122

Woese CR (1998) Default taxonomy: Ernst Mayr's view of the microbial world. Proc Natl Acad Sci USA 95:11043–11046

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci USA 87:4576–4579

Wolin MJ, Miller TL (1982) Interspecies hydrogen transfer: 15 years later. ASM News 48:561–565

Xiong J, Fischer WM, Inoue K, Nakahara M, Bauer CE (2000) Molecular evidence for the early evolution of photosynthesis. Science 289:1724–1730

Yoon HS, Golden JW (1998) Heterocyst pattern formation controlled by a diffusible peptide. Science 282:935–938

Zengler K, Richnow HH, Rossello-Mora R, Michaelis W, Widdel F (1999) Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 401:266–269

Zumft WG (1997) Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev 61:533–616

# 6 Prokaryote Characterization and Identification

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## Systematics of Prokaryotes*

### Some Definitions

Systematics can be defined as the scientific study of organisms with the ultimate goal to characterize and arrange organisms in an orderly manner. Systematics also might be defined as "the study of organismal diversity and interrelationships." As pointed out already by Cowan (1968), systematics includes taxonomy and aspects of ecology, biochemistry, microscopy, pathology, genetics, and molecular biology.

Taxonomy and systematics are often used synonymously, but it is more appropriate to regard taxonomy as a part of systematics (Tindall et al. 2007). Taxonomy was defined as "the art of biological classification" (Stanier et al. 1986) but also as the theoretical study of classification, including its bases, principles, and rules (Simpson 1961). As stated by Cowan (1968), taxonomy is traditionally divided into

1. *Classification*, the orderly arrangement of units into taxonomic groups
2. *Nomenclature*, the labeling of units defined by classification
3. *Identification* of unknowns to units defined by classification and labeled by nomenclature

Identification is the practical application on the foundation of classification and nomenclature. Classification and identification are sometimes confused but classification is rather a prerequisite for identification. Identification may be understood either transitively (identification of unknowns with units defined by classification) or intransitively (to describe the identity of a species as such and use it as a basis for classification) (Trüper and Schleifer 2006). Two functions of taxonomy have been considered by Stanier et al. (1986), the identification and description of basic taxonomic units or species and the devising of methods to arrange and catalogue these units.

*The basis of this section has been the excellent short review of Tindall et al. (2007) supplemented with further details given by Tindall et al. (2010)

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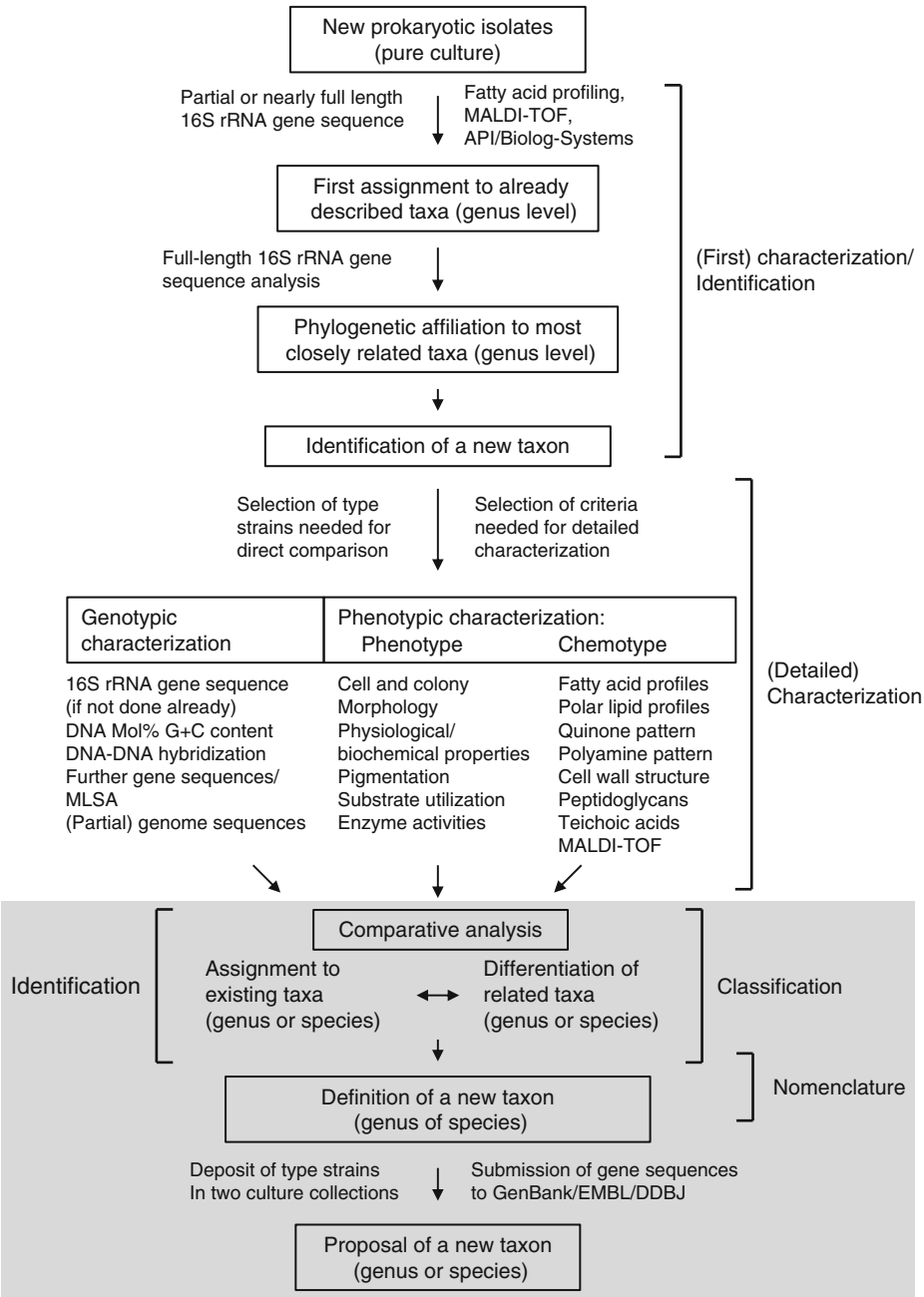


**Fig. 6.1**

**Flow diagram represents the steps currently performed for taxonomic characterization and description of new taxa. Furthermore the flow diagram outlines the interrelationship of characterization, identification, classification and nomenclature in taxonomy. Parts of the diagram were adapted from Rainey and Oren (2012) and Trüper and Schleifer (2006)**

The flow diagram in *Fig. 6.1* visualizes the interrelationship of the classification, nomenclature, and identification.

It should be the primary aim of taxonomy to provide a classification that can be used for a wide variety of purposes (including identification) and serving all microbiological disciplines. The basic physical object in microbiology to be classified is the organism, which is (in most cases) represented as pure bacterial or archaeal culture, which represents a population of ideally genotypically identical cells, with the cell as basic unit.

In contrast, taxonomic categories (including the "basic unit," the species) are mental representations sometimes defined as "units of knowledge" rather than physical objects.

There are many ways to classify organisms, and there is an ongoing debate about this issue, but in any case any resulting classification system remains an abstract (mental)

representation. Wayne et al. (1987) pointed out already that an ideal taxonomy would involve one system, which should be a hierarchical system, and in microbiology, the ultimate goal should be to establish a system that mirrors the taxonomic relationships as an "order in nature," which is now most often associated with "evolutionary order" back to the origin of life (Tindall et al. 2007).

A major step forward toward this goal was the analysis of the small subunit ribosomal RNA (16S rRNA) gene, which has clearly revolutionized prokaryotic taxonomic studies. For the first time, the establishment of a hierarchical taxonomic system on the basis of a molecular marker was possible.

A reliable classification system is a prerequisite for the scientific work with microorganisms to keep track of the large variety of those organisms. The currently applied classification is an *operational-based approach*, which depends more or less on data-driven analyses and is not based on a unifying theory.

A set of cutoff values and specific criteria are used for the delineation of group or taxa that share specific values of similarities. The most well-known "cutoff value" used for the differentiation of species is still the 70% DNA–DNA similarity value and the 5% difference in melting temperature of the respective DNA–DNA hybrids (Wayne et al. 1987).

But the currently applied system includes in addition to this cutoff value still a polyphasic approach (Colwell 1970; Vandamme et al. 1996), which comprises phenotypic, and further genotypic information. The phenotypic analysis includes morphological, physiological, and by definition also chemotaxonomic properties. As outlined below, phenotypic data, especially chemotaxonomic data, may also often reflect phylogenetic relationships of prokaryotes and are often suitable to assign and/or differentiate new taxa to related ones. The genotypic approach in contrast is based on genomic relationships and includes DNA–DNA hybridization (DDH) studies and more and more also comparative sequence analysis of homologous genes, which are used as phylogenetic markers (primarily the small ribosomal subunit, 16S rRNA gene). Different *theory-based concepts* have recently been brought into discussion to be used for classification as the ecotype-based (Koeppel et al. 2008) or the metapopulation-based concept (Achtman and Wagner 2008). Those concepts are important for the elucidation of specific microbes in an ecological and also evolutionary context, but it is problematic to apply those systems as tools for routine analysis for identification of the broad range of diversity of prokaryotes (Schleifer 2009; Kämpfer and Glaeser 2012). As previously outlined by Schleifer (2009), "it should be kept in mind that the end-users need a pragmatic classification system that can serve as a tool in routine identification. A classification that is of little use to microbiologists, no matter how sophisticated a scheme is, will soon be ignored or significantly modified (Staley and Krieg 1984)."

The practicability of the polyphasic approach has been documented in bacterial taxonomy (Vandamme et al. 1996; Schleifer 2009; Tindall et al. 2010), but there is a tendency to replace the sometimes time-consuming procedures by a genotypic, most often only single-step phylogenetic taxonomy based on the 16S rRNA gene and a few more gene sequences.

Genes and genomes, however, do not function on their own and can display their potential only within the cell as the basic unit of evolution (Tindall et al. 2007). It is the phenotype and the natural selection that "drive" evolution in a given environment. In this context, the "polyphasic taxonomic approach" is still important, and novel insights into genomes and other "omic" sciences should be brought in a more strict and detailed context with the phenotype (Kämpfer 2012).

## Nomenclature of Bacteria

The nomenclature of prokaryotes in a (hierarchical) taxonomic system is part of the general scheme for a system of all organisms developed already by Carl von Linné and published in 1735 in his *Systema Naturae*. To this system, the highest taxonomic ranks called "domains" were added after the discovery of the three domains of life by the pioneering work on the small subunit rRNA (16S rRNA) sequences of Woese and colleagues (e.g., Woese and Fox 1977; Stackebrandt and Woese 1981; Woese 1987; Woese et al. 1990).

Based on 16S rRNA gene data, all prokaryotes are classified into the domains "Archaea" or "Bacteria," which are subdivided in a hierarchical manner into the lower nonoverlapping ranks: "phylum," "class," "order," "family," "genus," and "species" (Brenner et al. 2001), and all these ranks are sometimes (not consistently) subdivided into lower ranks using the suffix "sub-" like, for example, "suborder" or "subspecies."

The basic unit of the taxonomic hierarchy is essentially the species. The definition of a prokaryotic species was and is still under discussion among microbiologist of different disciplines. Different "species definitions" and "species concepts" have been proposed (for a comprehensive review, see Rosselló-Mora and Amann 2001).

Cowan (1978) already addressed three meanings for the term species: a category (a mental representation), a taxonomic group, and a concept. The category indicates that the species is a taxonomic rank below the genus rank in a hierarchical system. Cowan (1968) even pointed out that a species is "a group of organisms defined more or less subjectively by the criteria chosen by the taxonomist to show the best advantage as far as possible and putting into practice his individual concept of what a species is." The usefulness of working with the concept of a species was not denied by Cowan, but he reminded the user that the species does not exist and does not represent a natural entity. It should be mentioned here that all three meanings given above are intrinsically connected. A description of a taxonomic group (or unit) and also of a concept is only possible if one uses a word for the category "species," which directly leads to the name of this category and, hence, nomenclature.

"Species" are regarded as the fundamental units in taxonomy, and microbial strains are most often assigned to the taxonomic rank "species" or "subspecies" and at least to the genus level. With this nomenclatural assignment (and simultaneously

the assignment of the meaning behind this name), the link of a mental representation (the taxonomic rank) to a "physical object" is most obvious. Type strains serve as reference points in the cases of the basic taxonomic unit species and subspecies.

The nomenclature of the "lower" ranks, that is, "family," "genus," "species," and "subspecies," is regulated by rules of the nomenclatural system, "The Bacteriological Code of Nomenclature" (Lapage et al. 1992), that has been developed several decades ago and serves microbiologists as a solid and indispensable foundation (Lapage et al. 1992; Skerman et al. 1989; Sneath 2005).

The 1990 revision of the *International Code of Nomenclature of Bacteria* supersedes all previous editions and has been applied from the date of publication in 1992 and consists of 7 general considerations, 9 principles, 65 rules (some supplemented by recommendations which do not have the force of rules), advisory notes (subdivided into suggestions for authors and publishers, advices regarding the quotations of authors, and names and the maintenance of type strains), 10 appendices, and the statutes of the International Committee on Systematic Bacteriology and the statutes of the Bacteriology and Applied Microbiology Division of the International Union of Microbiological Societies (IUMS).

The rules of the code give advice for the naming of taxa, nomenclatural types including their designation, the priority and the publication of the names, and the citation of authors and names. There are rules about changes in the names of taxa as a result of transference, union, or change in rank; about illegitimate and legitimate state of names and epithets; and the replacement, rejection, and conservation of names and epithets. A number of rules and Appendix 9 (Orthography) refer to the correct usage of Latin orthography. Since the revision of the Bacteriological Code, in 1990, Appendix 9 is stronger, incorporated in the code as a recommendation. In 2005, it has been accepted at the Plenary Meetings of the Judicial Commission and the ICSP during the IUMS Congress in San Francisco, CA, USA (July 23–29, 2005) that a new edition of the Appendix 9 should be available online: http://ijs.sgmjournals.org/cgi/content/full/59/8/2107 (Trüper and Euzéby 2009).

According to the general consideration 7, the word taxon (plural: taxa) is used in the code for any taxonomic group of organisms, and as laid down in Principle 6, the correct name of a taxon is based upon valid publication, legitimacy (named according to the rules), and priority of publication (for more details, see below).

Each taxon consists of one or more elements. For each named taxon of the various taxonomic categories (*Table 6.1*), a nomenclatural "type" shall be designated, that is, the element of the taxon to which the name is permanently associated. Appendix 4 contains all conserved and rejected names of bacterial taxa, and Appendix 5, the Opinions of the Judicial Commission of the International Committee on Systematic Bacteriology, which have the same force as the rules. Appendix 10 of the International Code of Nomenclature of Bacteria deals with the infrasubspecific subdivision, which is only based on selected "utilities" but not based on DNA-DNA Hybridization (DDH)-based differentiation. In an infrasubspecific taxon, one strain or a set of strains shows similar or identical properties and therefore is treated as a taxonomic group. The term "infrasubspecific" is used to refer to the kind of taxa below subspecies.

Subdivisions usually contain the suffixes "-type," "-var," or "-form." However, in Appendix 10, it is recommended, to use the suffix "-var" instead of "-type" to avoid confusion with the use of the term "type" in nomenclatural context (Rule 15). The following suffixes for subdivisions are recommended, whereas suffixes given in parenthesis should be avoided: biovar (biotype, physiological type), chemovar, chemoform (chemotype), cultivar, forma specialis (special form), morphovar (morphotype), pathovar (pathotype), phagovar (phagotype, lysotype), phase, serovar (serotype), and state (Trüper and Schleifer 2006).

A "biovar" (instead of biotype or physiological type) is based on biochemical or physiological properties; a "chemovar" is based on the production of a specific chemical, and a "chemoform" (instead of chemotype) is based on the chemical constitution. A "cultivar" refers to special cultivation properties, and a "forma specialis" (instead of special from) refers to a parasitic, symbiotic, or commensal bacterium which can be distinguished by adaptation to a particular host or habitat (here the scientific name of the host in the genitive should be used for nomenclature). A "morphovar" (instead of morphotype) reflects specific morphological characteristics, a "pathovar" (instead of pathotype) refers to specific hosts, a "phagovar" (instead of phagotype) is linked to a specific bacteriophage, and a "serovar" (instead of serotype), to antigenic characteristics.

As pointed out by Tindall (1999), "the Bacteriological Code does not attempt to regulate either which methods are to be used, or which taxonomic interpretation of a given problem is correct (i.e., there is no regulated "official" taxonomy). The Bacteriological Code deals solely with the way in which names are assigned to organisms, and which of these are to be used."

This system has been in operation since 1 January 1980 and was developed in order to clarify uncertainties of about 40,000 names found in the scientific literature over the last hundreds of years (Tindall et al. 2007). With the publication of the Approved List of Bacterial Names (Skerman et al. 1980), only around 2,000 names were incorporated into the system.

From 1 January 1980 onward, the proposal of a name for a prokaryotic organism representing a novel taxon must be accompanied by specific criteria laid down in the revised wording of Rule 27 of the Bacteriological Code (Lapage et al. 1992; Sneath 2001; Tindall et al. 2006). These include the elements, that the new name or new combination is clearly stated and indicated as such (i.e., fam. nov., gen. nov., sp. nov., comb. nov., etc.), in order to show clearly the intention of the author(s) to create a new name, the presentation of the derivation (etymology) of a new name (and if necessary of a new combination), the description of the properties of the taxon, and the accessibility of all these information. The major element of the presented system is the process of valid publication of a name of a taxonomic rank (subspecies, species, genus, family), which constitutes a form of official registration/indexing of that name through a centralized system.

**Table 6.1**

**Overview of taxonomic ranks, respective suffixes, and appropriate types as listed in the bacteriological code. Overview of taxonomic ranks, their suffixes, and appropriate types as listed in the Bacteriological Code revisions 1975 and 1990 (Lapage et al. 1992). The depicted example was presented previously by Tindall et al. (2006). The ranks of subfamily, tribe, subtribe, and subgenus are not widely used at present**

| Taxonomic category | Latin suffix | Examples[a] | Type (example) |
|---|---|---|---|
| Order | *-ales* | *Pseudomonadales* | *Pseudomonas* (genus) |
| Suborder | *-ineae* | *Pseudomonadineae* | *Pseudomonas* (genus) |
| Family | *-aceae* | *Pseudomonadaceae* | *Pseudomonas* (genus) |
| Subfamily | *-oideae* | *Pseudomonadoideae*[b] | *Pseudomonas* (genus) |
| Tribe | *-eae* | *Pseudomonadeae* | *Pseudomonas* (genus) |
| Subtribe | *-ineae* | *Pseudomonadinae*[b] | *Pseudomonas* (genus) |
| Genus | | *Pseudomonas* | *Pseudomonas aeruginosa* (species) |
| Species | | *Pseudomonas aeruginosa* | RH 815 = ATCC 10145 = CCEB 481 = CCUG 551 = CCUG 28447 = CCUG 29297 = CFBP 2466 = CIP 100720 = DSM 50071 = IBCS 277 = IFO (now NBRC) 12689 = JCM 5962 = LMG 1242 = NCCB 76039 = NCIB (now NCIMB) 8295 = NCTC 10332 = NRRL B-771 = VKM B-588 |
| Subspecies | | *Pseudomonas aeruginosa* subsp. *aeruginosa*[c] | RH 815 = ATCC 10145 = CCEB 481 = CCUG 551 = CCUG 28447 = CCUG 29297 = CFBP 2466 = CIP 100720 = DSM 50071 = IBCS 277 = IFO (now NBRC) 12689 = JCM 5962 = LMG 1242 = NCCB 76039 = NCIB (now NCIMB) 8295 = NCTC 10332 = NRRL B-771 = VKM B-588 |

[a]In the presented examples (according to Tindall et al. 2006), the stem above the genus level is Pseudomonad

[b]However, subfamily and subtribe are included in the Bacteriological Code, revisions 1975 and 1990; the respective names are not validly published

[c]The subspecies name *Pseudomonas aeruginosa* subsp. *aeruginosa* is not validly published, but if *Pseudomonas aeruginosa* would be divided into subspecies, the name would be created automatically (Rule 46)

Furthermore, the nomenclatural type of the taxon must be designated. In addition, the name must appear in the International Journal of Systematic and Evolutionary Microbiology (IJSEM) and make reference to these requirements listed. This may be via original publication in the IJSB/IJSEM or via publication in another journal and inclusion of the relevant information in the validation lists (which must include reference to the type, taxonomic rank, and where the remaining information is to be found).

## Importance of Type Material

One of the most important criteria in classification is the designation of the nomenclatural type. As pointed out by Tindall (2008) and Tindall and Garrity (2008), the type serves as a reference point for a taxon, and in the case of the proposal of a novel species or subspecies, these types are represented by "physical objects," the type strains (Lapage et al. 1992). Those "representative" strains must be made available without restrictions to the scientific community.

Since January 2001 (De Vos and Trüper 2000; Labeda 2000), it is a requirement laid down in the Bacteriological Code that authors proposing novel species, novel subspecies, and new combinations have to provide evidence that types are deposited in at least two recognized culture collections in two different countries, and since August 2002, the IJSEM, as the official organ of the International Committee on Systematics of Prokaryotes (ICSP), has asked that authors provide documented evidence from the collections confirming deposition and availability of type strains. The accession numbers assigned to the strain by the culture collections must be quoted in the published description.

Rule 30(3a) states: "As of 1 January 2001 the description of a new species, or new combinations previously represented by viable cultures must include the designation of a type strain, and a viable culture of that strain must be deposited in at least two publicly accessible service collections in different countries from which subcultures must be available. The designations allotted to the strain by the culture collections should be quoted in the published description. Evidence must be presented that the cultures are present, viable, and available at the time of publication."

There have been several discussions about this requirement followed by the proposal of standards for strain deposits (Tindall 2008; Tindall and Garrity 2008; Kämpfer 2010), and there is clearly a necessity to ensure that higher standards are being applied to taxonomic work, enabling the key elements of verifying existing experimental data and expanding on the data set associated with authentic biological material (type strains) to be carried out.

### The Provisional Status *Candidatus*

The provisional status *Candidatus* was introduced in 1994 by Murray and Schleifer (1994) followed by Murray and Stackebrandt (1995) for certain putative taxa that could not be described in sufficient detail as a novel taxon. The designation *Candidatus* is not a rank but a status that is currently not formally recognized in the *International Code of Nomenclature of Bacteria*. According to the "Ad Hoc Committee for the re-evaluation of the species definition in bacteriology" (Stackebrandt et al. 2002), microbiologists are encouraged to use the status *Candidatus* for well-characterized but as-yet-uncultured prokaryotes.

The phylogenetic relatedness of the as-yet-uncultured prokaryote has to be determined and their authenticity should have been revealed by, for example, in situ probing using fluorescence in situ hybridization or other similar techniques. Beside genotypic information, phenotypic properties should also be included in the description of a *Candidatus* species including structural, metabolic, physiological, and reproductive features.

The names of the category *Candidatus* should be written as follows: *Candidatus* in italics and the subsequent name(s) in roman type with an initial capital letter for the genus name. The entire name should be in quotation marks (e.g., "*Candidatus* Magnospira bakii"). It was decided by the Judicial Commission of the International Committee on Systematics of Prokaryotes that the concept *Candidatus* should be mentioned in the main body of the Bacteriological Code; however, the names have still no standing in nomenclature (De Vos et al. 2005). Many Bacteria and Archaea, which have currently a *Candidatus* status (more than 200 in 2009) are endosymbionts or parasites of eukaryotes, which belong to the phyla and orders *Mollicutes*, *Chlamydiales*, and *Rickettsiales*, respectively. Other as-yet-cultured prokaryotes with a *Candidatus* status are only available as special enrichments or cocultures (e.g., syntrophic organisms, freshwater *Actinobacteria*) or occurring in unusual habitats, revealing unusual metabolic properties (Anammox, Fe-oxidizing bacteria).

### Minimal Standards

Minimal standards are documents which are compiled by experts within the framework of subcommittees set up within the International Committee on Systematic Bacteriology/International Committee on Systematics of Prokaryotes (ICSB/ICSP; Lapage et al. 1992) to provide detailed information how specific groups of organisms should be characterized. Their role is covered by Recommendation 30 (formerly Recommendation 30b) of Rule 30 of the Bacteriological Code (Lapage et al. 1992), as modified at the 1999 meetings of the ICSB and its Judicial Commission (De Vos and Trüper 2000; Labeda 2000). Really updated minimal standards are only available for a very restricted group of organisms covered by the subcommittees. Some updated minimal standards, for example, have been published for aerobic, endospore-forming bacteria (Logan et al. 2009), family *Flavobacteriaceae* (Bernardet et al. 2002), order *Halobacteriales* (Oren et al. 1997), family *Halomonadaceae* (Arahal et al. 2007), genus *Helicobacter* (Dewhirst et al. 2000), suborder *Micrococcineae* (Schumann et al. 2009), class *Mollicutes* (division *Tenericutes*, order *Mycoplasmatales*) (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mycoplasmatales* 1972; Brown et al. 2007), genus *Mycobacterium* (Lévy-Frébault and Portaels 1992), family *Pasteurellaceae* (Christensen et al. 2007), and *Staphylococci* (Freney et al. 1999).

## From the Beginning of Prokaryotic Classification and Towards a Modern Taxonomy

The consideration of the classification of prokaryotes from the beginning in the late nineteenth century until today illustrated the tremendous changes in the classification of prokaryotes, which was primarily affected by the development of new techniques. This topic will only be briefly covered in this chapter. It is addressed in further detail in another chapter in this volume.

At the end of the nineteenth century, Ferdinand Cohn used morphological features as different cell shapes (cocci, small or elongated rods, or spirals) to classify bacteria into six genera at that time as members of the plants. Based on the morphological similarities, Cohn (1875) grouped the "common bacteria" and the cyanobacteria (later on referred as blue-green algae) together to the Schizophyta. Pathogenic bacteria were in the focus of interest at that time which is reflected by the fact that many well-known pathogenic bacteria were described at the end of the nineteenth century. The pathogenic potential and general growth requirements were the most important taxonomic markers, which were used beside morphology to classify bacteria.

In the first half of the twentieth century, beside morphology, numerous biochemical and physiological features were used to classify and identify bacteria. Later on, enzymes and metabolic pathways were additionally elucidated. In the first edition of Bergey's Manual of Determinative Bacteriology (Bergey et al. 1923), phenotypic properties were used for the classification of bacteria as "typically unicellular plants," the so-called Schizomycetes. Even in the 7th edition of Bergey's Manual (Breed et al. 1957), bacteria were still classified as members of plants (Protophyta, primitive plants). As first propagated by A. Lwoff, R. Stanier and C. B. van Niel described in 1962 the division into prokaryotic (bacteria) and eukaryotic (animal, plants) organisms (Stanier and Van Niel 1962), and then in the eighth edition of the Bergey's Manual (Buchanan and Gibbons 1974), bacteria were no longer recognized as plants but assigned as members of the kingdom Prokaryotae. At that time, phenotypically, properties including gram-staining, morphology, and oxygen requirements were used to classify bacteria. However, the phenotype-based classification alone led to a grouping of phenotypically coherent but as nowadays well-known genetically different bacteria.

In the 1970s and 1980s, comprehensive sets of phenotypic data were used to classify bacteria by using numerical studies

(numerical taxonomy; Goodfellow 1977; Sneath 1971; Sneath and Sokal 1973, and several others). Numerical taxonomy improved the accuracy of phenotypic identification by increasing the number of tests used and the calculation of coefficients of similarities between strains and species (Sneath and Sokal 1973). The results of the numerical studies were tabulated in tables of t organisms versus n characters and specific operation taxonomic units (OTUs). The characters, which were compared, should come from various different categories of properties as morphology, physiology, or biochemistry and were equally weighted in the analyses. The number of common characteristics used was considered as a quantitative measurement of taxonomic relatedness, although this did not mean that the compared organisms were also phylogenetically related.

In the last 50 years, tremendous achievements have been made in the development of DNA-based analyses for the characterization of microorganisms. The classification based on genotypic methods enabled more and more the investigation of the phylogenetic relationships among prokaryotes. DNA-based analysis started with the estimation of the guanine and cytosine nucleosides ratio within the total genomic DNA followed by the introduction of DNA–DNA hybridization (DDH) methods of genomic DNA (Johnson and Ordal 1968), which is still recognized as the "genotypic standard" for the delineation of prokaryotic species (Wayne et al. 1987; Stackebrandt et al. 2002; Tindall et al. 2010). An important step forward was the development of the PCR-based sequencing technology (Saiki et al. 1988) and the introduction of the 16S rRNA gene as a molecular marker in taxonomy. Several other genes (e.g., *rpoB, gyrA, recA, hsp60, atpD*, just to name a few) were introduced as alternative or supporting molecular markers in prokaryotic taxonomy, and the rapid development of high-throughput sequencing technologies will lead to more and more genome-based genotypic analysis.

The work of C. R. Woese was a "milestone" for the start of genotype-based classification of prokaryotes. Carl Woese and coworkers were the first who demonstrated the usefulness of small subunit (SSU) rRNA as a universal phylogenetic marker to reflect phylogenetic relationships (Fox et al. 1977).

Data obtained from comparative sequence analyses of the small ribosomal subunit (the 16S rRNA gene sequence) provided for the first time a basis to study phylogenetic relationships among all bacteria. With the investigation of partial sequences of 16S ribosomal RNA (rRNA) genes, the *Archaea* (which were originally assigned as Archaebacteria) were found to constitute a unique group leading to the description of a separate kingdom (Woese and Fox 1977). Other phylogenetic markers including other ribosomal RNA genes, the 5S and 23S rRNA, or other macromolecules besides rRNAs, for example, the elongation factor Tu or the β-subunit of ATP-synthase (Schleifer and Ludwig 1989), confirmed the relationships obtained by the 16S rRNA gene sequence–based approach.

Differences in evolutionary rates in various groups of organisms and other considerations can be obstacles preventing the single use of gene sequences in delineating taxa. Therefore, still the integrated use of phenotypic and genotypic characteristics, that is, the *polyphasic taxonomy* (Colwell 1970) is necessary for the delineation of taxa at all levels from kingdom to genus (Murray et al. 1990). In the majority of cases, there is a good evidence in bacterial systematics of a congruence between the distribution of specific chemical markers and the relative position of species in phylogenetic trees. Chemotaxonomic markers can therefore help to assign new taxa to related taxa but can also help to delimit groups of related species (Murray et al. 1990; Tindall et al. 2010).

With the increasing importance of the 16S rRNA gene for delineation of bacterial taxa and the increase of available sequences for almost all taxa, Stackebrandt and Goebel (1994) tried to correlate numerous published DDH data with 16S rRNA gene sequence similarity data and came to the conclusion that the correlation plot of the two parameters was not linear. They pointed out that each method is strong in those areas of relationships in which the other method sometimes fails to reliably show relationships. Sequence analysis of 16S rRNA genes is regarded as more suitable from the level of domains (starting at about 55% similarity) to genera or in some cases moderately related species, that is, below 97.5% rRNA sequence similarity. Above this value, DNA-DNA reassociation values were found to be very low (<20%) or as high as 100%. They concluded in addition that DDH remains the method for measuring the degree of relatedness between highly related organisms and provided evidence that at 16S rRNA gene sequence similarity values below about 97.5%, it was unlikely that two organisms shared more than 60–70% DNA similarity. In general, strong evidence was provided that sequence analyses of 16S rRNA genes are not the appropriate method to replace DNA–DNA reassociation for the delineation of species and measurement of infraspecies relationships.

Hence, a major critical point is the resolving power of the 16S rRNA gene sequence below the genus level because of its conserved structure. This means that branching pattern at the periphery of phylogenetic trees (sometimes at the genus level, but most often at the species level) cannot reliably reflect phylogeny in the sense of common ancestry (independent from the "treeing algorithm"). Organisms sharing very similar or even identical 16S rRNA gene sequences may be more diverse at the whole genome level than those having more variable positions (Stackebrandt and Goebel 1994).

The development of new sequencing technologies makes it now feasible to look in detail at the genome sequence because gene and genome sequences can be generated in a relatively short period of time, and it can be foreseen that the wealth of the new data can and will be used for a critical evaluation of the taxonomic system. Several new *genomic approaches*, based on total or partial genome comparisons, including concatenated sequences of all conserved or selected genes (summarized by Cole et al. 2010), confirmed the 16S-rRNA-gene-based hierarchical system at least at the genus level and above. As a consequence, the 16S-rRNA-gene-based hierarchical structure still remains for the time the backbone of prokaryotic systematics (Cole et al. 2010; Ludwig 2010).

Genome sequence–based studies start to overcome the problems associated with DDH experiments. The average nucleotide identity (ANI) index of shared genes between two genomes (Konstantinidis and Tiedje 2005) showed among several tested genomes-derived parameters the best correlation with DDH values. The first evaluation of ANI values was based on a pairwise genome comparison including all shared orthologous protein-coding genes (Konstantinidis and Tiedje 2005). Based on those studies, an ANI value of 94% was recommended that could reflect the bacterial species boundary of 70% DDH similarity. Similar results were observed by the comparison of genomes that were artificially sectioned into 1020 nucleotide fragments (Goris et al. 2007). Richter and Rosselló-Móra (2009) recommended the use of an even narrower boundary of 95–96% ANI to circumscribe prokaryotic species and showed that the comparison of at least 20% of genomes generated by simple randomly sequencing will be enough to determine reliable ANI values. Rosselló-Móra (2012) had a closer look to methods and their potential for a genome-based taxonomy.

In a recently published review, Klenk and Göker (2010) gave an *outlook to a genome-based classification of Archaea and Bacteria*. They concluded that microbial systematics and genomics have not been completely reconciled due to the intrinsic difficulties of inferring reasonable phylogenies from genome sequences, in part due to the significant role of lateral gene transfer (LGT) in microbial evolution. In addition, they pointed out that the consequences of using different methods for orthology detection, sequence alignment, and alignment filtering, and use of different reference sequences, may all have a significant effect of phylogenetic reconstructions and are not yet fully understood (Klenk and Göker 2010; Ludwig 2010). However, it can be foreseen, that the comparison of whole genomes will play a more and more important role in the classification of prokaryotes.

## Current Procedures for the Characterization and Identification of Bacterial and Archaeal Strains

The currently operational "polyphasic approach" includes phenotypic (including chemotaxonomic) and genotypic traits to characterize a prokaryotic taxa. DNA sequence–based methods are nowadays more and more the preferred method for the initial identification and characterization of a prokaryotic strain (see *Fig. 6.1*).

For classification, it is recommended to include more than just the type strain for the description of a new species (e.g., Christensen et al. 2001; Felis and Dellaglio 2007) because those characteristics can be variable within species, and this can only be determined by analyzing more than one strain. However this is only a recommendation and cannot be mandatory.

All properties, which are common, perhaps even unique for a strain, should be included in the description. Variable properties should be included as well and can be an indicator for subgroupings within a taxonomic group. Most often, the initial step to characterize a new bacterial isolate is the PCR amplification and sequencing of the 16S rRNA gene to determine its phylogenetic affiliation (*Fig. 6.1*). Expert-maintained web-based databases such as the search tool EzTaxon that contains the 16S rRNA gene sequences of all type strains enables a fast first assignment of a new strain to related taxa (Chun et al. 2007; updated version: Kim et al. 2012).

In some areas, phenotypic identification systems including fatty acid profiles or MALDI-TOF analyses and the investigation of physiological properties by commercial test systems are also often used for the identification of an unknown, especially in routine identification procedures. Here, also complete and updated respective databases are the prerequisites for this identification.

For the allocation of an unknown to a genus, detailed phylogenetic calculations based on nearly full-length 16S rRNA gene sequences are now in most cases "state of the art." This is followed by calculations of pairwise similarities and the construction of phylogenetic trees to determine the exact phylogenetic relationship of the new strain to closest related taxa. The results of 16S rRNA gene sequence similarity calculations often indicates whether or not the new strain may represent a new species or even a new genus and thereby provides the direction for the following detailed taxonomic investigations. At 16S rRNA gene sequence similarity levels below 97%, a new species or even genus assignment may be quite likely. Above 97% gene sequence similarities, further genotypic methods have to be applied to elucidate if the strain/strains represent a new species or has to be assigned to an existing one. In the current species concept, two organisms sharing a DNA–DNA hybridization value of higher than 70% represent the same species (Wayne et al. 1987; Stackebrandt et al. 2002). If 16S rRNA gene sequence similarities are below 95%, it is quite likely that a new genus has to be described (for more details, see below). If a new taxon will be described, the strains or at least the provisional type strain must be studied in great detail in order to provide evidence that the novel taxon can be clearly differentiated from closest related taxa also on the basis of phenotypic (including chemotaxonomic) characteristics. Distinguishing phenotypic differences is required for the description of a new species. If such differences are not found, groups of similar bacteria that appear to be genetically distinct should be described by other terms (e.g., genospecies/genomovars). An increasing number of taxonomic studies include the investigation of alternative genetic markers in addition to the phylogenetic analysis based on the 16S rRNA gene sequence to obtain a higher resolution at lower taxonomic ranks. Multilocus sequence analysis (MLSA) is most often used for this purpose including the investigation of a set of partial sequences of housekeeping genes. It has been suggested recently that MLSA may replace DDH analysis (see below). Also, other genotypic as genomic fingerprint methods are often applied to differentiate taxa at the lower taxonomic ranks, for example, strains within species.

Current recommendations for the characterization of new prokaryotic taxa have been summarized recently by Tindall et al. (2010). These recommendations may serve as general guidelines. If minimal standards are available for the respective taxa a new strain belongs to, those should also be considered in addition.

If a new species is assigned to an existing genus, differences of the new species and existing species within the genus have to be provided-if the genus is too heterogeneous (e.g., the genera *Bacillus and Clostridium*) or contains a large number of species (e.g., *Streptomyces*), then the authors must give scientific-base arguments for the selection of only a set of species of the genus for comparative analysis. The type species of the respective genus must be included. In the case where a new strain seems to represent a new genus, this genus has to be clearly differentiated from *all* "closely related" genera.

### Genotypic Traits

Genotypic and phenotypic methods that are typically applied in polyphasic taxonomic approaches for characterizing new taxa are summarized in ❯ *Fig. 6.1*.

*Genotypic* traits of an organism are those within its genetic material, the genome. The term genomic is convenient to refer to large amounts of information in the genome or derived from it very directly with few extraneous influences. Messenger RNA and ribosomal RNA can be regarded as formally intermediates between genotype and phenotype, but they reflect the genome so faithfully that they can in practice be treated as genotypic (Sneath 1989).

## Phylogenetic Assignment Based on 16S rRNA Gene Sequences

A 16S rRNA gene sequence–based approach alone cannot describe a new species but can provide the first evidence if a new isolate represents a new species or even a new genus. A 16S rRNA gene sequence similarity of ≤97% can be used as an indication for a new species. There are, however, many examples in the literature that two strains with less than 97% 16S rRNA gene sequence similarity are not members of the same species (Amann et al. 1992; Collins et al. 1991; Fox et al. 1992; Martinez-Murcia and Collins 1990; Martinez-Murcia et al. 1992). The prerequisite for such a conclusion is that the sequence comparison based on nearly full-length high-quality sequences and a well-performed sequence alignment (see below). If 16S rRNA gene sequence similarities are above 97% (over full pairwise comparisons), DNA–DNA hybridization or gene sequences showing a higher resolution must be considered.

16S rRNA gene similarity values (over full pairwise comparisons) of ≤95% gives often evidence that a new strain may represent a new genus.

While high attention was focused on the resolution power of the 16S rRNA gene at the species level, the delineation at the genus level based on16S rRNA gene sequence similarities is less clear. Within some genera, high 16S rRNA gene sequence similarities occur, for example, within the *Enterobacteriaceae* or *Actinomycetales*. In contrast, other genera contain species with lower sequence similarities, for example, the genus *Flavobacterium*.

Recently, some suggestions have been made for lower boundaries (Stackebrandt and Ebers 2006; Yarza et al. 2008; Tindall et al. 2010); however, it has to be mentioned again, the delineation of species cannot be only based on the 16S rRNA gene sequence similarities, and results obtained by several other methods have to be considered as well.

The importance of the phylogenetic analysis based on the 16S rRNA gene sequence as molecular marker for the identification and classification of prokaryotes makes it exceptionally important to perform this analysis as standardized as possible. Currently, no elaborative predictions are available, but several recommendations have been given (Tindall et al. 2010; Peplies et al. 2008).

Several critical steps have to be considered for the analysis of the 16S rRNA gene sequences, including the sequence qualities, sequence alignments, and the calculation of pairwise sequences similarities and of phylogenetic trees. Only high-quality nearly full-length sequences (at least 1,300 nucleotides) should be used for analysis. Not only the new sequence but also the reference sequences have to fulfill those requirements. Therefore, sequence qualities should be checked with caution before any analysis and especially before sequences are deposited in databases, used for a publication, or sent to culture collections for the deposited of the respective strains. Sequences should be checked for ambiguities, the consensus of primary and secondary structures, and for potential gene heterogeneities, which can occur by the direct sequencing of PCR fragments. It is recommended to use a set of well-aligned reference sequences for a final check of the sequence quality. The use of sequences available at the primary databases (GenBank/EMBL/DDBJ) has to be performed with caution because the databases contain several incorrect labeled and low-quality sequences, which have not undergone an official quality control before deposit.

The alignment itself is a critical step for all phylogenetic analysis because it is the basic for pairwise sequence similarity calculations and the construction of phylogenetic trees. It is highly recommended to use expert-maintained seed alignments that contain quality-checked sequences, as provided by ARB [www.arb-home.de], RDP [http://rdp.cme.msu.edu/], SILVA [www.arb-silva.de], or LTP [www.arb-silva.de/projects/living-tree/]. Reference sequences (of type strains) can also be downloaded from the databases and aligned in multiple alignment programs as ClustalW followed by a manual editing. However, those programs did not enable the inclusion of secondary structure information, as it is, for example, possible in ARB. Dependent on the investigated taxa, secondary structure information can be very helpful for correct alignments especially of highly variable regions of the 16S rRNA gene. The procedure of the sequence alignment should be stated clearly in a publication, and new sequences and the alignments of all sequences included in the analysis for a publication must be made available for editors and reviewers when a manuscript is submitted.

Pairwise nucleotide sequence similarity values of high-quality sequences should then also be performed with caution. It is recommended to perform the pairwise sequence similarity

calculations in ARB, PHYDIT, and jPHYDIT; local alignment programs as BLAST should not be used. Pairwise sequence similarities should not based on corrected evolutionary distances as, for example, calculated with the Jukes and Cantor, or several other evolutionary models. Here again, the procedure of the similarity calculations should be clearly stated.

Phylogenetic trees are the graphical representation of phylogenetic relationships of the new taxa to related taxa. All sequence used for the tree construction have to be full-length sequences.

The preferable methods for tree calculations are the maximum parsimony and maximum likelihood methods because both methods are based on evolutionary models. It is recommended to use distance-matrix-based treeing methods as the neighbor-joining methods only for first assignment of new taxa (Ludwig and Klenk 2001; Peplies et al. 2008; Tindall et al. 2010). In most studies, however, neighbor-joining methods are depicted in manuscripts because only a limited number of sequences could be investigated with the maximum likelihood method in a short time and bootstrap analyses are not possible by most maximum likelihood applications. However, there are tools for maximum likelihood analyses (PHYML, RAXML) available, which enable the inclusion of appropriate data sets and the application of bootstrap analysis. In general, different treeing methods should be applied in parallel to improve the calculated phylogenetic relationships.

It is recommended to use *sequence conservation profiles* determined for the group of interest and higher ranks as filters to recognize branch attraction effects, which may result from pleomorphic sites, and to test the stability of a tree topology (Ludwig and Klenk 2001; Peplies et al. 2008). The application of a filter results in the successive removing of alignment columns according to the variability of the positions. The most often applied filter is a 50% base frequency filter, which will exclude a complete alignment column if the frequency of the most abundant nucleotide is below 50% (Peplies et al. 2008). To proof the tree topology, the use of 30% and 40% filters are also recommended. If filters are used, tree topologies of trees generated with and without a filter have to be compared, and an alignment with the assignment of the filtered positions must be made available. The use of filters and the interpretations relying on the use of a filter must be clearly stated.

The choice of a reference out-group sequence can also have an important effect on the tree topology. Sequences used as out-groups should not be too distantly related to the investigated groups. Single distantly related organisms as out-group may lead to branch extraction.

An accurate performed analysis of 16S rRNA gene based data is the prerequisite for the interpretation of the phylogenetic relationships. However, the use of different treeing methods for the evaluation of the same data set does not identify effects such as gene transfer, convergent, or parallel evolution. Therefore, it is required to include other studies that can also reflect evolution including phylogenetic analysis based on alternative genes but also nonsequence-based methods.

The resolution power of the 16S rRNA gene is limited most often to distinction at the genus level. At the species level, other methods have to be considered if better resolution/distinction is required, and DDH and phylogenetic analysis of other molecular markers as single-gene analysis or in a set of genes have to be additional performed.

For the characterization of specific taxa and the differentiation of those taxa from closest related taxa, sometime signature nucleotides within the 16S rRNA gene sequence are determined and listed in a species description. The presence and absence of those signature nucleotides should be compared by the proposal of new species.

## DNA–DNA Hybridization

DNA–DNA and DNA–RNA hybridizations were introduced into prokaryotes systematics in the 1960s (Brenner et al. 1967; De Ley et al. 1966; Johnson and Ordal 1968; McCarthy and Bolton 1963; Pace and Campbell 1971; Palleroni et al. 1973). Later, DNA–RNA hybridizations were replaced by 16S rRNA gene sequencing because a good correlation was obtained for results of DNA–RNA experiments and 16S rRNA gene sequencing. DNA–DNA hybridization (or reassociation) (DDH) is still the recommended standard to confirm if strains that share more than 97% 16S rRNA gene sequence identity represent different or the same species. If the new taxon shows 97% 16S rRNA gene sequence similarity to more than one related species, DDH should be performed with all regarding type strains to ensure that there is enough dissimilarity to support classification of the strain(s) as a new taxon. DDH must also be performed in cases where the new taxon contains more than a single strain in order to show that all members of the taxon have a high degree of hybridization among each other and thereby indeed represent the same species.

A number of techniques can be used for DDH experiments. Most of them have been validated and show comparable results (Grimont et al. 1980; Rosselló-Móra 2006). It is recommended that the method (with all modifications) must be clearly cited and by the use of a novel method, the authors must provide evidence that the method produces comparable results to the established methods. As recommended by Tindall et al. (2010), the DDH data of the type strain of a proposed new species with the type strain(s) of the closest related species with validly published names (if >97% 16S rRNA gene sequence similarity occurs) and with all other strains of the proposed new species must be provided. Depending on the used method, at least one reciprocal value of the closest related species to the new type strain must be provided. If reciprocal values cannot be supplied, for example, by the use of spectrophotometric methods without DNA labeling, standard deviations of at least three analyses should be given.

The most often used DDH method in prokaryote systematics is the relative binding ratio (RBR) methods, where results are given in percentage binding or percentage hybridization. For the application of the RBR method in prokaryotic systematics, it was

recommended that only DNA from strains of at least 5 °C differences in melting temperature (deltaTm) should be compared. The temperature, which is used for DDH must be clearly stated. Commonly, hybridizations are performed at Tm-30 and/or Tm-15 (stringent conditions). The GC contents of the strains under study are normally used to calculate the melting temperature for each strain.

DDH results have to be critically considered; however, a DDH value of 70% has been recommended as species boundary (Brenner 1973; Johnson 1973, 1984; Wayne et al. 1987). This value should not be used strictly without considering other results (Ursing et al. 1995). All strains that cannot be clearly discriminated by a stable phenotypic property should be grouped into a single species. Hence, a single species may contain groups of strains with even less than 50% binding, which cannot be distinguishable by means of other properties tested at the time. In contrast, a single species may contain several genomic groups or genomovars (Ursing et al. 1995) that may be reclassified as new species if they can be clear and stable discriminated by phenotypic properties.

DNA–DNA hybridization is at the time of this writing still an important standard for the description of novel species (Tindall et al. 2010), but it is often criticized to be time-consuming, difficult to perform, and prone to high experimental errors (Rosselló-Móra 2006). With the increasing applicability of genomic sequencing technologies, it can be foreseen that this method will be replaced by methods relying on genomic information derived on sequence information. Currently applied techniques, which will have the potential to replace DDH, are the calculation of the average nucleotide identity (ANI) of two genomes, which are compared (see below). Recently, *multilocus sequence analysis* (MLSA) of approximately 5–12 housekeeping genes has been suggested as an alternative for the application of DDH (Gevers et al. 2005).

## DNA Base Composition

The guanine–cytosine (GC) content of DNA (DNA base composition) is an important character which is still required for the description of a new genus. Two strains differing by more than 10 mol% should not be considered as members of the same genus; however otherwise, a higher similarity of the DNA base composition does not necessarily imply that the two strains are closely related. It has been shown that the DNA base composition is a valuable character to distinguish between nonrelated bacteria, such as staphylococci (30–35 mol% GC) and micrococci (70–75 mol% GC).

## Phylogenetic Assignment Based on Alternative Phylogenetic Markers Including Protein Coding Genes

There are not many alternative genetic markers available that occur in all genomes. The comparative analyses of some of them, like the 23S rRNA gene, genes encoding translation elongation and initiation factors, RNA polymerase subunits, ATPase subunits, DNA gyrases, the RecA, and heat shock proteins give very similar tree topologies when compared with the topology of the 16S rRNA genes (Ludwig 2010). Rather conserved and ubiquitously distributed genes of the core genome are potential phylogenetic markers for the genotypic classification of less related prokaryotes. Thus, comparative sequence analysis of certain core genes, including rRNA genes, may be useful to obtain the phylogenetic relationships of higher taxa. For the classification of lower taxa, as the delineation of species, character genes may be suitable phylogenetic markers. Genes coding for key phenotypic differences may also play an important role to proof classification of bacteria and to enable a higher resolution of taxa especially at the species level.

## MultiLocus Sequence Analysis (MLSA)

*Multilocus sequence analysis* (MLSA) was outlined as an "intermediate resolution" between the 16S rRNA gene and genome-based approaches. MLSA is a method for the genotypic characterization of a selected group of prokaryotes, most often strains of one species; species of a genus, and also of closely related genera are investigated. Typically partial sequences of a set of 5–12 housekeeping genes are investigated and either compared as individual data sets or combined to concatenated sequences, which were then used for phylogenetic analysis.

MLSA based on multilocus sequence typing (MLST), which was introduced by Maiden et al. (1998) for epidemiological studies of pathogenic bacteria where it is currently one of the most successfully applied methods. In contrast to MLST, which is focused on the designation of sequence types based on base variation within sequences independent of the sequence similarities, MLSA is focused on the investigation of the phylogenetic relationship among closely related taxa. Sequence similarities are calculated and phylogenetic relationships are investigated by the construction of phylogenetic trees. Ideally, MLSA analysis is based on both DNA and amino acid sequences. Criteria for the selection of genes are that they have to be present in all investigated strains and they need to have conserved regions to generate primers for gene amplification but however have to provide enough sequence differences to distinguish between the investigated taxa. The selection of genes also depends on the taxa, which are studied. Different genes may be suitable if different species are investigated or if an intraspecies analysis is performed (Cole et al. 2010).

The advantage of MLSA to the 16S-rRNA-gene-sequence-based phylogeny is the use of protein coding housekeeping genes, which also have a conserved function but a higher degree of resolution than the 16S rRNA gene. The use of multiple genes provides more informative nucleotide sites and buffers against the distorting effects of recombination of one of the loci. MLSA enables the better phylogenetic resolution of deeply branching clusters and helps to delineate genotypic clustering within a genus or species (Soria-Carrasco et al. 2007). It was

recommended by Tindall et al. (2010) that if species delineation is based on MLSA schemes, then it has to be validated by DNA–DNA hybridization data.

The application of MLSA for taxonomic classification may be problematic for several reasons. A selection of a proper set of genes is not systematically evaluated. As it has been shown so far, different sets of genes are required for the classification of different taxa and lineages. An overall applicable set of genes does not exist. In contrast to the 16S rRNA genes, the design of primers for the amplification of housekeeping genes may be difficult or sometimes even impossible also for very closely related species or even strains within a species. A set of 5–12 genes has been recommended for MLSA studies (Stackebrandt et al. 2002; Tindall et al. 2010), but often, smaller sets including 3–5 genes are used, and even 12 genes correspond only to a minor fraction of the genome. Most often only small gene fragments are investigated, which leads to a restricted phylogenetic content. As already pointed out for 16S rRNA gene sequence analysis, the alignment is a critical step also in protein coding gene sequence analysis. Here, the open reading frames should be considered, and nucleotide sequences should be aligned according to the translated amino acid sequences. Especially if single amino acid deletions occur, amino-acid-based prealignments are often a prerequisite for a correct alignment and for reliable phylogenetic analysis. Often, only nucleotide sequences are considered, but the third codon positions however are more variable, and amino-acid-based sequence may be performed in parallel to prove the stability of the obtained phylogenetic relationships. MLSA analysis within a genus sometimes investigated with different MLSA schemes and most often not all species of a genus are included in the analysis. Often, only a selection of type strains of species of a genus or all genera under consideration are not available and thereby not considered by the delineation of new species. For example, the clinically relevant *Acinetobacter calcoaceticus—A. baumannii* complex (ABC-complex) has been intensively studied by even two different MLST schemes (Bartual et al. 2005; Wisplinghoff et al. 2008; Diancourt et al. 2010). Based on the MLST scheme published by Diancourt et al. (2010), a detailed phylogenetic analysis (MLSA) was performed leading to the proposal of two new species, *A. pittii* and *A. nosocomiales*, which represented the genomic species 3 and 13TU (within the ABC-complex). Despite those detailed analysis, MLSA has so far not been applied and evaluated for the whole genus *Acinetobacter* (Kämpfer and Glaeser 2012).

Recently, Jolley and Maiden introduced a combined taxonomic and typing approach, the ribosomal multilocus sequence typing (rMLST), which is based on the analysis of 53 bacterial ribosomal protein subunit (rps) gene loci. In contrast to other MLST/MLSA systems, the rMLST approach is promising to be suitable for the whole domain bacteria with a resolution down to the species or subspecies level (Jolley and Maiden 2010; Jolley et al. 2012). At least all of the genes are present in prokaryotes, widely distributed over the genome and represent proteins, and are thereby under stabilizing selection because of the functional conservation. However, so far the prerequisite for an rMLST approach are gene sequence data obtained by whole, draft, or partial genome sequences. In the future, the rMLST or rMLSA approach is promising to be "a natural extension to the 16S rRNA gene based analysis in microbial taxonomy" (Jolley et al. 2012).

## Genome-Based Analysis: GEBA Project: Toward a New Area in Taxonomy

The expected decrease in the costs for sequencing whole genomes, together with the technical advances that have been made recently, will make it more feasible that in the near future routine sequencing of more prokaryote genomes will be realistic. The detailed investigation of genome sequences may then overcome the problems associated with DDH experiments. However, these analyses and the interpretation of the data should be done very carefully. The key issue for taxonomic analysis is a reliable annotation of genes in a genome since identifying gene homologies (preferably orthologues) is of central importance.

In the last years, several genome indexes were established for pairwise genome comparison for taxonomy purposes. The average nucleotide identity (ANI, Konstantinidis et al. 2006) and maximal unique matches (MUM, Deloger et al. 2009) are the most common indexes, and it has been suggested that they will be able to substitute DDH. This may especially be true for ANI already in the near future. It was demonstrated that results of ANI and DDH correlate well and a range of about 95–96% ANI reflects the current boundary of 70% DDH similarity (Goris et al. 2007; Richter and Rosselló-Móra 2009). Goris et al. (2007) also performed the comparison of genomes that were artificially sectioned into 1020 nucleotide fragments and showed that the comparison of at least 20% of genomes generated by simple randomly sequencing will be enough to determine reliable ANI values.

So far, the number of genome sequenced type strains is still too small to apply genome sequence–based taxonomy. The *Genomic Encyclopedia of Bacteria and Archaea* (GEBA) project was the first project which was initiated for the genome sequencing of type strains of almost all bacteria and archaea (http://www.jgi.doe.gov/programs/GEBA/index.html). Most of genome sequencing projects so far were focused on the investigation of pathogens or strains of relevance in other context. The primary criterion for the selection of type strains in the initial phase of the GEBA project is their taxonomic position in the tree of life. Genome information obtained in the GEBA project will be made available by publishing information in a short genome report in the open access journal Standards in Genomics Sciences (http://standardsingenomics.org/index.php/sigen/index). In the currently ongoing pilot GEBA project, the JGI performs in collaboration with the DSMZ the sequencing of 100 bacterial and archaeal genomes. The selection of organisms from sequencing is based on their phylogenetic positions in the tree of life determined by 16S rRNA gene sequence analysis.

## The Problem of Horizontal Gene Transfer (HGT)

The increasing investigation of gene sequence and genome-based studies shows a remarkable occurrence of horizontal gene transfer (HGT) affecting genotypic analysis and impacts a clear resolution of bacteria of clusters of organisms which derive from a common ancestor. The dimension of the effect of gene flow, especially lateral gene transfer, on microbial evolution and thereby on microbial taxonomy is still unclear.

Investigations of potential HGT events between bacteria will get more and more important in microbial evolution. It has also been shown that changes in gene flow caused by ecological factors may even lead to an incipient merging of two bacterial species, as it has been demonstrated for *Campylobacter jejuni* and *C. coli* which reverses the process of speciation. It has been published recently that 80% of all genes were subjected to HGT at some point in their history (Dagan et al. 2008), even though most estimates are lower (Lienau and DeSalle 2009).

The gene content and gene order phylogenies of prokaryotes may be strongly affected by gene loss ("big/small genome attraction" and HGT; Wolf et al. 2001). Selection pressure may operate on whole sets of genes that are functionally linked to each other. In conclusion, Klenk and Göker (2010) summarized that genome-scale data will improve microbial taxonomy considerably once sufficient coverage of major lineages based on type strains has been obtained, and a more detailed insight into the processes given above is provided. They concluded further that if phenotypic information is integrated in a standardized manner in the course of such ventures, genomics as the basis of all -omics methods might open the door to a truly "holistic" approach to classification.

## Nucleic Acid Fingerprinting

Rapid and high-resolution differentiation of closely related taxa especially for the differentiation at the subspecies and/or strain level can be performed by genomic fingerprint analysis using different methods as amplified fragment-length polymorphism PCR (AFLP), random amplified polymorphic DNA (RAPD) analysis, repetitive element primed PCR (rep-PCR) using different primers that bind to highly conserved, repetitive DNA sequences distributed all over the genome, including repetitive extragenic palindromic (REP)-PCR, enterobacterial repetitive intergenic consensus sequences (ERIC)-PCR, and BOX-PCR (derived from the boxA element) and (GTG)5-PCR, and ribotyping. A standardized application to enable a comparison of results is only possible for AFLP and ribotyping. For other fingerprint methods, it is important that all strains under consideration are investigated in parallel. For the differentiation of a new species to related taxa, most of the fingerprint techniques are not suitable, but they can be applied to show if strains of new taxa are a clone or different representatives of a new species.

## Phenotypic Traits

Phenotypic characterizations, including morphology and physiology, belong to the oldest methods used in prokaryote systematics. But these methods are still very important for the initial characterization of a strain or a set of strains. Phenotypic traits are the observable characteristics that result from the expression of genes of an organism (Moore et al. 2010) but can largely be modulated by environmental or other conditions (e.g., growth conditions, like temperature, pH value, etc.). For phenotypic analysis, it is important that strains which under comparison should grow under the same conditions and cells/cultures in the same growth stage should be compared. A detailed and highly recommended book chapter on phenotypic characterization of prokaryotes with detailed references to methods has been provided by Tindall et al. (2007).

## Colony Morphology

Strains grown on media show different colony morphologies, including shape, size, color, and structure of the colony. Some strains form rapidly growing colonies, which spread out, while other strains form compact, slow growing colonies. Because each strain can show great morphological variation depending on temperature, pH, atmosphere, age, and growth medium, observations should be made on standardized media and growth conditions. Colony shape and size, surface, outer edge, production of slime, elevation, odor, opacity, swarming behavior, consistency, changing color, and any visible additional structures or features should be noted accurately as they are important parameters for the characterization of strains. Often, colony morphology on specific media helps to identify clinical isolates quickly. The color of colonies on agar surfaces or in deep agar-shake cultures may help in identification. Examples are *Serratia marcescens* within the *Enterobacteriaceae*, *Halobacterium* as an extremely halophilic bacterium, and phototrophic bacteria.

## Cell Shape and Cell Size

Cell shape and cell size vary greatly between species of Bacteria and Archaea. This becomes more and more apparent with the development of better methods to visualize prokaryotic cells and to analyze the structure of cells at the subcellular and molecular level. The most traditional tool for visualizing prokaryotic cells is the light microscope. But often, a higher resolution is needed to differentiate cell morphologies. Electron microscopy serves this purpose. The morphology of whole cells can best be differentiated with scanning electron micrographs, while inner structures, for example, the presence of cytoplasmic inclusions or internal membrane structures can be determined with transmission electron microscopy. Regardless of the method, cell shape and form should be described in detail and supported by appropriate photographs. Samples should be prepared by covering

the slides with thin layers of agar to get the best light microscope image results (Pfennig and Wagener 1986).

The two most common cell morphologies are rods and cocci. Rods can have a uniform size and rounded ends or the ends can have characteristic forms. Cocci often come with a regular spherical shape, but they can also show different shapes, for example, cocci, which have just divided can seem relatively flat. Other morphological forms are spirilla and spirochetes, which can differ in amplitude and wavelength, spirals, or vibrios. Next to this, cells can be stalked, sheathed, or divided by budding with its characteristic form. Cell shapes and forms are not only important taxonomic features; they can also play a role in medical microbiology to identify pathogenic microorganisms. Next to the shape of single cells, it should be noted if cells form aggregates, for example, myxobacteria, branching hyphae, mycelia, or sporangia, which are important features to differentiate actinomycetes. Other features that should be observed are, for example, apolar growth, the formation of prosthecae, budding, or branching as well as the possible undergoing of a life cycle.

## Staining Behavior of the Cell

One of the oldest staining procedures, which is still widely in use and which is an easy and fast method, is the Gram stain (Gram 1884). With Gram staining, cell wall structures of prokaryotes can be distinguished between cell walls where the dye complex is washed out (Gram negative) and cell walls where the dye complex is retained (Gram positive). Problems can arise if cells are too old because the Gram stain reaction alters with the age of cells. Organisms which have a defective cell wall also stain as Gram negative, although they might be Gram positive. Some species react Gram positive in the exponential growth phase and become Gram negative in the stationary phase. In certain groups, for example, the *Halobacteria*, cells have to be fixated before staining.

A staining method for organisms, which contain mycolic acids, is the acid fast staining. This staining procedure is used mainly for *Mycobacteria*. Cell components can be stained as well. Lipophilic cellular inclusions (e.g., polyhydroxybutyric acid) can be stained with Sudan black; extracellular polymers can be stained with India ink.

## Cellular Motility

The form and speed of mobility can differ between microorganisms; therefore, it should be described precisely. Motility can be differentiated, for example, between slow or rapid movements and motility caused by flagella or gliding. Some organisms developed their own characteristic form of motility, for example, myxobacteria, flexibacteria, *Cyanobacteria*, *Chloroflexaceae*, and *Beggiatoaceae*. Best results can be observed if fresh cultures are examined. Although one has to be aware that motility is difficult to observe and cells, which are not moving, are not necessarily "nonmotile." Nonmotile strains do not show motility at all and have no means of locomotion by flagella or gliding. If a cell is temporarily not moving, it should be described as "immotile."

## Flagellation

Flagellation of cells can differ regarding the position and the number of the flagella. Flagella can be polar, subpolar, or laterally inserted. They can occur in one location, as tufts of flagella, or distributed around the cell at different locations. Flagella cannot always be observed by light microscopy. In these cases, cells should be examined using electron microscopy. Sometimes, flagella may be lost during fixation of cells. Nevertheless, flagellation is still recommended as a criterion for the classification of bacteria. For further details, see the review by Rhodes (1965).

## Sporulation

Spores are divided into endospores and exospores. Organisms, which form endospores, should be examined regarding the position of the spores and their size in relation to the size of the vegetative cell. Endospores can be easily detected under the light microscope with phase contrast by examining living cultures or Gram staining. If this does not work well, a specific spore stain on the basis of malachite green can be used. Endospores often are formed under limited nutrient conditions and are an important criterion for identification and classification. For the stimulation of endospore growth, often selective media have to be used; details are summarized by Logan et al. (2009).

Exospores should be described regarding their form and other sporulation features, for example, the formation of sporangia. Shape and color are especially important for the identification of actinomycetes.

## Cellular Inclusions

Cellular inclusions are intracellular structures except spores, for example, polyhydroxybutyrate granules, gas vacuoles, phosphorus, and sulfur granules. They are visible under the light microscope and should be studied using appropriate methods like specific staining procedures. Cellular inclusions of different kinds are widespread among bacteria. Polyphosphate granules can be found in a variety of bacteria, but they play also an important role for the presumptive identification of clinically relevant bacteria like *Corynebacterium diphtheriae*. Polyphosphate are included in cells as volutine granules, which appear reddish when stained with toluidine blue due to their basophilic character. Another name for these volutine granules is metachromatic granules. Staining methods include the Albert's and Neisser's stain, where the polymetaphosphate bodies are blue-black after staining, while the cytoplasm stains light brown. Another cellular structures used for differentiation are

globules of elemental sulfur, which can often be found in phototrophic sulfur bacteria. Protein crystal on the other hand can be used to differentiate *Bacillus thuringiensis*.

## Pigmentation

Pigmentation of cells can be studied under the microscope or by analyzing the color of cell suspensions or colonies. The study of single cells is of less importance than the other two but can help to differentiate pigmented bacteria, for example, purple sulfur bacteria of the family *Chromatiaceae*, which have a conspicuous purple-red carotenoid okenone.

Of greater importance is the color of cell suspensions. With absorption spectra, the identification of pigment-containing bacteria, for example, phototrophic bacteria, is possible.

Cellular pigments can be soluble in water or organic solvents. Pigments, which can be found typically in cells, include carotenoids, flexirubins, (bacterio)chlorophyll, melanin, and pyocyanin. Several methods exist to identify these pigments. Carotenoids turn blue if concentrated sulfuric acid is added; flexirubins change their color reversible when observed under acid or alkaline conditions; (bacterio)chlorophyll can be solved in organic solvents and, after saponification, also in water; if treated with acids, (bacteria) phaeophytin is formed; and pyocyanin changes its color depending on the pH and fluoresces at 360 nm.

## Bacteriochlorophylls

The determination and differentiation of bacteriochlorophylls can be helpful in the classification of especially phototrophic bacteria (Oelze 1985). The majority of the species of the *Rhodospirillaceae, Ectothiorhodospiraceae*, and *Chromatiaceae* contain bchl *a* only. However, some of them possess bchl *b* instead.

In the *Chlorobiaceae* and *Chloroflexaceae*, bchl *c, d*, and *e* are the main pigments. They are always accompanied by small amounts of bchl *a*. Bchl *a* is also found in *Erythrobacter*, whereas *Heliobacterium chlorum* contains bchl *g*.

## Cytochromes

Bacterial cytochromes are involved in a wide variety of redox processes such as aerobic and anaerobic respiration and photosynthetic electron transfer. Most of the cytochromes are associated with the cytoplasmic membrane, but some exceptions have been determined. Some *c*-type cytochromes and various oxygenases are located within the cytoplasm or the periplasmic space. Cytochrome patterns of bacteria can be used as a valuable chemotaxonomic character since, unlike the mitochondrial respiratory chains of eukaryotes, bacterial respiratory chains contain a greater variation of different cytochromes (Jones and Poole 1985). Conventional difference spectrophotometry, preferably at low temperatures, is sufficient to determine the cytochrome pattern (Faller et al. 1980). It has been reported that the cytochrome pattern bcaa3 and o occurs predominantly in Gram-positive bacteria and the pattern bcdo and a1 is often found in Gram-negative bacteria (Jones and Poole 1985). However, other studies have revealed that cytochrome d is also present in some Gram-positive bacteria, in particular in micrococci and coryneform bacteria (Faller et al. 1980; Faller and Schleifer 1981). Cytochrome c is often absent in both Gram-negative and Gram-positive bacteria, and enterobacteria can be easily separated from pseudomonads since the former do not contain cytochrome *c* and therefore are oxidase negative.

When cytochrome patterns are used for taxonomic studies, it has to be taken into account that growth conditions (nutrition, growth phase) can influence quantitatively and, to a lesser extent, qualitatively the cytochrome content of a bacterial culture (Faller et al. 1980; Faller and Schleifer 1981). To assess the cytochrome-synthesizing capacity of a strain, cells from both the exponential and the stationary growth phase (as a minimum) have to be examined.

## Ultrastructural Characters

The ultrastructure of cells can be studied by electron microscopy, which has turned out to be useful in identification. With electron microscopy, cell wall layers, typical intracytoplasmic membrane systems, or characteristics, which differ between Gram-positive and Gram-negative cells, become visible. Intracytoplasmic membrane systems are especially important for the identification of phototrophic (cyanobacteria and purple bacteria), methylotrophic, and chemolithoautotrophic (thiobacilli and nitrifiers) bacteria. Chlorosomes are characteristic for *Chlorobiaceae* (Staehelin et al. 1978) and phycobilisomes for cyanobacteria. Carboxysomes are found in autotrophic bacteria as polyhedral bodies (Shively 1974) and are characterized by the polymerized form of the enzyme ribulose bisphosphate carboxylase, which appear as conspicuous polyhedral bodies in autotrophic bacteria only.

## Physiological Properties

In the past, identification of microorganisms was performed mainly by methods that we now refer to as "conventional procedures." Those included physiological reactions in tubed media and observation of physical characteristics, coupled with the already mentioned results of Gram staining, other morphological properties, and (sometimes) antimicrobial susceptibility profiles.

The physiological characterization of a new strain in comparison to related taxa includes the determination of enzyme activities, growth on specific substrates, and the determination of metabolic activity without associated growth (Tindall et al. 2010). Tindall et al. (2007) as well as Krieg and Padgett (2011) give excellent overviews for a broad range of routine tests, which can be applied for the physiological identification of prokaryotic taxa.

It should be determined if a new strain is phototrophic, chemoautotrophic, or chemoheterotrophic. The new taxa should furthermore be characterized with respect to the requirements of molecular oxygen and/or the growth in presence of molecular oxygen: aerobic, microaerophilic, anaerobic, and facultative anaerobic growth.

Important applied tests for the investigation of the presence and activity of respiratory enzymes are the oxidase and catalase tests.

Oxidases transfer electrons from a donor molecule to molecular oxygen and thereby occur most likely in aerobic and/or microaerophilic microorganisms, which use molecular oxygen as terminal electron acceptor. An artificial electron acceptor can be used as test reagent (e.g., N, N, N′,N′-tetramethyl-*p*-phenylenediamine (TMPD)) which turns to a colored compound after reaction. Bacteria harbor cytochromes, mostly associated with cytoplasmic membranes, which are involved in a wide variety of redox processes, as the aerobic or anaerobic respiration or photosynthetic electron transfers. Cytochrome *c* is often absent in both Gram-negative and Gram-positive bacteria. For example, enterobacteria can be distinguished from pseudomonads regarding the absence of cytochrome c (determined as oxidase negative bacteria) in the former ones. The comparison of cytochrome patterns can also be used for the differentiation of micrococci and staphylococci because the later one usually lack cytochrome *c* and *d*.

Catalases are enzymes that prevent oxidative damage caused by hydrogen peroxide via the disproportionation of hydrogen peroxide to water and oxygen. The presence and activity of catalase can be easily tested by "bubble formation" on cultures (grown on agar plates) after the addition of some drops of a 3% hydrogen peroxide solution.

Temperature-, pH-, and salinity (%w/v NaCl)-dependent growth range and growth optima are routinely performed for the characterization of new taxa. Optimal growth conditions often depend on the environmental condition of the original habitat of the strains under study and can thereby already differ between closely related taxa which were originally isolated from different environments, as, for example, freshwater (low salt content) or marine systems (high salt content). For the test of pH-dependent growth, a puffer should be used to stabilize the pH of the used medium. Buffers have to be selected critically regarding the puffer capacity, the use of a puffer as substrate, and the possible negative effect of a puffer to the tested organisms. The most often used buffer systems are phosphates buffers.

Carbon utilization or assimilation pattern is often a routine in test for the characterization of new taxa including the strains of new taxa and of related taxa which have to be compared in a respective study.

Nitrate reduction or denitrification, as well as the requirements of $CO_2$ or hydrogen for growth, or the use of $H_2$ as an energy and electron source for some autotrophic bacteria are metabolic properties which have to be tested for specific taxa. Several other physiological properties can and have to be tested dependent on the taxa of interest. Comparison to the characterization of related taxa or requirements listed in respective minimal standards (see above) can be used to determine which physiological tests are required for respective taxa.

The activity of a broad range of metabolically active enzymes are determined to characterize the metabolic proportion of a new strain, for example, activity of glycosidases (measurements by the used of chromogenic or fluorogenic substances linked to respective substrates), lipases, peptidases, phenylalanin deaminase, or phosphatases, to name a few, are determined.

The investigation of the oxidation and fermentation of different compounds (mostly sugars) as well as the determination of carbon substrate utilization pattern is mandatory for the characterization of new taxa.

Within the last decades, several methods simply miniaturized commonly used biochemical reactions into a more convenient format, and this system-dependent approach has become more and more standard, especially for the identification of clinically relevant bacteria. These methods are based on sets of substrates that are carefully selected for their positive and negative reactions. These patterns create metabolic profiles that are compared with established databases, in most cases by numerical procedures.

The numerical identification procedures developed by Sneath and coworkers in the 1960s and 1970s (Sneath and Sokal 1973) are either manual or automated. For all systems combined from carefully selected tests, the backbone of accuracy is the strength and utility of the database. Databases are constructed by using known and well-characterized strains and include the type strains of most taxa. The major disadvantage of this approach is that atypical microorganisms often cannot be reliably identified by these commercial systems. Furthermore, the number of species included in the databases may vary from just a few for some manual assays to thousands for automated instruments.

Database maintenance must be a continuous process and software upgrades incorporating major taxonomic changes must be provided by the manufacturer at regular intervals. This poses more and more a problem because the majority of novel species in the last 10 years have been proposed on a single strain (the type strain) only. Hence, the physiological diversity of these species are unknown and cannot be considered in the databases.

System identifications are nowadays computer-based and done by algorithm-based decision making. In some cases, the list of identifications is compiled into a preprinted index, which may then be consulted to manually convert the organism's biochemical profile number into an identification result. In many cases, Bayes' theorem, or modifications of it, is often the basis of algorithm construction from data matrices.

It is important to note here that microbiologists should not always become dependent upon these likelihoods and percentages when interpretive judgment would suggest an alternative taxonomic conclusion. Bacteria may not react in specific tests as expected in a commercial system, and even though a legitimate result is produced (e.g., lactose-positive *Salmonella* spp. or $H_2S$-positive *Escherichia coli*), these results may be misleading.

Physiological and/or biochemical tests are in the majority of cases determined by the reactions of individual organisms with different substrates in a system that gives often a color change or a change in turbidity in case of a positive reaction. The accuracy of these reactions is heavily dependent upon the users following exactly the directions of the manufacturer regarding the preparation of the inoculum, adjustment of the inoculum density, the incubation conditions, and test interpretation.

Many tests rely upon one or a combination of several indicators. These may include (1) pH changes resulting from acid formation and/or alkalinization as a result of the utilization of the substrate, (2) turbidity changes resulting from the growth and utilization of the substrate, (3) enzymatic reactions on a chromogenic and/or fluorogenic substrate that allow the release of a colored or fluorescent compound, (4) tetrazolium-based indicators of metabolic activity (here often dehydrogenase activities) in the presence of a variety of carbon sources, (5) detection of volatile or nonvolatile acids. Additional tests for microbial identification that use other means of detecting a positive response for a given substrate may also be included.

## Numerical Taxonomy and Numerical Identification

Numerical taxonomy is the well-established means for the assessment and evaluation of phenotypic data (Goodfellow 1977; Sneath 1971; Sneath and Sokal 1973; Sokal and Sneath 1963; Hill 1974). In numerical studies, results are tabulated in a table of $t$ organisms versus $n$ characters, and operational taxonomic units (OTUs) are assigned for each individual strain. Characters under study should be independent and should come about equally from the various different categories of properties (morphology, physiology, biochemistry, serology, etc.). For statistical reasons, the total number of characters should be above 60. Whereas in conventional taxonomy, so-called characteristic tests and differentiating media are in use (with special importance for identification), numerical taxonomy principally allots the same weight to each character because there is no logical alternative that would allow independence from the personal opinion of the scientist.

The number of common characters is considered as a quantitative measure for the taxonomic relationship (not phylogenetic "relatedness"). As an important consequence, not all members (species) of a group must have one special property in common. Similarities are quantitatively expressed by the matching coefficient of Sokal and Michener (Skerman 1967), usually expressed in percent:

$$S_{SM} = \frac{\text{Sum of positive and negative matches}}{\text{Total number of tests}}$$

The similarities between each OTU and every other OTU under study are set out in tables in the form of a triangular similarity matrix (also called a Sneath diagram). By forming linkage groups and performing cluster analyses, the numerical taxonomist finally arrives at a rearranged matrix that depicts linkage clusters. Another way to express relations between OTUs after an analysis is by the use of dendrograms. Kaneko (1979) introduced the correlative similarity coefficient as a new criterion for forming dendrograms. Other coefficients for numerical taxonomy have been evaluated by Austin and Colwell (1977).

Detailed information about the use of computer analysis in numerical taxonomy can be obtained from Sneath (1972, 1977, 1979a, b, c, 1980a, b, c). Computer-based identification systems have also been described, for example, by Edwards (1978); Kellogg (1979); Schindler et al. (1979); Beers and Lockhard (1962); Gyllenberg (1965); Holmes and Hill (1985); and Lapage et al. (1970, 1973).

## New Ways for Phenotypic Characterization

Recently, more sophisticated phenotyping systems analyzing different cellular fractions have been introduced into systematics, for example, the matrix-assisted-laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Welker and Moore 2011; Moore and Rosselló-Móra 2011) or the high-field ion cyclotron Fourier transform mass spectroscopy (ICP-FT MS) technique, which are referred to metabolomics (Rosselló-Móra 2012). However, despite the advantages of these systems to generate high numbers of data, which can be stored in databases, the general restrictions of cultivation-based dependencies of the cultivation conditions also apply to these techniques.

## Chemotaxonomy

Chemotaxonomic characterization is based on the investigation of various structural cellular compounds of prokaryotes. The most important investigated structural compound are fatty acids, polar lipids, respiratory chinones, and for some extent pigments of the outer membrane(s), cytoplasmatic polyamines or the peptidoglycan, teichoic acids, mycolic acids, or lipopolysaccharides (LPS), which are compounds of the outer cell layer.

The order of the importance of specific chemical compounds for the characterization of new taxa primarily depended on the chemotaxonomic trades used for the characterization of closest related taxa. As outlined above, the identification of closest related taxa is at most often done by the phylogenetic affiliation based on nearly full-length 16S rRNA gene sequences.

## Cell Wall and Membrane Compounds

### Peptidoglycan

Peptidoglycan is the most predominant cell wall compound of both, Gram-negative and Gram-positive bacteria. Peptidoglycan is a heteropolymer of glycan strand with alternating ß-1,4-linked

residues of *N*-acetylglucosamine and *N*-acetylmuramic acid, which are cross-linked by short peptides. *N*-acetylmuramic acid is a derivative of glucosamine and occurs in cells specifically as a component of the peptidoglycan. Gram-negative bacteria harbor a monolayer of peptidoglycan, which is remarkable uniform in its composition. The multilayer peptidoglycan of Gram-positive bacteria is characterized by a great variety in the chemical composition. Therefore, the discriminatory power of peptidoglycan structures is apparently restricted to Gram-positive bacteria. Especially for *Proteobacteria* and *Bacteroidetes*, no characteristic variations could be determined (Schleifer and Kandler 1972).

The advantage of peptidoglycan is that they are thought to be less affected by mutations, which could alter the peptidoglycan structure, and is less affected by phenotypic variations (Schleifer et al. 1976).

The analysis of the peptidoglycan structure can be performed at different levels. At first, characteristic diamino acid in the cross-linking peptide can be determined. Analysis of the peptidoglycan type (A type: cross-linkage of the two peptide side chains via amino acid 3 of one peptide subunit to amino acid 4 of the other peptide subunit; B type: cross-linkage of the two peptide side chains via amino acid 2 of the one peptide subunit to amino acid 4 of the other peptide subunit), mode of cross-linkage (direct or interpeptide bridge and amino acids in the bridge), and complete amino acid composition provides more detailed information. B-type peptidoglycan is characteristic for all genera of the family *Microbacteriaceae* and members of the *Erysipelothrix*/*Holdemania* group. All other murein-containing bacteria so far analyzed exhibit the A type. The amino acid composition of the peptide side chain, including the characteristic diamino acid is usually common to all species of a genus. However, a higher degree of variability has been detected in the mode of cross-linkage between the peptide side chains, which often differ among species of certain genera (e.g., members of the genus *Microbacterium*) but may differ also between strains of a single species as reported for *M. luteus*. Analysis of the peptidoglycan structure is a requirement for all members of novel Gram-positive genera when they are described, and at least the amino acid composition should be provided for every novel Gram-positive species. In the majority of cases, the amino acid composition of the peptide side chain of the type species of a novel genus may be shared by future species assigned to the genus, and hence, it should be listed in the genus description as a characteristic trait. The complete composition of the peptidoglycan of a novel species of a genus that has previously been described should be in agreement with the characteristics of the genus and may provide differences in the interpeptide bridge useful for differentiation from other species. A list of peptidoglycan variations can be found at http://www.dsmz.de/species/murein.htm. This system is slightly different to that developed by Schleifer and Kandler (1972).

Pseudomurein, a characteristic peptidoglycan, has been detected in some Gram-positive members of the Archaea, in which *N*-acetylmuramic acid is replaced by *N*-acetyltalosuronic acid (König et al. 1982, 1983).

#### Lipopolysaccharides (LPS)

Few routine studies are now carried out, but it is evident from work over the past 6 decades that both the nature of the sugar present in lipid A as well as the nature of the fatty acids and the way they are linked (ester and/or amide linked) to the sugar is of significance (Hase and Rietschel 1976; Weckesser and Mayer 1988). The chain length of the fatty acids in the LPS may also differ significantly from those found in the polar lipids. When carrying out whole cell fatty acid analysis, the fatty acids from the LPS will also be extracted, and this should be borne in mind when interpreting the data.

#### Mycolic Acids

Mycolic acids are characteristic lipid compound of the cell envelope of mycobacteria and related high-GC Gram-positive bacteria (Brennan 1988). The main fraction of mycolic acids are ester-linked fraction of arabinogalactan which is attached to peptidoglycan.

For bacteria that produce mycolic acids, they can be used as addition taxonomic markers because it was shown that the phylogenetic relationships based on the 16S rRNA gene sequence analysis and the grouping based on the length of mycolate side chain correlated well with each other. It has also been of taxonomic relevance if taxa of groups, which are characterized by the content of mycolic acids, are lacking. As described above, mycolic acids can be detected by the staining with carbol fuchsin (acid fast staining) but also detailed investigates after extraction and derivatization by thin-layer chromatography (Dobson et al. 1985), HPLC (Willumsen et al. 2001), gas chromatography (Rainey et al. 1995; Müller et al. 1998; Torkko et al. 2003), or mass spectrometry (Fujita et al. 2005a, b).

#### Fatty Acids and Isoprenoid Side Chains

Almost all *Bacteria* contain fatty acids ester linked to the glycerol as typical membrane constituents. The comparison of fatty acid pattern can be used to assign new strains to related taxa because the fatty acid composition generally does not fluctuate significantly within a taxonomic group. Otherwise, differences obtained between closely related strains can also be used to differentiate related taxa from each other. But, it has to be critically question if, for example, branched chain fatty acids are reported in a group that otherwise synthesizes straight chain and unsaturated fatty acids. In a similar manner, the presence or absence of hydroxylated fatty acids is generally characteristic, and their unexpected absence or presence should also be treated with caution.

The MIDI system generally used for fatty acid analysis provides a comprehensive database set, which however is certainly not complete and thereby may give discrepancies by the identification that need to be clarified, for example, compounds that are currently not included in the database has to be assessed. In general, all components that constitute 1% or more of the fatty acids must be reported; if major peaks were not identified, they will not be included in the peak-naming table, but their presence must be reported and the equivalent chain length (ECL) should

be given. This will allow any future work on the elucidation of the structure to link to the ECL given in publications.

It is important to determine fatty acid patterns of strains, which should be compared, from cells that were cultured under identical condition (medium, temperature) and were in the same physiological growth stage prior to fatty acid extraction. Exceptions may be made if strains could not be grown under the same conditions. But this must then be carefully documented.

The MIDI system extracts fatty acids from intact cells (Miller 1982), and comparisons with work that has been carried out on the lipid fraction(s) extracted from cells or on fatty acid methyl ester mixtures that have previously been separated into different classes by thin layer chromatography are not sensible and falsify the interpretation fatty acid compositions.

Several *Bacteria* also synthesize ether-linked lipids in addition to fatty acids, which have either straight chains or simple branched (not isoprenoid) side chains or monounsaturated derivatives, with the point of unsaturation adjacent to the ether bond (i.e., plasmalogens or vinyl ethers). Fatty acid methyl ester for gas chromatographic fatty acid analysis is prepared under acidic conditions. Under those conditions, it comes to the hydrolysis of plasmalogens, and/or vinyl ethers and dimethyl acetals are formed, which elute with the fatty acid methyl esters. If the presence of those compounds are known or suspected, their presence/absence should be investigated and reported. However, gas chromatographic results have to be interpreted with care, as it has been reported by Tindall et al. (2010) that the presence of dimethly acetals can be misinterpreted in the presence of hydroxylated fatty acids, which elute with the same retention time (e.g., Moore et al. 1994; Helander and Haikara 1995).

Nonplasmalogenic mono- and diethers also occur in the members of the Bacteria (Langworthy et al. 1983; Rütters et al. 2001) but do not cochromatograph with mono- or diethers from members of the *Archaea*. But, however, similar methods can be applicable for analysis.

Specific modified fatty acids occur in a variety of polar lipids of some *Bacteria*, which, for example, are formed by condensation reactions with small molecular weight molecule as amino acids. Sphingolipids (Naka et al. 2003), capnines (Godchaux and Leadbetter 1984), and alkylamines (Anderson 1983) are well-known examples.

#### Hopanoids

Sterols are normally not synthesized by prokaryotes. There are only two genuine exceptions, namely, *Methylococcus capsulatus* and *Nannocystis exedens* (Tornabene 1985). However, in recent years, hopanoids and other polyterpenoids have been discovered in prokaryotes (Ourisson et al. 1987), which can be considered as sterol surrogates. Hopanoids have been found in various eubacteria. For instance, they are present in most cyanobacteria, methylotrophs, *Rhodospirillaceae*, acetic acid bacteria, and various other Gram-negative and Gram-positive eubacteria. They have not been found in archaebacteria, purple sulfur bacteria, or *Enterobacteriaceae*. Although archaebacteria do not contain hopanoids, they have phytanyl and bisphytanyl ethers that are also polyterpenoids.

#### Polar Lipids

In addition to the analysis of the fatty acids composition, polar lipids are independent chemotaxonomic markers for prokaryotic classification. Prokaryotes harbor a vast diversity of polar lipids associated with cellular membranes. Among those phosphate lipids are the most commonly known polar lipids; however, several others are also present, which are so far not all identified (Ratledge and Wilkinson 1988). For taxonomic purposes, polar lipid of strains are extracted and resolved by two-dimensional thin-layer chromatography. Standardized conditions are an indispensable prerequisite for those analyses. Several thin layer chromatograms have to be generated to obtain a detailed analysis for the polar lipid profile of an organism. At first, polar lipids are stained with a reagent that visualizes all polar lipids. It is recommended to scan those thin layer plates with high resolution (300 dpi) and to provide the polar lipid profiles as 8-bit gray-scale images with a size of 7 × 7 cm (Tindall et al. 2010).

Different spraying reagents have to be used to stain specific characteristic functional groups of polar lipids including phosphates, alpha-glycols, sugars, free amino groups, quaternary nitrogen, and primary and secondary amines. For most of the stains, separate thin layer plates have to be prepared. Based on the staining reactions and Rf values, various polar lipids can be identified. As it is known so far, membranes of Bacteria contain phospholipids, glycolipids, phosphoglycolipids, aminophospholipids, amino-acid-derived lipids, capnines, sphingolipids (glyco- or phosphosphingolipids), and also hopanoids. For *archaea*, only phospholipids, glycolipids, and phosphoglycolipids are known as membrane substitutions.

All identified polar lipids should be assigned on the polar lipid profile stained with the unspecific staining reagent. If unknown lipids were determined, which were stained by specific reagents, they should be termed accordingly, for example, as "unidentified phospholipids" or "glycolipid."

A specific characteristic for some taxa of Bacteria are polar lipids with hydroxylated fatty acids which can cause changes of Rf values compared to the respective compounds without hydroxylated fatty acids (Cox and Wilkinson 1989; Kroppenstedt et al. 1990; Kroppenstedt and Goodfellow 1991).

Spots of polar lipid profiles are often referred to be single compounds if they are, for example, labeled as phosphatidylglycerol. But however, it has to be considered that dependent on the investigated organism, a variety of fatty acids can be bound to glycerin, and each of the fatty acid combinations results in a new compound. Therefore, spots should be more appropriately treated as being a class of compounds. In any one organism, the spot labeled phosphatidylglycerol may contain several different compounds with different fatty acid compositions.

#### Respiratory Lipoquinone Systems

Respiratory lipoquinones (isoprenoid quinones) are widely distributed in both anaerobic and aerobic organisms, within the *Bacteria* and *Archaea*, and play an important role in electron transport. Based on the structural composition, two major

classes can be divided, the naphthoquinones (menaquinones) and benzoquinones (including ubiquinones, rhodoquinones, and plastoquinones). A third class includes derivate of benzothiophene derivatives, which however seemed to be restricted to members of the order *Sulfolobales* (Tindall 2005). Ubiquinones are wildly distributed in bacteria and eukaryotes whereas plastoquinones occur in plants, algae, and cyanobacteria.

Isoprenoid quinones can be easily degraded and therefore samples before and during extraction should be handled very carefully. Biomass and extracts should be flushed with $N_2$ before they are stored at −20 °C to prevent oxidation, and dimmed light condition is recommended for extraction to avoid photo-oxidation. The analysis of quinones is performed by HPLC. Before analysis, ubiquinones and menaquinones are often separated by thin layer chromatography (TCL) and purified from respective TLC plates.

Ubiquinones and menaquinones vary in the length of the isoprenyl units in polyprenyl side changes, which are denoted with Q-n or MK-n, with n representing the number of isoprenyl units. The side-chain lengths recorded to date is in the range of 5–15 isoprenoids units. The isoprenoid side chains of menaquinones can be stereospecific hydrogenated (saturated), which is marked by the appreciation $H_n$ with n representing the number of hydrogen in the isoprenoid side chain. Menaquinones then are assigned as MK-n($H_n$). (For more details, see da Costa et al. 2011.)

The occurrence of ubiquinones seems to be restricted to the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Betaproteobacteria* (Tindall 2005). If those are reported for other taxa, the results should be treated with caution. The most abundant ubiquinone in *Alphaproteobacteria* is Q-10, but taxa with Q-8 were also determined. The variety of ubiquinone in *Gammaproteobacteria* is higher, and characteristic ubiquinones are Q-7 to Q-14, whereas Q-10 was not determined as a single predominant ubiquinone in this class. For example *Legionella* spp. typically synthesize more than one predominant ubiquinone, with chain lengths between Q-10 and Q-14 (Collins and Gilbart 1983; Karr et al. 1982). Menaquinones and rhodoquinone are also produced by some taxa of the *Alphaproteobacteria* and *Gammaproteobacteria*. The relative amount of those lipoquinones found in the cells depends on the used growth substrates and the use of oxygen as a terminal electron acceptor.

For some methanotrophic bacteria, ubiquinones with modified side chains were determined (Collins 1994); in the majority of Bacteria and Archaea lineages, derivates of menaquinone were found, including demethyl-menaquinones, monomethylmenaquinones, dimethylmenaquinones, and menathioquinones (Collins 1985; Collins 1994; Tindall 2005).

Isoprenoid side chains in high-GC Gram-positive bacteria show different patterns of hydrogenation of menaquinones, whereas it is characteristic for members of the *Deltaproteobacteria* or *Halobacteria* that only one point of the side changes are saturated (Collins 1994).

## Cytoplasmatic Compounds

### Polyamines

Polyamines have been detected in a wide range of concentrations in most prokaryotes.

They are small molecular weight compounds located in the cytoplasm with a broad range of functions including the stabilization of DNA, involvement in gene expression, and intracellular compensation of extracellular changes in osmotic conditions (Feuerstein et al. 1991; Eraso and Kaplan 2009). Certain polyamines are also known to be covalently linked to the peptidoglycan of members of the *Sporomusa-Pectinatus-Selenomonas* evolutionary group (Hirao et al. 2000; Kamio et al. 1981a, b; Kamio and Nakamura 1987). Polyamine pattern can be used to support to differentiate and define taxonomic groupings (Busse and Auling 1988; Hamana and Matsuzaki 1992; Altenburger et al. 1997; Busse and Schumann 1999; Busse 2011). The most commonly detected polyamines are 1,3-diaminopropane, putrescine, 2-hydroxyputrescine, cadervarine, sym-norspermidine, spermidine, sym-homospermidine, and spermine.

The analysis of polyamines should be performed from cells of a standardized physiological age; it is recommended to harvest the cells at the late exponential growth phase because a high polyamine content is obtained in cells of this growth phase (Busse and Auling 1988). The analysis of polyamines is carried out by gradient HPLC with fluorescence detection after polyamine extraction by acidic hydrolysis and derivatization with dansylchloride (Scherer and Kneifel 1983). The content of polyamines is commonly given in µmol per g dry or wet weight of cell biomass. For classification, the identification of the predominant polyamine is the most important criteria (Busse 2011). The presence or absence of some minor compounds however can also be very important, for example, for the differentiation of species of closely related genera .

## Other Features of Taxonomic Value

It should be remembered that prokaryotes are chemically diverse, and the presence of compounds such as teichoic and teichuronic acids (Fischer 1988; Neuhaus and Baddiley 2003; Hancock 1994), arabinogalactans (Brennan 1988), and other heteropolysaccharides (Hancock 1994; König 1994; Kandler and Hippe 1977; Schleifer et al. 1982) or the substitution of sphingoglycolipids for lipopolysaccharides in members of the family *Sphingomonadaceae* all contribute to differentiating various taxonomic groups (see, e.g., Lechevalier and Lechevalier 1970) within the prokaryotes (members of the Bacteria and Archaea) and that this information is encoded somewhere on the genome. Rahman et al. (2009a, b) have provided such an overview of the distribution of teichoic and lipoteichoic acids in actinomycetes, linking that information to the underlying genetic information encoding critical steps in their biosynthesis.

## Conclusion

Moore et al. (2010) has pointed out recently that taxonomy serves many purposes. It should be stable and predictable. A most comprehensive phenotypic and genotypic characterization (in the framework of a polyphasic taxonomic approach) is still necessary in characterization and classification (which is a prerequisite of identification). As already pointed out by Wayne et al. (1987), an ideal taxonomy would involve one system (a hierarchical system), and in microbiology, the ultimate ambition would be to establish a system that mirrors the taxonomic relationships as an "order in nature," which is now most often associated with "evolutionary order" back to the origin of life. The circumscription of the taxonomic rank "species" provided by Stackebrandt et al. (2002) was given as an open and workable "definition" allowing the integration of new information and new developments (which can be expected in the era of "omics"): "A species is a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions." The introduction of quality standards is highly desirable. At present, both traditional and also novel phenotypic approaches are important for the characterization of novel species and genera. The development of new methods and the improvement of existing methods for phenotypic characterization should be encouraged. Genomic (and other omic) approaches will provide a rich source of data that should be carefully investigated in regard to the information behind. It is hoped that we may be able to provide a better picture of the vast biodiversity of the prokaryotic world based on a taxonomic scheme that reflects nature (Kämpfer 2012).

## References


Achtman M, Wagner M (2008) Microbial diversity and the genetic nature of microbial species. Nat Rev Microbiol 6:431–440

Altenburger P, Kämpfer P, Akimov VN, Lubitz W, Busse H-J (1997) Polyamine distribution in actinomycetes with Group B peptidoglycan and species of the genera *Brevibacterium*, *Corynebacterium*, and *Tsukamurella*. Int J Syst Bacteriol 47:270–277

Amann RI, Lin C, Key R, Montgomery L, Stahl DA (1992) Diversity among *Fibrobacter* isolates: towards a phylogenetic and habitat-based classification. Syst Appl Microbiol 15:23–31

Anderson R (1983) Alkylamines: novel lipid constituents in Deinococcus radiodurans. Biochim Biophys Acta 753:266–268

Arahal DR, Vreeland RH, Litchfield CD, Mormile MR, Tindall BJ, Oren A, Bejar V, Quesada V, Ventosa A (2007) Recommended minimal standards for describing new taxa of the family *Halomonadaceae*. Int J Syst Evol Microbiol 57:2436–2446

Austin B, Colwell RR (1977) Evaluation of some coefficients for use in numerical taxonomy of microorganisms. Int J Syst Bacteriol 27:204–210

Bartual SG, Seifert H, Hippler C, Luzon MA, Wisplinghoff H, Rodriguez-Valera F (2005) Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. J Clin Microbiol 43:4382–4390

Beers RJ, Lockhard WR (1962) Experimental methods in computer taxonomy. J Gen Microbiol 28:633–640

Bergey DH, Harrison FC, Breed RS, Hammer BW, Hantoon FM (eds) (1923) Bergey's manual of determinative bacteriology, 1st edn. Williams and Wilkins, Baltimore

Bernardet JF, Nakagawa Y, Holmes B (2002) Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. Int J Syst Bacteriol 52:1049–1070

Breed RS, Murray EGD, Smith NR (eds) (1957) Bergey's manual of determinative bacteriology, 7th edn. Williams & Wilkins, Baltimore

Brennan PJ (1988) *Mycobacterium* and other actinomycetes. In: Ratledge C, Wilkinson SG (eds) Microbial lipids, vol 1. Academic, London, pp 203–298

Brenner DJ (1973) Deoxyribonucleic acid reassociation in the taxonomy of enteric bacteria. Int J Syst Bacteriol 23:298–307

Brenner DJ, Martin MA, Hoyer BH (1967) Deoxyribonucleic acid homologies among some bacteria. J Bacteriol 94:486–487

Brenner DJ, Staley JT, Krieg NR (2001) Classification of prokaryotic organisms and the concept of bacterial speciation. In: Garrity GM (ed) Bergey's manual of systematic bacteriology, vol 1, 2nd edn. Springer, New York, pp 27–31

Brown DR, Whitcomb RF, Bradbury JM (2007) Revised minimal standards for description of new species of the class Mollicutes (division Tenericutes). Int J Syst Evol Microbiol 57:2703–2719

Buchanan RE, Gibbons NE (eds) (1974) Bergey's manual of determinative bacteriology, 8th edn. Williams & Wilkins, Baltimore

Busse H-J (2011) Polyamines. In: Rainey F, Oren A (eds) Taxonomy of prokaryotes, vol 38, Methods in microbiology. Elsevier, Amsterdam, pp 15–60

Busse H-J, Auling G (1988) Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. Syst Appl Microbiol 11:1–8

Busse H-J, Schumann P (1999) Polyamine profiles within genera of the class *Actinobacteria* with LL-diaminopimelic acid in the peptidoglycan. Int J Syst Bacteriol 49:179–184

Christensen H, Bisgaard M, Frederiksen W, Mutters R, Kuhnert P, Olsen JE (2001) Is characterization of a single isolate sufficient for valid publication of a new genus or species? Proposal to modify recommendation 30b of the bacteriological code (1990 revision). Int J Syst Evol Microbiol 51:2221–2225

Christensen H, Kuhnert P, Busse H-J, Frederiksen WC, Bisgaard M (2007) Proposed minimal standards for the description of genera, species and subspecies of the *Pasteurellaceae*. Int J Syst Evol Microbiol 57:166–178

Chun J, Lee J-H, Jung Y, Kim M, Kim S, Kim BK, Lim Y-W (2007) EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int J Syst Evol Microbiol 5:2259–2261

Cohn F (1875) Untersuchungen über Bacterien II. Beitr Biol Pflanz 1(3):141–208

Cole JR, Konstandinidis K, Farris RJ, Tiedje JM (2010) Microbial diversity and phylogeny: extending from rRNAs to genomes. In: Liu W-T, Jackson JK (eds) Environmental molecular microbiology. Caister Academic Press, Norfolk, pp 1–19

Collins MD (1985) Isoprenoid quinone analyses in bacterial classification and identification. In: Goodfellow M, Minnikin DE (eds) Chemical methods in bacterial systematics, vol 20, SAB technical series. Academic, London, pp 267–287

Collins MD (1994) Isoprenoid quinones. In: Goodfellow M, O'Donnell AG (eds) Chemical methods in prokaryotic systematics. Wiley, Chichester, pp 345–401

Collins MD, Gilbart J (1983) New members of the coenzyme Q series from the Legionellaceae. FEMS Microbiol Lett 16:251–255

Collins MD, Rodrigues U, Ash C, Aguirre M, Farrow JAE, Martinez-Murcia A, Phillips BA, Williams AM, Wallbanks S (1991) Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol Lett 77:5–12

Colwell RR (1970) Polyphasic taxonomy of bacteria. In: Iizuka H, Hasegawa T (eds) Culture collections of microorganisms. University of Tokyo Press, Tokyo, pp 421–436

Cowan ST (1968) A dictionary of microbial taxonomic usage. Oliver & Boyd, Edinburgh

Cowan ST (1978) Hill LR (ed) Dictionary of microbial taxonomy. Cambridge University Press, Cambridge

Cox AD, Wilkinson SG (1989) Polar lipids and fatty acids of *Pseudomonas cepacia*. Biochim Biophys Acta 1001:60–67

Da Costa MS, Albuquerque L, Nobre MF, Wait R (2011) The extraction and identification of respiratory lipoquinones of prokaryotes and their sue in taxonomy. In: Rainey F, Oren A (eds) Taxonomy of prokaryotes, vol 38, Methods in microbiology. Elsevier, Amsterdam, pp 197–206

Dagan T, Artzy-Randrup Y, Martin W (2008) Modular networks and cumulative impact of lateral transfer in prokaryote genome evolution. Proc Natl Acad Sci USA 105:10039–10044

De Ley J, Park IW, Tijtgat R, van Ermengem J (1966) DNA homology and taxonomy of *Pseudomonas* and *Xanthomonas*. Microbiology 42:43–56

De Vos P, Trüper HG (2000) Judicial Commission on the International Committee of Systematic Bacteriology. IXth International (IUMS) congress of bacteriology and applied microbiology. Minutes of the meetings, 14, 15, and 18 August 1999, Sydney, Australia. Int J Syst Evol Microbiol 50:2239–2244

De Vos P, Trüper HG, Tindall BJ (2005) Judicial Commission of the International Committee on Systematics of Prokaryotes. Xth international (IUMS) congress of bacteriology and applied microbiology. Minutes of the Meetings. Int J Syst Evol Microbiol 55:525–532

Deloger M, El Karoui M, Petit M-A (2009) A genomic distance based on MUM indicates discontinuity between most bacterial species and genera. J Bacteriol 191:91–99

Dewhirst FE, Fox JG, On SLW (2000) Recommended minimal standards for describing new species of the genus Helicobacter. Int J Syst Evol Microbiol 50:2231–2237

Diancourt L, Passet V, Nemec A, Dijkshoorn L, Brisse S (2010) The population structure of *Acinetobacter baumannii*: expanding multiresistant clones from an ancestral susceptible genetic pool. PLoS One 5:e10034

Dobson G, Minnikin DE, Minnikin SM, Parlett JH, Goodfellow M (1985) Systematic analysis of complex mycobacterial lipids. In: Goodfellow M, Minnikin DE (eds) Chemical methods in bacterial systematics, vol 20, SAB technical series. Academic, London, pp 237–266

Edwards JG (1978) Computer-assisted identification of unknown. In: Sharpe AN, Clark DS (eds) Mechanizing microbiology. Charles C. Thomas, Springfield, pp 280–292

Eraso JM, Kaplan S (2009) Regulation of gene expression by PrrA in *Rhodobacter sphaeroides* 2.4.1: role of polyamines and DNA topology. J Bacteriol 191(13):4341–4352

Faller AM, Schleifer KH (1981) Effect of growth phase and oxygen supply on cytochrome composition and morphology of *Arthrobacter crystallopoietes*. Curr Microbiol 6:253–258

Faller AM, Götz F, Schleifer KH (1980) Cytochrome patterns of staphylococci and micrococci and their taxonomic implications Zentralbl. Bakteriol Mikrobiol Hyg Abt I Orig C1:26–39

Felis GE, Dellaglio F (2007) On species descriptions based on a single strain: proposal to introduce the status species proponenda (sp. pr.). Int J Syst Evol Microbiol 57:2185–2187

Feuerstein BG, Williams LD, Basu HS, Marton LJ (1991) Implications and concepts of polyamine-nucleic acid interactions. J Cell Biochem 46(1):37–47

Fischer W (1988) Physiology of lipoteichoic acids in bacteria. Adv Microb Physiol 29:233–302

Fox GE, Pechman KR, Woese CR (1977) Comparative cataloging of 16S ribosomal ribonucleic acid: molecular approach to prokaryotic systematics. Int J Syst Bacteriol 27:44–57

Fox GE, Wisotzkey JD, Jurtshuk P Jr (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int J Syst Bacteriol 42:166–170

Freney J, Kloos WE, Hajek V, Webster JA, Bes M, Brun Y, Vernozy-Rozand C (1999) Recommended minimal standards for description of new staphylococcal species. Int J Syst Bacteriol 49:489–502

Fujita Y, Naka N, Doi T, Yano I (2005a) Direct molecular mass determination of trehalose monomycolate from 11 species of mycobacteria by MALDI-TOF mass spectrometry. Microbiology 151:1443–1452

Fujita Y, Naka N, McNeil MR, Yano I (2005b) Intact molecular characterization of cord factor (trehalose 6,6′-dimycolate) from nine species of mycobacteria by MALDI-TOF mass spectrometry. Microbiology 151: 3403–3416

Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ et al (2005) Re-evaluating prokaryotic species. Nat Rev Microbiol 3:733–739

Godchaux W, Leadbetter ER (1984) Sulfonolipids of gliding bacteria. Structure of the *N*-acylaminosulfonates. J Biol Chem 259:2982–2990

Goodfellow M (1977) Numerical taxonomy. In: Laskin AI, Lechevalier HA (eds) CRC handbook of microbiology, vol 1, 2nd edn. CRC Press, Cleveland, pp 579–596

Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM (2007) DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57:81–91

Gram C (1884) Ueber die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpärparaten. Forschitte Med 2:185–189

Grimont PAD, Popoff MY, Grimont F, Coynault C, Lemelin M (1980) Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. Curr Microbiol 4:325–330

Gyllenberg HG (1965) A model for computer identification of microorganisms. J Gen Microbiol 39:401–405

Hamana K, Matsuzaki S (1992) Polyamines as a chemotaxonomic marker in bacterial systematics. Crit Rev Microbiol 18:261–283

Hancock IC (1994) Analysis of cell wall constituents of Gram-positive bacteria. In: Goodfellow M, O'Donnell AG (eds) Chemical methods in prokaryotic systematics. Wiley, Chichester, pp 63–84

Hase S, Rietschel ET (1976) Isolation and analysis of the lipid A backbone. Lipid A structure of lipopolysaccharides from various bacterial groups. Eur J Biochem 63:101–107

Helander IM, Haikara A (1995) Cellular fatty acyl and alkenyl residues in *Megasphaera* and *Pectinatus* species: contrasting profiles and detection of beer spoilage. Microbiol 141:1131–1137

Hill LR (1974) Theoretical aspects of numerical identification. Int J Syst Bacteriol 24:494–499

Hirao T, Sato M, Shirahata AY, Kamio Y (2000) Covalent linkage of polyamines to peptidoglycan in *Anaerovibrio lipolytica*. J Bacteriol 182:1154–1157

Holmes B, Hill LR (1985) Computers in diagnostic bacteriology, including identification. In: Goodfellow M, Jones D, Priest FG (eds) Computer assisted bacterial systematics. Academic, London, pp 265–287

International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of Mycoplasmatales (1972) Proposal for minimal standards for descriptions of new species of the order *Mycoplasmatales*. Int J Syst Bacteriol 22:184–188

Johnson JL (1973) Use of nucleic-acid homologies in the taxonomy of anaerobic bacteria. Int J Syst Bacteriol 23:308–315

Johnson JL (1984) Nucleic acids in bacterial classification. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 1. Williams & Wilkins, Baltimore, pp 8–11

Johnson JL, Ordal EJ (1968) Deoxyribonucleic acid homology in bacterial taxonomy. The effect of incubation temperature and reaction specificity. J Bacteriol 95:893–900

Jolley KA, Maiden MC (2010) BIGSdb: scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 11:595

Jolley KA, Bliss CM, Bennett JS, Bratcher HB, Brehony C, Colles FM et al (2012) Ribosomal multilocus sequence typing: universal characterization of bacteria from domain to strain. Microbiology 158:1005–1015

Jones CW, Poole RK (1985) The analysis of cytochromes. In: Gottschalk G (ed) Methods in microbiology, vol 18. Academic, London, pp 285–328

Kamio Y, Nakamura K (1987) Putrescine and cadaverine are constituents of peptidoglycan in *Veillonella alcalescens* and *Veillonella parvula*. J Bacteriol 169:2881–2884

Kamio Y, Itoh Y, Terawaki Y (1981a) Chemical structure of peptidoglycan in *Selenomonas ruminantium*: cadaverine links covalently to the D-glutamic acid residue of peptidoglycan. J Bacteriol 146:49–53

Kamio Y, Itoh Y, Terawaki Y, Kusano T (1981b) Cadaverine is covalently linked to peptidoglycan in *Selenomonas ruminantium*. J Bacteriol 145:122–128

Kämpfer P (2010) The importance of phenotype for bacterial systematics. Bull BISMiS 1(1):7–15

Kämpfer P (2012) Systematics of prokaryotes: the state of the art. Antonie Van Leeuwenhoek 101(1):3–11

Kämpfer P, Glaeser SP (2012) Prokaryotic taxonomy in the sequencing era—the polyphasic approach revisited. Environ Microbiol 14:291–317

Kandler O, Hippe H (1977) Lack of peptidoglycan in the cell walls of *Methanosarcina barkeri*. Arch Microbiol 113:57–60

Kaneko T (1979) Correlative similarity coefficient: new criterion for forming dendrograms. Int J Syst Bacteriol 29:188–193

Karr DE, Bibb WF, Moss CW (1982) Isoprenoid quinones of the genus *Legionella*. J Clin Microbiol 15:1044–1048

Kellogg ST (1979) MICRID: a computer-assisted microbial identification system. Appl Environ Microbiol 38:559–563

Kim O-S, Cho Y-J, Lee K, Yoon S-H, Kim M, Na H et al (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716–721

Klenk H-P, Göker M (2010) En route to a genome-based classification of archaea and bacteria? Syst Appl Microbiol 33(4):175–182

Koeppel A, Perry EB, Sikorski J, Krizanc D, Warner A, Ward DM et al (2008) Identifying the fundamental units of bacterial diversity: a paradigm shift to incorporate ecology into bacterial systematics. Proc Natl Acad Sci USA 105:2504–2509

König H (1994) Analysis of archaeal cell envelopes. In: Goodfellow M, O'Donnell AG (eds) Chemical methods in prokaryotic systematics. Wiley, Chichester, pp 85–119

König H, Kralik R, Kandler O (1982) Structure and modifications of pseudomurein in Methanobacteriales. Zbl Bakt Hyg I Abt Orig C 3:179–191

König H, Kandler O, Jensen M, Rietschel ET (1983) The primary structure of the glycan moiety of the pseudomurein from *Methanobacterium thermoautotrophicum*. Hoppe-Seyler's Z Physiol Chem 364:627–636

Konstantinidis KT, Tiedje JM (2005) Genomic insights into the species definition for prokaryotes. Proc Natl Acad Sci USA 102:2567–2572

Konstantinidis KT, Ramette A, Tiedje JM (2006) The bacterial species definition in the genomic era. Philos Trans R Soc Lond B Biol Sci 361:1929–1940

Krieg NR, Padgett PJ (2011) Phenotypic and physiological characterization methods. In: Rainey F, Oren A (eds) Taxonomy of prokaryotes, vol 38, Methods in microbiology. Elsevier, Amsterdam, pp 15–60

Kroppenstedt RM, Goodfellow M (1991) The family *Thermomonosporaceae*. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) The prokaryotes, 2nd edn. Springer, New York, pp 1085–1114

Kroppenstedt RM, Stackebrandt E, Goodfellow M (1990) Taxonomic revision of the actinomycete genera *Actinomadura* and *Microtetraspora*. Syst Appl Microbiol 13:148–160

Labeda DP (2000) International Committee on Systematic Bacteriology. IXth international (IUMS) congress of bacteriology and applied microbiology. Minutes of the meetings, 14 an 17 August 1999, Sydney, Australia. Int J Syst Evol Microbiol 50:2245–2247

Langworthy TA, Holzer G, Zeikus JG, Tornabene TG (1983) Iso- and anteiso-branched glycerol diethers of the thermophilic anaerobes *Thermodesulfobacterium commune*. Syst Appl Microbiol 4:1–17

Lapage SP, Bascomb B, Willcox WR, Curtis MA (1970) Computer identification of bacteria. In: Baillie A, Gilbert RJ (eds) Automation, mechanization, and data handling in microbiology. Academic, London, pp 1–22

Lapage SP, Bascomb B, Willcox WR, Curtis MA (1973) Identification of bacteria by computer: general aspects and perspectives. J Gen Microbiol 77:273–299

Lapage SP, Sneath PHA, Lessel EF, Skerman VBD, Seeliger HPR, Clark WA (1992) International code of nomenclature of bacteria (1990 revision). American Society for Microbiology, Washington, DC

Lechevalier MP, Lechevalier H (1970) Chemical composition as a criterion in the classification of aerobic actinomycetes. Int J Syst Bacteriol 20:435–443

Lévy-Frébault VV, Portaels F (1992) Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species. Int J Syst Bacteriol 42:315–323

Lienau EK, DeSalle R (2009) Evidence, content and corroboration and the tree of life. Acta Biotheor 57:187–199

Logan NA, Berge O, Bishop AH, Busse H-J, De Vos P, Fritze D, Heyndrickx M, Kämpfer P, Rabinovitch L, Salkinoja-Salonen MS, Seldin L, Ventosa A (2009) Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. Int J Syst Evol Microbiol 59:2114–2121

Ludwig W (2010) Molecular phylogeny of microorganisms: is rRNA still a useful marker? In: Oren A, Papke RT (eds) Molecular phylogeny of microorganisms. Caister Academic Press, Norfolk

Ludwig W, Klenk H-P (2001) Overview: a phylogenetic backbone and taxonomic framework of prokaryotes. In: Garrity GM (ed) Bergey's manual of systematic bacteriology, 2nd edn. Springer, New York, pp 49–65

Maiden MC, Bygraves JA, Feil E, Morelli G, Russel JE, Urwin R et al (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA 95:3140–3145

Martinez-Murcia AJ, Collins MD (1990) A phylogenetic analysis of the genus *Leuconostoc* based on reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol Lett 70:73–84

Martinez-Murcia AJ, Benlloch S, Collins MD (1992) Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA-DNA hybridisations. Int J Syst Bacteriol 42:412–421

McCarthy BJ, Bolton ET (1963) An approach to the measurement of genetic relatedness among organisms. Proc Natl Acad Sci USA 50:156–164

Miller LT (1982) A single derivatization method for bacterial fatty acid methyl esters including hydroxy acids. J Clin Microbiol 16:584–586

Moore ERB, Rosselló-Móra R (2011) MALDI-TOF MS: a return to phenotyping in microbial identification? Syst Appl Microbiol 34:1

Moore LVH, Bourne DM, Moore WEC (1994) Comparative distribution and taxonomic value of cellular fatty acids in thirty-three genera of anaerobic Gram-negative bacilli. Int J Syst Bacteriol 44:338–347

Moore ERB, Mihaylova SA, Vandamme P, Krichevsky MI, Dijkshoorn L (2010) Microbial systematics and taxonomy: relevance for a microbial commons. Res Microbiol 161:430–438

Müller K-D, Schmid EN, Kroppenstedt RM (1998) Improved identification of mycobacteria by using the microbial identification system in combination with additional trimethylsulfonium hydroxide pyrolysis. J Clin Microbiol 36:2477–2480

Murray RGE, Schleifer KH (1994) Taxonomic notes: a proposal for recording the properties of putative taxa of prokaryotes. Int J Syst Bacteriol 44:174–176

Murray RGE, Stackebrandt E (1995) Taxonomic note: implementation of the provisional status Candidatus for incompletely described prokaryotes. Int J Syst Bacteriol 45:186–187

Murray RGE, Brenner DJ, Colwell RR, De Vos P, Goodfellow M, Grimont PAD, Pfennig N, Stackebrandt E, Zavarzin GA (1990) Report of the ad hoc committee on approaches to taxonomy within the Proteobacteria. Int J Syst Bacteriol 40:213–215

Naka T, Fujiwaraa N, Yanoc I, Maedaa S, Doed M, Minaminob M, Ikedab N, Katob Y, Watabee K, Kumazawaf Y, Tomiyasug I, Kobayashia K (2003) Structural analysis of sphingophospholipids derived from *Sphingobacterium spiritivorum*, the type species of genus *Sphingobacterium*. Biochim Biophys Acta 1635:83–92

Neuhaus FC, Baddiley J (2003) A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in Gram-positive bacteria. Microbiol Mol Biol Rev 67:686–723

Oelze J (1985) Analysis of bacteriochlorophylls. In: Gottschalk G (ed) Methods in microbiology, vol 18. Academic, London, pp 257–284

Oren A, Ventosa A, Grant WD (1997) Proposed minimal standards for description of new taxa in the order Halobacteriales. Int J Syst Evol Microbiol 47:233–238

Ourisson G, Rohmer M, Poralla K (1987) Prokaryotic hopanoides and other polyterpenoidsterol surrogates. Annu Rev Microbiol 41:301–333

Pace B, Campbell LL (1971) Homology of ribosomal ribonucleic acid of diverse bacterial species with *Escherichia coli* and *Bacillus stearothermophilus*. J Bacteriol 107:543–547

Palleroni NJ, Kunisawa R, Contopoulou R, Doudoroff M (1973) Nucleic acid homologies in the genus *Pseudomonas*. Int J Syst Bacteriol 23:333–339

Peplies J, Kottmann R, Ludwig W, Glöckner F-O (2008) A standard operating procedure for phylogenetic inference (SOPPI) using (rRNA) marker genes. Syst Appl Microbiol 31:251–257

Pfennig N, Wagener S (1986) An improved method of preparing wet mounts for photomicrographs of microorganisms. J Microbiol Methods 4:303–306

Rahman O, Dover LG, Sutcliffe IC (2009a) Lipoteichoic acid biosynthesis: two steps forwards, one step sideways? Trends Microbiol 17:219–225

Rahman O, Pfitzenmaier M, Pester O, Morath S, Cummings SP, Hartung T, Sutcliffe IC (2009b) Macroamphiphilic components of thermophilic actinomycetes: identification of lipoteichoic acid in *Thermobifida fusca*. J Bacteriol 191:152–160

Rainey FA, Klatte S, Kroppenstedt RM, Stackebrandt E (1995) Dietzia, new genus including *Dietzia maris* comb. nov., formerly *Rhodococcus maris*. Int J Syst Bacteriol 45:32–36

Rainey FA, Oren A (2012) Taxonomy of prokaryotes – introduction. Methods Microbiol 38:1–5

Ratledge C, Wilkinson SG (1988) Microbial lipids, vol 1. Academic, London

Rhodes ME (1965) Flagellation as a criterion for the classification of bacteria. Bacteriol Rev 29:442–465

Richter M, Rosselló-Móra R (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci USA 106:19126–19131

Rosselló-Móra R (2006) DNA-DNA reassociation methods applied to microbial taxonomy and their critical evaluation. In: Stackebrandt E (ed) Molecular identification, systematics, and population structure of prokaryotes. Springer, Heidelberg/Berlin, pp 23–50

Rosselló-Móra R (2012) Towards a taxonomy of bacteria and archaea based on interactive and cumulative data repositories. Environ Microbiol 14:318–334

Rosselló-Mora R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25:39–67

Rütters H, Sass H, Cypionka H, Rullkötter J (2001) Monalkylether phospholipids in the sulfate-reducing bacteria *Desulfosarcina vaiabilis* and *Desulforhabdus aminigenus*. Arch Microbiol 176:435–442

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Scherer P, Kneifel H (1983) Distribution of polyamines in methanogenic bacteria. J Bacteriol 154:1315–1322

Schindler J, Duben J, Lysenko O (1979) Computer-aided numerical identification of Gram-negative fermentative rods on a desk-top computer. J Appl Bacteriol 47:45–51

Schleifer K-H (2009) Classification of bacteria and archaea: past, present and future. Syst Appl Microbiol 32:533–542

Schleifer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36:407–477

Schleifer KH, Ludwig W (1989) Phylogenetic relationships among bacteria. In: Fernholm B, Bremer K, Jörnvall H (eds) The hierarchy of life. Elsevier, Amsterdam, pp 103–117

Schleifer KH, Hammes WP, Kandler O (1976) Effect of endogenous and exogenous factors on the primary structures of bacterial peptidoglycan. Adv Microb Physiol 13:245–292

Schleifer K-H, Steber J, Mayer H (1982) Chemical composition and structure of the cell wall of *Halococcus morrhuae*. Zbl Bakt Hyg I Abt Orig C 3:171–178

Schumann P, Kämpfer P, Busse H-J, Evtushenko LI, For the Subcommittee on the Taxonomy of the suborder Micrococcineae of the International Committee on Systematics of Prokaryotes (2009) Proposed minimal standards for describing new genera and species of the suborder Micrococcineae. Int J Syst Bacteriol 59:1823–1849

Shively JM (1974) Inclusion bodies of prokaryotes. Annu Rev Microbiol 28:167–187

Simpson GG (1961) Principles of animal taxonomy. Columbia University Press, New York

Skerman VBD (1967) A guide to the identification of the genera of bacteria, 2nd edn. Williams & Wilkins, Baltimore

Skerman VBD, McGowan V, Sneath PHA (1980) Approved lists of bacterial names. Int J Syst Bacteriol 30:225–420

Skerman VDB, McGowan V, Sneath PHA (1989) The approved lists of bacterial names. Int J Syst Bacteriol 30:225–420

Sneath PHA (1971) Theoretical aspects of microbiological taxonomy. In: Pérez-Miravete A, Peláez D (eds) Recent advances in microbiology. Asociacióon Mexicana de Microbiología, Mexico City, pp 581–586

Sneath PHA (1972) Computer taxonomy. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 7A. Academic, London, pp 29–98

Sneath PHA (1977) The maintenance of large numbers of strains of microorganisms, and the implications for culture collections. FEMS Microbiol Lett 1:333–334

Sneath PHA (1979a) BASIC program for a significance test for two clusters in Euclidian space as measured by their overlap. Comput Geosci 5:143–155

Sneath PHA (1979b) BASIC program for identification of an unknown with presence-absence data against an identification matrix of percent positive characters. Comput Geosci 5:195–213

Sneath PHA (1979c) BASIC program for character separation indices from an identification matrix of percent positive characters. Comput Geosci 5:349–357

Sneath PHA (1980a) BASIC program for the most diagnostic properties of groups from an identification matrix of percent positive characters. Comput Geosci 6:21–26

Sneath PHA (1980b) BASIC program for determining the best identification scores possible for the most typical example when compared with an identification matrix of percent positive characters. Comput Geosci 6:27–34

Sneath PHA (1980c) BASIC program for determining overlap between groups in an identification matrix of percent positive characters. Comput Geosci 6:267–278

Sneath PHA (1989) Analysis and interpretation of sequence data for bacterial systematics—the view of a numerical taxonomist. Syst Appl Microbiol 12:15–31

Sneath PHA (2001) Bacterial nomenclature. In: Garrity GM (ed) Bergey's manual of systematic bacteriology, vol 1, 2nd edn. Springer, New York, pp 83–88

Sneath PHA (2005) The preparation of the approved lists of bacterial names. Int J Syst Evol Microbiol 55:2247–2249

Sneath PHA, Sokal RR (1973) Numerical taxonomy, the principles and practice of numerical classification. Freeman, San Francisco

Sokal RR, Sneath PHA (1963) Numerical taxonomy. Freeman, San Francisco

Soria-Carrasco V, Valens-Vadell M, Peña A, Antón J, Amann R, Castresana J, Rosselló-Móra R (2007) Phylogenetic position of *Salinibacter ruber* based on concatenated protein alignments. Syst Appl Microbiol 30:171–179

Stackebrandt E, Ebers J (2006) Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 8:6–9

Stackebrandt E, Goebel BM (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, Woese CR (1981) Towards a phylogeny of the *Actinomycetes* and related organism. Curr Microbiol 5:197–202

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PAD, Kämpfer P, Maiden MCJ et al (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

Staehelin LA, Golecki JR, Fuller RC, Drews G (1978) Visualization of the supramolecular architecture of chlorosomes (chlorobium type vesicles) in freeze-fractured cells of *Chloroflexus aurantiacus*. Arch Microbiol 119:269–277

Staley JT, Krieg NR (1984) Classification of prokaryotic organisms: an overview. In: Murray RG, Brenner DJ, Bryant MP, Holt JG, Krieg NR, Moulder JW, Pfennig N, Sneath PHA, Staley JT (eds) Bergey's manual of systematic bacteriology, vol 1. Williams and Wilkins, Baltimore, pp 1–4

Stanier RY, Van Niel CB (1962) The concept of a bacterium. Arch Microbiol 42:7–35

Stanier RY, Ingraham JL, Wheelis ML, Paintes PR (1986) The microbial world, 5th edn. Prentice-Hall, Englewood Cliffs

Tindall BJ (1999) Misunderstanding the bacteriological code. Int J Syst Bacteriol 49:1313–1316

Tindall BJ (2005) Respiratory lipoquinones as biomarkers. In: Akkermans A, de Bruijn F, van Elsas D (eds) Molecular microbial ecology manual, 2nd edn. Kluwer, Dordrecht, Section 4.1.5, Supplement 1

Tindall BJ (2008) Confirmation of deposit, but confirmation of what? Int J Syst Evol Microbiol 58:1785–1787

Tindall BJ, Garrity GM (2008) Proposals to clarify how type strains are deposited and made available to the scientific community for the purpose of systematic research. Int J Syst Evol Microbiol 58:1987–1990

Tindall BJ, Kämpfer P, Euzéby JP, Oren A (2006) Valid publication of names of prokaryotes according to the rules of nomenclature: past history and current practice. Int J Syst Evol Microbiol 56:2715–2720

Tindall BJ, Sikorski J, Smibert RM, Krieg NR (2007) Phenotypic characterization and the principles of comparative systematics. In: Reddy CA, Beveridge TJ, Breznak JA, Marzluf G, Schmidt TM, Snyder LR (eds) Methods for general and molecular microbiology. ASM Press, Washington, DC, pp 330–393

Tindall BJ, Rosselló-Mora R, Busse H-J, Ludwig W, Kämpfer P (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. Int J Syst Evol Microbiol 60:249–266

Torkko P, Katila M-L, Kontro M (2003) Gas-chromatographic lipid profiles in identification of currently known slowly growing environmental mycobacteria. J Med Microbiol 52:315–323

Tornabene TG (1985) Lipid analysis and the relationship to chemotaxonomy. In: Gottschalk G (ed) Methods in microbiology, vol 18. Academic, London, pp 209–234

Trüper HG, Euzéby JP (2009) International code of nomenclature of prokaryotes. Appendix 9: orthography. Int J Syst Evol Microbiol 59(Pt 8): 2107–2113

Trüper HG, Schleifer KH (2006) Prokaryote characterization and identification. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (eds) The prokaryotes: a handbook on the biology of bacteria, vol 1, 3rd edn. Springer, New York, pp 58–79

Ursing J, Rosselló-Mora RA, Garcia-Valdez E, Lalucat J (1995) Taxonomic note: a pragmatic approach to the nomenclature of phenotypically similar genomic groups. Int J Syst Bacteriol 45:604

Vandamme P, Pot B, Gillis M, De Vos P, Kersters K, Swings J (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev 60:407–438

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Trüper HG (1987) Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37:463–464

Weckesser J, Mayer H (1988) Different lipid A types in lipopolysaccharides of phototrophic and related non-phototrophic bacteria. FEMS Microbiol Rev 54:1431–1454

Welker M, Moore ER (2011) Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology. Syst Appl Microbiol 34:2–11

Willumsen P, Karlson U, Stackebrandt E, Kroppenstedt RM (2001) *Mycobacterium frederiksbergense* sp. nov., a novel polycyclic aromatic hydrocarbon-degrading *Mycobacterium* species. Int J Syst Evol Microbiol 51:1715–1722

Wisplinghoff H, Hippler C, Bartual SG, Haefs C, Stefanik D, Higgins PG, Seifert H (2008) Molecular epidemiology of clinical *Acinetobacter baumannii* and *Acinetobacter* genomic species 13TU isolates using a multilocus sequencing typing scheme. Clin Microbiol Infect 14:708–715

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271

Woese CR, Fox G (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci USA 74:5088–5090

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposla for the domains: archaea. Bacteria and Eucarya. Proc Natl Acad Sci USA 87:4576–4579

Wolf Y, Rogozin I, Grishin N, Tatusov R, Koonin E (2001) Genome trees constructed using five different approaches suggest new major bacterial clades. BMC Evol Biol 1:8

Yarza P, Richter M, Peplies J, Euzeby J, Amann R, Schleifer KH, Ludwig W, Glöckner FO, Rossello-Mora R (2008) The all-species living tree project: A 16S rRNA-based phylogenetic tree of all sequenced type strains. Syst Appl Microbiol 31:241–250

# 7 Principles of Enrichment, Isolation, Cultivation, and Preservation of Prokaryotes

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## Introduction

Currently, a total of 9,409 prokaryotic species are recognized (as of January 2012, validly published names not including homotypic and heterotypic synonyms, comb. nov. and *nomina nova*; DSMZ 2012; Euzéby 2012). By comparison, the number of small subunit ribosomal RNA (SSU rRNA) gene sequences deposited in public databases keeps increasing exponentially (Pruesse et al. 2007; Yarza et al. 2008) and surmounted the species numbers already some 15 years ago (Fig. 7.1). Meanwhile, a total of 2,492,653 sequences are available of which 2,282,670 are prokaryotic whereas only 33,842 originate from cultured strains (SILVA 2012). In line with these cumulative data, culture-independent analyses of DNA reassociation kinetics and of 16S rRNA gene sequences in individual environmental samples also indicate that prokaryotic diversity is poorly represented by the species cultivated so far. Thus, estimates of bacterial species numbers in just one type of soil reached values of up to 53,000 (Sandaa et al. 1999; Roesch et al. 2007). Furthermore, molecular investigations of 16S rRNA gene sequences in natural bacterial assemblages typically yielded many more sequence types than those recovered by cultivation-based approaches (Fuhrman et al. 1992; Ward et al. 1992; Barns et al. 1994; DeLong et al. 1994; Hiorns et al. 1997; Kuske et al. 1997; Ludwig et al. 1997; Suzuki et al. 1997b; Gich et al. 2001; Béjà et al. 2002; Roesch et al. 2007). In light of these findings, the earlier estimates of the fraction of already cultured bacterial species of 12–20% (Wayne et al. 1987; Bull et al. 1992) or even the commonly cited estimate of 1% appears to be far too optimistic. Based on recent estimates of total bacterial species numbers ($10^7$–$10^9$; Dykhuizen 1998; Curtis et al. 2002), the value more likely ranges between 0.1% and 0.001% and may be even lower (compare the higher estimates of bacterial species numbers in Sogin et al. 2006; Harwood and Buckley 2008).

Concomitantly to the explosive increase of 16S rRNA gene sequence numbers, the number of full genome sequences in Genbank has approached 2,000 (NCBI 2012). Together, complete and incomplete genome sequencing projects listed in the Genome Online Database (GOLD 2012) amount to 11,221, of which 80% are prokaryotic. The increase in new genome sequencing projects now has reached a pace (>2,000 year$^{-1}$) that clearly surpasses the current rate of new species descriptions (622 species year$^{-1}$, from 2005 on) (Fig. 7.1). This implies that the rate of cultivation of novel bacterial species has become insufficient to tap the currently available potential of genome sequencing facilities. Obviously, the future discovery of novel metabolic pathways, regulation mechanisms, novel metabolic capacities, but also novel antibiotics (Jensen et al. 2007) will largely depend on an improved access to novel types of prokaryotes.

In most cases, the 16S rRNA gene inventories of natural bacterial communities differ from the sequences of strains isolated from the same or similar samples (e.g., Hiorns et al. 1997; Suzuki et al. 1997; Hugenholtz et al. 1998; Bernard et al. 2000). Even a large cultivation campaign during which 659 bacterial isolates were obtained from grassland soil did not yield any strain with a 16S rRNA sequence corresponding to the dominant strains in the natural community (Felske et al. 1999). Entire bacterial phyla such as the *Acidobacteria* that are highly abundant in the soil environment or the OP10 phylum (now Armatimonadetes) have resisted cultivation for a long time. At the same time, culture-independent methods have shown that uncultured phylotypes are physiologically active in situ (Bernard et al. 2000) and that active cells can constitute a high percentage of total cell counts (Ouverney and Fuhrman 1999; Cottrell and Kirchman 2000; Janssen et al. 2002).

The not-yet-cultured prokaryotes may exhibit a different physiology that does not match established cultivation methods (e.g., ultraoligotrophic growth characteristics, see section "Low Nutrient Concentrations, "Oligotrophic Bacteria," and "Ultramicrobacteria""). Indeed, key reactions in biogeochemical cycles often appear to be mediated not by the frequently isolated prokaryotes but rather by other prokaryotes that are phylogenetically unrelated and that have escaped cultivation for a long time or until now remained uncultured. Examples include members of the ***Nitrosospira*** group which (instead of ***Nitrosomonas*** spp.) catalyze nitrification in at least some environments, most notably in wastewater treatment plants as the world's largest biotechnological process (Hastings et al. 1998; Schramm et al. 1999). Other examples are ***Achromatium*** spp. and coccoid magnetotactic bacteria participating in the sulfur cycle (Spring et al. 1993), archaea which mediate anaerobic methane consumption (Hinrichs et al. 1999), autotrophic planktonic marine Crenarchaeota that oxidize ammonia and exhibit an extraordinary high affinity to ammonia (Hallam et al. 2006; Martens-Habbena et al. 2009) and novel type II methanotrophic bacteria oxidizing methane at atmospheric concentrations (Holmes et al. 1999; Roslev and Iversen 1999). Some environments harbor whole groups of entirely unknown bacteria and archaea, which most likely are important for the biogeochemical transformations (Hugenholtz et al. 1998). These include for instance members of the green filamentous bacteria (Coolen et al. 2002) or freshwater actinobacteria (Glöckner et al. 2000) that appear to utilize *N*-acetyl-D-glucosamine as commensals of aquatic chitin degraders (Beier and Bertilsson 2011). In addition, culture-independent analyses of large genome fragments retrieved from natural samples have uncovered bacteria present in the natural environment that have a previously unrecognized physiology (Béjà et al. 2000). Only in a few instances could numerically important bacterial species also be isolated (Ferris et al. 1996; Kalmbach et al. 1997).

With the aid of modern molecular methods, some information on the physiological capabilities of "not-yet-cultured prokaryotes" can be obtained even down to the single-cell level (Ouverney and Fuhrman 2000; Wagner et al. 2006; Beier and Bertilsson 2011). Though culture-independent techniques have improved, they still provide only limited insights into the physiology of prokaryotes. Therefore, the role of not-yet-cultured prokaryotes in the environment cannot be fully appreciated until these microorganisms are available for detailed physiological and molecular studies. Until now, additional and at the same time innovative novel cultivation efforts often still are

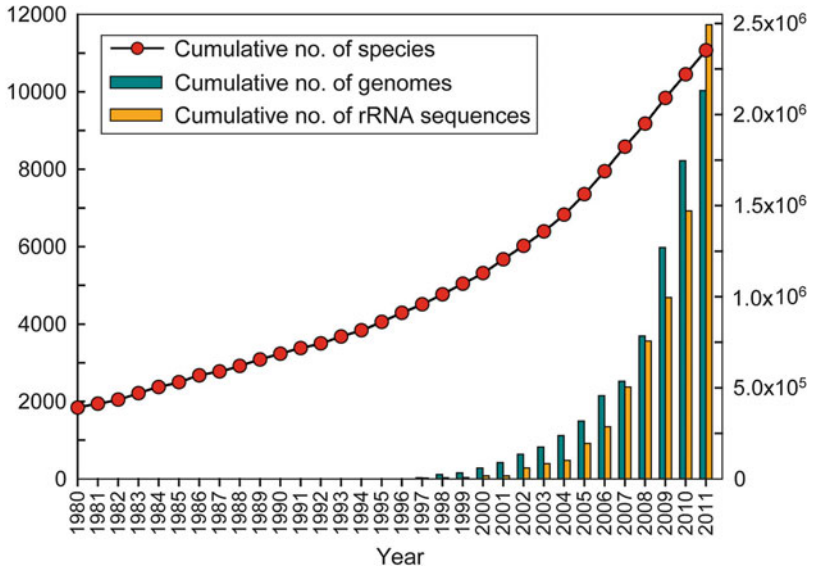


**Fig. 7.1**

**Increase in validly described species, numbers of small subunit ribosomal RNA sequences (SILVA 2012), and numbers of genome sequences (GOLD 2012) since the appearance of the approved list of bacterial names in 1980. This graph is an extended version of that in Overmann (2002b) and Yarza et al. (2008). Species data were obtained from Euzéby (2012) and the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ 2012), and include homotypic and heterotypic synonyms, comb. nov. and *nomina nova*. Ninety-two percent of the small subunit rRNA sequences are prokaryotic. Genome numbers include complete and incomplete sequencing projects; about 80% of the genomes are prokaryotic**

a precondition for gaining access to a large portion of microbial diversity. In a limited number of cases, abundant phylotypes (Rappe et al. 2002; Gich et al. 2005) and representatives of previously largely uncultured (sub)phyla such as *Acidobacteria* (Janssen et al. 2002; Koch et al. 2008; Eichorst et al. 2007), mesophilic Crenarchaea (Könneke et al. 2005), and members of the OP10 candidate phylum (Tamaki et al. 2011) could be isolated. Also, some symbiotic forms have become available, such as the symbionts of phototrophic consortia (Vogl et al. 2006).

Some of the isolates showed exceptionally high affinity toward growth substrates matching substrate concentrations in the natural environment (Button et al. 1998; Martens-Habbena et al. 2009). In most other cases, however, it has remained unclear which physiological traits prevented earlier isolation of these bacteria. In addition, the discrepancy between 16S rRNA gene inventories and cultivated phylotypes may simply be the result of the low number of cultivation attempts, since even conventional cultivation trials continue to yield novel phylotypes of bacteria (Pinhassi et al. 1997; Suzuki et al. 1997). The advent of 16S rRNA methodology in the mid-1990s permitted a significantly increased rate of discovery of novel species from a mere (144.0 ± 5.4) species year$^{-1}$ until the year 1994 to a meanwhile stable value of (621.9 ± 8.2) species year$^{-1}$ from the year 2005 on (*Fig. 7.1*), although the procedure for species descriptions has been streamlined and standardized considerably (Kämpfer et al. 2003).

Even at this increased rate, however, current cultivation efforts appear far too limited to adequately cover prokaryotic diversity because of systematic reasons. More importantly, a closer look at the cultivated fraction of prokaryotic diversity reveals that, to date, it is heavily biased toward Actinobacteria, Firmicutes, Alpha- and Gammaproteobacteria, and Bacteroidetes (Hugenholtz 2002; Handelsmann 2004) (*Fig. 7.2*). About 50% of the currently recognized bacterial phyla are still poorly covered by cultivated representatives (*Fig. 7.2*) (Schloss and Handelsman 2004). Furthermore, the majority of novel isolates is phylogenetically rather closely related to previously described species (i.e., >95% 16S rRNA gene sequence similarity; *Fig. 7.2*). Of the 472 novel species that were described in the *International Journal of Systematic and Evolutionary Microbiology*, only 19 exhibited a similarity of ≤90.4% in 16S rRNA gene sequence to the closest cultivated relative (*Fig. 7.2*) and hence represented novel higher taxa. Thus, rather than continuing to isolate the easily to obtain and phylogenetically and physiologically similar strains, a major effort is required to elucidate the genomic and physiological novelties of enigmatic groups of prokaryotes. Given the most likely vast number of total bacterial species (see above) and the highly time-consuming nature of cultivation trials, future cultivation-based approaches to microbial diversity need to be conducted in a much more targeted manner. Blind sampling of the environment does not necessarily yield a representative view of bacterial diversity and needs to be complemented by focusing on undersampled and geographically disjunct bacterial groups (Schloss and Handelsman 2004). Of the 19 novel species mentioned above, 10 were isolated from undersampled environments like geothermal soils, thermal springs, mud volcanos, oil

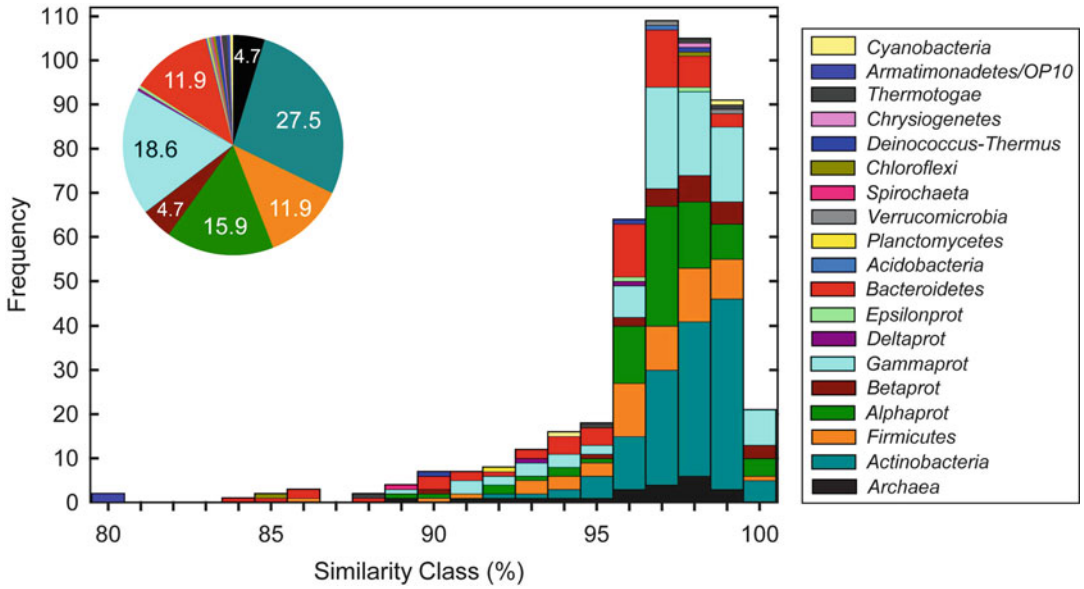


**Fig. 7.2**

**Similarity of 472 novel species described in the *International Journal of Systematic and Evolutionary Microbiology*, volume 61 (2011) with the phylogenetically closest cultured relative. Similarity is given as percentage sequence identity of the 16S rRNA gene. Insert depicts percentages of different (sub)phyla among the novel species**

production wells, an artesian well, sulfur mats, or cryptoendolithic microbial communities. Nine isolates were recovered in oligotrophic media and three from cocultures with *Escherichia coli* or amoeba.

In conclusion, the following strategies for an improved coverage of prokaryotic diversity by cultivation appear most promising:

- Development and further improvement of synthetic cultivation media that mimic the composition and ionic strength of the natural environment
- Increasing the number of systematic cultivation trials and improving their throughput by development of rapid screening tools to identify enrichments of phylogenetically deeply branching prokaryotes
- Determination of physiological traits by culture-independent methods such as MAR-FISH, SIP, nanoSIMS (Wagner et al. 2006), metatranscriptomics and metaproteomics, and stimulation experiments (Beier and Bertilsson 2011) or of the physiological potential by metagenomic (single-cell) analyses and development of targeted cultivation trials based on this information
- Improved long-term conservation protocols for reliable storage of unaltered bioresources
- Focusing -omics and physiological studies on selected novel isolates that are known to exhibit highly unusual metabolic traits

If cultivation approaches are not improved further, bacterial diversity and evolution might continue to remain largely uncharted, and prokaryotic diversity cannot be exploited in a systematic fashion for biotechnological purposes. The successful enrichment, isolation, and cultivation of prokaryotes critically depend on the choice of appropriate growth media and incubation conditions. Our present methods for the enrichment, isolation, and cultivation still are largely based on concepts developed first and foremost for the isolation of medically important bacteria (see section "Historical Perspective"). In the subsequent chapters, the requirements of prokaryotic cells for growth in the laboratory and the different existing current methods for their cultivation are discussed.

## Historical Perspective

The work of Louis Pasteur (1822–1895) marks the beginning of scientific microbiology and focused on the issue of spontaneous generation. The theory of spontaneous generation assumed that mice, frogs, and "lower" forms of life could develop spontaneously in decaying organic matter and mud. Pasteur's experiments with swan-necked flasks ruled out spontaneous generation and at the same time laid the foundations for aseptic manipulation and sterilization. Questioning the belief of eminent chemists of his time, Pasteur provided the first notion of the microbial nature of fermentation in milk (Pasteur 1862) and of anaerobic life. Other studies revealed that fermentation of sugar to alcohol, wine to vinegar, and the putrefaction of meat all were caused by microorganisms. Each particular kind of fermentation was accompanied by the development of a specific type of microorganism.

Until 1884, the bacterial pleomorphism versus monomorphism controversy was a central issue of microbiology. The description of rust and smut fungi and their morphological

changes during development had caused confusion among the early bacteriologists, and it was assumed that, depending solely on growth conditions, one and the same bacterium is able to appear in different forms (pleomorphic), can cause completely different diseases, and form totally different metabolic products. Of outstanding importance for our current thinking in microbiology is the contribution of Robert Koch (1843–1910), the founder of medical bacteriology. In his experimental work, Koch established a series of criteria, the so-called Koch's postulates, for the identification of causative agents of infectious diseases:

1. The microorganism should be constantly present in animals suffering from the disease but should not be present in healthy individuals.
2. The microorganism must be cultivated in pure culture outside the diseased animal.
3. Healthy animals infected with these pure cultures must display the characteristic disease symptoms.
4. The microorganism should be reisolated from the experimental animals and shown to be the same as the original.

In 1876, Koch isolated the anthrax bacillus from diseased cattle and conclusively demonstrated that the large nonmotile bacilli caused the disease. In 1873, Joseph Lister (1827–1912) had introduced the serial dilution technique to achieve pure cultures. However, this procedure did not yield pure cultures in the hands of every researcher. Pure culture techniques employing solid nutrient media containing gelatin were developed by Anton de Bary (1831–1888) and Oscar Brefeld (1839–1926) for the study of fungi (Bull and Slater 1982). Although the latter scientists succeeded in cultivating fungi on solid media, their techniques were not suited for the isolation and growth of bacteria for several reasons: various bacteria were found to liquefy gelatin, on hot days it melted spontaneously, and gelatin-based media could not be incubated at the temperature that most human pathogens needed to grow in a convenient time span. Starting out with cut surfaces of boiled potatoes and after adopting hydrated gelatin films as cultivation media, agar-solidified media (around 1881), covered culture dishes, and staining techniques were developed in the laboratory of Robert Koch and, in nearly unaltered form, have remained major tools in bacteriology and medical microbiology until today. After employing agar media, Koch announced his discovery of the tubercle bacillus as the causative agent of tuberculosis in 1882, at the time when this disease had many victims in Europe (Groeschel 1982). Two years later, he published the discovery of the cholera bacillus.

Subsequently, microorganisms were found to be also of crucial importance for the geochemical cycles. Ferdinand Cohn was the first to realize the role of microorganisms in the transformation of organic matter and inorganic substances on earth (Cohn 1872). The most significant contributions to the knowledge of the various types of microorganisms responsible for specific chemical transformations in nature (especially for the nitrogen and sulfur cycles) came largely from the laboratories of Sergius Winogradsky (1856–1953) and Martinus Willem Beijerinck (1851–1931). During the 1880s, Winogradsky advanced the concept of chemosynthesis on the basis of his studies of the colorless sulfur bacteria, ***Thiothrix*** and ***Beggiatoa*** (Winogradsky 1949), concluding that these aerobic bacteria obtain their energy for autotrophic growth by oxidizing reduced sulfur compounds (such as sulfides and sulfur) to sulfate.

The work of Beijerinck, with the contributions from Winogradsky, led to the development of the enrichment culture technique. Instead of directly isolating microorganisms from nature by exposing nonselective growth media to some environment and allowing chance to dictate what grew, Beijerinck proposed a different approach. Tailoring culture conditions to favor microbes with a particular metabolic activity usually leads to a rapid enrichment of the desired organism, even if its original numbers are very low in the sample (Van Iterson et al. 1940). One of the early examples of the application of this principle was the discovery and isolation of ***Spirillum*** (***Desulfovibrio***) ***desulfuricans***, described in a preliminary paper in 1894. Besides root nodule bacteria, ***Lactobacillus*** species, and others, Beijerinck also isolated the first pure cultures of aerobic nitrogen-fixing bacteria from a culture obtained by Winogradsky. Winogradsky applied enrichment of selective cultures for his research on sulfur bacteria and nitrifying bacteria and also used this technique to identify nitrogen-fixing bacteria in soil. In the years to come after Beijerinck, the success of the batch enrichment culture technique was demonstrated over and over again in the work of Kluyver, Van Niel, Stanier, and their students and associates (Veldkamp 1965; Van Niel 1967; Pfennig 1993). Very early, however, soil microbiologists noted a pronounced discrepancy between numbers of colony-forming bacteria and the numbers of cells that could be discerned by directly when observing the original samples. This led to the suggestion that the variety of bacteria in soil was so enormous that separating the different forms was impractical (Russell 1923).

At a time when the discovery of a bewildering variety of microbial forms and activities reached its height, Albert J. Kluyver (1884–1957) and H.J.L. Donker published a seminal paper on the unity in biochemistry (Kluyver and Donker 1926). This synthetic paper was based on an integration of the ideas of Carl Neuberg, Heinrich Wieland, Otto Warburg, and A. Harden. According to this concept, cells contained, in addition to a number of hydrolases, various oxidoreductases catalyzing the transfer of hydrogen from one molecule, the hydrogen (H) donor, to another, the H acceptor, along a gradient of energy. Hydrogen acceptors other than oxygen might also be used. The first report on the view that photosynthesis can be considered as a light-dependent reaction in which different substrates, specific for the different kinds of photosynthetic organisms, serve as H donors for the reduction of carbon dioxide came from Van Niel (1930). Van Niel (1967) described a general formula of photosynthesis. His work was extended greatly by N. Pfennig and his students, who grew many of the anaerobic photosynthetic bacteria in pure culture (Pfennig 1993).

Early attempts to devise truly anoxic culturing techniques included the use of deep-agar shake tubes gassed with hydrogen, the use of pyrogallol to remove oxygen, sealed tubes, and the

isolation of colonies by picking with capillary pipettes. Still, the only anaerobes isolated until 1940 were sporeformers and several non-spore-forming bacteria of clinical importance, while identities of the majority of the anaerobic prokaryotes in sediments, soils, and the gastrointestinal tract remained unknown. This was in part due to the fact that techniques were insufficient to reach the necessary anaerobic conditions but also to the fact that the media used were not simulating the microbial habitats. Hungate (1950), the first student of Van Niel from the United States, developed an anoxic roll tube technique based on various approaches from the older literature and used habitat-simulating media in the isolation of cellulolytic rumen bacteria (Hungate 1966, 1969, 1985). The Hungate technique was very successful and has been widely adapted by microbiologists from various other fields (dentistry, sewage, and sediment microbiology).

Many soil bacteria were studied in pure culture after their discovery to examine their basic metabolism and metabolic capacities. Winogradsky emphasized that the conclusions drawn from laboratory studies on the behavior of bacteria in the natural habitat may be misleading because laboratory strains might only be artifacts, that is, selected by the chosen nutrient medium, temperature, aeration, and agitation. Consequently, he already warned against the use of bacterial strains from culture collections as typical wild-type cells and, together with some of his students, designed methods to study bacteria in situ, within their natural habitats. Winogradsky's concepts on soil microbiology and on the differentiation between autochthonous and allochthonous populations in the soil are the basis of relevant, current research.

The majority of physiological investigations have been limited to pure laboratory cultures. According to the statement of Oscar Brefeld, work with impure cultures yields nothing but nonsense and ***Penicillium glaucum*** (Brefeld 1881). However, Koch's methods may also have delayed the study of microbial interactions as they occur in complex natural microbial communities, for example, in soil (Winogradsky 1949). In fact, interactions between microorganisms under natural conditions may lead to transformations that are unknown from pure cultures. The shortcomings of pure culture techniques are illustrated best by the interspecies hydrogen transfer described first by M.J. Wolin in 1975, as well as by the inhibition of growth of a ***Micrococcus*** culture by a chance ***Penicillium*** contaminant (Fleming 1929), which led to the discovery of antibiotics. For an in-depth understanding of microbial physiology, microbial transformations therefore have to be studied also in natural mixed microbial communities.

## Prokaryotic Growth

### Parameters of Prokaryotic Growth

Bacteria multiply in media of appropriate composition and in the presence of suitable substrates. The rate of growth is dependent on substrate concentration, temperature, pH, and osmolarity and can be assessed from the increase in bacterial cell number or in bacterial biomass. This distinction has to be made since under certain conditions, growth is not completely balanced, and the increase in biomass is not paralleled by an increase in cell numbers or vice versa. As an example, cells in the early stationary phase can undergo reductive division whereby cell numbers continue to increase while an almost constant overall biomass of the culture is maintained (Kolter et al. 1993).

Upon inoculation of bacterial cells into fresh growth medium, a period without growth is frequently observed (the so-called lag phase) during which adaptation of the cells occurs by, for example, synthesis of cellular enzymes. Afterward, a phase of exponential growth occurs until cell growth is limited by substrate availability and by accumulation of toxic metabolic products or unfavorable pH (stationary phase). The stationary phase is then typically followed by a phase of prokaryotic biomass reduction due to lysis of cells. If changes in bacterial cell numbers $N$ are followed during the exponential phase, the specific division rate $\nu$ and the generation time $g$ are used for the mathematical description of growth ($N_0$ denoting the initial cell number, and $t$ the time of incubation):

$$N = N_0 \cdot 2^{\nu t} \tag{7.1}$$

$$g = \frac{1}{\nu} \tag{7.2}$$

In a growing culture, the concentration of prokaryotic biomass $X$ increases autocatalytically (a first-order reaction), that is, the instantaneous biomass changes $dX/dt$ are proportional to the biomass present (Lengeler et al. 1999). Here the specific growth rate $\mu$ and the doubling time $t_d$ are used to describe prokaryotic growth:

$$\frac{dX}{dt} = \mu \cdot X \tag{7.3}$$

and hence

$$X = X_0 \cdot e^{\mu t} \tag{7.4}$$

The biomass doubling time is then calculated according to

$$t_d = \frac{\ln 2}{\mu} \tag{7.5}$$

Whereas $g = t_d$, the two parameters $\mu$ and $\nu$ are related according to

$$\mu = \nu \cdot \ln 2 \tag{7.6}$$

The specific growth rate is determined by the substrate concentration which, based on the theoretical consideration of Monod (Monod 1942, 1950), can be described by

$$\mu = \mu_{max} \cdot \left( \frac{S}{K_s + S} \right) \tag{7.7}$$

where $\mu_{max}$ is the maximum value of the specific growth rate attained at unlimiting substrate concentrations $S$ and $K_s$ (the saturation constant numerically equal to the substrate concentration at which $\mu = 0.5\ \mu_{max}$).

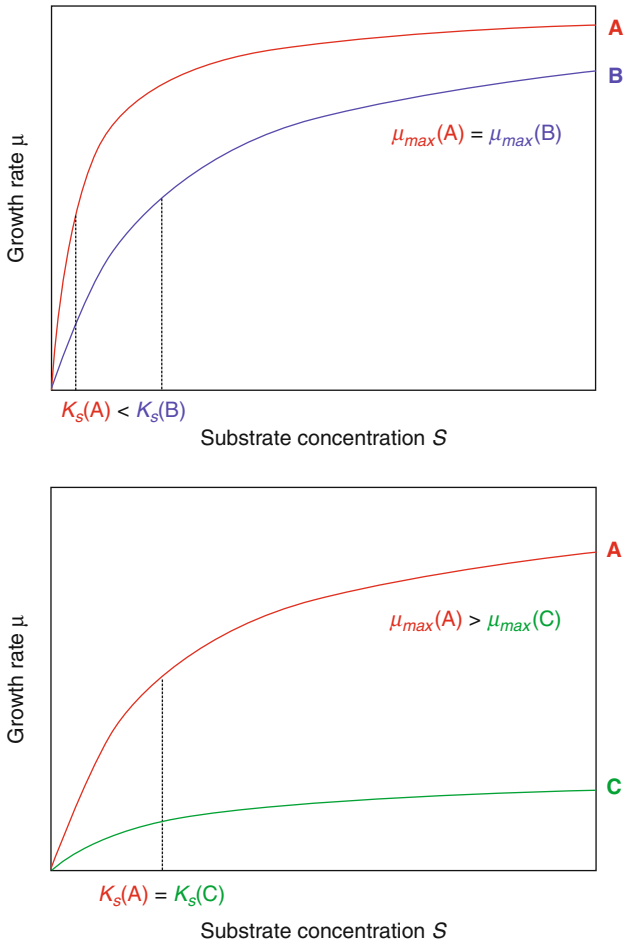


**Fig. 7.3**
**Significance of the substrate affinity of growth ($\mu_{max}/K_s$, i.e., the initial slope of the μ vs. S curve) for three species A, B, and C. Comparison of substrate-dependent specific growth rates for two strains A and B with the same maximum growth rate but different $K_s$ values (*upper panel*) and for two strains A and C with the same $K_s$ value but different maximum growth rates $\mu_{max}$ (*bottom*)**

For comparison of growth characteristics between different cultures, the $K_s$ value has been frequently invoked as a measure of affinity for a given substrate. At similar $\mu_{max}$, microorganisms with a lower $K_S$ indeed have a selective advantage (Fig. 7.3, upper panel). However, the competitive advantage of microorganisms is also determined by the value of its $\mu_{max}$ relative to that of its competitors. Thus, prokaryotes with similar $K_s$ values may differ substantially in their growth rate due to their different affinity (Fig. 7.3, bottom panel), which is calculated as the ratio $\mu_{max}/K_s$ (Schut et al. 1997). As an example, the recently isolated "*Candidatus Nitrosopumilus maritimus*" exhibits an extraordinarily high affinity of ammonium uptake and capable of depleting ammonium below the detection limit of 10 nM while reaching maximum growth rates similar to the low affinity ammonia-oxidizing bacteria (Martens-Habbena et al. 2009). The $K_S$ value for ammonia for this archaeon is 0.132 μM and thus higher than that of ammonium assimilation of some diatoms, but the specific affinity of "*Candidatus Nitrosopumilus maritimus*" surpasses that of the oligotrophic diatom *Thalassiosira pseudonana* by a factor of 30.

## Modes of Prokaryotic Growth

One of the most pronounced differences between prokaryotes and eukaryotes is the high metabolic versatility of the former. Among the outstanding metabolic properties of prokaryotes is the use of inorganic electron donors for energy generation, anaerobic growth, and the fixation of molecular nitrogen. In eukaryotes, these properties are either restricted to a few species or completely lacking. Novel ways of energy generation continue to be uncovered (Zengler et al. 1999; Schink and Friedrich 2000; Ettwig et al. 2010).

According to the concept of unity in biochemistry, redox reactions are central to life. Only a few exceptions exist to the rule that biological energy is produced in redox processes (Thauer et al. 1977). Such catabolic nonredox processes comprise the arginine fermentation in ***Enterococcus faecalis***, xanthine fermentation in ***Clostridium cylindrosporum***, and the pyruvate fermentation to acetate and formate in ***Proteus rettgeri***. In these cases, substrates are metabolized by lytic rather than redox reactions, and energy is conserved by substrate level phosphorylation. Also, photophosphorylation in ***Halobacterium halobium*** proceeds without the participation of an electron chain.

In the case of chemotrophic prokaryotes, the energy-driving cellular processes is derived from an exergonic chemical reaction (Table 7.1), during which an exogenously supplied substrate reduces an external electron acceptor (e.g., during respiration with oxygen, nitrate, or sulfate). Alternatively, a metabolic product of the substrate reduces an electron acceptor formed intracellularly from the substrate (fermentation).

The standard free energy change of a specific type of microbial metabolism can be assessed from the difference between the standard redox potential of the electron donors ($E_0'$[don]) and that of the electron acceptors ($E_0'$[acc]; tabulated in Thauer et al. 1977), according to

$$\Delta G_0' = -n \cdot F \cdot \Delta E_0' = -n \cdot F \cdot [E'_0\,(\text{acc}) - E'_0\,(\text{don})] \quad (7.8)$$

where $n$ is the number of electrons transferred and $F$ is the Faraday constant (96.48 kJ·V$^{-1}$).

The standard free energy change of a reaction can also be calculated from the free energy of formation ($G_f^0$) of the products and reactants (Thauer et al. 1977) and, in case of energy-yielding reactions involving $H^+$, the number of protons formed ($m$) and the free energy of formation of a proton at pH 7 and 25°C ($\Delta G_f'[H^+]$):

$$\begin{aligned}\Delta G'_0 &= \sum G_f^0\,(\text{Products}) - \sum G_f^0\,(\text{reactants}) \\ &\quad + m \cdot \Delta G_f\,(H^+) \\ &= \sum G_f^0\,(\text{products}) - \sum G_f^0\,(\text{reactants}) \\ &\quad + m \cdot (-39.83\ \text{kJ})\end{aligned} \quad (7.9)$$

**Table 7.1**
**Classification of microbial metabolism on the basis of energy source, the type of electron donor, and the type of carbon source**

| Energy source | | | | |
|---|---|---|---|---|
| Chemical | *Chemo-* | | | |
| Light | *Photo-* | | | |
| Electron donor | | | | |
| Inorganic | | *-litho-* | | |
| Organic | | *-organo-* | | |
| Carbon source | | | | |
| $CO_2$ | | | *-auto-* | |
| Organic | | | *-hetero-* | |
| Both | | | *-mixo-* | |
| | | | | *-troph* |

(Madigan et al. 2000a). It has been suggested that microbes can exploit a reaction for growth, if the Gibbs free energy is $\geq$20 kJ·(mol substrate)$^{-1}$ and hence sufficient for the translocation of one proton across the cytoplasmic membrane (Schink 1991, 1997). However, experiments with syntrophic cultures indicate that even smaller amounts of energy may be utilized and that syntrophic associations with methanogens metabolize near thermodynamic equilibrium at values as little as $\delta G' = -4.5$ kJ·(mol substrate)$^{-1}$ (Scholten and Conrad 2000; Jackson and McInerney 2002). In ecological niches where available energy is at a minimum, prokaryotes are hence capable of maximizing energy conservation when thermodynamic constraints begin to limit substrate degradation. It has been suggested that the underlying mechanisms control changes in cellular phosphorylation potential or the electron-motive membrane potential of the syntrophically metabolizing bacteria and that these mechanisms may be triggered by signals from their hydrogen-scavenging partner (Jackson and McInerney 2002).

In contrast to chemotrophs, phototrophic prokaryotes exploit electromagnetic energy for growth. During photosynthesis, light absorption converts a cellular pigment (the reaction center chlorophyll or bacteriochlorophyll) from a weak to a strong reductant. Electrons are transferred to associated electron carriers and—during cyclic electron transport (cyclic photophosphorylation)—finally returned to the oxidized reaction center pigment. As a principal difference from chemotrophic prokaryotes, phototrophic prokaryotes can convert light energy into chemical energy (a proton gradient) without the need of an external electron donor.

Organic or inorganic carbon compounds represent the most important nutrients for prokaryotic growth. Traditionally, prokaryotes have been divided into two major groups with respect to their carbon requirement, namely, autotrophs and heterotrophs. Autotrophic prokaryotes assimilate carbon dioxide as the principal source of cellular carbon. It has to be noted, however, that some inorganic carbon-fixing enzymes, such as acetyl-CoA carboxylase or propionyl-CoA carboxylase (present in certain autotrophs), as well as anaplerotic enzymes such as phosphoenolpyruvate (PEP) carboxylase or biosynthetic enzymes such as carbamoyl phosphate synthetase fix bicarbonate instead of $CO_2$ (Neuhard and Kelln 1996; van der Meer et al. 2001). As a rule, these microbes use light or the oxidation of inorganic compounds for the generation of metabolic energy; hence, they grow as photolithoautotrophs or chemolithoautotrophs (*Table 7.1*), respectively. Facultative autotrophs can also grow at the expense of organic carbon compounds, whereas mixotrophs can utilize $CO_2$ and organic carbon compounds simultaneously. One and the same strain can grow chemolithoautotrophically under one set of environmental conditions but may switch to chemoorganoheterotrophic growth under different conditions. Consequently, the metabolic types listed in *Table 7.1* are not mutually exclusive but may occur in the same prokaryote. In the case of heterotrophic bacteria, organic carbon compounds are used not only for biomass synthesis but also for energy generation. Approximately half of the organic carbon compounds are assimilated by aerobic microorganisms. Fermenting bacteria assimilate significantly less, typically 10–20%, while the remaining carbon substrate is required as an energy source.

The redox state of carbon in biomass (in $<C_4H_8O_2N>$, on average $-0.25$) is more reduced than many carbon sources utilized by prokaryotes. This situation is most pronounced in the case of autotrophic growth, but also during growth on organic carbon sources such as formate, glycolate, malate, fumarate, oxaloacetate, or citrate. The substrates used in microbial metabolism therefore not only serve to generate energy in the case of chemotrophic species, but also yield reducing power for the cells to carry out a variety of reductive processes during synthesis of new cell biomass. In the classification of microbial metabolism, the (inorganic or organic) nature of the electron donor for these reductive processes is considered (*Table 7.1*).

## Requirements for Prokaryotic Growth

### General Composition of the Prokaryotic Cell

For the understanding of the thermodynamics of bacterial growth and as a basis for the design of appropriate growth media, knowledge of the composition of prokaryotic biomass is essential. By comparing the ratio of macroelements in a medium recipe with that in bacterial biomass, it can be determined which of the elements will limit growth of the cells in a culture. Of the more than 100 elements that appear in the periodic table, some 35–40 are considered essential. Six nonmetals (C, O, H, N, S, and P), together with four metals (K, Mg, Fe, and Ca) comprise an average of 98% of the dry weight of prokaryotes. A good first approximation of the molar

**Table 7.2**

**Macroelements and their physiological functions**

| Element | % dry weight | mM[a,b] | Physiological functions |
|---|---|---|---|
| C | 48–59[c] | | Main constituent of organic cellular material |
| O | 13.1–23.9[c] | | Organic material and cytoplasmic water |
| H | 6.4–8[c] | | Organic material and cytoplasmic water |
| N | 13.6–14.7[c] | | Proteins, nucleic acids, and coenzymes |
| S | 0.9–1.4[c] | | Cysteine, methionine in proteins, coenzymes thiamine pyrophosphate, coenzyme A, biotin, and α-lipoic acid |
| P | 2–5.4[d] | | Nucleic acids, nucleotides, phospholipids, teichoic acids, and coenzymes |
| K | 0.03–1.4 | 18–800 *4500* | Predominant monovalent cation in cytoplasm, maintenance of cell osmolarity, cofactor of some enzymes (e.g., pyruvate kinase, peptidyl transferase, L-malate dehydrogenase; Walderhaug et al. 1987) |
| Mg | 0.007–0.05 | 6–46[e,f] | Predominant intracellular divalent cation, cofactor of many enzymes (e.g., kinases), in phosphate esters (coordinating phosphoryl oxygen atoms), ribosomes, membranes, and cell wall |
| Ca | 0.00006–0.06 | 0.01–9.5[g] | Present in exoenzymes (α-amylases and proteases) and in cell walls, role in transformation, $Ca^{++}$-dipicolinate is an important component of endospores, possibly in many putative EF-hand $Ca^{++}$-binding proteins such as calsymin |
| Na | *1.45* | *1400* | Bacterial oxaloacetate, glutaconyl-CoA, and methylmalonyl-CoA decarboxylases; NADH-quinone reductase of halophilic *Vibrio* spp. (Skulachev 1987), transport, $H_4$MPT: CoM methyltransferase of methanogenic archaea |
| Cl | 0.05 | 31–*1600*[f,h] | Essential for active uptake of compatible solutes and flagella formation in halophiles, for example, *H. halophilus* |
| Fe | 0.003-0.02[i]<br>1.8[k] | 1.2–7.7 | Present in cytochromes, ferredoxins, and other Fe–S proteins, cofactor in enzymes (some dehydratases) |

*Abbreviations*: CoA, coenzyme A; NADH, reduced beta-nicotinamide adenine dinucleotide; and $H_4$MPT: CoM, reduced molybdopterin coenzyme M

[a]Interconversion between % dry weight and mM was done based on the cytosolic volume of *E. coli* of 0.9 μL · (mg protein)$^{-1}$, equivalent to 0.45 μL · (mg dry mass)$^{-1}$ (Gangola and Rosen 1987)

[b]Intracellular concentrations in halophilic archaea or bacteria are given in italics

[c]From Norland et al. (1995) and Battley (1995)

[d]From Damoglou and Dawes (1968) and Norland et al. (1995)

[e]From Schmidt et al. (1971); up to 60 mM in moderately halophilic eubacteria (Shindler et al. 1977)

[f]From Battley (1995)

[g]In *E. coli* depending on extracellular concentrations (Gangola and Rosen 1987). The intracellular concentration of free $Ca^{2+}$ is 90–700 nM

[h]In *H. halophilus* (Roeßler and Müller 1998). Chloride has been shown to be obligatory also for growth of *Paracoccus denitrificans*, *Aeromonas hydrophila*, *Escherichia coli*, *Proteus mirabilis*, *P. vulgaris*, *Vibrio fischeri*, *Bacillus megaterium*, *B. subtilis*, *Staphylococcus aureus*, *Corynebacterium glutamicum*, and *Thermus thermophilus* (Roeßler and Müller 2002)

[i]Niehaus et al. (1991), calculated from their value of 1.6–6.9 μmol Fe · (g protein)$^{-1}$, and Braun (1997), based on the cellular amount of iron ions of $10^5$ cell$^{-1}$ and a cytosolic volume of 0.14 μm$^3$. Intracellular volume calculated from a protein content of *E. coli* of 156 fg · cell$^{-1}$ (Neidhardt and Umbarger 1996) and the cytosolic volume given in footnote (a). Similar values are also found in Abdul-Tehrani et al. (1999)

[k]Rouf (1964)

composition of prokaryotic biomass is given by the formula $<C_4H_8O_2N>$, although a more elaborate formula, namely, $C_4H_{6.4}O_{1.5}NP_{0.09}S_{0.024}$, has been published (Battley 1995). The total ionic constituents of the cytoplasm account for 1% of its dry weight. Consequently, the so-called macroelements (*Table 7.2*) are needed in relatively high concentrations in the growth medium. Most bacteria do not require Na, although many marine bacteria, certain phototrophs, and some strict anaerobes require this element. Chloride has been found to be obligatory for the growth of a variety of bacteria (*Table 7.2*). It should be noted, however, that certain gliding bacteria, for example, the marine filamentous sulfate-reducing bacterium ***Desulfonema magnum***, strictly require calcium concentrations of ≥4 mM (Widdel et al. 1983). This appears to be related to a higher $Ca^{2+}$ requirement of gliding motility (Castenholz 1973; Burchard 1980). While total iron content of bacterial cells is in the millimolar range, concentrations of free iron ions are in the tens of micromolar range (Yamamoto et al. 2004). Yet, certain bacteria grow independently of iron. The *Lactobacilli* were the first of these iron-independent organisms identified.

Their metabolic independence of iron explains their ability to grow in milk which represents an iron-restricted medium due to the high concentrations of the iron-binding protein lactoferrin. The genome sequence of the spirochete *Borrelia burgdorferi* does not seem to encode iron proteins, and the spirochete *Treponema pallidum* also lacks requirement for iron (Andrews et al. 2003).

The range of organic carbon compounds utilizable by heterotrophs is vast; virtually any compound synthesized by biological processes, as well as many xenobiotica (compounds synthesized in the laboratory which do not originate in nature), can be degraded by microbes. Different species of heterotrophic prokaryotes utilize considerably different numbers and kinds of carbon substrates. Some, such as the pseudomonads, are versatile and are known to utilize over 100 different carbon compounds as the sole source of carbon and energy. Their substrates include carbohydrates, sugar acids, polyols, fatty acids, primary alcohols, amino acids, and aromatic substances. In contrast to these versatile bacteria, several groups exist that are limited in their ability to decompose organic compounds. In this category are the obligate methylotrophs that only use methane, methanol, dimethyl ethers, and a few other compounds. Highly specialized species are restricted to the use of only one type of organic carbon substrate, for example, ***Bacillus fastidiosus*** to uric acid. Many chemoorganotrophs still require carbon dioxide in small amounts for anaplerotic reactions, for example, the synthesis of oxaloacetate by PEP carboxylase, PEP carboxykinase, PEP carboxytransphosphorylase, and by pyruvate carboxylase (Wood-Werkman reactions). Since carbon dioxide is produced during catabolism of organic compounds, it does not normally become a limiting nutritional factor. However, some bacteria, such as ***Neisseria*** and ***Brucella***, require higher concentrations of carbon dioxide (up to 10%) in the atmosphere for good growth in organic media, a need that must be considered in isolating and cultivating such organisms.

Oxygen and hydrogen are derived from water and, in the case of heterotrophic bacteria, from the organic carbon source. Molecular $O_2$ is required in only few exceptions, where an OH group is introduced by mono- or dioxygenases during the biosynthesis of cell constituents. Remarkably, exogenous oxygen does not seem to be mandatory in all cases. The recently discovered "*Candidatus Methylomirabilis oxyfera*" couples anaerobic methane oxidation with the reduction of nitrite to dinitrogen, apparently by conversion of two intermediate nitric oxide molecules to dinitrogen and oxygen. The latter is then used to oxidize methane (Ettwig et al. 2010). In cellular material, nitrogen is incorporated in the reduced form as amino groups. Many prokaryotes are capable of assimilatory nitrate reduction and therefore grow with nitrate as nitrogen source. A number of aerobic and anaerobic bacteria are capable of nitrogen fixation and thus grow in media devoid of a combined nitrogen source. Some prokaryotes, such as anoxygenic phototrophs, depend on reduced nitrogen in the form of ammonium salts. Others require amino acids or oligo- or polypeptides as a source of cellular nitrogen.

A large fraction of phosphorus is bound in RNA. As a result, phosphorus demand increases with the specific growth rate of the cells. No reduced phosphorus compounds are stable, and cellular phosphorus is in the oxidation state of phosphate. Hence, inorganic phosphate is the usual source of this element for microbial nutrition. Since phosphate is frequently also used as a pH buffer, it is added in excess of the growth requirement. However, high concentrations of inorganic phosphates may be growth inhibitory to at least some aquatic bacteria (Bartscht et al. 1999). Organic phosphorus sources such as glycerophosphate are an alternative supply.

Cellular sulfur is mostly in the reduced state and present in the sulfur-containing amino acids cysteine and methionine. Many bacteria are capable of assimilatory sulfate reduction and thus can utilize sulfate to satisfy the sulfur requirements for growth. Other prokaryotes, such as about half of the strains of anoxygenic phototrophic bacteria or methanogenic archaea, require sulfur in a reduced form as either sulfide or an organic compound with a thiol group, such as cysteine.

Potassium is the principal inorganic cation in the cell and reaches a concentration of about 300 mM in the cytoplasm. Although much of it is bound in the ribosomes, it is also a cofactor of some enzymes, is required for carbohydrate metabolism, and is involved in many transport processes and osmoregulation. Usually, an inorganic potassium salt ($K_2SO_4$ or $KH_2PO_4$) is added to the growth medium to satisfy this requirement.

The magnesium requirement of bacteria is principally that of bacterial ribosomes. Magnesium also functions as an enzyme cofactor and is present in cell walls and membranes. It is usually supplied as magnesium sulfate. Iron is required (though not by ***Borrelia burgdorferi***) at micromolar concentrations, whereas the trace elements listed in ❯ *Table 7.3* occur in only minute fractions of between $10^{-6}$% and 0.008% dry weight in the bacterial cell (Rouf 1964). Trace elements are toxic at higher concentrations and thus should be present in growth media at concentrations between 0.01 and 1 μM. The presence of some trace elements can actually prevent the growth of certain prokaryotes. Thus, tungsten (the heaviest metal with well-documented functions in bacteria) is misincorporated into the nitrogenase of spirochetes from the termite hindgut resulting in an inactive enzyme and in this manner prevents diazotrophic growth in laboratory culture (Lilburn et al. 2001). In this case, tungsten has to be removed and increased amounts of molybdenum (final concentrations of 5 μM) have to be added to the medium. Many iron-oxidizing bacteria are strongly inhibited by tungstate (Sugio et al. 2001).

Since divalent and trivalent metal cations tend to form insoluble hydroxides or phosphates at neutral to alkaline pH, they may become unavailable to bacteria in growth media. Concentrated stock solutions of inorganic salts therefore are often kept anoxically at acidic pH, and minimal amounts of complexing agents (ethylenediamine-N,N,N′,N′-tetraacetic acid [EDTA] and nitrilotriacetic acid [NTA]) are incorporated.

In addition, a variety of microelements (Mn, Co, Cu, Mo, Zn, Ni, V, and B) is required (❯ *Table 7.3*).

**Table 7.3**
**Microelements: their source and physiological functions**

| Element | Source | Physiological functions |
|---|---|---|
| Mn | $Mn^{2+}$ | Superoxide dismutase, photosystem II, some enzymes (PEP carboxykinase and citrate synthase) |
| Co | $Co^{2+}$ | Coenzyme $B_{12}$-containing enzymes (glutamate mutase and methylmalonyl-CoA mutase) |
| Cu | $Cu^{2+}$ | Cytochrome oxidase, plastocyanin, nitrite reductase, oxygenases, and superoxide dismutases[a] |
| Mo | $MoO_4^{2-}$ | Nitrate reductase, nitrogenase, xanthine dehydrogenase, and formate dehydrogenase |
| Zn | $Zn^{2+}$ | Carbonic anhydrase, alcohol dehydrogenase, alkaline phosphatase, aldolase, RNA and DNA polymerase, and proteinases |
| Ni | $Ni^{2+}$ | Urease, hydrogenase, and cofactor $F_{430}$ |
| V | $VOSO_4$ | V-dependent nitrogenase, and bromoperoxidase |
| Se | $SeO_3^{2-}$ | Formate dehydrogenase, hydrogenase, and glycine reductase in clostridia |
| W | $WO_4^{2-}$ | Some formate dehydrogenases (commensurate with its higher bioavailability replaces Mo in some anaerobic bacteria) |
| B | $H_3BO_3$ | Formation of heterocysts, akinetes in cyanobacteria; antibiotics (boromycins, tartrolons) in *Streptomyces*, *Sorangium*; possibly cross-linking of pyranoses in cellular envelope,[b] and AI-2 (cyclic borate diester)[c,d] |

*PEP* phosphoenolpyruvate, *V-dependent* vanadium-dependent, *AI-2* autoinducer-2
[a]Certain cyanobacteria such as *Synechococcus* are very sensitive to Cu, which therefore has to be omitted from the respective growth media
[b]Loomis and Durst (1992)
[c]However, boric acid acts as a bacteriostatic agent under certain circumstances (Lum and Meers 1989)
[d]Chen et al. (2002)

Certain prokaryotes, in particular strict anaerobes, often also require selenium and tungsten. Recently, the requirement for boron has been recognized as being rather widespread, since many bacteria employ the AI-2 autoinducer, a cyclic borate diester.

## Growth Factors

Many bacteria grow with a single carbon compound added to the medium. Additional organic compounds often stimulate the growth of microorganisms when present in small concentrations. These so-called growth factors represent building blocks of major cell constituents, and their addition to the medium therefore decreases the need for de novo biosynthesis in the cell, if the compounds can be transported into the cell. Generally, the growth factors recognized are (1) amino acids, (2) purines and pyrimidines, and (3) vitamins. The latter are required only in small amounts (on the order of $mg \cdot L^{-1}$, or between 0.1 and 1 μM). The biological function of a number of vitamins is listed in *Table 7.4*. Synthetic stock solutions of vitamins should be kept dark, cold, anoxic, and sterile (by filtration) and at a slightly acidic pH. They should be added to the medium after autoclaving the latter.

However, many prokaryotes obligately depend on the presence of growth factors in the medium because the cells are unable to synthesize all cell constituents from a single carbon source. In more rare cases, unusual compounds such as porphyrins (hemin in the case of ***Haemophilus*** spp.), short-branched fatty acids (e.g., 2-methyl-*n*-butyric acid in the case of ***Methanobacterium ruminantium***) or straight-chain fatty acids, mevalonic acid, cholesterol (***Mycoplasma***), choline, betaine, and polyamines are required. Some vitamins are required only during growth on specific substrates (such as $B_{12}$ by ***Escherichia coli***). Essential growth factors may include a few vitamins, such as biotin and *p*-aminobenzoic acid in the case of ***Clostridium kluyveri***, or a large variety of compounds (as in the case of *Lactobacilli*, which grow in media supplemented with peptone, Tween 80, acetate, and $Mn^{2+}$ salts [MRS-medium]). Many lactic acid bacteria including ***Leuconostoc*** have a requirement for $Mn^{2+}$ (Boyaval 1989). Yeast extract is often employed as a convenient source of most vitamins and also amino acids. Freshly prepared yeast autolysate has been found to be superior to commercially available dried preparations (Leadbetter et al. 1999; Vogl et al. 2006). However, the addition of growth factors such as yeast extract or casamino acids also clearly inhibits growth of some photolithoautotrophic or chemolithoautotrophic bacteria even at very low concentrations of 0.01% (w/v) (Overmann and Pfennig 1989).

In many instances, the nature of additional growth factors required for the growth of fastidious prokaryotes is not known. Besides yeast extract, also fresh rumen extract, fermented rumen extract or swine manure, or filtered hindgut homogenates of beetles larvae (Geissinger et al. 2009) have been successfully employed. Sterilized rumen extract contains a number of volatile, particularly short-branched, fatty acids, but also vitamins and hemin. Sludge supernatant is another complex source of supplines but has been applied less frequently. The polyol Tween 80 (polyoxyethylene sorbitan monooleate) can be used as a water-soluble source of the long-chain fatty acid oleate (*cis*-9-octadecenoic acid) and in addition consists of linoleate, palmitate, and stearate as minor compounds. Cold soil extract has been observed to stimulate growth of certain soil bacteria.

Growth factors may also stimulate microbial growth in an indirect fashion. Recently, evidence has accumulated for a stimulatory role of humic substances and extracellular quinones (e.g., anthraquinone-2,6-disulfonate) in dissimilatory iron reduction (Lovley and Blunt-Harris 1999). These redox-active compounds can serve as electron shuttles between

**Table 7.4**
**Functions of vitamins and related compounds in prokaryotes**

| Vitamin | Physiological functions |
|---|---|
| *p*-Aminobenzoic acid | Precursor of tetrahydrofolic acid involved in one-carbon transfer |
| Biotin | Carbon dioxide fixation and release |
| Coenzyme M | Methane formation |
| Folic acid | Tetrahydrofolic acid involved in one-carbon metabolism |
| Lipoic acid[a] | Transfer of acyl groups, for example, prosthetic group of pyruvate dehydrogenase complex |
| Thiamine ($B_1$) | Thiamine pyrophosphate is prosthetic group of decarboxylases and transketolases |
| Riboflavin ($B_2$) | Precursor of FMN and FAD, prosthetic group of flavoproteins, and redox reactions |
| Pyridoxine ($B_6$) | Pyridoxal phosphate coenzyme of transaminases and amino acid decarboxylases |
| Cyanocobalamin ($B_{12}$) | Molecular rearrangement reactions (e.g., glutamate mutase) |
| Pantothenic acid | Precursor of CoA, prosthetic group of acyl carrier proteins, and participant in metabolism of fatty acids |
| Nicotinic acid | Precursor of NAD and $NADP^+$ and found in many dehydrogenases |
| Vitamin K | Precursor of menaquinone and electron carrier in respiratory chains |
| Hemin | Precursor of cytochromes, and involved in redox reactions |

*FMN* flavin mononucleotide, *FAD* flavin adenine dinucleotide, *CoA* coenzyme A, *NAD* nicotinamide adenine dinucleotide, *NADP* nicotinamide adenine dinucleotide phosphate
[a]Dithiooctanoic acid
From Gottschalk (1985)

the cells of dissimilatory iron-reducing bacteria and their insoluble electron acceptors like Fe(III) oxides. In addition, organic ligands such as EDTA, NTA, *n*-methyliminodiacetic acid, ethanol diglycine, deferoxamine, or phosphates, if added to growth media, make Fe(III) more available and increase rates of microbial reduction significantly (Lovley et al. 1996b).

## pH

In natural environments, the pH value varies from below 1 in acidic springs to over 11 in soda lakes (Brock 1978; Grant and Tindall 1986; Madigan et al. 2000a). Prokaryotes can be isolated from these environments and are capable of growing at such pH values (Brock 1978; Langworthy 1978; Horikoshi and Akiba 1982; Krulwich and Guffanti 1989). Species with optima for growth below pH 5.5 are usually called acidophiles and can grow even at pH values of 1 (e.g., ***Acidithiobacillus*** and ***Sulfolobus***). Prokaryotes with optima above pH 8.5 are called alkaliphiles and grow up to values of pH 11.5 (e.g., ***Sporosarcina pasteurii***). Many enzymes and structural components in these extremophiles cannot function properly at the very high or low pH values found in their external environment, and the intracellular pH is kept at relatively constant values (Krulwich and Guffanti 1983, 1989; Padan and Schuldinger 1986; Matin 1990). In general, the internal pH of acidophiles is regulated to a value of 6.0–7.0, and alkaliphiles maintain an internal pH 1–2 units lower than the external value.

The majority of natural environments possess pH values between 5 and 9, and the pH optima for the growth of most prokaryotes (neutrophiles) fall well within this range. Neutrophiles keep the internal pH slightly more alkaline than this value outside. For example, ***Escherichia coli*** regulates its internal pH at 7.4–7.8 during growth over the external pH range of 5.0–9.0 (Slonczewski and Foster 1996). A much increased cultivation success for methanotrophs, Acidobacteria, and methanogenic Archaea from northern acidic wetlands has been attained by employing low-ionic-strength, low-nutrient acidic (pH 4.0–5.5) media (Dedysh 2011).

During the growth of microorganisms in cultures, pronounced pH changes can occur because organic acids (especially during fermentation) and ammonia from nitrogen-containing compounds are produced. When exposed to pH values at the upper or lower end of the permissive range of growth, prokaryotes may exhibit metabolic properties not displayed at normal pH values. A well-known example is the shift toward production of nonacidic end products by fermentative bacteria (Graham and Lund 1983; Gottwald and Gottschalk 1985; Ferchichi et al. 1986; Huang et al. 1986; Forsberg 1987; Hommes et al. 1989). Weak organic acids also influence the cytoplasmic pH in a direct manner since in their undissociated form they are lipophilic and therefore rapidly diffuse through cell membranes, ultimately conducting hydrogen ions along the transmembrane gradient. As a result, the intracellular pH is lowered. At low pH values, the effect of extracellular pH in microbial growth is therefore magnified in the presence of organic acids. Hence, permeant acids such as benzoic, propionic, and sorbic acids are inhibitors of microbial growth (and in fact are used as food preservatives), and their potency increases with decreasing pH (Ingraham and Marr 1996). However, the sensitivity of bacterial cells to low pH values is less pronounced in the stationary phase (Ingraham and Marr 1996).

In high-nutrient-strength complex media, the various acidic and basic functional groups of organic molecules often provide sufficient buffering capacity. In mineral media and low-nutrient-strength complex media, continuing acidification or alkalinization of the growth medium will rapidly lead to arrest of growth of prokaryotes. Therefore, the pH has to be maintained within the permissive range for growth using appropriate organic or inorganic buffer systems (*Table 7.5*). Below pH 3, the actual concentration of hydronium ions becomes high enough to obviate the need for buffering.

**Table 7.5**
**pH buffers used for culturing prokaryotes**

| Buffer | $pK_a$ | Buffering range | Concentration | References |
|---|---|---|---|---|
| HOMOPIPES | 4.55 (37 °C) | 4.0–5.0 | | Slonczewski and Foster (1996) |
| MES | 5.96 (37 °C) | 5.5–6.5 | | Slonczewski and Foster (1996) |
| $CO_2(H_2CO_3)/NaHCO_3$[a] | 6.35 (25 °C) | 5.4–7.8 | 30 mM | Widdel and Bak (1992) |
| PIPES | 6.66 (37 °C) | 6.0–7.0 | | Slonczewski and Foster (1996) |
| $KH_2PO_4/K_2HPO_4$ | 7.2 (25 °C) | 5.8–7.8 | 10 mM | Bast (2001) |
| MOPS | 7.2 (25 °C) | 6.5–7.7 | 10 mM | Bartscht et al. (1999) |
| | 7.01 (37 °C) | | | Slonczewski and Foster (1996) |
| HEPES | 7.5 (25 °C) | 6.8–8.2 | 10 mM | Bartscht et al. (1999) |
| Tris · HCl | 8.08 (25 °C) | 7.2–9.0 | 10 mM | Bast (2001) |
| TAPS | 8.11 (37 °C) | 7.5–8.5 | | Slonczewski and Foster (1996) |
| CAPSO | 9.43 (37 °C) | 9.0–10.0 | | Slonczewski and Foster (1996) |
| CAPS | 10.08 (37 °C) | 9.5–10.5 | | Slonczewski and Foster (1996) |
| $NaHCO_3/Na_2CO_3$ | 10.4 (25 °C) | 8.5–11.0 | 50 mM | Horikoshi and Akiba (1982) |

*CAPS* 3-(cyclohexylamino)-1-propanesulfonic acid, *CAPSO* 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid, *HEPES* *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid], *HOMOPIPES* homopiperazine-*N*,*N*′-bis-2(ethanesulfonic acid), *MES* 2-(*N*-morpholino)ethanesulfonic acid, *MOPS* 3-[*N*-morpholino] propanesulfonic acid, *PIPES* piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid), *TAPS* 3-[*N*-Tris(hydroxymethyl)methylamino]propanesulfonic acid, *Tris · HCl* tris [hydroxymethyl]aminomethane
[a]Applicable in closed gas-tight vessels only

Not all buffers listed can be applied for the isolation and cultivation of all prokaryotes. Phosphate buffers, although widely employed, inhibit the growth of bacteria from low-nutrient environments such as freshwater lakes even at comparatively low concentrations of 10 mM (Bartscht et al. 1999) and were also found to act inhibitory for Acidobacteria from acidic wetlands (Dedysh 2011). At higher concentrations (≥30 mM), phosphate buffers are inhibitory also to many other microorganisms (Bast 2001). A second disadvantage of phosphate buffers is their tendency to form precipitates with $Ca^{2+}$, $Mg^{2+}$, and $Fe^{3+}$ ions already at rather low concentrations (e.g., 7 mM $PO_4^{3-}$ and 0.7 mM $Ca^{2+}$). In these cases, the $Ca^{2+}$ concentration needs to be decreased to 200 µM, which (as calculated from the cellular $Ca^{2+}$ content; *Table 7.2*) still permits a sufficient cell yield of ≥7 g dry weight·$L^{-1}$ (for exceptions such as gliding bacteria, see section "General Composition of the Prokaryotic Cell"). The sulfonate buffers listed in *Table 7.5* have been found to be useful in several instances (Slonczewski and Foster 1996; Bartscht et al. 1999). Substituted amines such as TRIS (tris [hydroxymethyl]aminomethane) and triethanolamine should be avoided as they cross the cytoplasmic membrane in their deprotonated form. In general, the ionic form of a buffering substance is less toxic than its undissociated form, since the charged molecules penetrate the cellular membrane much more slowly. Consequently, cationic buffers (e.g., TRIS) are used at pH values below their p*K*, while anionic buffers are employed at a pH above their p*K* value. In addition to the buffers listed above, alginic acid has been employed at a concentration of 20–50 mg $L^{-1}$ to maintain pH of growth media at 4.0–5.0 (Pankratov and Dedysh 2011).

For the preparation of low pH solid media, silica gel has to be employed as solidifying agent (see section "Treatment of Growth Media").

## Osmolarity

Microorganisms differ considerably in the amount of water they require in their immediate surroundings (Brown 1976; Kushner 1978; Yancey et al. 1982). At higher concentrations of aqueous solutions, forces between solutes and solvent lead to a lower "effective concentration"—the so-called activity—of water. Availability of water is measured as the water activity $a_w$, which is defined by the mole fraction of water $N_w$ and the activity coefficient $\gamma_w$ according to Schopfer and Brennicke (1999)

$$a_w = \gamma_w \cdot N_w \tag{7.10}$$

According to Raoult's law, the water activity in a dilute solution is equal to the ratio of the vapor pressure $P$ of the solution relative to the vapor pressure $P_0$ of pure water at the same temperature:

$$a_w = \frac{P}{P_0} = \frac{n_1}{n_1 + n_2} \tag{7.11}$$

Here, $n_1$ is the number of moles of the solvent and $n_2$ the number of moles of ideal solute. Values of water activity range between

0 and 1. The majority of microorganisms known grow well only at values between 0.9 and 1.0 (Nichols et al. 1999), as they are commonly found in aquatic and soil environments. Freshwater media used for routine cultivation usually have $a_w$ values $\geq$0.99, whereas seawater (3.5% NaCl) has a value of approximately 0.98. Salt lakes in which ***Halobacterium*** and ***Halococcus*** thrive are saturated with NaCl, and $a_w$ values are around 0.75. The lowest value reported at which growth of microorganisms still occurs is 0.61 for the xerophytic fungus ***Xeromyces bisporus***, which can grow on dry foods.

Since the chemical potential of water $\mu_w$ is determined by the chemical potential of the pure solvent $\mu_w^\circ$ and the water activity by

$$\mu_w = \mu_w^\circ + RT \ln \ a_w \tag{7.12}$$

(where $R$ is the gas constant and $T$ the absolute temperature), higher concentrations of solutes decrease the chemical potential of water. Most prokaryotes maintain an internal activity of water lower than that of the surrounding medium. At the same time, the cytoplasmic membrane is highly permeable to water, and bacteria have a high surface-to-volume ratio. Essentially, the chemical potential of intracellular water must be equal to that outside. As a result of the lower intracellular water activity, a tendency exists for water to enter the cell. The direct consequence is osmosis, defined as diffusion of solvent (water) through a semipermeable membrane in the direction of a decreasing chemical potential of the solvent. During this process, the free enthalpy of the entire system decreases.

Osmotic pressure $\pi$ is the hydrostatic pressure (in MPa) that must be applied to a solution of lower chemical potential of the solvent to stop net diffusion of solvent through the semipermeable membrane

$$\pi \cdot V_w^\circ = -RT \cdot \ln \ a_w \tag{7.13}$$

with $V^\circ_w$ being the partial molal volume of water. In diluted solutions, Eq. 7.13 can be simplified (with $N_2 = n_2/(n_1 + n_2)$; $\ln a_w = \ln(1 - N_2) - N_2$; and $n_2 \ll n_1$):

$$\pi = \frac{R \cdot T}{V_w^\circ} \cdot N_2 \approx \frac{R \cdot T}{V_w^\circ} \cdot \frac{n_2}{n_1} = R \cdot T \cdot c_2 \tag{7.14}$$

where $c_2$ denotes the concentration of the solute. Hence, a concentration of 0.1 M of a solute at 25°C results in an osmotic pressure of 2.48 bar or 0.248 MPa. If $n$ different solutes are present, the osmotic pressure amounts to

$$\pi = R \cdot T \cdot \sum_i n_i c_i \tag{7.15}$$

The presence of a cell wall permits bacteria to maintain the intracellular osmotic pressure, termed "turgor pressure." Maintenance of this turgor pressure is essential for the growth and division of the prokaryotic cell. It is therefore understandable that bacteria regulate their turgor pressure over a wide range of extracellular values of $a_w$. Gram-negative bacteria have turgor pressures of 0.3–0.6 MPa, while Gram-positive bacteria such as ***Bacillus*** sp. maintain a turgor pressure of up to 2 MPa (Reed and Walsby 1985; Overmann et al. 1991; Ingraham and Marr 1996; Lengeler et al. 1999).

Two major strategies have been found by which prokaryotes adjust intracellular $a_w$ during changes of extracellular salt concentrations. Aerobic, extremely halophilic Archaea (Halobacteriales) and anaerobic, halophilic bacteria (Haloanaerobiales) accumulate inorganic ions, especially $K^+$ and $Cl^-$ (Oren 1986, 1999; Galinski and Trüper 1994). As a special adaptation to the resulting high intracellular ion concentrations, these prokaryotes contain salt-adapted or even salt-requiring enzymes that contain a large fraction of polar and acidic amino acids (especially aspartate and glutamate) but only a low fraction of hydrophobic amino acids. Aspartate and glutamate residues bind intracellular water especially well. Consequently, correct folding of these enzymes occurs despite low intracellular water concentrations (Oren 1999).

At lower external osmolalities ($\leq$1 Osm), turgor pressure in ***E. coli*** is also mainly regulated by increasing intracellular $K^+$ concentrations (and its counterion, glutamate) parallel to an increase in external osmolality. The intracellular concentration of $Na^+$ does not vary directly with external osmolality, and intracellular concentrations of the divalent polyamine putrescine are reduced by excretion of this compound into the medium, thereby decreasing the intracellular concentration from 50 to 5 mM. It has been suggested that putrescine bound to nucleic acids is displaced by high $K^+$ concentrations (Csonka and Epstein 1996).

As a second type of osmoregulation, small soluble organic molecules are accumulated upon an increase in extracellular salt concentrations. This type is found widely distributed among bacteria. The solutes accumulated do not strongly interfere with cellular metabolism and have thus been termed "compatible solutes" (Brown and Simpson 1972; Brown 1976). In addition, these molecules stabilize cellular proteins and increase their solubility (Cayley et al. 1992; Bolen 2001). Thus, *E. coli* cells accumulate trehalose (*O*-α-D-glucosyl[1 → 1]-α-D-glucoside), accompanied by a reduction in the concentration of $K^+$ and glutamate. It has been suggested that this decrease in ionic strength relieves the inhibition of transcription and translation and that the cytoplasmic membrane is stabilized by interaction of compatible solutes with the polar head groups of the phospholipids (Ingraham and Marr 1996). Various compatible solutes, zwitterionic derivatives of amino acids, sugars, or polyols, have been identified and include proline, glutamate, glutamine, alanine, α-aminobutyrate, glycine betaine (*N*,*N*,*N*-trimethylglycine), ectoine, $N^\epsilon$-acetyl-β-lysine, $N^\delta$-acetylornithine, β-dimethylsulfoniopropionate, sucrose, and choline (Csonka and Epstein 1996). Some of these, especially proline, glycine betaine, and choline, are also termed "osmoprotectants," since they can be added to the growth medium where they are taken up by the bacterial cells and alleviate osmotic stress effects through direct interactions with intracellular macromolecules. It has also been proposed that glycine betaine increases the fraction of free water in the cell, and it is believed that osmoprotectants stabilize and protect enzymes by being excluded from the protein surface (Cayley et al. 1992). Thus, tolerance of

***E. coli*** toward high external osmolalities can be increased further by the addition of osmotic protectants such as proline, glycine betaine, choline (which is metabolized to glycine betaine), trimethyl-γ-aminobutyrate, β-alanine betaine, taurine betaine, carnitine, or even MOPS (3-[*N*-morpholino] propanesulfonic acid) to the growth medium (Csonka and Hanson 1991). The latter is a buffer commonly used in growth media, which has been shown to accumulate in ***E. coli*** at high osmolality (Cayley et al. 1989). Glycine betaine is the most effective osmotic protectant of ***E. coli*** and ***Salmonella typhimurium***. Accumulation of glycine betaine reduces the concentration of endogenous trehalose as well as ionic solutes.

The osmoregulatory potential varies between microorganisms, which can be divided according to their osmotic tolerance. This classification has been used most often for NaCl tolerance. Nonhalophiles thus are organisms capable of growth at NaCl concentrations of >0.2 M, whereas moderate halophiles and marine species usually grow from 0.2 to 3.5 M NaCl, and extreme halophiles grow from 1 to 5.5 M NaCl (Kushner 1978; Yancey et al. 1982; Epstein 1986; Imhoff 1986; Larsen 1986).

## Temperature

Prokaryotes can grow at temperatures between −17°C or even lower (Morita 1975; Baross and Morita 1978; Mazur 1980; Carpenter et al. 2000) and +113°C (in the case of the archaeon ***Pyrolobus fumarii***; Blochl et al. 1997; Stetter 2001). The upper limit for microbial growth is suggested to be between 110°C and 150°C owing to constraints on the thermostability of several essential cell components. The half-life for ATP under these conditions is <1 s, and it is ∼1 ms for polynucleotides (Bernhardt et al. 1984; White 1984; Jaenicke 1988). At the other extreme, over 80% of the volume of the biosphere permanently resides at a temperature below 5°C which is mainly due to a rather constant temperature of about 2°C in two-thirds of deep ocean water (Graumann and Marahiel 1996). The cryosphere (i.e., the part of the planet that is permanently frozen) covers 15% of the Earth's surface, and 33% of the surface is seasonally covered by snow. Other, artificially, low-temperature environments encompass refrigerated appliances and products. Viable bacteria have been discovered in snow, glacier ice, and permafrost soils and even in 1-million-year-old and 3,600-m deep Antarctic ice layers. Physiological activity has been detected down to temperatures as low as −33°C which has been attributed to the presence of 50-nm-thin films of liquid at the surface of ice crystals and to microdiameter liquid water veins caused by ionic impurities in the crystal structure (Carpenter et al. 2000; Karl et al. 1999; Bidle et al. 2007; Christner 2010; Achberger et al. 2011). For bacteria belonging to the ***Deinococcus-Thermus*** group, evidence has been obtained for low rates of bacterial DNA and protein synthesis at ambient subzero temperatures of −12°C to −17°C (Carpenter et al. 2000).

Individual strains can grow over a temperature range of 10°C to maximally 60°C (usually 30–35°C; Wiegel and Ljungdahl 1986; Brock 1987; Wiegel 1990). On the basis of their minimum, optimum, and maximum temperatures for growth (the so-called cardinal temperatures $T_{min}$, $T_{opt}$, $T_{max}$), organisms are currently divided into four major groups: psychrophiles (also called stenopsychrophiles; Cavicchioli 2006) ($T_{opt} \leq 20°C$), mesophiles ($T_{opt}$ between 20°C and 42°C), thermophiles ($T_{opt}$, 42–70°C), and hyperthermophiles ($T_{opt}$, >70°C). In addition, the term "psychrotolerant" (also called eurypsychrophile; previously "psychrotrophic") has been coined to describe those prokaryotes capable of growing at temperatures between 0°C and 5°C but reach maximum temperatures of growth exceeding 25°C (Morita 1975). Different temperature ranges for psychrophilic and psychrotolerant prokaryotes have been used, however (Isaksen and Jørgensen 1996).

Generally, the optimum temperature for growth is only a few degrees Celsius lower than $T_{max}$. Consequently, it is advisable to perform routine cultivation at incubation temperatures below $T_{opt}$. It is very difficult to determine the minimum growth temperature, as doubling times can become extremely long at the low end of the temperature range.

As a rule of thumb, the rate constants of chemical reactions increase by a factor of about 2 (the so-called $Q_{10}$ value; range, 1.5–4) when the temperature is increased by 10°C. This exponential dependence on temperature exists for biochemical reactions (e.g., firefly bioluminescence reaction) and likewise for microbial metabolism (e.g., sulfate reduction; Bak and Pfennig 1991; Isaksen and Jørgensen 1996) and microbial growth. Hence, the specific growth rate doubles with a 10°C increase in temperature for many bacteria. Like rate constants of chemical reactions, the temperature dependence of the microbial growth rate between the minimum and optimum temperature can be described by the Arrhenius equation (Arrhenius 1889):

$$\mu = A \cdot e^{-\frac{E_a}{R \cdot T}} \tag{7.16}$$

in which μ represents the specific growth rate, $E_a$ the activation energy, $R$ the gas constant (8.31 J $K^{-1}$ $mol^{-1}$), $T$ the temperature (in K), and the constant $A$ the collision or frequency factor (in $h^{-1}$), which in chemical reactions describes the collision frequency and orientation of reacting molecules. If applied to bacterial growth or physiological activity, $E_a$ does not represent activation energy in the chemical sense but rather a measure of the temperature response of the bacteria. Consequently, $E_a$ has sometimes also been termed "the temperature characteristic" (King and Nedwell 1984). This equation permits an assessment of the temperature dependence of growth or physiological activity within certain limits. If the logarithm of μ is plotted versus the reciprocal of $T$, a straight line is obtained with a negative slope of $-E_a/2.303 \cdot R$ (Ingraham 1962; Harder and Veldkamp 1971; Ratkowsky et al. 1983):

$$\ln \mu = -\frac{E_a}{R} \cdot \frac{1}{T} + \ln A \tag{7.17}$$

Typical $E_a$ values for the specific growth rate or the physiological activity of bacteria are in the range of 23–132 kJ $mol^{-1}$ (Bak and Pfennig 1991; Ingraham and Marr 1996;

Knoblauch et al. 1999). On the basis of equation (7.3), the $Q_{10}$-value can be calculated according to:

$$Q_{10} = \exp\left[\frac{E_a \cdot 10}{R \cdot T(T+10)}\right] \quad (7.18)$$

At the low, and especially at the high, temperature end, the data of the logarithmic plot of log μ versus $T^{-1}$ deviate from linearity. In the case of psychrophiles, the slope of the Arrhenius plot is linear down to 0°C, whereas for psychrotolerants, it deviates from linearity at about 5°C, and for mesophilic bacteria, it tends to deviate from a straight line at even higher temperatures (Harder and Veldkamp 1971). At high temperatures, the growth rate decreases sharply due to the thermal inactivation of enzymes and disruption of the membrane structure. It should be mentioned that alternative models for the relationship between temperature and growth rate constant have been developed which fit the experimental data of many bacterial strains more precisely (Ratkowsky et al. 1983).

One important factor that determines minimum and maximum temperatures for growth is the fluidity of the membrane lipids, affected particularly by the ratio of monounsaturated and saturated fatty acids, their length, and by proportion of cyclic fatty acids (Russel 1984; Herbert 1986; Russel and Fukunaga 1990; Jones et al. 2002). Cold adaptation is related to incorporation of larger proportions of unsaturated fatty acids. Fatty acids containing one or more double bonds take a more expanded conformation than fatty acids with saturated bonds and cause less dense packing of the lipids and a higher fluidity of the membrane (Feller 2007). If the viscosity of the membrane cannot be maintained within certain limits, it may ultimately become leaky to ions at high temperatures. A second factor is the solute transport capability of the membrane (Baxter and Gibbons 1962; Rose and Evison 1965), which is dependent on its fluidity. Thirdly, the forces governing the formation of proper tertiary and quartenary structure of proteins (Jaenicke 1988) limit bacterial growth especially in the high temperature range. The first enzyme inactivated (e.g., homoserine transsuccinylase in ***Escherichia coli***; Ingraham and Marr 1996) determines $T_{max}$. Hence, the latter can be increased when the product of this critical enzyme is supplied exogenously (methionine in ***E. coli***). Several lines of evidence suggest that the inability to initiate translation and hence to synthesize protein determines the minimum temperature for growth (Ingraham and Marr 1996). Finally, an important factor determining maximum and minimum growth temperatures is the temperature sensitivity of regulatory mechanisms, which in case of malfunctioning may cause fatal imbalances in cellular metabolism.

At very high temperatures >80°C, proton circuits can no longer be maintained owing to a high proton permeability of the cytoplasmic membrane. Hyperthermophiles switch to energy coupling via $Na^+$ since the permeability of the membrane to this ion is less affected by high temperature (Lengeler et al. 1999). Also, the pattern of proteins changes significantly with growth temperatures outside the normal range. Changes that occur at higher temperatures are under the control of the heat shock response. About 20 proteins, many of them molecular chaperones or proteases, exhibit a large (10–20-fold) but transient increase in synthetic rate upon temperature upshift (Gross 1996).

In mesophilic bacteria, a specific group of 14 cold shock proteins (none of which is a heat shock protein) is produced during the period of growth cessation following a shift from 37°C to 10°C in ***E. coli***, and at least 75 proteins participate in the cold shock response of ***Bacillus subtilis*** (Graumann and Marahiel 1996). Considerable evidence suggests that the inability to synthesize protein determines the minimum temperature of growth in ***E. coli*** and that the sensitive step is the initiation of translation (Ingraham and Marr 1996; Graumann and Marahiel 1996). Additional limiting factors of growth at low temperature are the fluidity of the cytoplasmic membrane and rate of local melting of DNA by RNA polymerase (Graumann and Marahiel 1996). In psychrophiles, cell membranes tend to contain more unsaturated fatty acids and short-chain fatty acids than membranes of mesophiles (Bhakoo and Herbert 1979; Chan et al. 1971). Finally, the affinity for substrate uptake is decreased at low temperatures (Nedwell and Rutter 1994). Psychrophiles synthesize enzymes with high catalytic activities at low temperatures (Feller et al. 1994b; Trimbur et al. 1994) and produce more enzymes upon a decrease in temperature (Feller et al. 1994a). The lowest optimum temperature for an extracellular enzyme recovered from a bacterial culture was 20°C for a protease. The corresponding bacterial isolate produced the maximum amount of protease at −1°C, thereby counteracting the effects of very low temperatures on the catalytic efficiency of the enzyme (Huston et al. 2000). Chitobiase and leucine-aminopeptidase in situ even have lower $T_{opt}$ values of 15°C (Huston et al. 2000). Enhancement of the catalytic activity of psychrophilic enzymes is attributed to an increased flexibility of some of their structural components. Thus, a higher content of non-charged polar amino acids, particularly glutamine and threonine, and hydrophobic amino acids, and fewer charged residues in the solvent-accessible area were detected in psychrophilic Archaea, which probably destabilize the surface of these proteins and reduce the activation energy of the protein-substrate transition state and increase catalytic efficiency at low temperatures (Cavicchioli 2006). At the same time, these features lead to a reduction of their thermostability. Cold-active enzymes thus exhibit a pronounced heat lability and sensitivity to protein denaturants (Lonhienne et al. 2000). As another aspect of psychrophily, posttranscriptional modification of tRNA is much less pronounced in psychrophiles than in mesophiles. However, significantly higher levels of dihydrouridine occur in psychrophiles as compared to psychrotolerants or mesophiles (Dalluge et al. 1997). This latter finding has been explained by the high conformational flexibility, which is maintained by a high dihydrouridine content of tRNA molecules.

Not only the overall specific growth rate but also the physiology of a given strain changes upon changes in incubation temperature. Examples are the formation of different fermentation products (Jung et al. 1974), change in yield and maintenance coefficients (Brooke et al. 1989), altered specific extracellular xylanase activity (Suh et al. 1988), changes in the

affinity for $H_2$ and acetate consumption by ***Methanosarcina barkeri*** (Westermann et al. 1989), changes in the content of unsaturated or branched fatty acids in membrane lipids (Russel 1984; Kaneda 1991), and formation of secondary metabolites like pigments, for example, prodigiosin formed only below 30°C by ***Serratia marcescens*** (Burkhardt 1992). Thus, different enzymes in one and the same prokaryotic strain appear to exhibit considerable differences in thermostability.

Besides the increased content of unsaturated fatty acids of the cytoplasmic membrane, an increased flexibility of tRNAs, and the lower temperature optima of enzymes, bacterial adaptations to low-temperature environment also include the formation of cyroprotectants such as soluble carbohydrates and polyalcohols and the synthesis of antifreeze proteins or ice-binding proteins. These latter adaptations occur in bacteria that are frequently exposed to subzero temperatures. The sea-ice bacterium *Colwellia psychrerythraea* produces extracellular polymeric substances that were proposed to act as cryoprotectant enabling survival in brine-filled ice veins (Cavicchioli 2006). The antifreeze protein (AFP) of *Marinomonas primoryensis* lowers the freezing point of water further than any of the other known AFP of fish or insects (Garnham et al. 2008). Due to their specific tertiary structure, AFPs (in a broader sense also called "ice-structuring proteins" or "ice-binding proteins") adsorb to the surface of the ice crystals, resulting in a thermodynamically less favorable mode of ice crystal growth. AFPs often lower the freezing point, whereas the melting point remains largely unaltered (thermal hysteresis). They are highly effective and can protect cells at concentrations 200–500-fold lower than NaCl but without increasing significantly the osmotic pressure of the medium (Crevel et al. 2002). The irreversible binding of the 34-kDa bacterial AFP of *Marinomonas primoryensis* to ice crystals is enabled by the highly ordered structure of hydrogen bonds that result in an array of ice-like surface waters and by hydrophobic interactions (Garnham et al. 2011). Even if freezing is not prevented, ice-structuring proteins protect cells during freezing and thawing. They inhibit recrystallization and the formation of large ice crystals in an highly effective manner, stabilizing the initially formed small ice crystals and thereby preventing mechanical damage of the cellular envelope (Chao et al. 1996; Tomczak et al. 2003). Such ice-binding proteins have been detected in *Actinobacteria*, *Firmicutes*, *Bacteroidetes* (e.g., *Flavobacterium*), and *Proteobacteria* (*Pseudomonas*, *Marinomonas*, and *Colwelia* spp.) (Christner 2010).

If some simple procedural precautions are observed, psychrophilic microorganisms can be readily isolated from natural environments. Besides using precooled pipettes, media, diluents, etc., the inoculum should not be exposed to lethal temperatures above 20°C (room temperature). The number of known psychrophiles and psychrotolerant bacteria increases steadily (i.e., by 24 novel species in the year 2011). The isolates obtained belong to the Archaea, low G + C Gram-positive bacteria, high G + C Gram-positive bacteria, alpha-, beta-, gamma-, and delta-subclasses of the Proteobacteria (and include purple nonsulfur bacteria, methanotrophs, and sulfate-reducing bacteria), and the ***Bacteroidetes*** (Bowman et al. 1997c; Gosink and Staley 1995; Knoblauch and Jørgensen 1999; Knoblauch et al. 1999; Madigan et al. 2000a). An exemplary list covering the diversity of recently described psychrophilic bacteria is presented in *Table 7.6*.

Most temperate environments, like deeper parts of the water column of lakes and deeper soil strata, are permanently below 20°C. It therefore appears possible that bacteria in these environments are adapted to these lower temperatures. Indeed, first experiments indicate that the fraction of lake water bacteria growing in artificial liquid media reaches maximum values at 16°C (Bussmann et al. 2001). Hence, the preferred temperature used for enrichments of freshwater or marine planktonic bacteria is 15°C. Some psychrotolerant bacteria have been isolated from packed food after storage at 2–4°C for several weeks (Broda et al. 2000; Kato et al. 2000). Like psychrophilic bacteria, psychrotolerants also fall in various phylogenetic groups such as low G + C Gram-positive bacteria (including acetogens; Nozhevnikova et al. 2001), high G + C Gram-positive bacteria, Proteobacteria, Bacteroidetes, but also methanogenic archaea (Nozhevnikova et al. 2001; *Table 7.6*). Sulfate-reducing bacteria isolated from cold sediments are psychrotolerants with respect to growth rate and show a $T_{opt}$ of 18–19°C, whereas the maximum growth yield was attained at much lower temperatures of 0°C and 12°C (Isaksen and Jørgensen 1996). However, the sulfate-reducing activity of bacteria in Antarctic sediments had a temperature optimum well above the in situ temperature, exhibiting a mesophilic response (Isaksen and Jørgensen 1996). Consequently, the temperature response of a given bacterial strain cannot be judged solely on the basis of respiratory activity or growth rate alone.

## Hydrostatic Pressure

Besides temperature, osmolarity, and pH, hydrostatic pressure has been shown to directly influence the growth of prokaryotes. Hydrostatic pressure is a decisive environmental variable in the deep sea. At nearly 11,000 m, the Challenger Deep is the deepest known oceanic site where pressure values greater than 100 MPa (1,000 atm) exist. Since the oceans cover about 71% of the earth's surface at a mean depth of 3,700 m, the high-pressure, cold habitat (37 MPa, $\leq$5°C) represents the largest portion of the biosphere by volume.

As early as 1872, the Challenger expedition revealed the occurrence of living material from depths of at least 8,000 m. Twelve years later, the bacteria found in samples from the deep sea were shown to be more pressure tolerant than terrestrial species (Marquis and Matsumura 1978). These findings have now been confirmed. Barophilic microorganisms, recently also termed "piezophilic," are defined as those well adapted to growth at high pressure (ZoBell and Johnson 1949; Jannasch and Taylor 1984), and hence they exhibit optimum growth rates at elevated pressures. Barophilic prokaryotes are usually found below a depth of 2,000 m in the ocean. At high hydrostatic pressures and 2°C, doubling times as short as 7 h have been observed (Yayanos 1986). Obligate barophiles grow only at

**Table 7.6**

**Some psychrophilic and psychrotolerant bacteria described to date**

| Group | Species | Strain | References |
|---|---|---|---|
| Psychrophiles | | | |
| Archaea | *Methanogenium frigidum* | SMCC 459W$^T$ | Franzmann et al. (1997) |
| Low G + C Gram+ | *Bacillus marinus* | DSM 1297$^T$ | Ruger et al. (2000) |
| | *Sporosarcina psychrophila* | DSM 3$^T$ | Euzéby 2001 |
| High G + C Gram+ | *Alpinimonas psychrophila* | DSM 23737$^T$ | Schumann et al. (2012) |
| | *Arthrobacter psychrolactophilus* | ATCC 700733$^T$ | Loveland-Curtze et al. (1999) |
| | *Clostridium vincentii* | DSM10228$^T$ | Mountfort et al. (1997) |
| | *Cryobacterium psychrophilum* | NCIMB 2068$^T$ | Suzuki et al. (1997) |
| | *Ditzia psychralcaliphila* | NCIMB13777$^T$ | Yumoto et al. (2002) |
| | *Frigoribacterium faeni* | DSM 10309$^T$ | Kämpfer et al. (2000) |
| | *Subtercula boreus* | DSM 13056$^T$ | Mannisto et al. (2000) |
| | *Subtercula frigoramans* | DSM 13057$^T$ | Mannisto et al. (2000) |
| Bacteroidetes | *Flavobacterium frigidarium* | ATCC 700810$^T$ | Humphry et al. (2001) |
| | *Flavobacterium xueshanense* | NBRC 106479$^T$ | Dong et al. (2011) |
| | *Flavobacterium urumqiense* | NBRC 106480$^T$ | Dong et al. (2011) |
| | *Gelidibacter algens* | ACAM 536$^T$ | Bowman et al. (1997b) |
| | *Hymenobacter psychrophilus* | DSM 22290$^T$ | Zhang et al. (2011b) |
| | *Polaribacter franzmannii* | ATCC 700399$^T$ | Gosink et al. (1998) |
| | *Polaribacter filamentus* | ATCC 700397$^T$ | Gosink et al. (1998) |
| | *Polaribacter irgensii* | ATCC 700398$^T$ | Gosink et al. (1998) |
| | *Psychroflexus torquis* | ACAM623$^T$ | Bowman et al. (1998a) |
| | *Psychroserpens burtonensis* | ACAM 188$^T$ | Bowman et al. (1997b) |
| Alphaproteobacteria | *Devosia psychrophila* | DSM 22950$^T$ | Zhang et al. (2012) |
| | *Devosia glacialis* | LMG 26051$^T$ | Zhang et al. (2012) |
| | *Sphingomonas alpina* | DSM 22537$^T$ | Margesin et al. (2012) |
| Betaproteobacteria | *Glaciimonas immobilis* | DSM 23240$^T$ | Zhang et al. (2011a) |
| | *Polaromonas vacuolata* | 34-P$^T$ | Irgens et al. (1996) |
| | *Polaromonas glacialis* | DSM24062$^T$ | Margesin et al. (2011) |
| | *Polaromonas cryoconiti* | DSM 24248$^T$ | Margesin et al. (2011) |
| | *Rhodoferax antarcticus* | ATCC700587$^T$ | Madigan et al. (2000b) |
| Gammaproteobacteria | *Acinetobacter calcoaceticus* | LP009 | Pratuangdejkul and Dharmsthiti (2000) |
| | *Glaciecola pallidula* | ATCC 700757$^T$ | Bowman et al. (1998b) |
| | *Glaciecola punicea* | ATCC 700756$^T$ | Bowman et al. (1998b) |
| | *Methylosphaera hansonii* | ACAM 549$^T$ | Bowman et al. (1997a) |
| | *Moritella marina* | ATCC 15381$^T$ | Urakawa et al. (1998) |
| | *Psychrobacter pacificensis* | IFO 16270$^T$ | Maruyama et al. (2000) |
| | *Psychromonas antarcticus* | DSM 10704$^T$ | Mountfort et al. (1998) |
| | *Shewanella frigidimarina* | ATCC 700753$^T$ | Bozal et al. (2002) |
| | *Shewanella gelidimarina* | ATCC 700752$^T$ | Bowman et al. (1997c) |
| | *Shewanella livingstonensis* | LMG 19866$^T$ | Bozal et al. (2002) |
| | *Thiocapsa* sp. | Ant.Rd | Madigan (1998) |
| Deltaproteobacteria | *Desulfofaba gelida* | DSM 12344$^T$ | Knoblauch and Jørgensen (1999) |
| | *Desulfofrigus fragile* | DSM 12345$^T$ | Knoblauch and Jørgensen (1999) |
| | *Desulfofrigus oceanense* | DSM 12341$^T$ | Knoblauch and Jørgensen (1999) |
| | *Desulfotalea arctica* | DSM 12342$^T$ | Knoblauch and Jørgensen (1999) |
| | *Desulfotalea psychrophila* | DSM 12343$^T$ | Knoblauch and Jørgensen (1999) |

**Table 7.6 (continued)**

| Group | Species | Strain | References |
|---|---|---|---|
| Psychrotolerants | | | |
| Archaea | *Methanococcoides burtonii* | DSM 6242$^T$ | Franzmann et al. (1992) |
| Bacteroidetes | *Flavobacterium sinopsychrotolerans* | JCM 16398$^T$ | Xu et al. (2011) |
| | *Gelidibacter* sp. | IC158 | Nichols et al. (1999) |
| | *Pedobacter arcticus* | A12$^T$ | Zhou et al. (2012) |
| | *Psychroflexus gondwanense* | ACAM 48$^T$ | Bowman et al. (1998b) |
| Low G + C Gram+ | *Acetobacterium tundrae* | DSM 9173$^T$ | Simankova et al. (2000) |
| | *Carnobacterium funditum* | DSM 5970$^T$ | Franzmann et al. (1991) |
| | *Clostridium gasigenes* | DSM 12272$^T$ | Broda et al. (2000) |
| | *Lactobacillus bavaricus* | DSM 20269$^T$ | Euzéby (2001) |
| | *Lactobacillus algidus* | JCM 10491$^T$ | Kato et al. (2000) |
| High G + C Gram+ | *Arthrobacter flavus* | MTCC 3476$^T$ | Reddy et al. (2000) |
| | *Arthrobacter livingstonensis* | DSM 22825$^T$ | Ganzert et al. (2011a) |
| | *Arthrobacter cryotolerans* | DSM 22826$^T$ | Ganzert et al. (2011a) |
| | *Arthrobacter globiformis* | DSM 20124$^T$ | Euzéby (2001) |
| | *Brevibacterium* | NCIMB 13216 | Nedwell and Rutter (1994) |
| | *Leifsonia psychrotolerans* | DSM 22824$^T$ | Ganzert et al. (2011b) |
| | *Micrococcus agilis* | | Siebert and Hirsch (1988) |
| | *Micrococcus roseus* | | Siebert and Hirsch (1988) |
| | *Modestobacter marinus* | DSM 45201$^T$ | Xiao et al. (2011) |
| Betaproteobacteria | *Hydrogenophaga pseudoflava* | NCIMB 13215 | Nedwell and Rutter (1994) |
| Gammaproteobacteria | *Colwellia chukchiensis* | DSM 22576$^T$ | Yu et al. (2011) |
| | *Pseudomonas alcaliphila* | IAM 14884$^T$ | Yumoto et al. (2001) |

*DSM* Deutsche Sammlung von Mikroorganismen; *ATCC* American Type Culture Collection; *NCIMB* National Collections of Industrial Food and Marine Bacteria; *ACAM* Australian Collection of Antarctic Microorganisms; *IFO* Institute for Fermentation Culture Collection; *LMG* Universiteit Gent, Laboratorium voor Mikrobiologie, Gent, Belgium; *JCM* Japanese Collection of Microorganisms; *MTCC* Microbial Type Culture Collection at Institute of Microbial Technology, Chandigarh, India; *IAM* Institute of Applied Microbiology, University of Tokyo

pressures exceeding 0.1 MPa (1 atm). In studies of more than 100 bacterial strains isolated from depths between 2,000 and 7,000 m, obligate barophile has been detected only in isolates from ≥6,350 m (Yayanos 1986). The maximum pressure permitting growth of an obligate barophile was determined as 115 MPa (Deming et al. 1988). Some barophilic bacteria grow at pressures >100 MPa (in one case even 130 MPa, a value reached nowhere in the ocean; Yayanos 1986). Many abyssal and hadal prokaryotes were shown to be barophilic (Yayanos 1986). On the contrary, barotolerant microorganisms, which are also abundant at great depth, grow fastest at 0.1 MPa and more slowly as hydrostatic pressure is increased. Barophilic bacteria isolated from the deep sea are also psychrophiles ($T_{opt}$ 8–10°C). However, the maximum temperature of growth ($T_{max}$) is higher at high pressure (Yayanos 1986). On the other hand, barophilic bacteria show barotolerant properties if cultured not at 10–15°C, but at 4°C, which corresponds to the actual temperature of their environment, and decreasing substrate concentrations induce a more efficient barophilic response in certain deep-sea psychrophiles (Wirsen and Molyneaux 1999).

Pressure-retaining devices have been designed which allow the sampling of barophilic prokaryotes without decompression (Jannasch et al. 1976; Yayanos 1978). Phylogenetically, many barophilic bacteria fall in the gamma-subclass of Proteobacteria (DeLong et al. 1997). The major genera of cultivated barophiles include the γ-proteobacterial genera ***Shewanella***, ***Photobacterium***, ***Colwellia***, and ***Moritella***. In addition, members of other physiological and phylogenetic groups have been described, for example, the sulfate-reducing ***Desulfovibrio profundus*** (Bale et al. 1997). Most characterized barophiles are thus closely related to shallow-water marine bacteria. Most of the prokaryotes isolated are also psychrophiles, and some are capable of growing at very low nutrient concentrations (Deming and Colwell 1985; Deming 1986). The latter observation indicates that barophiles are not confined to nutrient-rich niches, such as fecal pellets and inside higher organisms, but are also found free in the water column and in the sediment. Since at least some barophilic prokaryotes are oligotrophs, high-pressure continuous culture techniques had to be developed which now permit a study of barophilic prokaryotes at pressures of up to

71 MPa and at low and precisely controlled nutrient concentrations (Jannasch et al. 1996; Wirsen and Molyneaux 1999).

High pressures affect different aspects of cell structure and metabolism, such as membrane structure, transcription, translation, and the quarternary structure of enzymes, and hence their activity (Marquis 1976; Marquis and Matsumura 1978; MacDonald 1984; DeLong and Yayamos 1986; Morita 1986; Wirsen et al. 1987; Jaenicke 1988; Somero 1992; Welch et al. 1993). On the basis of direction of the change in molecular volume, biochemical reactions can be slowed down or accelerated. In fact, increased hydrostatic pressure can accelerate the fructose bisphosphate reaction in a barophilic organism, while decreasing it in a non-barophilic one (Hochachka et al. 1972). However, most of the biological reactions are slowed down at pressures of 30 MPa or more (Ingraham and Marr 1996). ***Escherichia coli*** is moderately barotolerant and withstands a maximum pressure of 56 MPa in complex medium, whereas strains of *Lactococcus lactis* subsp. *cremoris* are unaffected by 200 MPa and inactive at pressures as high as 400–800 MPa (Malone et al. 2002). Five MPa cause a detectable decrease in growth rate. The pressure sensitive steps are polysome formation (Schulz et al. 1976) and translocation. Genetic changes in ribosome structure can increase the barotolerance of ***E. coli*** (Ingraham and Marr 1996).

Several molecular adaptations are thought to be required for barophily. High pressure and low temperature in the deep-sea habitat affect the physicochemical properties of the cytoplasmic membrane by tighter packing and by restricting the rotational motion of acyl chains. Changes in membrane fluidity (which is negatively influenced by high pressures) are known to occur through changes in the ratio of unsaturated over saturated fatty acids in membrane phospholipids (MacDonald 1984; DeLong and Yayamos 1986; Wirsen et al. 1987), similarly to responses to temperature changes. Many piezophiles in the deep sea contain high proportions of unsaturated fatty acids in the cytoplasmic membrane. In some bacteria, these proportions increase concomitantly with increasing growth pressure. Omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) are characteristics of piezophilic and psychrophilic bacteria. In the psychrophilic piezophile *Shewanella violacea* DSS12 that was isolated from a water depth of 5,110 m, eicosapentaenoic acid prevents the cytoplasmic membrane from becoming hyperfluid and largely maintains membrane stability against changes in hydrostatic pressure (Usui et al. 2012). This permits *S. violacea* to grow optimally at 30 MPa.

The barophilic bacterium ***Photobacterium profundum*** strain SS9 preferentially synthesizes a 37-kDa protein, designated OmpH in response to an elevated pressure of 290 atm. (29 MPa). On the basis of amino acid sequence comparison, OmpH is an outer membrane porin and possibly especially adapted to a high pressure (Bartlett et al. 1989), and it may represent a member of a high-pressure regulon (Bartlett and Welch 1995). Control of the abundance of OmpH is probably regulated at the transcriptional level. Unusual putative regulatory sequences have been found upstream of the *ompH* gene (Bartlett et al. 1993; Bartlett and Welch 1995). In addition to hydrostatic pressure, OmpH is induced by carbon starvation and subject to catabolite control (Bartlett and Welch 1995). Additional pressure-regulated operons have been identified, and for instance comprise a gene involved in the assembly of the cytochrome *bd* complex (Li et al. 1998). Alternative RNA polymerase σ factors (e.g., the *rpoE* gene product) and modifiers are involved in genetic regulation by hydrostatic pressure (Chi and Bartlett 1995; Nakasone et al. 1998).

High hydrostatic pressures also affect the bacterial cytoskeleton. Cells of *E. coli* form filaments and fail to produce septa for cell division at 40 MPa, indicating that cell division is directly affected under these conditions. Based on evidence from in vitro experiments, it has been suggested that high hydrostatic pressure results in dissociation of the bacterial cytoskeleton and growth of the cells (Ishii et al. 2004). Finally, hydrostatic pressure appears to activate cell wall hydrolase activity or to increase cell wall accessibility to the enzyme (Malone et al. 2002).

## Treatment of Growth Media and Equipment

### Types of Culture Media

Conventional bacteriological culture media are provided as either liquid broths or solid media. Liquid media are used in studies of growth and metabolism in which homogenous media conditions are mandatory. Usually, optical density can be followed easily in liquid media and subsamples for the analysis of substrates and metabolic products can be withdrawn. In addition, many bacteria, especially from planktonic samples, do not appear to grow on solid media and have to be isolated in liquid dilution series. To maintain an adequate supply of oxygen, cultures of aerobic prokaryotes need to be shaken vigorously, leaving a large head space (gas phase volume).

Solid media were originally designed for the enumeration and isolation of bacteria but are now also used routinely for general culture work. On solid media, colony morphology and other properties (such as swarming over the agar surface) can be easily observed. Extracellular enzymes originating from the cells but diffusing into the surrounding noncolonized media can be detected as a result of their action on insoluble substrates (cellulose, starch, and lipid emulsions), which can be maintained evenly distributed in solid as opposed to liquid media. Hydrolysis of starch can be visualized after flooding plates with iodine, since only the intact starch molecules produce the characteristic deep-blue to purple color. Lipids can be stained with Sudan black. Furthermore, the effects of antibiotic substances on colony growth can be tested on solid media. Solidifying agents include agar, Gelrite, and silica gel. Agar is used most frequently and is a sulfated polygalactan (D-galactose and 3,6-anhydrogalactose linked by 1 → 3 and 1 → 4 bonds) produced by marine red algae of the genera ***Gelidium***, ***Pterocladia***, and ***Gracilaria***. This polymer is degraded by only very few bacteria such as some ***Cytophaga***, ***Pseudomonas***, and

***Vibrio*** spp. Unusual is the large difference between the temperatures for melting (~100°C) and solidification (~40°C). Consequently, many temperature-sensitive constituents of the media, for example, vitamins, may be added at temperatures slightly above 40°C. Agar-containing media should not be adjusted to pH values <6.0 before sterilization, because the agar may be hydrolyzed. If lower pH values are required, the adjustment should be done by aseptic addition of acid after heat treatment or, alternatively, solid silica gel media should be used. Agar may contain variable amounts of impurities such as Ca, Mg, and other minerals (Bromke and Hammel 1987). One means of reducing soluble nonpolymeric contaminants is the repeated washing in distilled water (e.g., five times in double-distilled water, using 300% of the final volume; Widdel and Bak 1992). Alternatively, agar has been purified by subsequent extraction with acetone and ethanol. For direct plating of oligotrophs from the marine environment and if even the washed agar inhibits growth, a glass filter may be used as a substitute.

Various oligotrophic bacteria have been found to be unable to grow on agar-containing media (Giovannoni and Stingl 2007; Dedysh 2011). Gellan gum (or Gelrite, Phytagel, Sigma-Aldrich) is another alternative for solidification of microbial media. Harris (1985) proposed gellan gum as an agar substitute since agar at high concentrations may be toxic to methanogens. Gellan gum (which is produced by strains of ***Sphingomonas*** spp.) is an anionic acidic heteropolysaccharide consisting of glucose, glucuronic acid, and rhamnose that overcomes some of the toxic effects that agar has on some groups of microorganisms (Ferris et al. 1996; Liesack et al. 1997) and starts to solidify upon addition of $Ca^{++}$; the concentration of divalent cations influences gel strength and solidification temperature (Bast 2001). However, the polymerization of gellan gum requires the addition of substantial concentrations of $Ca^{++}$ that potentially inhibit growth of bacteria adapted to oligotrophic conditions. The effect of gellan gum does not seem to be restricted to oligotrophic bacteria, however. Thus, substituting agar with gellan gum resulted in a significantly improved cultivation success of bacteria from a eutrophic freshwater sediment (Tamaki et al. 2005). Most notably, gellan gum has recently been employed simultaneously as the sole growth substrate for the isolation of *Chthonomonas calidirosea*, a novel member of the Armatimonadetes (the former OP10 phylum) (Lee et al. 2011).

Silica gel media have been developed for use when solid media free of any organic contamination are needed, low pH is desired, or agar-degrading microorganisms are to be cultivated (Funk and Krulwich 1964; Bast 2001).

Media may affect the growth of microorganisms nonselectively or selectively. Selective media favor the growth of only some bacteria by the inclusion of a particular substrate as a carbon/energy source, by the presence or absence of specific nutrients (e.g., nitrogen or vitamins), or by the presence of compounds with differential toxicity. Inhibitory compounds include (1) antibiotics with rifampicin (2.4), kanamycin (5.0), erythromycin (50), penicillin (50), tetracycline (100), and fosfomycin (100 [maximum concentrations in $mg \cdot L^{-1}$]) acting against bacteria, and cycloheximide (100), tunicamycin (0.25), colchicine (20), or cordycepin (25) inhibiting eukarya; (2) dyes (such as crystal violet, methylene blue, or brilliant green) inhibit many Gram-positive bacteria; (3) high concentrations of glycine and LiCl, which permit the growth of many staphylococci but not that of physiologically similar other bacteria; (4) bile salts, which permit the growth of enteric bacteria but not that of many other bacteria; and (5) bromoethane sulfonate (BES) for inhibition of methanogenic archaea, or sodium molybdate for the inhibition of sulfate-reducing bacteria. However, the inhibitory effects vary among different phylogenetic groups of bacteria. Despite their alleged broad range action, certain antibiotics can thus be used to selectively enrich particular phylogenetic groups of bacteria. Quite early, it was noted that kanamycin favors the enrichment of Flavobacteria (Flint 1985) and rifampicin is routinely used to enrich for spirochetes (Stanton and Canale-Parola 1979). Recent examples for selective cultivation of novel types of bacteria include the isolation of a fermentative spirochete from sulfur mats in the presence of rifampicin (Dubinina et al. 2011), the isolation of the phylogenetically deeply branching betaproteobacterium *Parasutterella secunda* in oxacillin-containing media (Morotomi et al. 2011), and the isolation of *Fibrisoma limi*, a novel type of Bacteroidetes, as well as two novel types of thermophilic aerobic heterotrophs of the phylum *Chloroflexi* in the presence of kanamycin (Filippini et al. 2011; Yabe et al. 2011). Similarly, lithium toxicity varies among microorganisms and has been added for the selective growth of *Bifidobacterium* spp. (Lapierre et al. 1992).

Selective media are especially used for the isolation, detection, and recognition of pathogenic bacteria from mixed cultures but are equally important for the isolation of selected groups of slow-growing bacteria from environmental samples. In a strict sense, however, all media are at least slightly selective.

Differentiating media are designed to distinguish one type of microorganism from another in a mixed culture. A differentiating medium contains a special ingredient that changes during growth of a certain type of bacterium. They are designed to differentiate between morphologically or physiologically similar microorganisms (e.g., hemolytic reaction on blood agar, urease, production of acetoin, and reduction of tellurite). Differentiating media can be selective or nonselective.

Assay media are used for the quantification of organic substances such as vitamins, amino acids, and growth factors in bioassays, in which the growth response of a certain organism requiring the factor is directly proportional to the concentration of the factor under investigation.

## Media Preparation and Sterilization

Heat-labile supplements such as serum, vitamins, or growth factors (e.g., freshly prepared yeast extract or fermented rumen fluid) are added to the basal medium after sterilization to avoid deterioration. This is also a recommended practice for compounds that might react with other medium ingredients during

autoclaving. Glucose and other sugars, when autoclaved with salts such as phosphate, may form inhibitory sugar phosphates. The carbonyl groups of reducing sugars react with free amino groups of primary amines and may result in the formation of toxic Maillard reaction (or "browning" reaction) products such as furfurals or furaldehyde. Oxygen or oxidation products accelerate Maillard reactions. Reducing agents such as cysteine and sulfide will be oxidized by other medium ingredients during autoclaving and form toxic radicals (Carlsson et al. 1979; Cypionka et al. 1985) and thus have to be added separately afterward. The formation of mineral precipitates (e.g., struvite or $MgNH_4PO_4$; Schink et al. 2002) can often be avoided by separate sterilization of solutions of the calcium, magnesium, and/or iron salts, which are added to the cooled medium. Addition of chelating agents such as EDTA helps to prevent the precipitation in some cases. In many instances, the microelements are present in adequate amounts as contaminants of the mineral salts used in media or as contaminants of glassware and water. In several cases, however, microelements need to be added separately to the growth media. Dispensing of media is usually carried out after cooling to below 50°C to avoid condensation.

Treatments can be distinguished on the basis of their effect on total and viable cell numbers with exposure time. Bacteriostatic substances inhibit growth of bacterial cells, while total cell numbers and the viable cell count (determined, e.g., by plating of cells after diluting out the inhibitory substance) remain constant. Bactericidal compounds lead to a decrease, hence an irreversible damage, of viable cells, while total cell numbers remain constant. Finally, bacteriolytic compounds lead to a decrease in viable as well as total cell numbers owing to prokaryotic cell lysis.

Sterilization—the complete inactivation or removal of microorganisms—is achieved by applying heat or irradiation (physical methods) or by treatment with toxic compounds and gases (chemical methods). Gases and liquids can also be sterilized by filtration through filters with extremely small pores (preferably $\leq$0.1 μm). Disinfection is a procedure that results in the inactivation of only a fraction of the microbial population from an object or from a culture and is most often used to inactivate pathogens. When a pure culture is exposed to a lethal agent, the kinetics of death are exponential, that is, when the logarithm of the number of survivors (usually determined by counting colony-forming units on a suitable medium) is plotted against time, a straight line is obtained whose downward slope is called the "death rate." The time course of killing is described by the *D*-value, which gives the decimal reduction time, that is, the time it takes for a tenfold reduction in the microbial population at a particular temperature. The *Z*-value is the number of degrees that the temperature must be raised to reduce the *D*-value tenfold, hence

$$Z = \frac{T_1 - T_2}{\log D_1 - \log D_2} \qquad (7.19)$$

The actual number of survivors is then determined by the initial size of the population and the death rate. Differences exist among microbial species in their resistance to heat, radiation, and other treatments. Hence, sterilization procedures need to be designed to kill the most resistant forms of life, namely, the endospores of Gram-positive bacteria. Of the latter, ***Moorella thermoacetica*** forms unusually heat-resistant endospores which reach decimal reduction times of up to 111 min at 121°C (Byrer et al. 2000). Consequently, purified suspensions of bacterial endospores are used as indicators for the effectiveness of the sterilization process. Routine sterilization procedures are designed to provide a wide margin so that the chance of having even a single survivor is less than one in a million.

Direct heat, dry heat, and moist heat are the three most common methods of sterilization. For sterilization by direct heat, objects are exposed to an open flame, and the adhering microorganisms are quickly burned. Small equipment such as inoculating needles and loops, forceps, open ends of culture tubes, and Pasteur pipettes are routinely flame-sterilized. In addition, combustion is the method of choice for destruction of disposables and contaminated wound dressings in hospitals. When employing a Bunsen burner for sterilization, it has to be kept in mind that air supply must be regulated such that the flame generated is completely blue (not yellow-orange) and that the point where the highest temperature is reached is found atop of the inner (dark-blue) cone within the flame. A yellow color of the flame indicates the presence of sooty particles, hence incomplete combustion and lower temperatures. Dry heat is used to sterilize empty glassware and other heat-resistant objects such as laboratory instruments, surgical tools, glass syringes, needles, mineral oils, and dry powders of heat-stable substances and involves baking at 170°C for 2 h in a hot-air oven. The objects should be protected from subsequent contamination by wrapping them in aluminum foil prior to sterilization.

Obviously, dry sterilization cannot be used to sterilize liquids, which would boil at temperatures above 100°C at atmospheric pressure. Also, the method is not suited for the sterilization of heat-sensitive materials such as cotton wool, plastics, and rubber. In addition, heat conduction is less rapid in moist air, and dried bacterial cells and spores intrinsically have a higher heat resistance than wet cells and wet spores, which makes sterilization by dry heat a more time-consuming process. Therefore, moist heat is the most effective and most commonly used method for sterilization. Autoclaving denotes a heat treatment with a water-saturated atmosphere under pressure. In principle, the autoclave represents a type of pressure cooker in which a pressure of 1 atm. and a temperature of 121°C are reached simply by heating or by inflow of preformed pressurized steam. Only if the autoclave chamber is completely filled with steam can a temperature of 121°C be reached. This temperature, provided a sufficient exposure time is chosen, is sufficient to kill even bacterial endospores. Therefore, the temperature has to be monitored when checking the reliability of the sterilization procedure. As a rule of thumb, 50 min of autoclaving are sufficient to sterilize 1 L of liquid even when contaminated with endospore-forming bacteria (but compare ***Moorella thermoacetica***). After sterilization, the steam is allowed to

escape slowly to prevent boiling of liquids, which would otherwise occur upon a sudden drop in pressure.

Pasteurization is a mild treatment with moist heat employed to control spoilage of food products and to extend their shelf life without significant decay of heat-labile constituents such as vitamins. One treatment is the low-temperature-long-time (LTLT) procedure in which, for example, milk is heated for 30 min at 63°C. In the more frequently used high-temperature-short-time (HTST) or flash procedure, heating lasts for 20 s at a temperature of 72°C followed by rapid cooling to minimize undesirable changes in taste and nutrient content. Pasteurization reduces the number of viable cells by between 97% and 99% and is intended to eliminate pathogens. It was introduced by L. Pasteur to control the spoilage of wine. However, even vegetative cells of certain non-spore-forming bacterial species, such as ***Microbacterium lacticum***, ***Enterococcus*** spp., and ***Coxiella burnetii***, are capable of surviving pasteurization and lead to subsequent food spoilage.

Filtration is the method of choice for the sterilization of heat-sensitive liquids and gases, which are passed through filter material with pores small enough to retain the microorganisms. Membrane filters consisting of mixed cellulose esters, polycarbonate, polytetrafluoroethylene bonded to polyethylene, or polypropylene are most frequently used for sterilization of liquids. Pore size of membrane filters is precisely determined during manufacture, and different pore sizes between 0.05 and 12 μm are currently available. Passage time is inversely related to pore-size diameter, and permeability can be affected by the chemical or electrostatic properties of the filtrate. In some instances (such as natural water samples containing larger concentrations of dissolved organic matter present in colloidal form), filters will be rapidly clogged. The application of prefilters with larger pore sizes will reduce clogging of the membrane. Certain prokaryotes, such as ***Flexibacter*** (Little et al. 1987), mycoplasmas, or spirochetes, may pass membrane filters. For safe sterilization, 0.1-μm-pore-size membrane filters should be employed since the cells of some prokaryotes are known to have diameters of around 0.2 μm. In most cases, viruses cannot be removed by filtration, however. Depth filters clog less rapidly and are therefore frequently used for the clarification of liquids. Depth filters consist of a matrix of randomly oriented fibers bound together in a tortuous maze of flow channels. However, depth filters differ considerably from membrane filters since organisms are trapped within the matrix and gradually go through and hence eventually contaminate the filtrate. This is not the case for dry (sterilized) cotton plugs which therefore can be employed for the sterilization of gases. Also, high-efficiency particulate air filters (HEPA) have become available commercially for the filtration of large volumes of air, for example, to supply clean rooms or laminar flow cabinets. Also, glass pipettes plugged with cotton wool or plastic pipette tips equipped with sterile filters should be used in the laboratory, especially where dealing with medically important bacteria.

Chemical sterilization is the method of choice for sterilizing solid objects that cannot be treated without damage by physical methods, such as certain disposable plasticware, plastic tubing, surgical supplies (sponges), optical equipment, and samples of plant material (if sterile seeds are required; Shockey and Dehority 1989). While polypropylene plasticware can be autoclaved without problems, polyethylene polymers, polystyrole, and some polysulfonates or polyfluoroethylene materials are more temperature sensitive. Microorganisms are killed by exposing them to toxic chemicals, mostly propylene oxide, β-propiolactone, or the most widely used ethylene oxide (EtO). The latter is an alkylating agent that reacts with hydroxyl-, sulfhydryl, and amino groups in proteins and nucleic acids. Ethylene oxide volatilizes above 10.8°C, and sterilization involves an exposure of materials for at least 4 h to EtO gas in a closed chamber, after which they must be thoroughly flushed for 8–12 h with sterile inert gas or air. EtO is explosive, flammable, causes skin burns, and is highly toxic. Therefore, nonflammable mixtures of EtO and freon or carbon dioxide with the same microbicidal activity as EtO alone are in use.

For decontamination of laboratory surfaces, clear phenolics and hypochlorites (3%) are most commonly used, but alcohols (ethanol at a final concentration of 70%) or mixtures of alcohols and formaldehyde and iodophores are most effective against spores.

Ionizing radiation is employed in large laboratories for the sterilization of heat-sensitive solid objects such as powdered pharmaceuticals, disposable plastics, or clothing. Because it has the power of penetrating solids, radiation is also used to retard or eliminate spoilage of foods (Murray 1989). Gamma radiation emitted by a radioactive cobalt source is most commonly applied. A high dose of 2.5 Mrad is sufficient to kill microorganisms, spores, and viruses, but chemical changes in media are possible. Gamma rays interact with water molecules to produce ions ($OH^-$) and free radicals ($OH^\bullet$) that can significantly alter and destroy many different biomolecules in the cell.

## Handling of Glassware and Equipment

An important element of the handling of glassware is its proper cleaning. New glassware needs special treatment for the removal of free alkali. Detergents alone often are not sufficient to remove adsorbed compounds that may later inhibit growth especially of fastidious bacteria from natural bacterioplankton. Soaking in 0.2 N HCl often alleviates these problems. Detergents such as Mucasol® have been found to aid in proper cleaning of glassware. Glassware should be rinsed thoroughly with bidistilled water after treatment with detergents, since even traces of the latter are inhibitory to certain bacteria such as certain cyanobacteria or marine oligotrophs (Gottschal et al. 1991).

In extreme cases, in which prokaryotes from oligotrophic environments are to be isolated, it is necessary to rigorously clean water samplers, storage containers, and culture vessels with acid followed by repeated washings with ultrapure deionized or double quartz-distilled water (Waterbury 1991). Soaking in appropriate detergent for 1 week and subsequent rinsing with especially clean water followed by 1 week of soaking in 0.5 N HCl and repeated washing with clean water has been

recommended for the culturing of cyanobacteria (Waterbury 1991). At least in some cases, such as obligate oligotrophic marine heterotrophs or cyanobacteria of the genera ***Prochlorococcus*** or ***Trichodesmium***, special precautions against traces of organic or inorganic contaminants are mandatory (Chisholm et al. 1992; Orcutt et al. 2002; Giovannoni and Stingl 2007). Non-glass systems consisting of Teflon® vessels are used in which natural seawater is sterilized by gentle tyndallization in a microwave. Furthermore, Millipore Q water is employed for dilution and cultures grown in Nalgene polycarbonate flasks.

Because of the potential danger involved in working with infectious microorganisms, irrespective of their known pathogenic nature, the requirements for sterile working conditions and safe handling of contaminated glassware and equipment should be rigid. All used glassware and other materials should be autoclaved first before unloading in the wash-up room.

Sterile rooms and sterile cabinets supplied with gas, electricity, sterile air, and ultraviolet (UV) irradiation are useful, because they considerably reduce the possibility of air contamination. For the sterilization of inoculation rooms and other work areas, UV irradiation (wavelength, 260 nm) is often applied. Laminar flow (clean air) cabinets are designed to provide a work area that is protected from the environment and are useful for preventing airborne contamination when handling sterile media, such as during aseptically dispensing sterile fluids and culture media. Filtered air is passed in a vertical or horizontal unidirectional (laminar) flow through the cabinet. This air is made sterile by filtering through high-efficiency air filters that can remove particles down to 0.3 µm. In one type of cabinet, the flow of filtered air is directed toward the front. In a second type of cabinet, the flow occurs vertically downward, forming a curtain of sterile air and is subsequently in part recirculated. For decontamination of laminar flow hoods, 3% hypochloric acid can be used. Laminar flow hoods are certified for handling microorganisms of different hazard classes. For handling bacteria from natural samples and with unknown properties, safety class II cabinets should be used.

### Removal and Exclusion of $O_2$, Cultivation of Anaerobes

The fundamental methodology for the cultivation of anaerobic prokaryotes is the Hungate technique (Stewart and Bryant 1988; Widdel and Bak 1992). Modifications of the original Hungate technique include the use of butyl rubber stoppers (Hungate 1966) and of serum bottles closed with crimp-closure aluminum seals holding butyl rubber stoppers (Miller and Wolin 1974), the syringe technique (Macy et al. 1972), and the use of pressurized tubes and vessels for the culture of methanogens (Balch and Wolfe 1976). Usually, anoxic media contain a bicarbonate-$CO_2$ buffer system (*Table 7.5*), mineral salts, and a reducing agent. Depending on the requirements of the prokaryotes to be cultivated, clarified rumen fluid or low concentrations of yeast extract are added. Resazurin can be used as a redox indicator, provided it does not inhibit growth of the prokaryotes as in the case of many phototrophic sulfur bacteria.

Reductants frequently employed are sulfide or cysteine. A sulfur-free reducing agent is titanium (III) citrate. Owing to complex formation between titanium and citrate, $Ti(OH)_3$-precipitate formation is minimized (Zehnder and Wuhrman 1976). The solution can be prepared by adding 5 mL of a 15% (w/v) titanium (III) chloride solution to 50 mL of a 0.2 M sodium citrate solution, followed by neutralization with saturated sodium carbonate. Moench and Zeikus (1983) reported an easy method for preparing this reductant with the use of nitriloacetic acid (NTA) instead of citrate as the complexing agent. However, NTA inhibits growth of certain prokaryotes. Furthermore, titanium (III) citrate may be inhibitory to anaerobic prokaryotes at low growth rates (Wachenheim and Hespell 1984). In cases where organic reducing agents are to be avoided and free sulfide is toxic to the prokaryotes, amorphous ferrous sulfide has been employed as a reducing agent. This reductant can be easily prepared in the laboratory and reacts much more rapidly with $O_2$ than does either soluble sulfide or cysteine (Brock and O'Dea 1977). After preparation, media are dispensed anoxically as described by Widdel and Bak (1992).

Three-electrode poised-potential amperometric culture systems have been developed which consist of a platinum counter electrode, a platinum working electrode, and an AgCl-Ag reference electrode connected to a potentiostat (Emde et al. 1989; Emde and Schink 1990; Ohmura et al. 2002). These systems can be used to grow anaerobic prokaryotes at a carefully controlled constant redox potential despite continuing redox reactions and flow of electrons to the working electrode. During growth experiments, the redox potential of the growth medium and the electron flow between working and counter electrodes can be recorded.

Even fastidious anaerobic prokaryotes such as methanogenic archaea can now be cultivated on solid agar media, if anaerobic chambers are employed (Leedle and Hespell 1980). This adaptation of the technique for anaerobes permits the application of replica-plating techniques to ecological and genetic studies of bacterial populations from anaerobic habitats. Sufficient $CO_2$ (10–20%) should be present in the gas phase to maintain the pH of the medium if a bicarbonate buffer system is used.

## Culture Systems

### Batch Culture

The typical sequence of lag phase, exponential phase, and stationary phase (see section "Prokaryotic Growth") is observed when prokaryotes are grown in batch culture. In this most frequently employed culture system, cells grow suspended in a medium containing sufficient carbon and energy sources and other required nutrients to allow growth at maximum rate for a limited period of time. Since fresh supply of essential components and removal of metabolic waste products do not occur after inoculation, batch cultures are closed systems, and cells grow in a continuously changing environment, which shifts from a nonlimiting supply of nutrients to conditions of starvation. Because of its relative simplicity and ease of operation, the

batch culture is nevertheless most widely used as a routine procedure for propagation of bacteria both in research and industry. Various degrees of control can be obtained by regulating important parameters such as pH, temperature, and oxygen tension.

A high-throughput variant of the batch culture technique in combination with high-throughput screening has recently become popular to recover novel types of bacteria from the natural environment, particularly aquatic oligotrophs (see section ❯ Low Nutrient Concentrations, "Oligotrophic Bacteria," and "Ultramicrobacteria"). In the "dilution-to-extinction" approach, a large number of small volumes of appropriate media are prepared in microtiter plates. As another measure to generate a large number of positive cultures, each well is inoculated with 1–5 cells instead of preparing dilution across the microtiter plates (Connon and Giovannoni 2002). Inoculation of the wells can be automated by use of, for example, the Microdrop® pipetting robot (Bruns et al. 2003a). Instead of monitoring growth by classical approaches such as turbidity measurements, membrane arrays are prepared from cultures and bacterial cells are detected by fluorescence microscopy (Connon and Giovannoni 2002). This provides a much higher sensitivity ($10^3$ cell $mL^{-1}$) for the detection of low-density oligotrophic cultures than standard optical density measurements (typically $10^5$–$10^6$ cells $mL^{-1}$). If the cultivation success in the particular growth medium is lower (i.e., 1–10%), the wells of microtiter plates can be inoculated with about 50–200 cells to yield a sufficiently large number of positive cultures (Gich et al. 2005). Cultivation success can be calculated from the number of dividing cells and the total number of cells inoculated (Bruns et al. 2003a) (see section ❯ "Isolation of Prokaryotes"). Inoculating a larger number of cells also enables microbial interactions to be established which in some cases leads to the enrichment of additional and previously uncultured bacterial types that depend on accompanying microorganisms.

## Fed-Batch Culture

A further elaboration of the batch cultivation technique was developed especially for certain industrial fermentations (Yoshida et al. 1973; Pirt 1974, 1975). In fed-batch cultures, a continuous supply of fresh medium is fed to a batch culture as soon as the substrate concentrations drop to low (sometimes growth-limiting) levels. As a result, prokaryotic cells continue to grow. Since no medium is allowed to flow out, the culture volume and cell biomass increase. At the same time, the ratio of biomass per amount of nutrients entering the vessel increases such that nutrient limitation becomes more severe over time and the microbial growth rate decreases continuously. Ultimately, fed-batch culture systems provide the means to largely extend the transition between the exponential and stationary growth phases. For some industrial fermentations, these very conditions have proven vital for optimal production of metabolites, for example, citric acids, penicillin, some enzymes, and alcohols (Yoshida et al. 1973; Esener et al. 1981; Cleland and Enfors 1983; Gond et al. 1986; Dawson et al. 1988; Kole et al. 1988). Especially for industrial fermentations, the possibility of turning a fed-batch culture into a "repeated fed-batch culture" by withdrawing part of the culture volume at regular time intervals allows the productive growth phase in principle to be extended indefinitely. The major difference with truly continuous culture systems remains that the volume is not kept constant, thus introducing permanent transience in growth rate, which is possibly essential for certain metabolic activities.

## Recycling Fermenter, Dialysis Culture, Retentostat, and Recyclostat

To overcome the problem of permanently changing volumes and concentrations of metabolic end products, the recycling fermenter was introduced. In this type of fermenter, either complete or partial recycling of the biomass is accomplished, but the culture liquid is continuously replenished with fresh medium and removed at the same rate. This is achieved by a cross-flow type of external membrane that retains prokaryotic cells while allowing spent medium to leave the fermenter (Müller and Babel 1996; Ahn et al. 2001). Typically, cross-flow dialysis membranes with large total membrane areas together with peristaltic pumps are employed for efficient and rapid filtration without clogging of the membrane (Pörtner and Märkl 1998). This type of fermenter has proven an especially valuable tool to obtain cultures of high cell density since inhibitory metabolic products such as short-chain fatty acids or alcohols are removed. Another important use is to study growth rate dependence of microbial metabolism at very long generation times (i.e., very low specific growth rates; Chesbro et al. 1979; van Verseveld et al. 1984; Chesbro 1988; Bulthuis et al. 1989; Müller and Babel 1996). Under natural conditions, the transition from exponential to stationary phase is expected to occur much more gradually than in conventional batch cultures (Mason and Egli 1993). Hence, physiological properties, such as maintenance energy requirements and resuscitation, of cells at extreme substrate limitation can be studied best in retentostats (Mason and Egli 1993; Tappe et al. 1996, 1999). A very simple means of prolonging the transition time between the exponential and stationary phase is through biphasic culture. In this type of culture, a bottom gel layer containing 4% agar is overlain by liquid medium. This arrangement results in a rapid exponential growth based on the nutrients in the liquid, and a subsequent prolonged deceleration phase of growth when cells utilize substrates slowly diffusing out of the agar layer (Chesbro 1988).

## Chemostat

The main difference between continuous culture and other batch-type cultures is that it is a typical open system in which fresh nutrient medium is added at a constant rate to a well-mixed culture while the volume is kept constant through an overflow device (❯ *Fig. 7.4*). In general, this culture system is

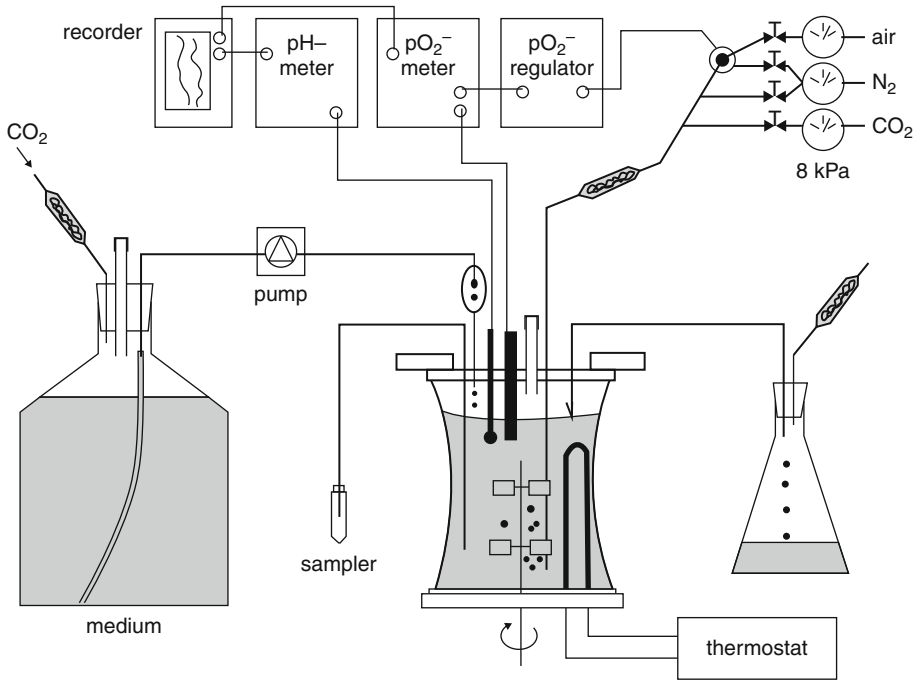


**Fig. 7.4**

**Example for a chemostat arrangement designed for the continuous cultivation of anaerobic or microaerophilic bacteria. The partial pressure of oxygen can be set to any value between 0% and 21%. A simple regulation of pH is possible by manually controlling the $CO_2$ flow into the chemostat culture with a high precision needle valve and using a $CO_2$/bicarbonate buffering system (From Overmann and Pfennig 1992)**

designed to provide a culture growing permanently in an exponential fashion at a constant submaximum growth rate. The growth rate is dictated by the rate at which the limiting nutrient is fed to the culture. In this type of arrangement, the continuous culture is termed "a chemostat." In continuous culture approaches with a chemostat, the continuously changing conditions characteristic of a batch culture are eliminated (Veldkamp and Kuenen 1973), and relatively large populations of prokaryotic cells with constant physiological state can be maintained in the presence of low concentrations of a limiting nutrient, which resemble those under natural conditions. It has to be acknowledged, however, that in certain natural systems, nutrient supply and cell removal are not closely linked; these environments therefore do not exhibit steady-state conditions (Wirsen and Molyneaux 1999). Chemostats offer a tremendously powerful tool for studying the physiology and ecology of prokaryotes. Its characteristics render the chemostat one of the most widely used culturing devices for studying microbial metabolism under carefully controlled environmental conditions in both pure and mixed cultures (Tempest 1970; Veldkamp 1977; Tempest and Neyssel 1978; Matin 1981; Kuenen and Harder 1982; Gottschal 1986, 1990; Gottschal and Dijkhuizen 1988; Overmann and Pfennig 1992; Van den Ende et al. 1996, 1997).

In a chemostat, growth of prokaryotes is determined by the dilution rate $D$, defined as the rate of nutrient supply $F$ (dimension: volume per time) divided by the volume of the culture vessel $V$, and by the concentration of the limiting nutrient in the reservoir medium $S_R$ (Monod 1950; Novick and Szilard 1950; Herbert et al. 1956; Tempest 1970; Pirt 1975; Calcott 1981; Gottschal 1990). The combined effect of growth and dilution by the inflowing medium will eventually result in a steady state in which no further change in biomass concentration $X$ occurs:

$$\frac{dX}{dt} = (\mu - D) \cdot X = 0 \tag{7.20}$$

At this point, the specific growth rate exactly balances the dilution rate, and therefore a chosen rate of culture dilution fixes the specific growth rate of the culture at a value below $\mu_{max}$. When the specific growth rate is described by the Monod equation (Monod 1942)

$$\mu = \mu_{max} \cdot \frac{S}{K_s + S} \tag{7.21}$$

in which $K_s$ is the half-saturation constant for growth, the actual substrate concentration $S$ in the culture is fixed at a low, rate-limiting value. In the steady state, the substrate concentration and biomass density are

$$S = K_s \cdot \frac{D}{\mu_{max} - D} \tag{7.22}$$

$$X = Y \cdot (S_R - S) \tag{7.23}$$

if the biomass yield $Y$ (the amount of biomass formed per substrate consumed) remains constant over the range of dilution rates employed.

For the optimal production of some microbial metabolites, a continuous culture system is required in which the cells pass through various stages of growth (Ricica and Dobersky 1981; Thompson et al. 1983; Parkes and Senior 1988). In these coupled systems, two or more chemostats are arranged serially, by connecting the outlet of the first chemostat to the inlet of the second. Conditions vary between the different chemostat vessels, for example, with respect to dilution rate, $O_2$ supply, temperature, or substrate supply. Culturing different prokaryotes in coupled chemostats for instance permits the study of sequential mineralization of recalcitrant organic compounds. Two-stage cascade chemostats are also employed to investigate predator-prey relationships, in which bacteria grown in the first stage are transferred to a second one to serve as food for bacterivorous protozoa (Jost et al. 1973; Swift et al. 1982; Sambanis and Fredrickson 1987; Simek et al. 1997; Pernthaler et al. 2001).

Even more complex, but rarely used, systems are the bidirectionally linked multistage chemostats, which permit the mutual exchange of bacteria and/or nutrients. In a "gradostat" (Lovitt and Wimpenny 1981), a series of chemostats (linked by tubing) is fed from both sides with media of different, sometimes complementary, compositions. The inoculated bacteria will be exposed to different physicochemical conditions in the different chemostat vessels. In "bidirectional compound diffusion-linked chemostats," the various chemostat vessels are not linked by tubing but through membranes, permitting diffusion of solutes but not bacteria (Keith and Herbert 1985). Multistage chemostats are especially suited to study prokaryotes under conditions prevailing in the natural habitat, since heterogeneous conditions of environmental parameters and interactions between different species can be reproduced with these systems.

For growth studies at extremely low nutrient concentrations, the chemostat is not a suitable tool since extremely low dilution rates ($\ll 0.5\ h^{-1}$) give rise to inhomogeneities due to mixing problems and low steady-state biomass concentrations. As a more realistic approach, a retentostat can be employed which offers the advantage of studying physiological properties of cells at extreme substrate limitation (Mason and Egli 1993; Tappe et al. 1999).

### Redox-Controlled Sulfidostat

As a special type of continuous culture, the redox-controlled sulfidostat permits the cultivation of phototrophic sulfur-oxidizing bacteria under constant concentrations of hydrogen sulfide (Sánchez et al. 1996). In this type of culture, a constant concentration of sulfide is maintained despite light intensity variations that affect photosynthetic rate and hence sulfide oxidation. A redox controller modulates the rate at which the medium is pumped into the culture and therefore governs the dilution rate. In a similar manner, the system can adjust to new rates of sulfide oxidation caused by changes in light intensity.

### Auxostat

An essential property of chemostat cultures is that their rate of growth is fixed by the rate at which fresh medium is fed to the culture. Although this rate can be varied over a considerable range of values, steady state cannot be obtained near $\mu_{max}$, and washout of the cells occurs at the critical dilution rate (Pirt 1975). Furthermore, unbalanced growth (and in some cases washout) may occur when inhibitory metabolites accumulate or potentially toxic substrates are used. An alternative for obtaining controlled growth at an appreciable and constant cell density in such cases is by switching over to some type of internal control of the rate of medium supply. Such a control must be based on a growth-dependent parameter. The first continuous culture with internal control was named "turbidostat" because the feedback control was based on measurements of culture turbidity (Myers and Clark 1944; Bryson and Szybalski 1952). However, a continuous and accurate measurement of turbidity represents a major problem in these systems because of wall growth, inhomogeneity of the culture, etc. Therefore, other parameters directly dependent on the culture density are measured with electrodes in more recent systems. Parameters include $CO_2$, $O_2$, pH, redox potential, fermentation products, and sulfide (Watson 1969; Martin and Hempfling 1976; Oltmann et al. 1978; Kjaergaard and Jørgensen 1979; Schauer et al. 1982; Cypionka 1986; de la Broise and Durand 1989). These more recent designs have proven reliable, and their use is most rewarding in studying growth of microbes in the presence of inhibitory concentrations of substrates or products, and possibly also in selecting mutant strains exhibiting the highest growth rates under such conditions.

## Possible Reasons for "Nonculturability"

The titer of colony-forming units obtained from a given sample in almost all cases is significantly lower than the actual titer of prokaryotic cells in the sample as determined by culture-independent microscopic techniques. Only few exceptions have been published so far (e.g., Button et al. 1993). This observation has been termed the "great plate count anomaly" and is attributed to several factors. One simple explanation would be that only a fraction of the prokaryotic cells are culturable under a certain set of conditions such that no single medium will allow growth of all types of prokaryotes. Indeed, the efficiency of plate counting could be significantly increased when 25 different media were employed instead of a single medium (Balestra and Misaghi 1997). However, in most cases, the numerically dominant species of prokaryotes from natural samples are not recovered. Certain bacteria and archaea, among them the mesophilic Crenarchaeota which constitute a fraction of up to 34% of the prokaryotic plankton in subpolar or polar latitudes (DeLong et al. 1994), the clone T78 group of the green gliding bacteria (Coolen et al. 2002), or most of the Acidobacteria detected by molecular methods in soils (Barns et al. 1999), for a long time escaped cultivation in numerous different types of media.

It therefore appears reasonable to suggest that (1) cells of not-yet-cultured prokaryotes in natural samples are in a specific physiological state which prevents them from growing in conventional cultivation media and/or (2) the physiology of not-yet-cultured species of prokaryotes is fundamentally different from that of known prokaryotes such that cultivation methods applied do not meet the requirements for growth.

## Physiological State of Prokaryotic Cells

### Starvation Response

The gene product of *rpoS*, namely, the transcription factor $\sigma^S$, is involved in cellular responses to a diverse number of stresses (Loewen et al. 1998). Induction of *rpoS* results in an increased survival of cells under unfavorable conditions (Munro et al. 1995). Accordingly, and dependent on the growth state of the cells, RpoS ($\sigma^S$) can positively influence the culturability of ***E. coli*** and ***Salmonella typhimurium*** in oligotrophic seawater (Munro et al. 1995). However, $\sigma^S$ is also involved in the transition to stationary phase, and cAMP (in a complex with the cAMP receptor protein [CRP-cAMP]) acts as a negative regulator of the transcription of *rpoS* (Loewen et al. 1998). Addition of extracellular cAMP has the same effect (Lange and Hengge-Aronis 1991). Scavenging transporters (such as LamB), which are regulated by cAMP or endoinduction, are turned on at higher substrate concentrations than RpoS-dependent functions (Notley and Ferenci 1996). Therefore it appears feasible that, by addition of extracellular cAMP, cells could be maintained more easily in a nutrient scavenging state and hindered from entering the protective stationary-phase response, which potentially could facilitate cultivation in the absence of other stress factors. In fact, addition of cAMP to low-nutrient liquid artificial freshwater or marine media resulted in significant increase of cultivation success compared to controls containing AMP (Bruns et al. 2002, 2003b).

### Presence of Dead Cells, Prevention, and Reversal of Cellular Damage

The presence of nongrowing cells has been shown in laboratory cultures of ***Enterobacter aerogenes***, which at maximum doubling times of 100 h contained up to 50% of cells not capable of growing on agar plates (Tempest et al. 1967). Since culture-independent methods such as microautoradiography or the direct viable count technique have revealed that up to 50%, and in some cases even 90%, of the prokaryotic cells may be metabolically active (Kogure et al. 1979; Fry 1990; Karner and Fuhrman 1997), dead cells may actually not be present at high numbers in the natural environment. However, elongating cells may be capable of completing only a limited number of division cycles, which prevents detection of growth by standard methods (Binnerup et al. 1993). This limitation of division cycles can be caused by injury (e.g., to the cell envelope, proteins, or DNA; Barer and Harwood 1999), caused for instance by oxidative stress. Hence, dormant cells of ***Micrococcus luteus*** are permeant to certain fluorescent stains but restore the cytoplasmic membrane barrier upon resuscitation (Kaprelyants et al. 1996). Other factors that may limit multiplication are limiting nutrients or an (as yet unexplained) upper limit of cell density as described for ***Sphingomonas*** sp. strain RB2256 (Schut et al. 1997). Injury of DNA may activate the SOS response which includes expression of SulA, a protein that interacts with the tubulin-like protein FtsZ, thereby preventing septation and thus resulting in a filamentous growth of cells (Bi and Lutkenhaus 1993; Walker 1996). Detoxifying enzymes like catalase and superoxide dismutase are involved in the prevention of oxidative stress. Heat shock proteins, peptide methionine sulfoxide reductase, and glutathione reductase are involved in the reversal of damage within the cell (Dukan and Nyström 1998; Barer and Harwood 1999).

### Dormancy

Dormancy is defined as a reversible state of low metabolic activity in which viability is maintained. This physiological state has been studied extensively for *Micrococcus luteus*. Incubation of stationary-phase cultures at room temperatures for several months results in large numbers of dormant cells. In the case of ***Micrococcus luteus***, dormant cells show reduced activity, as exemplified by uptake of the membrane energization-sensitive dye rhodamine-123 (Kaprelyants and Kell 1993). However, such temporarily nonculturable cells can be resuscitated in the presence of supernatants from growing ***M. luteus*** cultures (Kaprelyants et al. 1994, 1996). The agent responsible for resuscitation of dormant cells (Rpf, the resuscitation promoting factor) was identified as a 17-kDa protein exported by ***M. luteus*** (Mukamolova et al. 1998). In ***E. coli***, the protein SdiA regulates the *ftsQAZ* cluster of essential cell division genes and the $P_2$ promoter (Wang et al. 1991) and is a member of the LuxR subfamily of transcriptional activators. The expression of SdiA is regulated in turn by a factor released by growing cells into the medium (Garcia-Lara et al. 1996). The factor was identified as an *n*-acyl homoserine lactone (Sitnikov et al. 1996). Cell-cell signaling between cells of the same clone therefore may be important in regulating cell division.

### Substrate-Accelerated Death

If a growth-limiting substrate (e.g., glycerol, glucose, ribose, phosphate, or ammonia) is added in concentrations of $\geq$1–10 μM to cells previously starved for the same substrate (Postgate and Hunter 1963, 1964; Calcott and Postgate 1972), growth of the cells is inhibited. This phenomenon has been called "substrate-accelerated death." Cells challenged with this substrate exhibit low levels of cAMP, and in the presence of extracellular cAMP, substrate-accelerated death disappears (Calcott et al. 1972). Also, the addition of $Mg^{2+}$ protects cells

against substrate-accelerated death. Substrate-accelerated death has been reported to occur in ***Klebsiella***, ***Escherichia***, ***Streptococcus***, ***Azotobacter***, ***Arthrobacter***, and ***Mycobacteria*** (Calcott and Calvert 1981). The significance of substrate-accelerated death on recovery of bacteria from environmental samples remains unknown. However, it has recently been demonstrated that addition of cAMP at low concentrations (10 μM) in certain cases can significantly increase the culturability for natural samples (Bruns et al. 2002).

## The Viable but Nonculturable State

In addition, it has been proposed that certain bacteria may acquire a state termed "viable but nonculturable" (VBNC; Xu et al. 1982; Roszak and Colwell 1987). In the proposed VBNC state, bacterial cells, especially of pathogenic bacteria like ***Vibrio cholerae*** or ***Campylobacter jejuni*** (Bovill and Mackey 1997), are believed to become temporarily nonculturable until they are exposed to an environment that stimulates their resuscitation. In ***Vibrio vulnificus***, exposure of cells to temperatures around 4°C results in a decline of culturability without cell lysis (Oliver 1995). Temperature upshift results in a resuscitation of these nonculturable cells (Whitesides and Oliver 1997). Those cells that retain the capacity for resuscitation appear to maintain physiological activity. Over 35 species belonging to 17 proteobacterial genera have been reported to show this phenomenon (McDougald and Kjelleberg 1999). Replacement of the term VBNC by "temporarily nonculturable" or "not immediately culturable" has been proposed since it is unclear whether VBNC is the result of a specific programmed differentiation or an adaptive process (Barer and Harwood 1999). The view that the VBNC state is a single phenomenon and a valid operational concept has been challenged (Barer and Harwood 1999).

## Lysogenic Phages

Lysogenic phages may be induced upon plating onto agar media by SOS response-related induction (Barer and Harwood 1999), leading to a rapid decrease of culturability.

## Low Nutrient Concentrations, "Oligotrophic Bacteria," and "Ultramicrobacteria"

The concentration of nutrients critically affects the outcome of cultivation experiments (Pinhassi and Berman 2003). Whereas early in the history of microbiology the ionic of media were adjusted to concentrations commensurate with the natural environment, carbon-poor ("oligotrophic") media have only recently been used to tap the large diversity of bacterioplankton by systematic cultivation approaches. As an example, the concentrations of all major elements except carbon, nitrogen, and phosphorous in marine broth 2,216 that was established in 1941 (ZoBell 1941) mimic the average concentrations in seawater very well (Giovannoni and Stingl 2007), while organic carbon concentrations exceed those in seawater by 170 times, not considering the large fraction of recalcitrant organic matter in the natural environment. It should be noted that even terrestrial bacteria are limited by the availability of organic carbon substrates in some soils despite the much higher total organic carbon concentrations in these environments (Alden et al. 2001). Commensurate with this situation, strongly diluted nutrient broth yielded a significantly improved culturability of soil bacteria (Janssen et al. 2002; Koch et al. 2008; Dedysh 2011). Media supplemented with comparatively low concentrations of complex carbon sources like yeast extract and peptone (i.e., 0.25 g $L^{-1}$ each or less) have been established for the isolation of fastidious bacteria such as Planctomycetes (Schlesner 1986; Staley et al. 1992), for Armatimonadetes (the former OP10 phylum; Tamaki et al. 2011), and for novel types of freshwater sphingomonads (Chen et al. 2012). Using concentrations of $\leq$5 mg of organic carbon·$L^{-1}$, which are supplied as casamino acids, or employing straight filtered autoclaved seawater can increase the apparent cultivation success (determined as the ratio of most probable numbers in relation to total cell counts) up to 20–60% (Button et al. 1993; Eguchi et al. 2001) and has also allowed the isolation of first strains of the abundant marine photoautotroph *Prochlorococcus marinus* (Chisholm et al. 1992). Subsequently, a combination of miniaturization of culture volumes, molecular screening techniques, and clean techniques for handling of seawater (Connon and Giovannoni 2002) allowed the isolation of a considerable number of heterotrophic marine oligotrophs (Rappe et al. 2002; Cho and Giovannoni 2004). Alternatively, microencapsulation (Zengler et al. 2002) or separation of cells from the surrounding environment in diffusion chambers (Kaeberlein et al. 2002) or microbial culture chips (Ingham et al. 2007) allow an exchange of natural substrates at in situ concentrations and may provide initial enrichments of interesting novel bacteria.

In addition, the qualitative composition of carbon sources determines the cultivation success of naturally occurring prokaryotes. Complex media have been shown to yield higher numbers and a higher diversity of isolates than similar media containing only one defined carbon source at the same concentration: single substrates yielded almost exclusively strains of the gamma-subclass of Proteobacteria (Uphoff et al. 2001). For soil pseudomonads, nutrient-poor media containing only ~15 mg C·$L^{-1}$ have been shown to yield higher numbers of culturable cells than conventional organic-rich media (Aagot et al. 2001). Strongly diluted nutrient broth resulted in significantly increased numbers of Acidobacteria, Actinobacteria, α-Proteobacteria, low G + C Gram-positive bacteria, and Verrucomicrobia from soil samples (Janssen et al. 2002). This combined evidence suggests that the majority of heterotrophic prokaryotic cells in many natural samples require lower concentrations of organic carbon for growth. The cultivation success of freshwater planktonic bacteria could also be increased in media with reduced nitrogen and phosphorus concentrations. In the latter case, nutrient levels were decreased to match the maximal ambient levels in the planktonic environment (50 μM nitrogen

and 1.5 μM phosphorus; Eilers et al. 2001). In general, viable cell counts of freshwater or marine planktonic bacteria reached on agar-solidified media are orders of magnitude smaller than those obtained as most probable numbers in liquid extinction dilution series (Eguchi et al. 2001; Jaspers et al. 2001).

Ultramicrobacteria are defined by a cell volume of $<0.1\ \mu m^3$ (Eguchi et al. 2001) and prevail in oligotrophic marine waters (Schut et al. 1997). In this respect, an important question is whether ultramicrocells represent a distinct class of prokaryotes that maintain their small biovolume independently of their growth status or whether they are capable of becoming larger. In many bacterial species, nutrient deprivation results in pronounced changes of cell morphology and size (Torella and Morita 1981; Morita 1982; Amy and Morita 1983; Kjelleberg et al. 1993). Upon starvation, many nongrowing marine isolates produce ultramicrocells by reductive division, and these can have volumes as little as 1% that of rapidly growing cells. The resulting daughter cells retain measurable metabolic activity, at least in an initial period following their formation (Amy and Morita 1983). It appears likely that such ultramicrocells contribute significantly to observed populations of prokaryotes in ocean waters. However, in cells of other species, like for instance ***Sphingomonas alaskensis*** within the α-Proteobacteria (Eguchi et al. 2001; Vancanneyt et al. 2001) or the Verrucomicrobiales, a small cell volume ($0.03–0.06\ \mu m^3$) is a stable characteristic and independent of substrate concentrations (Schut et al. 1993; Janssen et al. 1997). Freshwater Actinobacteria are ultramicrobacteria that have recently been successfully enriched based on their cell size, employing 0.2-μm-pore-size filters in a prefiltration step to eliminate accompanying, faster-growing copiotrophic bacteria (Hahn et al. 2003). For successful laboratory cultivation of these bacteria, an acclimatization procedure was employed that encompassed an increase of the temperature in 2°C-steps to the final incubation temperature of 15°C and stepwise increases of the concentrations of organic carbon substrates in the medium were from 5 mg to 1 g $L^{-1}$. The freshwater Actinobacteria so far could be grown only slowly and as cocultures with other heterotrophic bacteria on agar plates, forming very small colonies (Hahn 2009). Ultramicrobacteria form a dominant fraction of freshwater and marine bacterioplankton where they represent typical oligotrophs. Another abundant group of obligate freshwater ultramicrobacteria are members of the betaproteobacterial *Polynucleobacter necessarius* cluster that are facultative oligotrophs (Hahn 2003). Small cell size does not always correlate with ultraoligotrophic characteristics, however. The recent isolation of the first representative of the termite group 1 phylum (now Elusimicrobia) was enabled by selecting for ultramicrobacteria through filtration of beetles gut homogenate through 0.2-μm-pore-size membrane filters but employing anoxic cultivation media with standard concentrations (i.e., 2 mM) of organic carbon substrates (Geissinger et al. 2009).

Although debated for a considerable time (Morita 1982), it was eventually recognized (Kuznetsov et al. 1979) that certain bacteria are capable of replicating at nutrient concentrations present in seawater but cannot grow in the standard high-nutrient microbiological media. Oligotrophic bacteria are defined as those that on first cultivation develop on media containing 1–15 mg $C{\cdot}L^{-1}$ (Kuznetsov et al. 1979). Consequently, such bacteria cannot be cultivated directly on nutrient-rich agar (Vancanneyt et al. 2001). Actually, the range of carbon concentrations reported for the pelagic and deep ocean is 30–200 μM, corresponding to 0.36–2.4 mg $C{\cdot}L^{-1}$ (Jannasch et al. 1996). Therefore, bacto yeast extract (Difco) is added from autoclaved stock solutions to a final concentration of 10–1 $mg{\cdot}L^{-1}$ (corresponding to 3.3–0.33 mg $C{\cdot}L^{-1}$ or 270–27 μM carbon; Jannasch et al. 1996) in media designed to recover marine pelagic prokaryotes. It has been demonstrated, however, that prolonged incubation over 1 year at 5–8°C or storage and monthly reculturing at 15°C can yield cultures which eventually can multiply on high-nutrient laboratory media (Eguchi et al. 2001; Vancanneyt et al. 2001). Obligate oligotrophs are prokaryotes that typically fail to grow at high substrate concentrations even after multiple passages in the laboratory. Examples are members of the alphaproteobacterial SAR11 clade or of the gammaproteobacterial OMG clade (Rappe et al. 2002; Cho and Giovannoni 2004). In contrast, facultative oligotrophs such as ***Sphingomonas alaskensis*** RB2256 can (eventually) be cultivated on standard microbiological media.

Oligotrophic bacteria may represent novel types of bacteria with unknown physiological properties. Indeed, the recently described ammonia-oxidizing archaeon "*Candidatus Nitrosopumilus maritimus*" exhibits the highest affinity for ammonia reported to date (Martens-Habbena et al. 2009). Notably, this archaeon is inhibited in growth at ammonia concentrations of ≥2 mM. Another example is the presence of a novel pathway for assimilation of dimethylsulfoniopropionate in "*Candidatus* Pelagibacter ubique" and other marine planktonic bacteria (Reisch et al. 2011). In contrast to all other (mostly copiotrophic) bacteria investigated to date, the marine ultramicrobacterium ***Sphingomonas alaskensis*** RB2256 exhibits a high level of inherent stress resistance toward oxidative stress (hydrogen peroxide [$H_2O_2$]), but no starvation-induced stress against $H_2O_2$ (Ostrowski et al. 2001). Stress resistance is negatively correlated to growth rate, but only marginal changes in catalase activity were observed; hence, other factors must be critical to the stress resistance of this strain.

## Growth on Multiple Substrates

In spite of the fact that bacteria in natural environments grow in the presence of a large diversity of compounds, most laboratory studies have focused on growth with single substrates. When bacteria are grown in batch culture with more than one growth substrate, sequential utilization of these substrates is often observed. In some cases, this results in a typical diauxic growth pattern (Monod 1942; Lengeler et al. 1999) in which the substrate that is used first represses the synthesis of enzymes required for the utilization of the other substrates. Only after (almost) complete utilization of the first substrate is growth on

the second one induced. It is noteworthy that sugars may cause catabolite repression of inducible enzyme systems even if the sugars are not utilized themselves (Pastan and Perlman 1969). In enteric bacteria, cAMP is part of the pleiotropic *crp* activation system which regulates most peripheral catabolic operons and carbohydrate transport systems, and it typically mediates carbon catabolite repression (Lengeler et al. 1999). Sugars transported by the phosphotransferase system (PTS) decrease intracellular cAMP levels which in turn prevents the expression of genes necessary for the uptake of non-PTS sugars. After depletion of glucose, intracellular cAMP concentrations rise from 0.3 up to 3 μM, and other sugars can be taken up and metabolized after a lag period required for the induction of the necessary genes. This regulatory pattern leads to a biphasic growth curve. The intermediate lag phase can be abolished by addition of extracellular cAMP when added at millimolar concentrations (Epstein et al. 1975), whereas the growth rate itself does not change (Okada et al. 1981). The involvement of cAMP in regulation of catabolic enzymes has also been demonstrated for a wide range of nonenteric bacteria, including other γ-Proteobacteria, the α-Proteobacteria, β-Proteobacteria, and the cyanobacteria (Botsford and Harman 1992).

However, this type of clear-cut diauxic pattern with a lag period between consumption of the first and the second substrate is by no means very common. More often, a gradual transition between the use of two (or more) substrates is observed, and in some cases, no enzyme repression is evident at all (Harder and Dijkhuizen 1982; Gottschal 1986). Moreover, it is questionable whether distinct preferences for one substrate would be functionally valuable in nutrient-poor natural environments with many different substrates available at the same time. Under nutrient limitation, one would rather expect organisms to develop physiological strategies enabling them to make use of several nutrients simultaneously. Growth in chemostats under limitation of mixtures of different substrates has provided ample evidence that a multitude of substrates, serving similar physiological functions, can be growth limiting at the same time (Harder and Dijkhuizen 1982; Egli et al. 1983; Gottschal 1986; Gottschal and Dijkhuizen 1988). Low $K_s$ values have been found in marine isolates (Schut et al. 1995). However, the reported $V_{max}$ values are generally so low that specific affinities and oligotrophic capacities are insufficient to allow growth on single substrates. In a marine coryneform bacterium, the presence of amino acids can enhance the uptake of glucose and lowers the threshold concentrations for growth (Law and Button 1977). On the other hand, growth on alanine of ***Sphingomonas alaskensis*** strain RB2256 occurred with a lower affinity in the presence of glucose (Schut et al. 1995).

Metabolically versatile bacteria exist that are specialized in using several different substrates at the same time if the latter are present at growth-limiting concentrations (Laanbroek et al. 1979; Dykhuizen and Davies 1980; Gottschal and Kuenen 1980; Beudeker et al. 1982; Legan and Owens 1988). Since these prokaryotes usually display lower maximum growth rates relative to more specialized species, metabolically versatile bacteria will in most cases be missed when batch-type enrichment techniques are used, even if mixtures of several substrates are present. Instead, chemostat cultures need to be employed for enrichment.

## Effect of Inhomogeneities

Inhomogeneities can have a profound effect on the physiology of prokaryotes. Many environments such as soils, sediments, and marine snow are highly heterogeneous, and therefore microbial substrates and bacterial productivity are distributed in microscale patches of variable concentration and size (Azam 1998; Ploug et al. 1999). Under such conditions, steep gradients, for example, of molecular oxygen, may result in a close proximity of aerobic and anaerobic species and transformations (Revsbech and Jørgensen 1986).

Attachment of prokaryotic cells has been shown to be of significance under some conditions. Some compounds are optimally metabolized only if oxygen-dependent and strictly anoxic steps are coupled by diffusion of metabolic products and thus proceed in close proximity. This principle has been demonstrated in an elegant study of the degradation of DDT (1,1,1-trichloro-2,2-bis[4-chlorophenyl]ethane), using calcium alginate beads for immobilization of bacteria (Beunink and Rehm 1988). In this system, the attached cells of ***Alcaligenes*** species and ***Enterobacter cloacae*** performed reductive dechlorination of DDT inside the beads, and the partly dechlorinated products were metabolized oxidatively. Since highly chlorinated compounds are dehalogenated more readily under anoxic conditions, this same principle may hold for many other halogenated xenobiotics as well.

Besides affinity for a given substrate and cell yield, attachment to solid surface is an additional determinant of the outcome of competition between prokaryotes with similar physiology. Attachment to solid surfaces can thus change the metabolic interrelations between competing bacteria. When growing in suspension, ***Pseudomonas putida*** R1 and ***Acinetobacter*** C6 compete for the substrate benzyl alcohol, with ***Acinetobacter*** largely outcompeting ***P. putida***. However, both strains formed stable and structured biofilms when glass as solid substrate was available (Christensen et al. 2002). Under the latter conditions, ***P. putida*** is capable of utilizing benzoate which ***Acinetobacter*** uses only inefficiently and therefore excretes to a large extend.

In the natural environment, nonpolar organic carbon substrates occur in the adsorbed state. In the adsorbed state, these substrates are often not directly available, and rates of desorption control the rate of degradation. However, some prokaryotes can degrade adsorbed substrates more rapidly than can be accounted for by the rates of desorption into the aqueous phase (Harms and Zehnder 1995). Prokaryotes thus differ in their capability of using adsorbed substrates (Guerin and Boyd 1997; Stringfellow and Aitken 1994; Crocker et al. 1995). Consequently, enrichment strategies, in which especially nonpolar substrates are offered in the adsorbed state, may provide a more

relevant low-bioavailability environment, and hence lead to the enrichment and isolation of novel types of prokaryotes (Tang et al. 1998; Grosser et al. 2000).

## Interactions with Other Microorganisms

Most likely, one considerable problem in current enrichment and cultivation techniques is that microbial interactions cannot be reproduced adequately (see ❯ "Alternative and Novel Concepts for Cultivation"). In nature, prokaryotes reach cell densities of about $10^6$ $mL^{-1}$ in most aquatic environments, $10^9$ $cm^{-3}$ in sediments, and $10^{11}$ $cm^{-3}$ in soils. If a homogenous distribution of the cells is assumed, the average cell-to-cell distance at these increasing densities would amount to 112, 10 and 1 µm, respectively. Over such small distances, transport of small molecules by molecular diffusion proceeds at a rapid rate and takes between microseconds and a few seconds (Overmann 2002a). Owing to the laws of three-dimensional diffusion, the flux of metabolites experienced in the vicinity of a prokaryotic cell by another one decreases to as little as 0.01% when the cell-to-cell distance increases to 10 µm (Overmann and Schubert 2002). This simple calculation indicates that (1) interactions between prokaryotes may influence their growth under natural conditions and most likely need to be considered in cultivation attempts of some not-yet-cultured bacteria and (2) a strong selective pressure must exist for interacting prokaryotic cells to maintain close spatial proximity.

At high cell densities, even monospecific associations of some bacteria exhibit physiological traits that differ from those of dilute cultures. In the case of quorum sensing, the excretion of autoinducer molecules signals high cell density and triggers light production, expression of virulence factors, and swarming (Fuqua and Greenberg 1998; Basler and Losick 2006) or prevents cell aggregation (Puskas et al. 1997). Similarly, myxobacteria exhibit complex social interactions: when deprived of nutrients, they enter into a complex developmental cycle that results in the formation of a multicellular fruiting body that contains myxospores (Reichenbach 1984). Other cases of monospecific associations have been described occasionally (Lins and Farina 1999). However, it has also been found that different types of bacteria can form associations. These comprise highly structured associations of defined composition, so-called consortia (Schink 1991; Overmann 2002a). In addition, less-structured assemblages, like microcolonies, netlike structures, biofilms, or aggregates of up to 18 different prokaryotic genera, have commonly been observed in natural samples (Weise and Rheinheimer 1978; Paerl 1982; Alldredge and Youngbluth 1985; van Gemerden et al. 1989; Dubinina et al. 1993; Kolenbrander and London 1993; Seitz et al. 1993; Overmann et al. 1996; Jacobi et al. 1997; Moissl et al. 2002). Least structured and hence difficult to detect are 10–100-µm-large patches of free-living bacterial cells, but evidence has accumulated for the existence of such inhomogeneities in the pelagic habitat (Krembs et al. 1998). Under certain natural conditions, the growth of prokaryotes is therefore not only influenced by the various physicochemical factors but also by interactions with other microorganisms. Cells may need to communicate with each other for growth. The metabolic interaction between the carbohydrate-fermenting *Streptococcus gordonii* and the lactic acid-fermenting *Veillonella atypica* not only involves the exchange of lactic acid but also involves signaling between both partners that leads to an increased expression of α-amylase by *S. gordonii*. In an open flow-through system (similar to human oral biofilms which contain both bacterial species), the diffusible signal functions only over short distances on the order of 1 µm (Egland et al. 2004). These experimental results emphasize the relevance of spatial organization for the metabolic activity and mutual control of bacteria.

So far, the compounds known to be exchanged between prokaryotes that can promote growth of otherwise unculturable bacteria include signal compounds (as in the case of quorum sensing) or cAMP (Bruns et al. 2002) or possibly oligopeptides (Nichols et al. 2008), growth factors such as vitamins (Graber and Breznak 2005), siderophores (Guan et al. 2000), essential nutrients (Tripp et al. 2008), carbon sources (Brown and Whiteley 2007), and other compounds directly involved in energy metabolism (mostly electron donors/electron acceptors such as hydrogen or inorganic sulfur compounds in syntrophic interactions). Signaling compounds also comprise small peptides such as the 5-amino-acid peptide LQPEV that at 3.5 nM induces growth of a *Psychrobacter* strain on standard media (Nichols et al. 2008). Besides enabling prokaryotes to perform novel syntrophic reactions, interactions can also lead to altered kinetics of microbial transformations. For example, the high-affinity oxidation of methane observed in soil could be reproduced in a coculture of a nonnovel methanotroph with a ***Variovorax*** strain (Dunfield et al. 1999). This finding indicates that the physiology and ecology of prokaryotes can only be completely appreciated if interactions with other prokaryotes are considered as well. The experimental setup to exploit interactions with living accompanying bacteria for the cultivation of novel types of bacteria is typically based on diffusion chambers (Kaeberlein et al. 2002; Nichols et al. 2008; ❯ *Fig. 7.5*, see section ❯ "Cocultivation"). An alternative approach is the microencapsulation technique (Zengler et al. 2002).

Overall, eight types of interspecies interrelationships can be distinguished based on the effect of each of the two populations (Atlas and Bartha 1993; ❯ *Table 7.7*), ranging from mutualistic to antagonistic interactions. It has to be kept in mind, however, that the different categories listed in ❯ *Table 7.7* represent only concepts, which in many cases fail to account for all facets of microbial interactions.

### Neutralism

This describes the situation of a complete lack of interaction between two populations. This situation is more likely to occur between populations with very different metabolic capabilities. However, neutralism is defined in a negative way and therefore is most difficult to verify experimentally. Most likely interactions

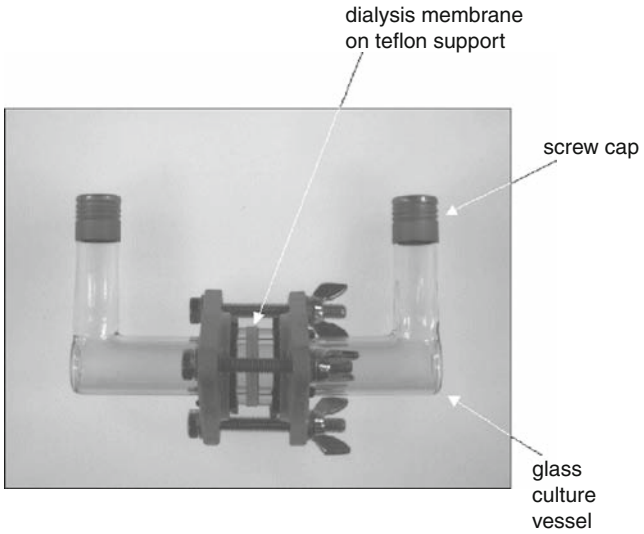


**Fig. 7.5**
**A simple dialysis coculture setup that permits the growth of two separate prokaryotic cultures while contact via a dialysis membrane is maintained. For growth of aerobic cultures, the screw caps are replaced by cotton plugs and the glass vessels are only half full. Homogeneous cell suspensions can be maintained by adding a small stirring bar to each of the two chambers and placing the set up on a magnetic stirrer**

**Table 7.7**
**Types of interspecies interactions**

| Name of interaction | Effect of interaction |
|---|---|
| Neutralism | Neither population affects the other |
| Competition | Populations inhibit each other when resources are in limiting supply |
| Amensalism | Population 1 is negatively affected by 2, but 2 is not affected by 1 |
| Parasitism | Population 1 consumes population 2 but usually not in a destructive manner |
| Predation | Population 1 consumes population 2 in a destructive manner |
| Commensalism | Population 1 benefits from population 2 without affecting it in a negative sense |
| Synergism (protocooperation) | Both populations benefit from the interaction, which is not obligatory |
| Mutualism (symbiosis) | Both populations benefit from the interaction, which is obligatory |

are absent at very low population densities and if prokaryotes form physiologically largely inactive resting stages (Atlas and Bartha 1993). However, it has been demonstrated that even endospores can affect the surrounding environment by the presence of extracellular enzymes (i.e., $Mn^{2+}$ oxidase; Francis and Tebo 2002).

## Competition

Since energy and nutrient sources are often present in limiting concentrations, competition for growth-limiting resources is one of the major types of interactions between cells of one population or between different prokaryotes, and it results in a reduction of growth rate. Eventually, it may lead to the exclusion of the slower-growing species, a process also termed "competitive exclusion" (Gause 1934; Hardin 1960). Since the introduction of continuous culture techniques which allowed cultivation under conditions of permanent nutrient limitation, the competition in mixed cultures has been studied under a great variety of environmental conditions (Powell 1958; Veldkamp and Jannasch 1972; Fredrickson 1977; Veldkamp 1977; Fredrickson and Stephanopoulos 1981; Kuenen and Gottschal 1982; Kuenen and Harder 1982; Gottschal and Dijkhuizen 1988; Visscher et al. 1992b). These studies were mostly concerned with simple and pure competition for single growth-limiting nutrient in the absence of other interactions, and the results supported the competitive exclusion principle. The outcome of the competition between two or more species is merely dependent on the shape of the μ versus *S* relationship of the competitors (*Fig. 7.2*). Those prokaryotes which reach a higher specific growth rate at a given substrate concentration always outcompete the slower-growing species. In some instances, the μ versus *S* curves cross, and as a result, the outcome of the competition depends on the dilution rate chosen. Apparently, certain species are much better adapted to growth at very low substrate concentrations (and exhibit a relatively low $\mu_{max}$ value), whereas others are more specialized in growth at high rates in the presence of high substrate concentrations. Examples have been reported for aerobic and anaerobic heterotrophs, chemolithotrophic species, and phototrophic organisms (Harder and Veldkamp 1971; Jannasch and Mateles 1974; Fredrickson 1977; Harder et al. 1977; Mur et al. 1977; Veldkamp 1977; Matin and Veldkamp 1978; Kristjansson et al. 1982; Lovley et al. 1982; Laanbroek et al. 1983, 1984; King 1984; Kuenen and Robertson 1984; Robinson and Tiedje 1984; Veldkamp et al. 1984; Legan et al. 1987; Legan and Owens 1988). Competition between microorganisms exists not only for organic carbon but also for nutrients like phosphate (Rhee 1972; Currie and Kalff 1984).

## Amensalism

The production of lactic acid by lactic acid bacteria and sulfuric acid by ***Thiobacillus thiooxidans***, which inhibit other non-acidophilic microorganisms, are examples of amensalistic relationships. Similarly, the inhibition of ***Salmonella enterica*** by acetate and propionate produced by ***Clostridium lactatifermentans*** at low pH (5.8) has been demonstrated (van der Wielen et al. 2002). At these low pH values, considerable acetate and propionate present as undissociated acids penetrate the cytoplasmic membrane and hence decrease the membrane potential (see section "pH").

Production of inhibitory compounds has been observed more frequently among particle-attached marine bacteria than their free-living counterparts and may be a mechanism to deter other potential colonizers from the nutrient-rich particle environment (Long and Azam 2001). Members of the marine *Roseobacter* clade are dominant primary colonizers of surfaces in the coastal environment (Dang and Lovell 2000) and show the strongest antagonistic activity (Long and Azam 2001). The marine antagonistic *Phaeobacter* strain 27-4, a member of the *Roseobacter* clade, has been found to synthesize tropodithietic acid and thiotropocin that inhibit *Vibrio anguillarum* and *Vibrio splendidus* (Bruhn et al. 2005) and hence may act as a probiotic against infection of fish larvae in commercial fish farms.

## Predation

The best-documented example for predatory prokaryotes is bacteria of the genus ***Bdellovibrio*** which attack Gram-negative bacteria by attaching to their prey, penetrating the cell wall, and subsequently multiplying within the periplasmic space. Multiplication of ***Bdellovibrio*** sp. occurs at the expense of cellular components of the host cell and leads to the formation of 4–20 daughter cells by segmentation within 2–3 h after infection (Stolp and Starr 1963, 1965; Varon and Shilo 1980; Shilo 1984). The cell content of the host is partially degraded and utilized. Although growth and survival of wild-type ***Bdellovibrio*** spp. is strictly dependent on the availability of appropriate prey cells, this parasite differs fundamentally from viruses in that it does not depend directly on the metabolic machinery of the host cell. ***Bdellovibrio*** species and similar bacteria appear to be widespread, having been isolated from many different aquatic and terrestrial ecosystems (Varon and Shilo 1980; Burnham and Conti 1984). Additional predatory bacteria that require cell contact have been described. ***Vampirococcus*** sp. is a nonmotile, Gram-negative anaerobic bacterium which occurs as an epibiont of phototrophic ***Chromatium*** spp. to which it adheres by means of specific attachment structures without penetrating the outer cell layers. Concomitant to growth and division of the epibiont, the host cell cytoplasm is degraded, leaving behind an almost empty cell envelope (Guerrero et al. 1986). A second type of predatory bacterium, ***Daptobacter*** sp., penetrates the cell envelope of cells of various genera of the Chromatiaceae and degrades the cytoplasm of its prey. The Gram-negative ***Daptobacter*** is a facultative predator, facultatively anaerobic and motile (Guerrero et al. 1986). Other bacteria can lyse prokaryotes without direct contact. An isolate of ***Stenotrophomonas maltophilia*** was found to lyse cells of ***Chlorobium limicola*** and several heterotrophic bacteria (Nogales et al. 1997). Finally, myxobacteria can cause lysis of susceptible strains at some distance, apparently with the aid of exoenzymes. The myxobacteria derive their nutrition from material released by the lysed cells.

## Commensalism

In the nonobligatory commensal relationship, one population benefits, for example, from growth factors excreted by a second population, while the latter remains unaffected. This type of relationship thus is unidirectional in character. Commensalism within one and the same population is of significance if, for instance, the growth substrates are insoluble (such as lignin or cellulose). These substrates are made available by extracellular enzymes, and while the exoenzymes themselves are kept at the cell surface (i.e., in the periplasmic space in the case of Gram-negative bacteria or by attachment to the cytoplasmic membrane in the case of Gram-positives), the resulting substrates often are rapidly lost by diffusion from the vicinity of single cells. In contrast, soluble products can be utilized at high efficiency if cell densities are high. As an example, ***Myxococcus xanthus*** does not grow on insoluble casein at cell densities lower than $10^3$ $mL^{-1}$, whereas growth rates increased with cell densities above this value (Rosenberg et al. 1977). This effect of cell density is not observed on prehydrolyzed casein.

Not in all cases does the limitation of two populations of prokaryotes by one substrate lead to competitive exclusion, but it can result in stable mixed cultures. The underlying reasons include the occurrence of additional interactions, especially commensalism and mutualism, the formation of self-inhibitory products, the presence of predators, selective adhesion, fluctuations in physical parameters (pH, temperature, light, and oxic/anoxic conditions), or an alternating supply of differing growth-limiting substrates (Bungay and Bungay 1968; Megee et al. 1972; Jost et al. 1973; Meers 1973; van Gemerden 1974; Meyer et al. 1975; Lee et al. 1976; Fredrickson 1977; de Freitas and Fredrickson 1978; Slater and Bull 1978; Gottschal et al. 1979; Miura et al. 1980; Bull and Slater 1982; Kuenen and Robertson 1984; Kuenen et al. 1985). For example, purple sulfur bacteria and colorless sulfur bacteria, which are expected to compete for $H_2S$ as electron-donating substrate, are found to thrive in high population densities in the same layer of microbial mats (Visscher et al. 1992a). This has been explained by the removal of oxygen by colorless sulfur oxidizers and the formation of incompletely oxidized inorganic sulfur intermediates, which then serve as alternative electron donor for the anoxygenic phototroph (Visscher et al. 1992b; van den Ende et al. 1996). Also, competition can be alleviated and stable cocultures obtained if conditions for competition do not exist over an entire 24-h period but only for a shorter time interval (van Gemerden 1974). This fact for instance can be exploited for the enrichment of more fastidious purple sulfur bacteria (like the large-celled ***Chromatium*** spp.) by using light/dark cycles instead of continuous light.

Commensalism between nonrelated bacteria has been detected in a wide variety of cases. In freshwater bacterioplankton, chitinolytic Flavobacteria colonize chitin particles which they hydrolyze and solubilize, thereby supporting uptake and utilization of *N*-acetyl-D-glucosamine by Actinobacteria of the ubiquitous freshwater AcI cluster (Beier and Bertilsson 2011).

As another example, ***Propionibacterium shermanii*** can grow in mixed continuous culture at the expense of lactate produced by ***Lactobacillus plantarum*** from glucose, the growth-limiting nutrient in the chemostat (Lee et al. 1976). Lactate was also the mediator in a commensal relationship between ***Streptococcus mutans*** and ***Veillonella alcalescens***. Both strains are commonly found in dental plaque and were shown to coexist in mixed continuous cultures supplemented with glucose as the limiting nutrient (Mikx and van der Hoeven 1975). In this mixed culture, the lactate produced by ***S. mutans*** was metabolized to acetate, propionate, and ethanol, which, owing to lower dissociation constants, might reduce the demineralization of tooth enamel. Also, consumption of lactate by a second organism (***Pseudomonas stutzeri***) under anaerobic conditions and in the presence of nitrate in a lactose-limited mixed chemostat culture results in a marked stimulation of the growth yield of ***Lactococcus cremoris*** (Otto et al. 1980). The latter is capable of an electrogenic lactate export during which two $H^+$ are translocated across the cytoplasmic membrane together with one lactate molecule, thereby generating a membrane potential. Since the driving force of this transport is the transmembrane lactate gradient, the stimulatory interaction is based on the very low external lactate concentration maintained by ***Pseudomonas***. This enables ***S. cremoris*** to gain more energy from the efflux of lactate than at higher external concentrations (Michels et al. 1979). Commensalistic interactions are also established in mixed cultures where either stimulatory compounds are formed or inhibitory compounds are being removed. As an example of the removal of inhibitory compounds, methane-consuming pseudomonads grew in a mixed culture with a ***Hyphomicrobium*** species, the latter removing small inhibitory amounts of methanol formed during methane oxidation (Wilkinson et al. 1974).

## Synergism

Synergism (protocooperation) occurs between ***Lactobacillus arabinosus*** and ***Enterococcus faecalis*** in a minimal medium (Nurmikko 1956) in which each organism is unable to grow on its own. The synergism is based on the fact that ***S. faecalis*** requires folic acid, which is produced by ***Lactobacillus***, whereas the latter requires phenylalanine, which is produced by ***Streptococcus***. Reciprocal stimulation also can occur in yogurt, where ***Lactobacillus bulgaricus*** produces amino acids that stimulate the growth of ***Streptococcus thermophilus***. The latter produces small quantities of formic acid, which in turn stimulates ***L. bulgaricus*** (Driessen 1981). Chemolithotrophic and phototrophic organisms growing with $CO_2$ as carbon source are known to excrete substantial quantities (>50%) of the carbon fixed as carbohydrates, peptides, amino acids, lipids, or vitamins (Clark and Schmidt 1966; Czeczuga 1968; Fogg 1971; Cohen et al. 1979; Jones 1982; Coveney 1982; Wolter 1982; Soendergaard et al. 1985; Bateson and Ward 1988). In these interactions, the benefit for the chemo- or photolithoautotrophic partner is often less evident and may comprise the supply of vitamins (Jones 1982), the reduction of oxygen concentration around heterocysts (Bunt 1961; Paerl 1978, 1982)—which has been disputed on physical grounds (Overmann 2002a)—and the formation of $CO_2$ (Lange 1971). A reciprocal relationship was also suggested in a mesophilic estuarine microbial mat system in which the cyanobacterium ***Microcoleus chthonoplastes*** excreted organic matter that stimulated growth of ***Thiocapsa roseopersicina***, which in turn prevented accumulation of excess amounts of hydrogen sulfide (de Wit and van Gemerden 1988).

Many different types of interspecies interactions occur in laminated microbial ecosystems, or so-called microbial mats, which are found on seashores, estuarine areas, salt marshes, and along the effluents from geothermal springs. Metabolic interactions include (1) the excretion of photosynthates and cell lysis of cyanobacteria as the primary colonizers, (2) respiration of the organic substances by heterotrophic bacteria, thereby generating anoxic conditions, (3) fermentation and reduction of sulfate by anaerobic bacteria, and (4) oxidation of the reduced sulfur compounds by anoxygenic phototrophs and chemolithotrophs (Stal et al. 1985). In addition, a multitude of interactions, most of them beneficial, occur between the different groups of prokaryotes (Overmann and van Gemerden 2000). In microbial mats, sulfate-reducing bacteria can reach their highest abundance at the surface, rather than at the deeper, permanently anoxic sediment layers. Accordingly, the sulfate-reducing bacterium ***Desulfovibrio desulfuricans*** has been found to be capable of syntrophic growth with the colorless sulfur-oxidizing bacterium ***Thiobacillus thioparus*** under oxygen limitation and with lactate as the electron-donating substrate (van den Ende et al. 1997). In this association, ***Thiobacillus*** removed molecular oxygen inhibiting ***Desulfovibrio*** and provided the latter with soluble polysulfide compounds as electron acceptor. ***Desulfovibrio*** in turn oxidized lactate to acetate, forming sulfide concomitantly, which served again as electron-donating substrate of ***Thiobacillus***.

## Syntrophic Interactions

Under certain conditions, synergism between different groups of anaerobic prokaryotes is essential for the degradation of organic matter. In contrast to aerobic decomposition, anaerobic mineralization involves the participation of different groups of prokaryotes with only a limited physiological flexibility. Consequently, a tight cooperation of mixed populations exists. In this multistep process, polymeric organic matter (such as cellulose, proteins, and lipids) is first hydrolyzed to oligomers and monomers that are subsequently fermented (Hungate 1960; Bryant 1976; Laanbroek and Veldkamp 1982; Wolin 1982; Nedwell 1984). The first step in anaerobic degradation of sugars, most amino acids, and other readily fermentable substrates is the formation of $H_2$, $CO_2$, formate, alcohols, acetate and other short-chain fatty acids, sulfide, and ammonia. In the presence

of oxidized sulfur compounds, further mineralization may proceed directly through activity of sulfate-reducing bacteria, which, as a group, can directly oxidize the (long-chain) fatty acids, amino acids, alcohols, aromatic compounds, and hydrogen (Widdel 1988). In the absence of sulfate, $CO_2$ will serve as the major electron acceptor resulting in the formation of methane. Methanogenic bacteria, however, have a very narrow range of substrates (mainly $H_2$, acetate, formate, methanol, and several methylamines; Oremland 1988). Hence, an additional group of prokaryotes, the "acetogenic" bacteria, are involved in the formation of suitable methane precursors. In the absence of sulfate, the subsequent fermentative degradation of some of the products like propionate, butyrate, or benzoate to hydrogen, $CO_2$, and acetate is an endergonic process under standard conditions, however. A syntrophic relation, the so-called interspecies hydrogen transfer, is established between hydrogen-producing bacteria (also called "obligate proton-reducing bacteria") on one hand and hydrogen-consuming prokaryotes such as methanogens on the other.

Anaerobic chemotrophic syntrophic cocultures are the only associations of prokaryotes that so far have been investigated in sufficient detail to understand the physiological basis of the interaction (Schink 1991). Interspecies hydrogen transfer in these cocultures is decreased to values below 10 Pa (Zehnder and Stumm 1988), which renders the oxidation of short-chain fatty acids exergonic. Thus, ***Syntrophomonas wolfei*** in coculture can metabolize even-numbered fatty acids such as butyrate, caproate, and caprylate to $H_2$ and acetate; odd-numbered fatty acids such as valerate and heptanoate are metabolized to propionate, acetate, and hydrogen (Dwyer et al. 1988; McInerney et al. 1979, 1981). ***Syntrophobacter wolinii*** was shown to degrade propionate to acetate, $CO_2$, and $H_2$ in coculture with an $H_2$-consuming ***Desulfovibrio*** species (Boone and Bryant 1980). Although acetate can be cleaved to $CH_4$ and $CO_2$ by several methanogenic species, a thermophilic acetate-oxidizing organism was shown to convert acetate to $CO_2$ and $H_2$ only in coculture with ***Methanobacterium thermautotrophicum*** (Zinder and Koch 1984). Benzoate and several other aromatic compounds were also shown to be degraded by cocultures of a proton-reducing species and methanogens, sulfate-reducing bacteria, or both (Mountfort and Bryant 1985; Dolfing and Tiedje 1986; Mountfort and Asher 1986; Szewzyk and Schink 1989). Although hydrogen represents the best-documented agent for transfer of reducing equivalents, formate serves the same purpose in species lacking hydrogenases (Thauer et al. 1975; Thiele and Zeikus 1988; Thiele et al. 1988). Syntrophic associations can involve even three different physiological types of prokaryotes, for example, two different sulfate-reducing bacteria and one methanogenic archaeon (Thiele and Zeikus 1988).

In other fermentations, proton reduction and subsequent hydrogen transfer is not a strict requirement since the reaction is exergonic under standard conditions, but interspecies hydrogen transfer shifts the fermentation pattern toward the production of more oxidized products plus hydrogen in contrast to the reduced fermentation products formed in pure culture. For example, ***Ruminococcus albus*** ferments glucose to acetate, $H_2$, and $CO_2$ (instead of to ethanol) if cocultured with ***Wolinella succinogenes***, which uses hydrogen as electron donor in the reduction of fumarate to succinate (Ianotti et al. 1973). Hydrogen transfer results in an additional energy gain for ***R. albus*** as more acetyl-CoA is converted to acetate in an ATP-yielding route. Shifts in fermentation products were also demonstrated for cocultures of ***Clostridium thermocellum*** and ***Methanobacterium thermautotrophicum*** (Weimer and Zeikus 1977).

A second type of syntrophic interaction has been found in cocultures of green sulfur bacteria with sulfur- or sulfate-reducing bacteria (Wolfe and Pfennig 1977; Biebl and Pfennig 1978; Warthmann et al. 1992). Under conditions of limitation by inorganic sulfur compounds as they prevail in freshwater ecosystems, the sulfide-producing bacterium relies on the activity of the sulfide-oxidizing phototroph for the availability of the electron acceptor, while the activity of the green sulfur bacteria is controlled by the activity of the sulfide-producing organism. In this way, a closed sulfur cycle is established through which each sulfur atom cycles many times (Pfennig 1980).

## Symbiosis

Mutual relationships between different prokaryotes render them capable of occupying niches that could not be occupied by either organism alone. "Symbiosis" in its original sense is a close association between different species of organisms (de Bary 1879). Currently, symbiosis is often defined as an obligatory and highly specific mutual relationship that benefits both partners (Atlas and Bartha 1993; ❯ *Table 7.7*). Most symbioses of prokaryotes known to date are with eukaryotes. Besides the endosymbiosis of chloroplasts (cyanobacteria) or α-mitochondria (Proteobacteria; Margulis 1981), the root nodule symbiosis of rhizobia with legumes is another well-studied example. In the latter association, the bacteria provide combined nitrogen and the plant host dicarboxylic acids. In their symbiosis with anaerobic protozoa, prokaryotes appear to act as hydrogen sinks in interspecies hydrogen transfer (Hackstein et al. 1999). In ectosymbiotic association with marine nematodes, especially adapted γ-Proteobacteria oxidize sulfide with concomitant $CO_2$ fixation (Ott et al. 1991), as do the endosymbionts of the same group in gutless marine oligochaetes (Dubilier et al. 1995). Cellulolytic nitrogen-fixing bacteria in shipworms (Bivalvia: Terebrenidae) provide exoenzymes necessary for the digestion of cellulose or keratene (Waterbury et al. 1983). Bacterial symbionts of insects synthesize nutrients such as vitamins (Aksoy 1995) or recycle amino acids (Douglas 1998). Intracellular bacterial symbionts of protozoa can provide toxins, which in turn are of selective value for the host in competition with protozoal competitors (Görtz and Brigge 1998). In other cases, the cytotoxicity of the protozoal host is increased by endosymbiotic bacteria (Fritsche et al. 1998). Most of the prokaryotic symbionts of higher organisms could not be cultured to date and thus have only been identified by 16S rDNA analyses.

In contrast to the hundreds of cases of symbiotic relationships between prokaryotes and eukaryotes, only very few examples of symbioses between different prokaryotes have been described to date (Overmann and Schubert 2002). However, evidence from very different environments, which range from the termite hindgut and dental plaque to the chemocline of stratified lakes and deep-sea sediments, indicates that many more symbiotic associations exist among prokaryotes (Overmann 2002a; Overmann and Schubert 2002). As indicated by microscopic studies, morphologically conspicuous associations of prokaryotes exist in the form of "consortia," in which two or more prokaryotes maintain a permanent cell-to-cell contact (Hirsch 1984; Schink 1991; Trüper and Pfennig 1971), and most likely represent the extreme case of a mutual interdependence of different prokaryotes. To date, some 16 different types of such consortia have been described (Huber et al. 2002; Overmann 2002a; Overmann and Schubert 2002) and are comprised of very different phylogenetic groups, for example, filamentous cyanobacteria associated with heterotrophic bacteria, associations between giant sulfur-oxidizing γ-Proteobacteria (***Thioploca***) and sulfate-reducing ***Desulfonema***, or the aggregates of sulfate-reducing bacteria with methanogenic archaea, which were detected in methane-hydrate-rich marine sediments. An additional novel type of association has been described (***Nanoarchaeum equitans***; Huber et al. 2002). Recently, advancements have been made toward a more detailed understanding of the structure and function of phototrophic consortia consisting of green sulfur bacterial epibionts surrounding a colorless motile central Betaproteobacterium (Fröstl and Overmann 1998, 2000; Overmann et al. 1998; Tuschak et al. 1999; Overmann and Schubert 2002).

## Enrichment Techniques

When the desired prokaryote is present in very small numbers, the enrichment culture technique is used to enable this particular type of microorganism to grow faster than all others in the sample. During selective enrichment, a natural sample harboring many different microorganisms is kept under conditions which favor the growth of a particular physiological type of prokaryote or a group of prokaryotes, thus allowing them to grow faster than accompanying physiologically different types of microorganisms (Schlegel and Jannasch 1967; Aaronson 1970; Norris and Ribbons 1970; Veldkamp 1977; Gerhardt et al. 1981; Poindexter and Leadbetter 1986; Austin 1988). Usually, no inhibitory ingredient is added to the medium, but the enrichment medium is designed to favor growth of the desired organism. The technique was essentially developed by microbiologists (e.g., Beijerinck and Winogradsky) early in the twentieth century. The enrichment technique is applied if (1) a prokaryote with particular metabolic properties is known to exist or (2) it is to be determined whether prokaryotes exist which grow under specific conditions, for example, high/low temperature, high osmolarity, extreme pH values, or with xenobiotic carbon substrates.

By far the most common method of enrichments is the batch culture technique, resulting in the selection of those prokaryotes that attain the maximum specific growth rate under the conditions chosen. Since at the same time, bacteria are growing for a substantial period of time at high substrate concentrations, batch culture enrichments frequently select for prokaryotes with high maximum growth rates and low substrate specificity (the so-called zymogenous organisms [Winogradsky 1949], also referred to as "*R*-strategists" [Andrews 1984; Andrews and Harris 1986]). It has been argued for many years that prokaryotes obtained in this way are not representative of those which dominate microbial transformations in the natural environment (Jannasch 1967a, b; Veldkamp and Jannasch 1972; Veldkamp 1977; Kuenen and Harder 1982; Gottschal 1986; Poindexter and Leadbetter 1986; Gottschal and Dijkhuizen 1988). Nevertheless, nonlimiting growth conditions possibly also occur under natural conditions at least as transient phenomena, for instance after the burial of organic matter (leaves and fecal pellets), during algal blooms, tidal flooding, or upon sudden changes in physicochemical conditions. Similarly, in gastrointestinal tracts, feeding may provide for conditions most suitable for a rapid response of opportunistic microbial species. The batch culture technique can be employed for the enrichment and isolation of *K*-strategists, if the latter outnumber the accompanying *R*-strategists in the original sample and are capable of (albeit slow) growth in the medium used. In these cases, serial dilution series are established, such that *R*-strategists are absent at higher dilutions and cannot overgrow the target prokaryotes (Ferris et al. 1996; Sekiguchi et al. 2001).

The second, less frequently employed technique is the use of a chemostat with one or more growth-limiting nutrients in the inflowing medium. Using this approach, organisms specialized in growing at very low substrate concentrations and usually exhibiting relatively low $\mu_{max}$ values and fairly high substrate specificities (so-called autochthonous prokaryotes or *K*-strategists) can be obtained and studied (Andrews 1984; Jannasch 1967a; Jannasch 1967b; Schlegel and Jannasch 1967; Harder et al. 1977; Kuenen and Harder 1982; Poindexter and Leadbetter 1986; Gottschal and Dijkhuizen 1988). Another advantage of continuous cultures systems over batch cultures is that physical and chemical parameters can be maintained constant over prolonged periods. Alternatively, these conditions may be changed in a controlled fashion, such as during light/dark cycles or oxic/anoxic transitions.

## Isolation of Prokaryotes

The purpose of isolation is the generation of a single clone, that is, a population consisting of bacteria all derived from a single cell. The strains obtained in this manner are an essential, but not sufficient (see section "Interactions with Other Microorganisms"), prerequisite for thorough, in-depth, and unambiguous studies of the ultrastructure, physiology, molecular biology, genetics, and autecology of prokaryotes. When free from other (contaminating) microorganisms, a culture is called "axenic." Not all individuals in this population need to be genetically identical as

mutation and selection of mutants can take place, but this heterogeneity is accommodated in the pure culture concept.

Since pure cultures almost never occur in nature, their preparation involves the physical separation of single cells from others, inoculation into sterile medium, and incubation under conditions allowing axenic growth. In general, samples are diluted by means of different methods and concomitantly placed on a nonselective medium. Either direct samples or aliquots from appropriate enrichments may be used for isolation of strains. Separation of single prokaryotic cells can be accomplished by streaking on solid media such as agar plates, separation in liquid media as occurs during preparation of pour plates, in liquid dilution series, or with the aid of micromanipulators. Each colony on solid media or culture developing in the highest positive dilutions potentially has grown from a single cell.

For inoculation with diluted sample material, a small amount of liquid ($\leq$100 µL for agar plates) containing not more than about 200 cells is brought on the surface of an agar plate and spread evenly by the use of a Drigalski spatula. Samples with higher cell densities, for instance material from cell colonies, are streaked on the surface with a sterile loop after dipping the latter in the sample. One of the most efficient ways of streaking is the "13-streak method" (Cypionka 1999), which involves intermittent sterilization of the loop and provides the highest chance to obtain single, well-isolated colonies from samples of widely different cell densities. Physically separated cells grow out into distinct populations visible on the solidified medium as small heaps or spots, called "colonies," varying in shape, color, and size. When streaking an alleged pure culture, all colonies should be identical in appearance, color, and texture, but in crowded parts of the plate, the size of the colonies will be smaller owing to nutrient depletion. Consequently, size difference as a criterion can only be applied to plates with well-separated (>5 mm) colonies. Motile and swarming prokaryotes cannot easily be isolated on agar plates unless the plates are thoroughly dried and chemicals (mostly detergents like Pril® at a concentration of 0.2% w/v, as used in Pril-Mannitol-Agar [Merck]; Pietzsch 1967; Reusse and Meyer 1972) added to disintegrate bacterial flagella and thus stop motility. Some bacteria cannot be cultivated or isolated on solid agar media and require sloppy agar or a liquid medium.

An alternate method is the inoculation of agar plates in the liquid stage, shortly before solidification (the so-called pour-plate technique). A known volume of a bacterial suspension is mixed with molten agar (kept at 42°C to just prevent solidification) and then poured into a sterile Petri dish where the mixture is allowed to solidify. With some experience, mixing can also be performed in the Petri dish itself. Upon incubation, cells will grow into colonies that mostly develop below the surface. The latter colonies often show a lenticular shape. Gas-forming colonies may produce cracks in the surrounding agar. The pour-plate technique is suited for the isolation of organisms requiring oxygen at pressures lower than atmospheric (microaerobic bacteria). It is not easy, however, to isolate or count subsurface colonies or to assess colony morphology as a criterion for culture purity. Also, some prokaryotes may be killed by exposure to the higher temperatures of the molten agar.

Anaerobic bacteria most frequently are isolated in deep-agar dilution series in prereduced media (see section "Removal and Exclusion of $O_2$, Cultivation of Anaerobes" in this chapter). The first tube is inoculated and mixed, and an aliquot (usually a tenth of the volume) is transferred to the following tube. In this manner, a serial dilution series is obtained, which is subsequently solidified by cooling in a 20°C water bath, rapidly gassed with $N_2$ or a mixture of $N_2/CO_2$, and sealed with gas-tight butyl rubber stoppers.

Liquid dilution series are preferentially employed for more sensitive bacteria that do not readily grow on agar-solidified media or for inocula with cell titers too high for direct plating on solid media. An additional advantage of this technique is that physical separation of bacterial cells in serial dilutions may promote growth of those bacteria which are otherwise inhibited by antibacterial substances (e.g., bacteriolytic enzymes) produced by the accompanying bacteria (Talamoto et al. 1994). Heterogeneous liquid samples are first homogenized by shaking or agitation in some sort of mechanical device, for example, a tube containing sterile glass beads, or in a mixer. A nontoxic detergent and complexing agents, for example, Tween 80 (final concentration, 0.05% v/v) and sodium pyrophosphate (10 mM in 10 mM *n*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] [HEPES] buffer), respectively, may be added for detachment of cells adhering to solids. Subsequently, serial dilutions (mostly in 1:10 dilution steps) are prepared in sterile medium. Directly after inoculation, each tube has to be mixed carefully, and a fresh glass pipette or pipette tip must be employed for the following dilution step. By inoculating tubes with cell suspensions so dilute that some tubes receive just one single cell or no cell at all, pure cultures may ultimately be obtained, and most probable numbers (MPN) of culturable bacteria can be calculated. This method is based on the assumption that bacteria are normally distributed in the liquid media. Numbers of viable cells from natural samples obtained in this way frequently surpass those obtained on agar media (Bartscht et al. 1999; Jaspers et al. 2001). However, the opposite was observed for soil samples, which yielded highest numbers of cultivated bacteria on improved solid media (Janssen et al. 2002). More recently, the MPN technique has been adapted and increasingly used to recover ultraoligotrophic and highly fastidious marine planktonic bacteria by dilution-to-extinction culturing. In these approaches, sterilized seawater was used as growth medium and permitted the isolation of *Sphingopyxis alaskensis* from cold, coastal oceanic samples (Button et al. 1993) and the enrichment of "*Candidatus Pelagibacter ubique*," the first cultured representative of the most abundant heterotrophic bacterioplankton group in the sea (Rappe et al. 2002).

One drawback of MPN dilution series when employed for the isolation of bacterial strains is that only few cultures can be obtained from the highest positive dilutions (i.e., based on the number of parallel dilution series, usually 3–8). However, provided the average viability for cells in a given sample is known, the number of pure cultures produced can be increased by orders of magnitude without increasing the total number of cultures inoculated. To this end, one appropriate size of inoculum is

chosen which statistically contains 0.5 viable cells for parallel inoculation of media. A very efficient way of automated inoculation is the MICRODROP® technique, in which microdroplets (volume of ~100 pL) of a cell suspension are generated by a piezoelectric actuator and positioned directly on agar plates (Schober et al. 1993) or into each well of microtiter plates filled with liquid media. If the procedure is automated, large numbers of inoculation can be performed very rapidly (e.g., 100 inoculations in less than a minute). If each droplet statistically contains 0.5 viable bacterial cells, the number of positive positions on the agar plate or of wells in a microtiter plate follows a Poisson distribution and the most probable number of culturable bacteria can be calculated from fraction $p$ of positive tubes (i.e., the number of positive tubes divided by the total number of tubes $n$) according to Button et al. (1993)

$$MNP = -\ln(1-p) \tag{7.24a}$$

The standard deviation is

$$\text{S.D.} = \sqrt{\frac{p}{[n\cdot(1-p)]}} \tag{7.24b}$$

A much more tedious isolation procedure involves the use of micromanipulator devices (Skerman 1968), which nowadays include computer-assisted electronic controls, and is used to physically separate single cells or morphologically defined symbiotic consortia under an inverted microscope (Fröhlich and König 1999; Fröstl and Overmann 2000). The isolated cells can then be transferred into sterile media. This latter method is especially valuable if morphologically conspicuous prokaryotes, which are easily overgrown by other microorganisms, are to be isolated. This procedure has been used to isolate cyanobacteria, myxobacteria, budding bacteria, and manganese-oxidizing bacteria and phototrophic consortia (Fröhlich and König 1999; Fröstl and Overmann 2000; Sly and Arunpairojana 1987).

## Alternative and Novel Concepts for Cultivation

The usually low cultivation success and the molecular detection of entire not-yet-cultured groups of prokaryotes (see section "Introduction" in this chapter) strongly indicate that specially adapted or novel techniques have to be developed for the isolation of not-yet-cultured prokaryotes from natural samples. Most importantly, novel or alternative cultivation techniques should account for those factors most likely responsible for the low culturability.

Meanwhile, state-of-the-art molecular techniques are frequently employed to identify phylotypes associated with biogeochemical transformations. Molecular approaches are not limited to monitor abundances of certain phylotypes but also include metagenomic techniques that helped to identify entirely unexpected physiological properties of selected phylotypes such as the widespread occurrence and utilization of proteorhodopsin by marine Proteobacteria (Béjà et al. 2000). Some information on the physiological capabilities of "not-yet-cultured prokaryotes" can now be obtained even down to the single-cell level (Ouverney and Fuhrman 2000; Wagner et al. 2006; Beier and Bertilsson 2011). The information gained by molecular approaches allows to develop even more targeted cultivation strategies. Chemotaxis assays have been developed that permit in situ testing of the response of particular bacteria to selected substrates and at the same time enrich for the desired bacterial type (Overmann 2005). It should be stressed, however, that recent progress in the successful cultivation (often also isolation) of previously unculturable prokaryotes cannot be attributed to a single technological breakthrough but rather an educated combination of different, often innovative, methods. The importance of applying a combination of different growth media rather than a single "universal" medium has already been demonstrated 15 years ago (Balestra and Misaghi 1997). A thorough analysis of a bacterial genome sequence may reveal media supplements required for axenic growth as in the case of the human pathogenic actinobacterium *Tropheryma whipplei*, the agent of Whipple's disease that is unable to synthesize several amino acids de novo. Subsequently, a cell-free culture medium for this bacterium could be established (Renesto et al. 2003). However, it will remain a challenging task to identify more complex requirements such as those based on oligotrophic properties or the dependence on biotic interactions with other microorganisms based on a genome sequence alone. Therefore, it is to be expected that careful and high-throughput growth experiments will remain a crucial element of cultivation trials also in the foreseeable future.

### Ionic Composition of the Growth Media

Fully synthetic media have been developed which correspond to the ionic strength of freshwater (Bartscht et al. 1999) or seawater (Schut et al. 1993; Sass et al. 2001). For the cultivation of soil prokaryotes, ionic concentrations have been adapted to those typically found in soil solution (Angle et al. 1991; Grosser et al. 2000; for Winogradsky's salt solution, compare Aagot et al. 2001). Synthetic seawater medium has been used in several investigations of the culturability of marine bacteria (Schut et al. 1993; Eguchi et al. 2001; Sass et al. 2001). The various recipes differ slightly with respect to trace element content, salt composition, and the type of buffer used, and they represent an improvement over other types of media employed previously. Reduction or elimination of phosphate and copper concentrations, respectively, has proven very successful for the isolation of "unculturable" cyanobacteria (Waterbury 1991). Recently, artificial freshwater media have been developed which facilitate the cultivation of planktonic bacteria from limnic sources (Bartscht et al. 1999; Jaspers et al. 2001).

### Exploring Novel Types of Metabolism

The potential of any reaction to drive prokaryotic growth can be predicted based on a calculation of the free energy change.

A negative value of the free energy change indicates that a postulated reaction can actually promote microbial growth. This information can then be used to tailor enrichments specifically for microorganisms that perform the transformation of interest. As one prominent example, anaerobic ammonium oxidation (the so-called anammox process) was predicted theoretically by Broda (1977) and later discovered to occur in certain members of the phylum Planctomycetes (Jetten et al. 1998). Similarly, the proper combination of untraditional electron donors and acceptors based on thermodynamical calculations has led to the successful enrichment and isolation of an anaerobic arsenite-oxidizing autotrophic gammaproteobacterium (Oremland et al. 2002) and a phosphite-oxidizing autotrophic deltaproteobacterium (Schink et al. 2002). Novel types of microbial reactions may also be postulated on the basis of measurements of concentration changes in potential microbial substrates and products in the environment (Zengler et al. 1999; Schink and Friedrich 2000).

## Cultivation-Independent Physiological Testing of Target Bacteria

The enrichment and isolation of numerically abundant prokaryotes or specific groups of interest can be followed by a phylogenetic screening of enrichments, for example, by fluorescent in situ hybridization (FISH; Kane et al. 1993; Spring et al. 2000). FISH has been used in a combined approach to identify potential growth substrates of target prokaryotes for subsequent enrichment and isolation experiments. In this approach, the uptake of radiolabeled substrates by target bacteria can be followed if microautoradiography is combined with FISH using oligonucleotide probes designed for selected prokaryotes (Lee et al. 1999; Cottrell and Kirchman 2000; Gray et al. 2000; Nielsen et al. 2000; Ouverney and Fuhrman 2000). Pitfalls of this technique are (1) that only one growth substrate can be tested at a time and (2) that the added substrate may be rapidly degraded by other (nontarget) microorganisms and that target prokaryotes are actually the only organisms present capable of assimilating the (unidentified) metabolites.

Stable isotope probing is a parallel approach based on labeling with $^{13}$C-labeled substrates of genomic DNA of selected prokaryotes. Active prokaryotes can subsequently be identified by 16S rDNA sequencing of the heavy DNA (Radajewski et al. 2000). A limitation of the latter technique is that (1) only those substrates can be tested which are utilized by very few types of prokaryotes (e.g., $C_1$-compounds), (2) a large excess of labeled substrate and long incubation times are necessary to maximize $^{13}$C-uptake and obtain sufficiently heavier genomic DNA—possibly resulting in pronounced shifts in microbial community structure, and (3) unknown intermediates may be rapidly generated by other bacteria, thus preventing a correct identification of potential growth substrates.

Thirdly, prokaryotes stimulated by defined substrates can be identified on the basis of the incorporation of bromodeoxyuridine (BrdU) into genomic DNA by metabolically active cells, subsequent immunocapture of BrdU-labeled DNA, and sequencing of the corresponding 16S rDNA sequences (Urbach et al. 1999). An important limitation of the latter approach is that not all prokaryotes assimilate BrdU, even when metabolically active.

As a fourth approach to identify potential growth substrates and optimize growth conditions for yet-to-be-cultured prokaryotes, specific oligonucleotide probes can be developed which are directed against the internal transcribed spacer region of the rRNA (*rrn*) operon and used for FISH to detect selectively, physiologically active cells (Cangelosi and Brabant 1997; Licht et al. 1999; Oerther et al. 2000; Schmid et al. 2001). Lately, the quantification of ITS transcripts via qPCR was also used to monitor physiological activity in situ (Marschall et al. 2010). All of the above techniques in principle can be employed to perform substrate tests for target prokaryotes under close to in situ conditions without prior cultivation of the microorganism of interest.

## Types and Concentrations of Growth Substrates

Sometimes, specific substrates can selectively promote the growth of numerically abundant bacteria (González et al. 1997). However, target bacteria are frequently slow-growing *K*-strategists and hence easily overgrown by the more rare but rapidly dividing large-celled *R*-strategist bacteria during enrichment in the presence of higher substrate concentrations. Consequently, one novel approach for the isolation of bacteria from natural samples is the utilization of low substrate concentrations of carbon substrates added to synthetic media of appropriate ionic strength (see section "Ionic Composition of Growth Media"). In addition, reduction of the concentrations of N and P compounds (which at certain times may represent the growth-limiting natural substrates) has been found to significantly increase colony counts (Eilers et al. 2001). An alternative approach is to employ sterile filtered seawater directly (Button et al. 1993; Connon and Giovannoni 2002; Rappe et al. 2002), which already contains naturally occurring organic carbon substrates as potential substrates for the growth of prokaryotes. Media for the cultivation of soil bacteria can be supplemented with cold soil extract (Olsen and Bakken 1987) as a carbon and energy source or as a source of supplies. In some instances, application of low-nutrient media has yielded comparably high cultivation success (up to 60%; Button et al. 1993). Application of complex mixtures of organic carbon substrates from natural sources can also increase growth of selected groups of anaerobic bacteria. For example, anaerobically prepared sterilized sludge or sediment slurries resulted in higher MPN of sulfate-reducing bacteria as compared to conventional media (Vester and Ingvorsen 1998).

As an alternative, typical *K*-strategists can be preconcentrated and potential *R*-strategists selectively excluded by a prefiltration step during which the inoculum is filtered through sterile 0.8-µm- or 0.2-µm-pore-size membrane filters. This treatment can significantly increase the enrichment

efficiency of certain target bacteria. As an example, members of the ***Holophaga/Acidobacterium*** lineage or the freshwater *Actinobacterium* cluster could be successfully enriched from freshwater lake sediments or lake water, respectively, using prefiltration (Spring et al. 2000; Hahn et al. 2003).

Especially adapted cultivation techniques have been developed to meet the exact physiological requirements of fastidious bacteria. Gradient plates or gradient tubes represent a simple means to cultivate prokaryotes dependent on two substrates, each present at a defined and very narrow concentration range. Examples include chemolithotrophic sulfur-oxidizing bacteria, which are naturally occurring in highly stratified environments such as the chemocline of stratified lakes or sediments. In such cases, artificial agar-stabilized oxygen-sulfide-countergradients have been used very successfully to isolate for the first time ***Beggiatoa alba*** or magnetotactic cocci (Nelson et al. 1986; Meldrum et al. 1993). In a second type of system, a quartz sand core is employed for gradient stabilization and is sandwiched between a lower anoxic sulfide-containing compartment and an upper oxic compartment (benthic gradient chamber; Pringault et al. 1996). As a great advantage of such gradient systems, the bacteria of interest (by means of their own physiological activity) precisely create the necessary environmental conditions for growth (Jørgensen 1982).

The testing of novel classes of substrates has led to the discovery of novel physiological capabilities of known bacterial species or even to the enrichment and isolation of novel bacteria of a broad phylogenetic diversity (Coates et al. 2002). Many investigations have focused on the role of humic substances on bacterial growth. Humic substances consist of a skeleton of alkyl or aromatic units which are cross-linked mainly by oxygen and nitrogen groups and which carry carboxylic acid, phenolic and alcoholic hydroxyls, ketone, and quinone groups (Schulten et al. 1991). Contrary to the conservative view of humic substances as being refractory, they in fact can be utilized in different ways by a large number of prokaryotes. Besides the utilization as growth substrates by ***Rhodococcus*** spp. (Goodfellow 1992a), and probably also by ***Actinoplanes*** and related genera (Vobis 1992), ***Microbispora***, ***Streptosporangium*** (Goodfellow 1992b), and ***Pedomicrobium*** spp. (Poindexter 1992), humic substances represent redox-active compounds that act as electron carriers in abiotic as well as biotic redox reactions (Coates et al. 2002). The redox-active moieties in these reactions are the quinone groups (Lovley et al. 1996a; Scott et al. 1998). Humic compounds can act as an electron acceptor for respiratory and fermentative bacteria belonging to diverse bacterial lineages, such as the high G + C Gram-positive bacteria (***Propionibacterium freudenreichii***), γ-Proteobacteria (***Shewanella algae***), δ-Proteobacteria (***Geobacter metallireducens***), or the ***Holophaga/Acidbacterium*** division (e.g., ***Geothrix fermentans***, Benz et al. 1998; Coates et al. 1998; Lovley et al. 1999). Humic compounds as water-soluble carriers can shuttle electrons between microorganisms and insoluble terminal electron acceptors (e.g., Fe(III) oxides; Lovley et al. 1996). In addition, humic substances have been shown to be utilized as electron-donating substrates during respiration with nitrate or fumarate, while organic carbon compounds (e.g., acetate) are assimilated heterotrophically (Lovley et al. 1999; Coates et al. 2002). This latter type of metabolism has been shown for ***S. alga***, ***G. metallireducens***, and ***G. fermentans*** but also for the denitrifying bacterium ***Paracoccus denitrificans***, the α-Proteobacterium ***Agrobacterium*** strain PB, the β-Proteobacteria ***Dechloromonas agitata***, ***Dechloromonas*** strain JJ, ***Azoarcus*** strain HA, the γ-Proteobacteria ***Pseudomonas*** strain BU, ***Pseudomonas*** strain NMX, and ***Marinobacter*** strain SBS, and a γ-Proteobacterium (strain KC; Lovley et al. 1999; Coates et al. 2002). These microorganisms are capable of exploiting a part of the large pool of humic substances in soils and sediments and gain a competitive advantage over prokaryotes that depend on limited organic compounds (e.g., acetate) as energy source.

## Cocultivation

Often, positive interactions between cells of one clone are required for growth of a single cell. This is exemplified by the fact that in the case of fastidious prokaryotes, small inocula can result in an extended lag period or a complete failure of growth. This effect has been attributed to the presence of low-molecular-weight metabolic intermediates excreted by living cells whose introduction into fresh medium depends on the size of the inoculum. This type of isolation difficulties can be remedied by preparing a sterile filtrate of spent enrichment culture medium and including this filtrate as a major constituent of the new medium on which single cells of fastidious microorganisms are to be isolated (Atlas and Bartha 1993).

As a third point of concern, evidence has accumulated that different prokaryotes may actually depend on phylogenetically nonrelated partner bacteria for a successful enrichment (see section "Interactions with Other Microorganisms"). For example, bacteria of the ***Holophaga/Acidobacterium*** lineage in enrichment cultures as well as natural microbial communities are frequently accompanied by Alphaproteobacteria of the ***Beijerinckia*** group (Felske et al. 1998; Nogales et al. 1999; Radajewski et al. 2000; Spring et al. 2000). Another example is the not-yet-cultured Betaproteobacterium associated with green sulfur bacteria in phototrophic consortia (Fröstl and Overmann 2000) and to date not enriched or grown in pure culture. Double culture vessels for cocultivation of different bacteria have been described earlier (Jannasch and Mateles 1974; Wirsen and Jannasch 1978). Meanwhile, simple incubation devices have been developed which permit the growth of two different prokaryotes in coculture but separate them by a membrane permeable to metabolites. These devices range from simple O-rings covered with membrane filters (Kaeberlein et al. 2002) over commercially available tissue culture inserts (Nichols et al. 2008) to specific cultivation vessels that facilitate cocultivation for aerobic but also anaerobic bacteria (*Fig. 7.5*).

The addition of cAMP, which represents one type of bacterial signal compound at least in some cases, resulted in a significant increase of culturability in natural samples (Bruns et al. 2002).

An isolated ***Roseovarius*** strain exhibited increased cell yield in the presence of cAMP. Similarly, the addition of pyrophosphate to cultures of ***E. coli*** has been proposed to confer upon the cells a better capacity to use carbon sources, induce biosynthetic processes, and enhance stationary-phase survival of ***E. coli*** cells (Biville et al. 1996).

### Chemical Protection

Upon exposure to heat, ethanol, or osmotic stress, bacterial cells may stop growing while their metabolism continues. As a result of this uncoupling, a burst of free radical production occurs which may be lethal to the cells. Exponentially growing bacterial cells are significantly more sensitive to this so-called suicide response than cells in the stationary phase (Aldsworth et al. 1999). Entry of ***E. coli*** into stationary-phase growth leads to expression of genes for resistance mechanisms under the control of the alternative sigma factor $\sigma^s$, and they include those for resistance against oxidative stress such as *katE* (Loewen and Hengge-Aronis 1994). In addition, the presence of oxidizing compounds, in particular molecular oxygen, itself can cause an inhibition of sensitive bacteria. Facultative anaerobes and microaerophiles can be grown only under reduced oxygen tension. Under full oxygen tension, these bacteria form cocultures with aerobes that lower concentrations in liquid enrichments or colonies on agar media. Isolation of target bacteria then requires reduced oxygen tension. The recovery after starvation stress can be significantly enhanced by inclusion of catalase in the agar medium even for bacteria like *Escherichia coli* that should itself be capable of $H_2O_2$ detoxification (Mizunoe et al. 1999). At least in some bacteria, cellular damage by oxidative stress can be prevented by the addition of $H_2O_2$-degrading compounds such as catalase, sodium pyruvate, or α-oxoglutaric acid (Martin et al. 1976; Brewer et al. 1977; Mossel et al. 1980; Mizunoe et al. 1999). The addition of ascorbic acid to the growth medium or lowering of the oxygen partial pressure has similar effects (Jannasch and Mateles 1974). The addition of chemical protectants or antioxidants may thus improve the recovery of abundant bacteria from natural samples. Accordingly, addition of activated charcoal as another scavenger of toxic oxygen forms increased the number of colony-forming units in samples from the deep terrestrial subsurface (Stevens 1995).

### Solid Surfaces

Solid surfaces may lead to stimulation of cell division and growth of starved bacteria (Kjelleberg et al. 1982, see section "Effect of Inhomogeneities"). So far, however, the capability of attachment has rarely been exploited as a selective factor for the enrichment and isolation of novel types of prokaryotes. The cover slip cultivation technique was originally developed for the growth of streptomycetes for microscopic examination (Kawato and Shinobu 1959) and subsequently established for the isolation of aquatic Planctomycetes (Schlesner 1994). This method uses sterilized glass cover slips that are inserted vertically into sterilized agar (often water or soft agar) at the bottom of a Petri dish. For the inoculation and growth of oligotrophic aquatic prokaryotes, the agar is overlaid with 10 mL of sample water and incubated for several weeks. For monitoring of growth, individual cover slips can be recovered and inspected by phase contrast light microscopy. Also, the presence of specific bacterial target groups can be investigated by fluorescence in situ hybridization (Dedysh 2011). Recently, polyurethane foam soaked with various agar media was employed during in situ incubation experiments and shown to trap a large variety of different marine bacteria (Yasumoto-Hirose et al. 2006).

Gliding bacteria, like the green sulfur bacterium *Chloroherpeton thalassium*, require a solid matrix for growth. For initial isolation, washed soft agar at a final concentration of 0.8% has been found to be the most suitable matrix. On this matrix, spreading of the filaments during growth leads to a fluffy appearance of the colonies. A gelling agent needs to be added even to pure cultures for growth, however. Good results have been obtained by adding Gelrite (4% w/v stock solution) to a final concentration of 0.025% which produces a visible increase in viscosity of the liquid medium and significantly stimulates cell division of the gliding bacteria. In a similar fashion, the filamentous gliding multicellular sulfate-reducing Desulfonema spp. can be maintained on insoluble aluminum phosphate precipitate which can readily be generated in the anoxic growth medium by addition of $AlCl_3$ and $Na_2CO_3$ (Widdel et al. 1983).

## Conservation of Prokaryotic Cultures

For future studies, as reference for standardized assays and tests, for taxonomic purposes, and as valuable stock for biotechnological applications, strains of prokaryotes need to be maintained and preserved over extended periods of time. During repeated cultivation cycles, mutations can arise that change major properties of strains. As a prominent example, Bdellovibrio strains that are typically host dependent for growth can acquire the ability for independent growth. For long-term maintenance, cultures are therefore routinely stored in a lyophilized or deep-frozen form to prolong their viability and to reduce changes due to the occurrence of mutations. Genetic changes may even during conservation, however, since conservation also selects for adapted variants among the same cell population and genetic drift can lead to rapid genetic changes upon transfer of culture small volumes after conservation.

### Maintenance of Working Cultures

Usually, maintenance of working cultures requires the periodic transfer of strains to fresh minimal media. Aerobic strains are streaked on cotton-plugged agar slants or agar plates, whereas

stab cultures in agar deeps are produced for facultative anaerobes or microaerophiles and closed by screw caps. Obligate anaerobes are transferred to fresh liquid media. After cultivation, cultures are stored in the refrigerator in the dark at 5–8°C to reduce metabolic activity while maintaining viability. The frequency of transfer should be kept to a minimum, but it has to be determined separately for each organism: some require transfer after just a few days, while some sporeformers can survive in liquid cultures for years. During storage, agar slants and stab cultures must be prevented from drying. To avoid selection of mutants, it is advisable to subculture from the whole plate or slant and not just from one colony.

For different physiological groups of bacteria, specific maintenance requirements exist. Working cultures of rumen bacteria and other fermentative anaerobes can be maintained in slants or sloppy agar (0.5–0.7% [w/v]) prepared from nonselective media. The cultures are incubated at 39°C until growth is just apparent and then stored at 4°C. Such cultures remain viable for a few weeks at least, although the viable count is decreased. Purple sulfur bacteria of the family Chromatiaceae are precultured in sulfide-containing media until the sulfide is depleted and intracellular sulfur has been formed as the intermediate oxidation product in the cells; cultures in this phase of growth have a chalky appearance macroscopically in reflected light. Subsequently, cultures are transferred to the dark at 4°C and can be stored for at least 3 months. Green sulfur bacteria (Chlorobiaceae) are maintained best after the accumulated sulfur has also been depleted. However, cultures of many microorganisms kept at 4°C still show slow growth and turnover of cellular components. Special preservation of cultures is required for extended storage and to prevent genetic and metabolic alterations.

## Preservation of Stock Cultures

### Spore-Forming Bacteria

One method of long-term preservation for spore-forming bacteria is based on the use of sterile soil or sand. A sample of soil or sand is sterilized in a screw-capped bottle by autoclaving for several hours on at least two successive days. Then, 1 mL of the suspension of the organism is added, and the contents dried in a vacuum desiccator with the cap of the tube loosely closed. When the contents are dry, the cap is closed tightly and the bottle stored in the refrigerator. Even easier is the use of sterile filter paper discs or strips soaked with the bacterial suspension. The discs are stored as described for sand above. One may keep several discs in one screw-cap container and remove one disc at a time, taking care not to contaminate the remaining discs.

### Lyophilization

Lyophilization (Tindall 2007) is often preferred over cryoconservation (see below) due to the simpler handling procedure. However, not all bacteria can be preserved by this method (e.g., purple sulfur bacteria [Chromatiaceae]) (Pfennig and Trüper 1989). For example, about 65% of the culture stocks held in the DSMZ culture collection are provided as lyophilized cultures.

Freshly grown cells are harvested by centrifugation and placed in an ampoule containing suspending medium and frozen at −60°C to −80°C. Rapid freezing is often accomplished in a metal chamber with dry ice (solid $CO_2$) and ethanol. The water is then removed from the frozen state directly by sublimation in a vacuum (<1 Pa). This prevents formation of a liquid water phase. Freeze-dryers consist of a manifold connected to a vacuum pump. The ampoule is sealed and stored in a cool place. The suspending medium is critical for the rate of survival during the freeze-drying process and for the rate of death of dried bacteria during storage. Among the most commonly used suspending media are serum plus 30% glucose nutrient broths or sterile skimmed milk fortified with 5% sucrose, sterilized in 3-mL amounts. Survival rates of Gram-positive and Gram-negative bacteria and yeasts during the freeze-drying procedure range between 8% and 85% and for subsequent long-term archiving range between 85% and 98% (Miyamoto-Shinohara et al. 2006). More robust bacterial strains could thus be conserved over 50 years in the freeze-dried state (Gherna and Reddy 2007).

A percentage of survivors of freeze-thaw stress may lose the ability to form colonies on a minimal salts-glucose medium. In ***E. coli***, this effect was not due to a particular nutrient that was absent but rather to a perturbation of the cell control system since cyclic GMP or ppGpp singly or in combination partly restored the efficiency of plating on a minimal medium of frozen-thawed cells. Mutagenic effects of freeze-thaw stress have been shown to be related to single-stranded breaks in DNA, an effect that might be similar to that of ionizing radiation. The damage can be repaired in nutrient media. In ***E. coli***, both the *uvrA*-, *polA*-dependent (excision repair) and the error-prone *rec*-, *polA*-dependent (recombinational repair) DNA repair pathways are required for repair of freeze-thaw-induced DNA damage.

### Freezing and Ultrafreezing

Cultures of rumen bacteria can be kept viable for up to one year by adding 20% (v/v) glycerol as a cryoprotectant and storage at −20°C (Teather 1982). Freezing and archiving of bacterial strains at −70°C has been successful for less fastidious bacteria and for time periods of 1–3 years (Gherna and Reddy 2007). Novel approaches comprise loading of porous glass or ceramic beads that are frozen directly without culture supernatant. Individual beads can then be retrieved while effectively avoiding thawing of the entire sample.

Extra- and intracellular ice crystal formation represent the major problems during freezing and ultrafreezing of living cells. During the freezing process, ice crystal formation in the

extracellular matrix results in an osmotic gradient across the cytoplasmic membrane which during slow freezing rates causes an osmotic loss of water and osmotic stress of the cells. Addition of extracellular cryoprotectants interferes with ice crystal growth and decreases the temperature of ice crystal formation. Extracellular cryoprotectants that have been tested are skimmed milk powder (20% w/v), sucrose (12% w/v) or other sugars (raffinose, lactose, trehalose), sugar alcohols (inositol, sorbitol; 10% w/v), glycine betaine, methanol, ethylen glycol, propandiole, 2-methyl-2,4-pentandiole, and polymers such as dextran, polyglycol, hydroxyethyl starch, methyl cellulose, polyvinylpyrrolidone, Ficoll, hyaluronan, bovine serum albumin, or activated charcoal (Hubalek 2003; Gherna and Reddy 2007).

Rapid freezing prevents water to leave the bacterial cell and but results in the formation of intracellular ice crystals that also may cause damage and permeabilization of the cytoplasmic membrane similar to extracellular crystals. Membrane-permeable cryoprotectants such as glycerol or dimethyl sulfoxide bind intracellular water, raise the osmotic pressure and hence interfere with intracellular ice crystal formation. Typical concentrations applied for glycerol are 10–25% (v/v) and for dimethyl sulfoxide are 5–10% (v/v). Due to the slow diffusion of glycerol through the cytoplasmic membrane, the cryoprotective effect of glycerol is only weak. DMSO is highly effective in promoting vitrification (i.e., formation of a viscous glassy aggregate state of water without ice crystals) of the cells but at the same time is cytotoxic. Therefore, DMSO must be removed rapidly upon thawing of the cells and before cultivation.

Another complication of cryoconservation is oxidative stress. Antioxidative protecting enzymes are damaged by freezing which may result in peroxidation of membrane phospholipids. In eukaryotic cells, formation of toxic oxygen species has been prevented by addition of reducing agents such as ascorbate (Tarin and Trounson 1993) or detoxifying enzymes such as superoxide dismutase (Dinara et al. 2001).

In order to promote vitrification of bacterial cells, rapid cooling is typically performed in the presence of cryoprotectants and at the temperature of liquid nitrogen (−196°C) or in the gaseous phase of liquid nitrogen (−150°C) which simultaneously excludes molecular oxygen (Hespell and Bryant 1981; Pfennig and Trüper 1989). Successful cryoconservation protocols comprise the use of narrow glass capillaries that are sealed directly after filling with culture suspension and subsequently dropped into liquid nitrogen (Hippe 1991; Tindall 2007). Yet, different strains of the same bacterial species can perform very differently during conservation, and an increasing number of novel isolates cannot be conserved using established protocols.

## Culture Collections

A complete list of culture collections worldwide may be obtained from the World Data Center for Microorganisms maintained by the World Federation for Culture Collections (WFCC) at (http://www.wfcc.info/ccinfo/collection/).

## References


Aagot N, Nybroe O, Nielsen P, Johnsen K (2001) An altered *Pseudomonas* diversity is recovered from soil by using nutrient-poor *Pseudomonas*-selective soil extract media. Appl Environ Microbiol 67:5233–5239

Aaronson S (1970) Experimental microbial ecology. Academic, New York

Abdul-Tehrani H, Hudson AJ, Chang YS, Timms AR, Hawkins C, Williams JM, Harrison PM, Guest JR, Andrews SC (1999) Ferritin mutants of *Escherichia coli* are iron deficient and growth impaired, and fur mutants are iron deficient. J Bacteriol 181:1415–1428

Achberger AM, Brox TI, Skidmore ML, Christner BC (2011) Expression and partial characterization of an ice-binding protein from a bacterium isolated at a depth of 3,519 m in the Vostok ice core, Antarctica. Front Microbiol 2:255

Ahn WS, Park SJ, Lee SY (2001) Production of poly (3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant *Escherichia coli*. Biotechnol Lett 23:235–240

Aksoy S (1995) *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidia* sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of Tsetse flies. Int J Syst Bacteriol 45:848–851

Alden L, Demoling F, Baath E (2001) Rapid method of determining factors limiting bacterial growth in soil. Appl Environ Microbiol 67:1830–1838

Aldsworth TG, Sharman RL, Dodd CER (1999) Bacterial suicide through stress. Cell Mol Life Sci 56:378–383

Alldredge AL, Youngbluth MJ (1985) The significance of macroscopic aggregates (marine snow) as sites for heterotrophic bacterial production in the mesopelagic zone of the subtropical Atlantic. Deep-Sea Res 32:1445–1456

Amy PS, Morita RY (1983) Starvation-survival patterns of sixteen freshly isolated open-ocean bacteria. Appl Environ Microbiol 45:1109–1115

Andrews JH (1984) Relevance of r-and K-theory to the ecology of plant pathogens. In: Klug MJ, Reddy CA (eds) Current perspectives in microbial ecology. ASM Press, Washington, DC, pp 1–7

Andrews JH, Harris RF (1986) r-and K-selection and microbial ecology. In: Marshall KC (ed) Advanced microbiology and ecology, vol 9. Plenum, New York, pp 1–7

Andrews SC, Robinson AK, Rodriguez-Quinones F (2003) Bacterial iron homeostasis. FEMS Microbiol Rev 27:215–237

Angle JS, McGrath SP, Chaney RL (1991) New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution. Appl Environ Microbiol 57:3674–3676

Arrhenius S (1889) Über die Reaktionsgeschwindigkeit bei der Inversion von Rohrzucker durch Säuren. Z Phys Chem 4:226–248

Atlas RM, Bartha R (1993) Microbial ecology, 3rd edn. Benjamin/Cummings, Redwood City

Austin B (1988) Methods in aquatic bacteriology. Wiley, Chichester

Azam F (1998) Microbial control of oceanic carbon flux: The plot thickens. Science 280:694–696

Bak F, Pfennig N (1991) Microbial sulfate reduction in littoral sediment of Lake Constance. FEMS Microbiol Ecol 85:31–42

Balch WE, Wolfe RS (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethane sulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl Environ Microbiol 32:781–791

Bale SJ, Goodman K, Rochelle PA, Marchesi JR, Fry JC, Weightman AJ, Parkes RJ (1997) *Desulfovibrio profundus* sp. nov., a novel barophilic sulfate-reducing bacterium from deep sediment layers in the Japan Sea. Int J Syst Bacteriol 47:515–521

Balestra GM, Misaghi IL (1997) Increasing the efficiency of the plate counting method for estimating bacterial diversity. J Microbiol Methods 30:111–117

Barer MR, Harwood CR (1999) Bacterial viability and culturability. Adv Microb Physiol 41:93–137

Barns SM, Fundyga RE, Jeffries MW, Pace NR (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci USA 91:1609–1613

Barns SM, Takala SL, Kuske CR (1999) Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. Appl Environ Microbiol 65:1731–1737

Baross JA, Morita RY (1978) Microbial life at low temperatures: ecological aspects. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, London, pp 9–17

Bartlett DH, Welch TJ (1995) *ompH* gene expression is regulated by multiple environmental cues in addition to high pressure in the deep-sea bacterium *Photobacterium* species strain SS9. J Bacteriol 177:1008–1016

Bartlett D, Wright M, Yayanos AA, Silverman M (1989) Isolation of a gene regulated by hydrostatic pressure in a deep-sea bacterium. Nature 342:572–574

Bartlett DH, Chi E, Wright ME (1993) Sequence of the *ompH* gene from the deep-sea bacterium *Photobacterium* SS9. Gene 131:125–128

Bartscht K, Cypionka H, Overmann J (1999) Evaluation of cell activity and of methods for the cultivation of bacteria from a natural lake community. FEMS Microbiol Ecol 28:249–259

Basler BL, Losick R (2006) Bacterially speaking. Cell 125:237–246

Bast E (2001) Mikrobiologische methoden, 2nd edn. Spektrum Akad, Berlin

Bateson MM, Ward DM (1988) Photoexcretion and fate of glycolate in a hot spring cyanobacterial mat. Appl Environ Microbiol 54:1738–1743

Battley EH (1995) An apparent anomaly in the calculation of ash-free dry weights for the determination of cellular yields. Appl Environ Microbiol 61: 1655–1657

Baxter RM, Gibbons NE (1962) Observations on the physiology of psychrophilism in a yeast. Can J Microbiol 8:511–517

Beier S, Bertilsson S (2011) Uncoupling of chitinase activity and uptake of hydrolysis products in freshwater bacterioplankton. Limnol Oceanogr 56:1179–1188

Béjà O, Aravind L, Koonin EV, Suzuki MT, Hadd A, Nguyen LP, Jovanovich SB, Gates CM, Feldman RA, Spudich JL, Spudich EN, DeLong EF (2000) Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. Science 289:1902–1906

Béjà O, Suzuki MT, Heidelberg JF, Nelson WC, Preston CM, Hamadas T, Eisen JA, Fraser CM, DeLong EF (2002) Unsuspected diversity among marine aerobic anoxygenic phototrophs. Nature 415:630–633

Benz M, Schink B, Brune A (1998) Humic acid reduction by *Propionibacterium freudenreichii* and other fermenting bacteria. Appl Environ Microbiol 64:4507–4512

Bernard L, Schäfer H, Joux F, Courties C, Muyzer G, Lebaron P (2000) Genetic diversity of total, active and culturable marine bacteria in coastal seawater. Aquat Microb Ecol 23:1–11

Bernhardt G, Lüdemann H-D, Jaenicke R, König H, Stetter KO (1984) Biomolecules are unstable under "black smoker" conditions. Naturwissenschaften 71:583–586

Beudeker RF, Gottschal JC, Kuenen JG (1982) Reactivity versus flexibility in thiobacilli. Antonie Van Leeuwenhoek 48:39–51

Beunink J, Rehm HJ (1988) Synchronous anaerobic and aerobic degradation of DDT by an immobilized mixed culture system. Appl Microbiol Biotechnol 29:72–80

Bhakoo M, Herbert RA (1979) The effects of temperature on the fatty acid and phospholipid composition of four obligately psychrophilic *Vibrio* spp. Arch Microbiol 121:121–127

Bi E, Lutkenhaus J (1993) Cell division inhibitors SulA and MinCD prevent formation of the FtsZ ring. J Bacteriol 175:1118–1125

Bidle KD, Lee SH, Marchant DR, Falskowski PG (2007) Fossil genes and microbes in the oldest ice on Earth. Proc Natl Acad Sci USA 104:13455–13460

Biebl H, Pfennig N (1978) Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. Arch Microbiol 117:9–16

Binnerup SJ, Jensen DF, Thordal-Christensen H, Sorgensen J (1993) Detection of viable, but non-culturable *Pseudomonas fluorescens* DF57 in soil using a microcolony epifluorescence technique. FEMS Microbiol Ecol 12:97–195

Biville F, Laurent-Winter C, Danchin A (1996) In vivo positive effects of exogenous pyrophosphate on Escherichia coli cell growth and stationary phase survival. Res Microbiol 147:597–608

Blochl E, Rachel R, Burggraf S, Hafenbradl D, Jannasch HW, Stetter KO (1997) *Pyrolobus fumarii*, gen. nov. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. Extremophiles 1:14–21

Bolen DW (2001) Protein stabilization by naturally occurring osmolytes. Methods Mol Biol 168:17–36

Boone DR, Bryant MP (1980) Propionate degrading bacterium *Syntrophobacter wolinii* sp. nov., gen. nov. from methanogenic ecosystems. Appl Environ Microbiol 33:1162–1169

Botsford JL, Harman JG (1992) Cyclic AMP in prokaryotes. Microbiol Rev 56:100–122

Bovill RA, Mackey BM (1997) Resuscitation of "non-culturable" cells from aged cultures of *Campylobacter jejuni*. Microbiology 143:1575–1581

Bowman JP, McCammon SA, Brown JL, Nichols PD, McMeekin TA (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 Evol Microbiol 47:670–677

Bowman JP, McCammon SA, Nichols DS, Skerratt JH, Rea SM, Nichols PE, McMeekin TA (1997b) *Shewanella gelidimarina* sp. nov. and *Shewanella frigidimarina* sp. nov., novel Antarctic species with the ability to produce eicosapentaenoic acid (20:5w3) and grow anaerobically by dissimilatory Fe (III) reduction. Int J Syst Bacteriol 47:1040–1047

Bowman JP, McCammon SA, Skerratt JH (1997c) *Methylosphaera hansonii* gen. nov., sp. nov., a psychrophilic, group I methanotroph from Antarctic marine-salinity, meromictic lakes. Microbiology 143:1451–1459

Bowman JP, McCammon SA, Brown JL, McMeekin TA (1998a) *Glaciecola punicea* gen. nov., sp. nov., and *Glaciecola pallidula* gen. nov., sp. nov.: psychrophilic bacteria from Antarctic sea-ice habitats. Int J Syst Evol Microbiol 48:1213–1222

Bowman JP, McCammon SA, Lewis T, Skerratt JH, Brown JL, Nichols DS, McMeekin TA (1998b) *Psychroflexus torquis* gen. nov., sp. nov., a psychrophilic species from Antarctic sea ice, and reclassification of *Flavobacterium gondwanense* (Dobson et al. 1993) as *Psychroflexus gondwandense* gen. nov., comb. nov. Microbiology 144:1601–1609

Boyaval P (1989) Lactic acid bacteria and metal ions. Lait 69:87–113

Bozal N, Montes MJ, Tudela E, Jimenez F, Guinea J (2002) *Shewanella frigidimarina* and *Shewanella livingstonesis* sp. nov. isolated from Antarctic coastal areas. Int J Syst Evol Microbiol 52:195–205

Braun V (1997) Avoidance of iron toxicity through regulation of bacterial iron transport. Biol Chem 378:779–786

Brefeld O (1881) Botanische Untersuchungen Über Schimmelpilze: Culturmethoden. Felix, Leipzig

Brewer DG, Martin SE, Ordal ZJ (1977) Beneficial effects of catalase or pyruvate in a most-probable-number technique for the detection of *Staphylococcus aureus*. Appl Environ Microbiol 34:797–800

Brock TD (1978) Thermophilic microorganisms and life at high temperatures. Springer, New York

Brock TD (1987) Introduction: an overview of the thermophiles. In: Brock TD (ed) Thermophiles: general molecular and applied microbiology. Wiley, New York, pp 1–16

Brock TD, O'Dea K (1977) Amorphous ferrous sulfide as a reducing agent for culture of anaerobes. Appl Environ Microbiol 33:254–256

Broda E (1977) Two kinds of lithotrophs missing in nature. Z Allg Mikrobiol 17:491–493

Broda DM, Saul DJ, Lawson PA, Bell RG, Musgrave DR (2000) *Clostridium gasigenes* sp. nov., a psychrophile causing spoilage of vacuum-packed meat. Int J Syst Evol Microbiol 50:107–118

Bromke B, Hammel JM (1987) Gelatin as a complete endogenous source of calcium for *Serratia marcescens* protease activity. J Microbiol Methods 6:253–256

Brooke AG, Watling EM, Attwood MM, Tempest DW (1989) Environmental control of metabolic fluxes in thermotolerant methylotrophic *Bacillus* strains. Arch Microbiol 151:268–273

Brown AD (1976) Microbial water stress. Bacteriol Rev 40:803–846

Brown AD, Simpson JR (1972) Water relations of sugar-tolerant yeasts: the role of intracellular polyols. J Gen Microbiol 72:589–591

Brown SA, Whiteley M (2007) A novel exclusion mechanism for carbon resource partitioning in *Aggregatibacter actinomycetemcomitans*. J Bacteriol 189: 6407–6414

Bruhn JB, Nielsen KF, Hjelm M, Hansen M, Brescani J, Schulz S, Gram L (2005) Ecology, inhibitory activity, and morphogenesis of a marine antagonistic bacterium belonging to the *Roseobacter* clade. Appl Environ Microbiol 71:7263–7270

Bruns A, Cypionka H, Overmann J (2002) Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central Baltic Sea. Appl Environ Microbiol 68:3978–3987

Bruns A, Hoffelner H, Overmann J (2003a) A novel approach for high throughput assays and the isolation of planktonic bacteria. FEMS Microbiol Ecol 45:161–171

Bruns A, Nübel U, Cypionka H, Overmann J (2003b) Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. Appl Environ Microbiol 69:1980–1989

Bryant MP (1976) The microbiology of anaerobic degradation and methanogenesis with special reference to sewage. In: Schlegel HW, Barnea J (eds) Microbial energy conversion. Erick Goltze KG, Göttingen, pp 107–1167

Bryson V, Szybalski W (1952) Microbial selection. Science 116:45–51

Bull AT, Slater JH (1982) Microbial interactions and community structure. In: Bull AT, Slater JH (eds) Microbial interactions and communities, vol 1. Academic, London, pp 13–44

Bull AT, Goodfellow M, Slater JH (1992) Biodiversity as a source of innovation in biotechnology. Annu Rev Microbiol 46:219–252

Bulthuis BA, Koningstein GM, Stouthamer AH, van Verseveld HW (1989) A comparison between aerobic growth of *Bacillus licheniformis* in continuous culture and partial-recycling fermenter, with contributions to the discussion on maintenance energy demand. Arch Microbiol 152:499–507

Bungay HR, Bungay ML (1968) Microbial interactions in continuous culture. Adv Appl Microbiol 10:269–290

Bunt JG (1961) Nitrogen-fixing blue-green algae in Australian rice soils. Nature 192:479–480

Burchard RP (1980) Gliding motility of bacteria. Bioscience 30:157–162

Burkhardt F (1992) Mikrobiologische diagnostik georg. Thieme, New York

Burnham JC, Conti SF (1984) Genus *Bdellovibrio*. In: Krieg NR (ed) Bergey's manual of systematic bacteriology, vol 1. Williams and Wilkins, Baltimore, pp 118–124

Bussmann I, Philipp B, Schink B (2001) Factors influencing the cultivability of lake water bacteria. J Microbiol Methods 47:41–50

Button DK, Schut F, Quang P, Martin R, Robertson BR (1993) Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. Appl Environ Microbiol 59:881–891

Button DK, Robertson BR, Lepp PW, Schmidt TM (1998) A small, dilute-cytoplasm, high-affinity, novel bacterium isolated by extinction culture and having kinetic constants compatible with growth at ambient concentrations of dissolved nutrients in seawater. Appl Environ Microbiol 64: 4467–4476

Byrer DE, Rainey FA, Wiegel J (2000) Novel strains of *Moorella thermoacetica* form unusually heat-resistant spores. Arch Microbiol 174:334–339

Calcott PH (1981) The construction and operation of continuous cultures. In: Calcott PH (ed) Continuous cultures of cells, vol 1. CRC Press, Boca Raton, pp 13–26

Calcott PH, Calvert TJ (1981) Characterization of 3′:5′cyclic AMP phosphodiesterase in *Klebsiella aerogenes* and its role in substrate accelerated death. J Gen Microbiol 122:313–321

Calcott PH, Postgate JR (1972) On substrate-accelerated death in *Klebsiella aerogenes*. J Gen Microbiol 70:115–122

Calcott PH, Montague W, Postgate JR (1972) The levels of cyclic AMP during substrate accelerated death. J Gen Microbiol 73:197–200

Cangelosi GA, Brabant WH (1997) Depletion of pre-16S rRNA in starved *Escherichia coli* cells. J Bacteriol 179:4457–4463

Carlsson J, Granberg GPD, Nyberg GK, Edlund M-BK (1979) Bactericidal effect of cysteine exposed to atmospheric oxygen. Appl Environ Microbiol 37: 383–390

Carpenter EJ, Lin S, Capone DG (2000) Bacterial activity in South Pole snow. Appl Environ Microbiol 66:4514–4517

Castenholz RW (1973) Movements. In: Carr NG, Whitton BA (eds) The biology of blue-green algae. Blackwell, London, pp 320–339

Cavicchioli R (2006) Cold-adapted archaea. Nat Rev Microbiol 4:331–343

Cayley S, Record MT, Lewis BA (1989) Accumulation of 3-[*N*-morpholino] propanesulfonate by osmotically stressed *Escherichia coli* K-12. J Bacteriol 171:3597–3602

Cayley S, Lewis BA, Record MT (1992) Origins of osmoprotective properties of betaine and proline in *Escherichia coli* K-12. J Bacteriol 174:1586–1595

Chan M, Himes RH, Akagi JM (1971) Fatty acid composition of thermophilic, mesophilic, and psychrophilic clostridia. J Bacteriol 106:876–881

Chao H, Davies PL, Carpenter JF (1996) Effects of antifreeze proteins in red blood cell survival during cryopreservation. J Exp Biol 199:2071–2076

Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL, Hughson FM (2002) Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415:545–549

Chen H, Jogler M, Rohde M, Klenk H-P, Busse H-J, Tindall B, Spröer C, Overmann J (2012) Reclassification and amended description of *Caulobacter leidyi* as *Sphingomonas leidyi* comb. nov., and emendation of the genus *Sphingomonas*. Int J Syst Evol Microbiol (in press)

Chesbro W (1988) The domains of slow bacterial growth. Can J Microbiol 34:427–435

Chesbro WR, Evans T, Eifert R (1979) Very slow growth of *Escherichia coli*. J Bacteriol 139:625–638

Chi E, Bartlett DH (1995) An rpoE-like locus controls outer membrane protein synthesis and growth at cold temperatures and high pressures in the deep-sea bacterium *Photobacterium* sp. strain SS9. Mol Microbiol 17:713–726

Chisholm SW, Frankel SL, Goericke R, Olson RJ, Palenik B, Waterbury JB, West-Johnsrud L, Zettler ER (1992) *Prochiorococcus marinus* nov. gen. nov. sp.: an oxyphototrophic marine prokaryote containing divinyl chlorophyll a and b. Arch Microbiol 157:297–300

Cho JC, Giovannoni SJ (2004) Cultivation and growth characteristics of a diverse group of oligotrophic marine Gammaproteobacteria. Appl Environ Microbiol 70:432–440

Christensen BB, Haagensen JAJ, Heydorn A, Molin S (2002) Metabolic commensalism and competition in a two-species microbial consortium. Appl Environ Microbiol 68:2495–2502

Christner BC (2010) Bioprospecting for microbial products that affect ice crystal formation and growth. Appl Microbiol Biotechnol 85:481–489

Clark C, Schmidt EL (1966) Effect of mixed culture on *Nitrosomonas europaea* simulated by uptake and utilization of pyruvate. J Bacteriol 91:367–373

Cleland N, Enfors SO (1983) Control of glucose fed batch cultivations of *E. coli* by means of an oxygen stabilized enzyme electrode. Eur J Appl Microbiol Biotechnol 18:141–147

Coates JD, Ellis DJ, Blunt-Harris EL, Gaw CV, Roden E, Lovley DR (1998) Recovery of humic-reducing bacteria from a diversity of environments. Appl Environ Microbiol 64:1504–1509

Coates JD, Cole KA, Chakraborty R, O'Connor SM, Achenbach LA (2002) Diversity and ubiquity of bacteria capable of utilizing humic substances as electron donors for anaerobic respiration. Appl Environ Microbiol 68:2445–2453

Cohen Y, de Jonge I, Kuenen JG (1979) Excretion of glycolate by *Thiobacillus neapolitanus* grown in continuous culture. Arch Microbiol 122:189–194

Cohn F (1872) Über bacterien, die kleinsten lebenden. Wesen Carl Habel, Berlin

Connon SA, Giovannoni SJ (2002) High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl Environ Microbiol 68:3878–3885

Coolen MJL, Cypionka H, Smock A, Sass H, Overmann J (2002) Ongoing modification of Mediterranean Pleistocene sapropels mediated by prokaryotes. Science 296:2407–2410

Cottrell MT, Kirchman DL (2000) Natural assemblages of marine proteobacteria and members of the Cytophaga-Flavobacteria cluster consuming low-and high-molecular-weight dissolved organic matter. Appl Environ Microbiol 66:1692–1697

Coveney MF (1982) Bacterial uptake of photosynthetic carbon from freshwater phytoplankton. Oikos 38:8–20

Crevel RWR, Fedyk JK, Spurgeon MJ (2002) Antifreeze proteins: characteristics, occurrence and human exposure. Food Chem Toxicol 40:899–903

Crocker FH, Guerin WF, Boyd SA (1995) Bioavailability of naphthalene sorbed to cationic surfactant-modified smectite clay. Environ Sci Technol 29: 2953–2958

Csonka LN (1989) Physiological and genetic responses of bacteria to osmotic stress. Microbiol Rev 53:121–147

Csonka LN, Epstein W (1996) Osmoregulation. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*, 2nd edn. ASM Press, Washington, DC, pp 1210–1223

Csonka LN, Hanson A (1991) Prokaryotic osmoregulation: genetics and physiology. Annu Rev Microbiol 45:569–606

Currie DJ, Kalff J (1984) Can bacteria outcompete phytoplankton for phosphorus? A chemostat test. Microb Ecol 10:205–216

Curtis TP, Sloan WT, Scannell JW (2002) Estimating prokaryotic diversity and its limits. Proc Natl Acad Sci USA 99:10494–10499

Cypionka H (1986) Sulfide-controlled continuous culture of sulfate-reducing bacteria. J Microbiol Methods 5:1–9

Cypionka H (1999) Grundlagen der mikrobiologie. Springer, New York

Cypionka H, Widdel F, Pfennig N (1985) Survival of sulfate-reducing bacteria after oxygen stress, and growth in sulfate-free oxygen-sulfide gradients. FEMS Microbiol Ecol 31:39–45

Czeczuga B (1968) An attempt to determine the primary production of the green sulphur bacteria, *Chlorobium limicola* Nads, (Chlorobacteriaceae). Hydrobiologia 31:317–333

Dalluge JJ, Hamamoto T, Horikoshi K, Morita RY, Stetter KO, McCloskey JA (1997) Posttranscriptional modification of tRNA in psychrophilic bacteria. J Bacteriol 179:1918–1923

Damoglou AP, Dawes EA (1968) Studies on the lipid content and phosphate requirement for glucose-and acetate-grown *Escherichia coli*. Biochem J 110:775–781

Dang HY, Lovell CR (2000) Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. Appl Environ Microbiol 66:467–475

Dawson MW, Maddox IS, Boag IF, Brooks JD (1988) Application of fed-batch culture to citric acid production by Aspergillus niger: the effects of dilution rate and dissolved oxygen tension. Biotechnol Bioeng 32:220–226

De Bary A (1879) Die Erscheinung der Symbiose Naturforschung Versammlung Cassel

de Freitas MJ, Fredrickson AG (1978) Inhibition as a factor in the maintenance of the diversity of microbial ecosystems. J Gen Microbiol 106:307–320

de la Broise D, Durand A (1989) Osmotic, biomass, and oxygen effects on the growth rate of *Fusarium oxysporum* using a dissolved oxygen controlled turbidstat. Biotechnol Bioeng 33:699–705

De Wit R, van Gemerden H (1988) Interactions between phototrophic bacteria in sediment ecosystems. Hydrobiol Bull 22:135–145

Dedysh SN (2011) Cultivating uncultured bacteria from northern wetlands: knowledge gained and remaining gaps. Front Microbiol 2:184

DeLong EF, Yayamos AA (1986) Biochemical function and ecological significance of novel bacterial lipids in deep-sea prokaryotes. Appl Environ Microbiol 51:730–737

DeLong EF, Ying Wu K, Prézelin BB, Jovine RVM (1994) High abundance of Archaea in Antarctic marine picoplankton. Nature 371:695–697

DeLong EF, Franks DG, Yayanos AA (1997) Evolutionary relationships of cultivated psychrophilic and barophilic deep-sea bacteria. Appl Environ Microbiol 63:2105–2108

Deming JW (1986) Ecological strategies of barophilic bacteria in the deep ocean. Microbiol Sci 3:205–207

Deming JW, Colwell RR (1985) Observations of barophilic microbial activity in samples of sediments and intercepted particulates from the Demerara abyssal plain. Appl Environ Microbiol 50:1002–1006

Deming JW, Somers LW, Straube WL, Swartz DG, MacDonell MT (1988) Isolation of an obligately barophilic bacterium and description of a new genus, *Colwellia* gen. nov. Syst Appl Microbiol 10:152–160

Dinara S, Sengoku K, Tamate K et al (2001) Effects of supplementation with free radical scavengers on the survival and fertilization rates of mouse cryopreserved oocytes. Human Reprod 16:1976–1981

Dobell C (1932) Anthony van Leeuwenhoek and his "little animals". Harcourt Brace, New York

Dolfing J, Tiedje JM (1986) Hydrogen cycling in a three-tiered food web growing on the methanogenic conversion of 3-chlorobenzoate. FEMS Microbiol Ecol 38:293–298

Dong K, Liu H, Zhang J, Zhou Y, Xin Y (2011) *Flavobacterium xueshanense* sp. nov. and *Flavobacterium urumqiense* sp. nov., two psychrophilic bacteria isolated from the China No. 1 glacier. Int J Syst Evol Microbiol 62:1151–1157

Douglas AE (1998) Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu Rev Entomol 43:17–37

Driessen FM (1981) Protocooperation of yoghurt bacteria in continuous cultures. In: Buschell ME, Slater JH (eds) Mixed culture fermentations. Academic, London, pp 99–120

Dubilier N, Giere O, Distel DL, Cavanaugh CM (1995) Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm (Oligochaeta, Annelida) by phylogenetic 16S rRNA sequence analysis and in situ hybridization. Appl Environ Microbiol 61:2346–2350

Dubinina GA, Leshcheva NV, Grabovich MY (1993) The colorless sulfur bacterium *Thiodendron* is actually a symbiotic association of spirochetes and sulfidogens. Microbiology 62:432–444

Dubinina GA, Grabovich MY, Leshcheva NV, Rainey FA, Gavrish E (2011) *Spirochaeta perfilievii* sp. nov., an oxygen-tolerant, sulfide-oxidizing, sulfur- and thiosulfate-reducing spirochaete isolated from a saline spring. Int J Syst Evol Microbiol 61:110–117

Dukan S, Nyström T (1998) Bacterial senescence: stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. Genes Dev 12:3431–3441

Dunfield PF, Liesack W, Henckel T, Knowles R, Conrad R (1999) High-affinity methane oxidation by a soil enrichment culture containing a type II methanotroph. Appl Environ Microbiol 65:1009–1014

Dwyer DF, Weeg-Aerssens E, Shelton DR, Tiedje JM (1988) Bioenergetic conditions of butyrate metabolism by a syntrophic, anaerobic bacterium in coculture with hydrogen oxidizing methanogenic and sulfidogenic bacteria. Appl Environ Microbiol 54:1354–1359

Dykhuizen DE (1998) Santa Rosalia revisited: why are there so many species of bacteria? Antonie Van Leeuwenhoek 73:25–33

Dykhuizen D, Davies M (1980) An experimental model: bacterial specialists and generalists competing in chemostats. Ecology 61:1213–1227

Egland PG, Palmer RJ, Kolenbrander PE (2004) Interspecies communication in *Streptococcus gordonii-Veillonella atypica* biofilms: signaling in flow conditions requires juxtaposition. Proc Natl Acad Sci USA 101:16917–16922

Egli T, Lindley ND, Quayle JR (1983) Regulation of enzyme synthesis and variation of residual methanol concentration during carbon limited growth of *Kloeckera* ap. 2201. on mixtures of methanol and glucose. J Gen Microbiol 129:1269–1281

Eguchi M, Ostrowski M, Fegatella F, Bowman J, Nichols D, Nishino T, Cavicchioli R (2001) Sphingomonas alaskensis AFO1, an abundant oligotrophic ultramicrobacterium from the North Pacific. Appl Environ Microbiol 67:4945–4954

Eichorst SA, Breznak JA, Schmidt TM (2007) Isolation and characterization of soil bacteria that define *Terriglobus* gen. nov., in the phylum Acidobacteria. Appl Environ Microbiol 73:2708–2717

Eilers H, Pernthaler J, Peplies J, Glöckner FO, Gerdts G, Amann R (2001) Isolation of novel pelagic bacteria from the German Bight and their seasonal contributions to surface picoplankton. Appl Environ Microbiol 67: 5134–5142

Emde R, Schink B (1990) Oxidation of glycerol, lactate, and propionate by *Propionibacterium freudenreichii* in a poised-potential amperometric culture system. Arch Microbiol 153:506–512

Emde R, Swain A, Schink B (1989) Anaerobic oxidation of glycerol by Escherichia coli in an amperometric poised-potential culture system. Appl Microbiol Biotechnol 32:170–175

Epstein W (1986) Osmoregulation by potassium transport in *Escherichia coli*. FEMS Microbiol Rev 39:73–78

Epstein W, Rothman-Denes LB, Hesse J (1975) Adenosine 3′:5′-cyclic monophosphate as mediator of catabolite repression in *Escherichia coli*. Proc Natl Acad Sci USA 72:2300–2304

Esener AA, Roels JA, Kossen NW (1981) Fed batch culture: modelling and application in the study of microbial energetics. Biotechnol Bioeng 22:1851–1871

Ettwig KF, Butler MK, LePaslier D, Pelletier E, Mangenot S, Kuypers MMM et al (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464:543–548

Euzéby JP, Tindall BJ (2001) Nomenclatural type of orders: corrections necessary according to Rules 15 and 21a of the Bacteriological Code (1990 Revision),

and designation of appropriate nomenclatural types of classes and subclasses. Request for an opinion. Int J Syst Evol Microbiol 51(Pt 2):725–727

Euzéby JP (2012) List of bacterial names with standing in nomenclature. http://www.bacterio.cict.fr

Evans CG, Herbert D, Tempest DW (1970) The continuous cultivation of microorganisms. 2. Construction of a chemostat. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 2. Academic, London, pp 277–327

Feller G (2007) Life at low temperatures: is disorder the driving force? Extremophiles 11:211–216

Feller G, Narinx E, Arpigny JL, Zekhnini Z, Swings J, Gerday C (1994a) Temperature dependence of growth, enzyme secretion and activity of psychrophilic Antarctic bacteria. Appl Microbiol Biotechnol 41:477–479

Feller G, Payan F, Theys F, Quian M, Haser R, Gerday C (1994b) Stability and structural analysis of α-amylase from the Antarctic psychrophile *Alteromonas haloplanctis* A23. Eur J Biochem 222:441–447

Felske A, Wolterink A, van Lis R, Akkermans ADL (1998) Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). Appl Environ Microbiol 64:871–879

Felske A, Wolterink A, van Lis R, de Vos WM, Akkermans ADL (1999) Searching for predominant soil bacteria: 16S rDNA cloning versus strain cultivation. FEMS Microbiol Ecol 30:137–145

Ferchichi M, Hemme D, Bouillaune C (1986) Influence of oxygen and pH on methanethiol production from L-methionine by *Brevibacterium linens* CNRZ 918. Appl Environ Microbiol 51:725–729

Ferris MJ, Ruff-Roberts AL, Kopczynski ED, Bateson MM, Ward DM (1996) Enrichment culture and microscopy conceal diverse thermophilic *Synechococcus* populations in a single hot spring microbial mat habitat. Appl Environ Microbiol 62:1045–1050

Filippini M, Kaech A, Ziegler U, Bagheri HC (2011) *Fibrisoma limi* gen. nov., sp. nov., a filamentous bacterium isolated from tidal flats. Int J Syst Evol Microbiol 61:1418–1424

Fleming A (1929) On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*. Br J Exp Pathol 10:226–236

Flint KP (1985) A note on a selective agar medium for the enumeration of *Flavobacteriu*m species in water. J Appl Bacteriol 59:561–566

Fogg GE (1971) Extracellular products of algae in freshwater. Arch Hydrobiol Beih Ergebn Limnol 5:1–25

Forsberg CW (1987) Production of 1,3-propanediol from glycerol by *Clostridium acetobutylicum* and other *Clostridium* species. Appl Environ Microbiol 53:639–643

Francis CA, Tebo BM (2002) Enzymatic manganese(II) oxidation by metabolically dormant spores of diverse *Bacillus* species. Appl Environ Microbiol 68:874–880

Franzmann PD, Höpfl P, Weiss N, Tindall BJ (1991) Psychrotrophic, lactic acid-producing bacteria from anoxic waters in Ace Lake, Antarctica: *Carnobacterium funditum* sp. nov. and *Carnobacterium alterfunditum* sp. nov. Arch Microbiol 156:255–262

Franzmann PD, Springer N, Ludwig W, Conway de Macario E, Rohde M (1992) A methanogenic archaeon from Ace Lake, Antarctica: *Methanococcoides burtonii* sp. nov. Syst Appl Microbiol 15:573–581

Franzmann PD, Liu Y, Balkwill DL, Aldrich HC, Conway de Macario E, Boone DR (1997) *Methanogenium frigidum* sp. nov., a psychrophilic $H_2$-using methanogen from Ace Lake Antarctica. Int J Syst Bacteriol 47:1068–1072

Fredrickson AG (1977) Behaviour of mixed cultures of microorganisms. Annu Rev Microbiol 31:63–87

Fredrickson AG, Stephanopoulos G (1981) Microbial competition. Science 213:972–979

Fritsche TR, Sobek D, Gautom RK (1998) Enhancement of in vitro cytopathogenicity by *Acanthamoeba* spp. following acquisition of bacterial endosymbionts. FEMS Microbiol Lett 166:231–236

Fröhlich J, König H (1999) Rapid isolation of single microbial cells from mixed natural and laboratory populations with the aid of a micromanipulator. Syst Appl Microbiol 22:249–257

Fröstl JM, Overmann J (1998) Physiology and tactic response of the phototrophic consortium "*Chlorochromatium aggregatum*". Arch Microbiol 169:129–135

Fröstl JM, Overmann J (2000) Phylogenetic affiliation of the bacteria that constitute phototrophic consortia. Arch Microbiol 174:50–58

Fry JC (1990) Direct methods and biomass estimation. In: Grigorova R, Norris JR (eds) Methods in microbiology, vol 22. Academic, London, pp 41–85

Fuhrman JA, McCallum K, Davis AA (1992) Novel major archaebacterial group from marine plankton. Nature 356:148–149

Fuhrman JA, McCallum K, Davis AA (1993) Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. Appl Environ Microbiol 59:1294–1302

Funk HB, Krulwich TA (1964) Preparation of clear silica gels that can be streaked. J Bacteriol 88:1200–1201

Fuqua C, Greenberg EP (1998) Self perception in bacteria: quorum sensing with acylated homoserine lactones. Curr Opin Microbiol 1:50–58

Galinski EA, Trüper HG (1994) Microbial behaviour in salt-stressed ecosystems. FEMS Microbiol Rev 15:95–108

Gangola P, Rosen BP (1987) Maintenance of intracellular calcium in *Escherichia coli*. J Biol Chem 262:12570–12574

Ganzert L, Bajerski F, Mangelsdorf K, Lipski A, Wagner D (2011a) *Arthrobacter livingstonensis* sp. nov. and *Arthrobacter cryotolerans* sp. nov., salt-tolerant and psychrotolerant species from Antarctic soil. Int J Syst Evol Microbiol 61:979–984

Ganzert L, Bajerski F, Mangelsdorf K, Lipski A, Wagner D (2011b) *Leifsonia psychrotolerans* sp. nov., a psychrotolerant species of the family *Microbacteriaceae* from Livingston Island, Antarctica. Int J Syst Evol Microbiol 61:1938–1943

Garcia-Lara J, Shang LH, Rothfield LI (1996) An extracellular factor regulates expression of sdiA, a transcriptional activator of cell division genes in *Escherichia coli*. J Bacteriol 178:2742–2748

Garnham CP, Gilbert JA, Hartman CP, Campbell RL, Laybourn-Parry J, Davies PL (2008) A $Ca^{2+}$-dependent bacterial antifreeze protein domain has a novel β-helical ice-binding fold. Biochem J 411:171–180

Garnham CP, Campbell RL, Dabies PL (2011) Anchored clathrate waters bind antifreeze proteins to ice. Proc Natl Acad Sci USA 108:7363–7367

Gause GF (1934) The struggle for existence. Williams and Wilkins, Baltimore

Geissinger O, Herlemann DPR, Mörschel E, Maier UG, Brune A (2009) The ultramicrobacterium "*Elusimicrobium minutum*" gen. nov., sp. nov., the first cultivated representative of the termite group 1 phylum. Appl Environ Microbiol 75:2831–2840

Genomes Online Database (GOLD) (2012) http://www.genomesonline.org/cgi-bin/GOLD/index.cgi?page_requested=Statistics

Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (1981) Manual of methods for general bacteriology. American Society for Microbiology, Washington, DC

Gherna RL, Reddy CA (2007) Culture preservation. In: Reddy CA (ed) Methods for general and molecular microbiology. ASM Press, Washington, DC, pp 1019–1033

Gich F, Garcia-Gil J, Overmann J (2001) Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. Arch Microbiol 177:1–10

Gich F, Schubert K, Bruns A, Hoffelner H, Overmann J (2005) Specific detection, isolation and characterization of selected, previously uncultured members of freshwater bacterioplankton. Appl Environ Microbiol 71:5908–5919

Giovannoni S, Stingl U (2007) The importance of culturing bacterioplankton in the 'omics' age. Nature 5:820–826

Glöckner FO, Zaichikov E, Belkova N, Denissova L, Pernthaler J, Perthaler A, Amann R (2000) Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria. Appl Environ Microbiol 66:5053–5065

Gond O, Engasser JM, Matta-El-Amouri C, Petitdemange H (1986) The acetone butanol fermentation on glucose and xylose. II: Regulation and kinetics in fed batch cultures. Biotechnol Bioeng 28:167–175

González JM, Mayer F, Moran MA, Hodson RE, Withman WB (1997) *Sagittula stellata* gen. nov., sp. nov., a lignin-transforming bacterium from a coastal environment. Int J Syst Bacteriol 47:773–780

Goodfellow M (1992a) The family Nocardiaceae. In: Balows A, Trüper HG, Dworkin M, Schleifer K-H (eds) The prokaryotes, 2nd edn. Springer, New York, pp 1188–1213

Goodfellow M (1992b) The family Streptosporangiaceae. In: Balows A, Trüper HG, Dworkin M, Schleifer K-H (eds) The prokaryotes, 2nd edn. Springer, New York, pp 1115–1138

Görtz H-D, Brigge T (1998) Intracellular bacteria in protozoa. Naturwissenschaften 85:359–368

Gosink JJ, Staley JT (1995) Biodiversity of gas vacuolate bacteria from Antarctic sea ice and water. Appl Environ Microbiol 61:3486–3489

Gosink JJ, Woese CR, Staley JT (1998) *Polaribacter* gen. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov. and *P. filamentus* so. 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 Evol Microbiol 48:223–235

Gottschal JC (1986) Mixed substrate utilization by mixed cultures. In: Leadbetter ER, Poindexter JS (eds) Bacteria in nature, vol 2. Plenum, New York, pp 261–292

Gottschal JC (1990) Different types of continuous culture in ecological studies. In: Norris JR, Grigorova R (eds) Methods in microbiology, vol 22. Academic, London, pp 87–124

Gottschal JC, Dijkhuizen L (1988) The place of the continuous culture in ecological research. In: Wimpenny JWT (ed) Handbook of laboratory model systems for microbial ecosystems, vol 1. CRC Press, Boca Raton, pp 19–79

Gottschal JC, Kuenen JG (1980) Selective enrichment of facultatively chemolithotrophic thiobacilli and related organisms in continuous culture. FEMS Microbiol Lett 7:241–247

Gottschal JC, Morris JG (1981) The induction of acetone and butanol production in cultures of *Clostridium acetobutylicum* by elevated concentrations of acetate and butyrate. FEMS Microbiol Lett 12:385–389

Gottschal JC, de Vries S, Kuenen JG (1979) Competition between the facultatively chemolithotrophic *Thiobacillus* A2, an obligately chemolithotrophic *Thiobacillus* and a heterotrophic *Spirillum* for inorganic and organic substrates. Arch Microbiol 121:241–249

Gottschal JC, Harder W, Prins RA (1991) Principles of enrichment, isolation, cultivation, and preservation. In: Balows A, Trüper HG, Dworkin M, Schleifer K-H (eds) The prokaryotes, 2nd edn. Springer, New York, pp 149–196

Gottschalk G (1985) Bacterial metabolism. Springer, New York

Gottwald M, Gottschalk G (1985) The internal pH of *Clostridium acetobutylicum* and its effect on the shift from acid to solvent formation. Arch Microbiol 143:42–46

Graber JR, Breznak JA (2005) Folate cross-feeding supports symbiotic homoacetogenic spirochetes. Appl Environ Microbiol 71:1883–1889

Graham AF, Lund BM (1983) The effect of alkaline pH on growth and metabolic products of a motile, yellow-pigmented *Streptococcus* sp. J Gen Microbiol 129:2429–2435

Grant WD, Tindall BJ (1986) The alkaline saline environment. In: Herbert RA, Codd GA (eds) Microbes in extreme environments. Academic, London, pp 25–54

Graumann P, Marahiel MA (1996) Some like it cold: response of microorganisms to cold shock. Arch Microbiol 166:293–300

Gray ND, Howarth R, Pickup RW, Jones JG, Head IM (2000) Use of combined microautoradiography and fluorescence in situ hybridization to determine carbon metabolism in mixed natural communities of uncultured bacteria from the genus *Achromatium*. Appl Environ Microbiol 66:4518–4522

Groeschel DHM (1982) The etiology of tuberculosis: a tribute to Robert Koch on the occasion of the centenary of his discovery of the tubercle bacillus. ASM News 48:248–250

Gross CA (1996) Function and regulation of the heat shock proteins. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*, 2nd edn. ASM Press, Washington, DC, pp 1382–1399

Grosser RJ, Friedrich M, Ward DM, Inskeep WP (2000) Effect of model sorptive phases on phenanthrene biodegradation: different enrichment conditions influence bioavailability and selection of phenanthrene-degrading isolates. Appl Environ Microbiol 66:2695–2702

Guan LL, Onuki H, Kamino K (2000) Bacteria growth stimulation with exogenous siderophore and synthetic *N*-acyl homoserine lactone autoinducers under iron-limited and low-nutrient conditions. Appl Environ Microbiol 66:2797–2803

Guerin WF, Boyd SA (1997) Bioavailability of naphthalene associated with natural and synthetic sorbents. Water Res 51:1504–1512

Guerrero R, Pedros-Alió C, Esteve I, Mas J, Chase D, Margulis L (1986) Predatory prokaryotes: predation and primary consumption evolved in bacteria. Proc Natl Acad Sci USA 83:2138–2142

Hackstein JH, Akhmanova A, Boxma B, Harhangi HR, Voncken FG (1999) Hydrogenosomes: eukaryotic adaptations to anaerobic environments. Trends Microbiol 7:441–447

Hahn MW (2003) Isolation of strains belonging to the cosmopolitan *Polynucleobacter necessarius* cluster from freshwater habitats located in three climatic zones. Appl Environ Microbiol 69:5248–5254

Hahn MW (2009) Description of seven candidate species affiliated with the phylum Actinobacteria, representing planktonic freshwater bacteria. Int J Syst Evol Microbiol 59:112–117

Hahn MW, Lünsdorf H, Wu Q, Schauer M, Höfle MG, Boenigk J, Stadler P (2003) Isolation of novel ultramicrobacteria classified as Actinobacteria from five freshwater habitats in Europe and Asia. Appl Environ Microbiol 69:1442–1451

Hallam SJ, Mincer TJ, Schleper C, Preston CM, Roberts K, Richardson PM, DeLong EF (2006) Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine Crenarchaeota. PLoS Biol 4:e95

Handelsmann J (2004) Metagenomics: application of genomics to uncultured microorganisms. Microbiol Mol Biol Rev 68:669–685

Harder W, Dijkhuizen L (1982) Strategies of mixed substrate utilization in microorganisms. Philos Trans R Soc Lond B Biol Sci 297:459–480

Harder W, Veldkamp H (1971) Competition of marine psychrophilic bacteria at low temperatures. Antonie Van Leeuwenhoek 37:51–63

Harder W, Kuenen JG, Matin A (1977) Microbial selection in continuous culture. J Appl Bacteriol 43:1–24

Hardin G (1960) The competitive exclusion principle. Science 131:1292–1297

Harms H, Zehnder AJB (1995) Bioavailability of sorbed 3-chlorodibenzofuran. Appl Environ Microbiol 61:27–33

Harris JE (1985) Gelrite as an agar substitute for the cultivation of mesophilic *Methanobacterium* and *Methanobrevibacter* species. Appl Environ Microbiol 50:1107–1109

Harwood C, Buckley M (2008) The uncharted microbial world: microbes and their activities in the environment. A report from the American Academy of Microbiology. American Academy of Microbiology, Washington, DC, 37 p

Hastings RC, Saunders JR, Hall GH, Pickup RW, McCarthy AJ (1998) Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. Appl Environ Microbiol 64:3674–3682

Herbert RA (1986) The ecology and physiology of psychrophilic microorganisms. In: Herbert RA, Codd GA (eds) Microbes in extreme environments. Academic, London, pp 1–23

Herbert D, Elsworth R, Telling RC (1956) The continuous culture of bacteria: a theoretical and experimental study. J Gen Microbiol 14:601–622

Hespell RB, Bryant MP (1981) The genera Butyrivibrio, Succinivibrio, Lachnospira and Selenomonas. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds) The prokaryotes. Springer, New York, pp 1479–1494

Hinrichs K-U, Hayes JM, Sylva SP, Brewer PG, DeLong EF (1999) Methane-consuming archaebacteria in marine sediments. Nature 398:802–805

Hiorns WD, Methé BA, Nierzwicki-Bauer SA, Zehr JP (1997) Bacterial diversity in Adirondack Mountain lakes as revealed by 16S rRNA gene sequence analysis. Appl Environ Microbiol 63:2957–2960

Hippe H (1991) Maintenance of methanogenic bacteria. In: Kirsop BE, Doyle A (eds) Maintenance of microorganisms and cultured cells, 2nd edn. Academic, London, pp 101–114

Hirsch P (1984) Microcolony formation and consortia. In: Marshall KC (ed) Microbial adhesion and aggregation: Dahlem Konferenzen. Springer, New York, pp 373–393

Hochachka PW, Moon TW, Mustafa T (1972) The adaptation of enzymes to pressure in abyssal and midwater fishes. In: Sleigh MA, Macdonald AG (eds) The effects of pressure on living organisms. Academic, London, pp 175–195

Holmes AJ, Roslev P, McDonald IR, Iversen N, Henriksen K, Murrell JC (1999) Characterization of methanotrophic bacterial populations in soil

showing atmospheric methane uptake. Appl Environ Microbiol 65: 3312–3318

Hommes RWJ, Postma PW, Tempest DW, Neijssel OM (1989) The influence of the culture pH value on the direct glucose oxidation pathway in *Klebsiella pneumoniae* NCTC 418. Arch Microbiol 151:261–267

Hooke R (1665) Micrographia: or some physiological descriptions of minute bodies made by magnifying glasses with observations and inquiries thereupon. John Martyn and James Allestry, London

Horikoshi K, Akiba T (1982) Alkalophilic microorganisms. Springer, New York

Huang L, Forsberg CW, Gibbins LN (1986) Influence of external pH and fermentation products on *Clostridium acetobutylicum* intracellular pH and cellular distribution of fermentation products. Appl Environ Microbiol 51:1230–1234

Hubalek Z (2003) Protectants used in the cryopreservation of microorganisms. Cryobiology 46:205–229

Huber H, Hohn MJ, Rachel R, Fuchs T, Wimmer VC, Stetter KO (2002) A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. Nature 417:63–67

Hugenholtz P (2002) Exploring prokaryotic diversity in the genomic era. Genome Biol Rev 3:0003.1–0003.8

Hugenholtz P, Pitulle C, Hershberger KL, Pace NR (1998) Novel division level bacterial diversity in a yellowstone hot spring. J Bacteriol 180:366–376

Humphry DR, George A, Black GW, Cummings SP (2001) *Flavobacterium frigidarium* sp. nov., an aerobic psychrophilic, xylanolytic and laminariolytic bacterium from Antarctica. Int J Syst Evol Microbiol 51:1235–1243

Hungate RE (1950) The anaerobic mesophilic cellulolytic bacteria. Bacteriol Rev 14:1–49

Hungate RE (1960) Microbial ecology of the rumen. Symposium: selected topics in microbial ecology. Bacteriol Rev 24:353–364

Hungate RE (1966) The rumen and its microbes. Academic, New York

Hungate RE (1969) A roll tube method for cultivation of strict anaerobes. In: Norris JR, Ribbons WD (eds) Methods in microbiology, vol 3B. Academic, London, pp 117–132

Hungate RE (1985) Anaerobic biotransformations of organic matter. In: Leadbetter ER, Poindexter JS (eds) Bacteria in nature, vol 1. Plenum, New York, pp 39–96

Huston AL, Krieger-Brockett BB, Deming JW (2000) Remarkably low temperature optima for extracellular enzyme activity form Arctic bacteria and sea ice. Environ Microbiol 2:383–388

Ianotti EL, Kafkewitz D, Wolin MJ, Bryant MP (1973) Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes*. J Bacteriol 114:1231–1240

Imhoff JF (1986) Osmoregulation and compatible solutes in eubacteria. FEMS Microbiol Rev 39:57–66

Ingham CJ, Sprenkels A, Bomer J, Molenaar D, van der Berg A, van Hylckama Vlieg JET, de Vos WM (2007) The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. Proc Natl Acad Sci USA 104:18217–18222

Ingraham JL (1962) Temperature relationships. In: Gunsalus IC, Stanier RY (eds) The bacteria, vol 4. Academic, New York, pp 265–296

Ingraham JL, Marr AG (1996) Effect of temperature, pressure, pH, and osmotic stress on growth. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella typhimurium*. ASM Press, Washington, DC, pp 1570–1578

Irgens RL, Gosink JJ, Staley JT (1996) *Polaromonas vacuolata* gen. nov., sp. nov., a psychrophilic, marine gas vacuolate bacterium from Antarctica. Int J Syst Evol Microbiol 46:822–826

Isaksen MF, Jørgensen BB (1996) Adaptation of psychrophilic and psychrotrophic sulfate-reducing bacteria to permanently cold marine environments. Appl Environ Microbiol 62:408–414

Ishii A, Sato T, Wachi M, Nagai K, Kato C (2004) Effects of high hydrostatic pressure on bacterial cytoskeleton FtsZ polymers in vivo and in vitro. Microbiology 150:1965–1972

Jackson BE, McInerney MJ (2002) Anaerobic microbial metabolism can proceed close to thermodynamic limits. Nature 415:454–456

Jacobi CA, Aßmus B, Reichenbach H, Stackebrandt E (1997) Molecular evidence for association between the *Sphingobacterium*-like organism "*Candidatus comitans*" and the myxobacterium *Chondromyces crocatus*. Appl Environ Microbiol 63:719–723

Jaenicke R (1988) Molecular mechanisms of adaptation of bacteria to extreme environments. Forum Microbiol 11:435–440

Jannasch HW (1967a) Enrichment of aquatic bacteria in continuous culture. Arch Mikrobiol 59:165–173

Jannasch HW (1967b) Growth of marine bacteria at limiting concentrations of organic carbon in seawater. Limnol Oceanogr 12:264–271

Jannasch HW, Mateles RI (1974) Experimental bacterial ecology studied in continuous culture. Adv Microb Physiol 11:165–212

Jannasch HW, Taylor CD (1984) Deep-sea microbiology. Annu Rev Microbiol 38:487–514

Jannasch HW, Wirsen CO, Taylor CD (1976) Undecompressed microbial populations from the deep sea. Appl Environ Microbiol 32:360–367

Jannasch HW, Wirsen CO, Doherty KW (1996) A pressurized chemostat for the study of marine barophilic and oligotrophic bacteria. Appl Environ Microbiol 62:1593–1596

Janssen PH, Schuhmann A, Mörschel E, Rainey FA (1997) Novel anaerobic ultramicrobacteria belonging to the Verrucomicrobiales lineage of bacterial decent isolated by dilution culture from anoxic rice paddy soil. Appl Environ Microbiol 63:1382–1388

Janssen PH, Yates PS, Grinton BE, Taylor PM, Sait M (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

Jaspers E, Nauhaus K, Cypionka H, Overmann J (2001) Multitude and temporal variability of ecological niches as indicated by the diversity of cultivated bacterioplankton. FEMS Microbiol Ecol 36:153–164

Jensen PR, Williams PG, Oh DC, Zeigler L, Fennical W (2007) Species-specific secondary metabolite production in marine actinomycetes of the genus *Salinispora*. Appl Environ Microbiol 73:1146–1152

Jetten MSM, Strous M, van de Pas-Schoonen KT, Schalk J, van Dongen UGJM, van de Graaf AA, Logemann S, Muyzer G, van Loosdrecht MCM, Kuenen JG (1998) The anaerobic oxidation of ammonium. FEMS Microbiol Rev 22:421–437

Jones AK (1982) The interaction of algae and bacteria. In: Bull AT, Slater JH (eds) Microbial interactions and communities. Academic, London, pp 189–247

Jones SL, Drouin P, Wilkinson BJ, Morse PD (2002) Correlation of long-range membrane order with temperature-dependent growth characteristics of parent and a cold-sensitive, branched-chain-fatty-acid-deficient mutant of *Listeria monocytogenes*. Arch Microbiol 177:217–222

Jørgensen BB (1982) Ecology of the bacteria of the sulphur cycle with special reference to anoxic-oxic interface environments. Philos Trans R Soc Lond B Biol Sci 298:543–561

Jost JL, Drake JF, Fredrickson AG, Tsuchiya HM (1973) Interactions of *Tetrahymena pyriformis*, *Escherichia coli*, *Azotobacter vinelandii* and glucose in a minimal medium. J Bacteriol 113:834–840

Jung L, Jost R, Stoll E, Zuber H (1974) Metabolic differences in *Bacillus stearothermophilus* grown at 55°C and 37°C. Arch Microbiol 95:125–138

Junge K, Eicken H, Deming JW (2004) Bacterial activity at −2 to −20°C in Arctic wintertime sea ice. Appl Environ Microbiol 70:550–557

Kaeberlein T, Lewis K, Epstein SS (2002) Isolating the "uncultivable" microorganisms in pure culture in a simulated natural environment. Science 296:1127–1129

Kalmbach S, Manz W, Szewyk U (1997) Isolation of new bacterial species from drinking water biofilms and proof of their in situ dominance with highly specific 16S rRNA probes. Appl Environ Microbiol 63:4164–4170

Kämpfer P, Rainey FA, Andersson MA, Nurmiaho Lassila EL, Ulrych U, Busse HJ, Weiss N, Mikkola R, Salkinoja-Salonen M (2000) *Frigoribacterium faeni* gen. nov., sp. nov., a novel psychrophilic genus of the family Microbacteriaceae. Int J Syst Evol Microbiol 50:355–363

Kämpfer P, Buczolits S, Albrecht A, Busse H-J, Stackebrandt E (2003) Towards a standardized format for the description of a novel species (of an established genus): *Ochrobactrum gallinifaecis* sp. nov. Int J Syst Evol Microbiol 53: 893–896

Kane MD, Poulsen LK, Stahl DA (1993) Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide probes designed from

environmentally derived 16S rRNA sequences. Appl Environ Microbiol 59:682–686

Kaneda T (1991) Iso-fatty and anteiso-fatty acids in bacteria-biosynthesis, function, and taxonomic significance. Microbiol Rev 55:288–302

Kaprelyants AS, Kell DB (1993) Dormancy in stationary-phase cultures of *Micrococcus luteus*—flow cytometric analysis of starvation and resuscitation. Appl Environ Microbiol 59:3187–3196

Kaprelyants AS, Mukamolova GV, Kell DB (1994) Estimation of dormant *Micrococcus luteus* cells by penicillin lysis and by resuscitation in cell free spent culture medium at high dilution. FEMS Microbiol Lett 115:347–352

Kaprelyants AS, Mukamolova GV, Davey HM, Kell DB (1996) Quantitative analysis of the physiological heterogeneity within starved cultures of *Micrococcus luteus* by flow cytometry and cell sorting. Appl Environ Microbiol 62:1311–1316

Karl DM, Bird DF, Björkman K, Houlihan T, Shackelford R, Tupas L (1999) Microorganisms in the accreted ice of Lake Vostok, Antarctica. Science 286:2144–2147

Karner M, Fuhrman JA (1997) Determination of active bacterioplankton: a comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. Appl Environ Microbiol 63:1208–1213

Kato Y, Sakala RM, Hayashidani H, Kiuchi A, Kaneuchi C, Ogawa M (2000) *Lactobacillus algidus* sp. nov., a psychrophilic lactic acid bacterium isolated from vacuum-packaged refrigerated beef. Int J Syst Evol Microbiol 50:1143–1149

Kawato M, Shinobu R (1959) On *Streptomyces herbaricolor* nov. sp.; supplement: a simple technique for the microscopical observation. Mem Osaka Univ Lib Arts Educ 8:114

Keith SM, Herbert RA (1985) The application of compound bi-directional flow diffusion chemostats to the study of microbial interactions. FEMS Microbiol Ecol 31:239–248

King GM (1984) Utilization of hydrogen, acetate, and "noncompetitive" substrates by methanogenic bacteria in marine sediments. Geomicrobiol J 3:275–306

King D, Nedwell DB (1984) Changes in the nitrate-reducing community of an anaerobic saltmarsh sediment in response to seasonal selection by temperature. J Gen Microbiol 130:2935–2941

Kjaergaard L, Jørgensen BB (1979) Redox potential as a state variable in fermentation systems. Biotechnol Bioeng Symp 9:85–94

Kjelleberg S, Humphrey BA, Marshall KC (1982) Effect of interfaces on small, starved bacteria. Appl Environ Microbiol 43:1166–1172

Kjelleberg S, Flardh KBG, Nystrom T, Moriarty DJW (1993) Growth limitation and starvation in bacteria. In: Ford TE (ed) Aquatic microbiology: an ecological approach. Blackwell, Oxford, pp 298–320

Kluyver AJ, Donker HJL (1926) Unity in biochemistry. Chem Zelle Gewebe 13:134–190

Knoblauch C, Jørgensen BB (1999) Effect of temperature on sulphate reduction, growth rate and growth yield in five psychrophilic sulphate-reducing bacteria from Arctic sediments. Environ Microbiol 1:457–467

Knoblauch C, Jørgensen BB, Harder J (1999) Community size and metabolic rates of psychrophilic sulfate-reducing bacteria in arctic marine sediments. Appl Environ Microbiol 65:4230–4233

Koch IH, Gich F, Dunfield PF, Overmann J (2008) *Edaphobacter modestus* gen. nov., sp. nov., and *Edaphobacter aggregans* sp. nov., two novel acidobacteria isolated from alpine and forest soils. Int J Syst Evol Bacteriol 58:1114–1122

Kogure K, Simidu U, Taga N (1979) A tentative direct microscopic method for counting living marine bacteria. Can J Microbiol 25:415–420

Kole MM, Draper I, Gerson DF (1988) Protease production by *Bacillus subtilis* in oxygen controlled, glucose fed-batch fermentations. Appl Microbiol Biotechnol 28:404–408

Kolenbrander PE, London J (1993) Adhere today, here tomorrow: oral bacterial adherence. J Bacteriol 175:3247–3252

Kolter R, Siegele DA, Tormo A (1993) The stationary phase of the bacterial life cycle. Annu Rev Microbiol 47:855–874

Könneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB, Stahl DA (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437:543–645

Krembs C, Juhl AR, Long RA, Azam F (1998) Nanoscale patchiness of bacteria in lake water studied with the spatial information preservation method. Limnol Oceanogr 43:307–314

Kristjansson JR, Schönheit P, Thauer RK (1982) Different Km values for hydrogen of methanogenic bacteria and sulfate reducing bacteria: an explanation for the apparent inhibition of methanogenesis by sulfate. Arch Microbiol 131:278–282

Krulwich TA, Guffanti AA (1983) Physiology of acidophilic and alkalophilic bacteria. Adv Microb Physiol 24:173–214

Krulwich TA, Guffanti AA (1989) Alkalophilic bacteria. Annu Rev Microbiol 43:435–463

Kuenen JG, Gottschal JC (1982) Competition among chemolithotrophs and methylotrophs and their interactions with heterotrophic bacteria. In: Bull AT, Slater JH (eds) Microbial interactions and communities, vol 1. Academic, London, pp 153–187

Kuenen JG, Harder W (1982) Microbial competition in continuous culture. In: Burns RG, Slater JH (eds) Experimental microbial ecology. Blackwell, Oxford, pp 342–367

Kuenen JG, Robertson LA (1984) Competition among chemolithotrophic bacteria under aerobic and anaerobic conditions. In: Klug MJ, Reddy CA (eds) Current perspectives in microbial ecology. ASM Press, Washington, DC, pp 306–313

Kuenen JG, Robertson LA, van Gemerden H (1985) Microbial interactions among aerobic and anaerobic sulfur-oxidizing bacteria. Adv Microbiol Ecol 8:1–59

Kushner DJ (1978) Life in high salt and solute concentrations: halophilic bacteria. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, London, pp 318–368

Kuske CR, Barns SM, Busch JD (1997) Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographical regions. Appl Environ Microbiol 63:3614–3621

Kuznetsov SI, Dubinina GA, Laptev NA (1979) Biology of oligotrophic bacteria. Annu Rev Microbiol 33:377–387

Laanbroek HJ, Veldkamp H (1982) Microbial interactions in sediment communities. Philos Trans R Soc Lond B Biol Sci 297:533–550

Laanbroek HJ, Smit AJ, Klein Nulend G, Veldkamp H (1979) Competition for L-glutamate between specialized and versatile *Clostridium* species. Arch Microbiol 120:61–66

Laanbroek HJ, Geerlings HJ, Peynenburg AACM, Siesling J (1983) Competition for L-lactate between *Desulfovibrio*, *Veillonella*, and *Acetobacterium* species isolated from anaerobic intertidal sediments. Microb Ecol 9:341–354

Laanbroek HJ, Geerlings HJ, Sijtsma L, Veldkamp H (1984) Competition for sulfate and ethanol among *Desulfobacter*, *Desulfobulbus*, and *Desulfovibrio* species isolated from intertidal sediments. Appl Environ Microbiol 47: 329–334

Lange W (1971) Enhancement of algal growth in cyanophyta-bacteria systems by carbonaceous compounds. Can J Microbiol 17:303–314

Lange R, Hengge-Aronis R (1991) Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol Microbiol 5:49–59

Langworthy TA (1978) Microbial life in extreme pH values. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, New York, pp 279–317

Lapierre L, Undeland P, Cox LJ (1992) Lithium chloride-sodium propionate agar for the enumeration of bifidobacteria in fermented dairy products. J Dairy Sci 75:1192–1196

Larsen H (1986) Halophilic and halotolerant microorganisms—an overview and historical perspective. FEMS Microbiol Rev 39:3–7

Law AT, Button DK (1977) Multiple-carbon-source-limited growth kinetics of a marine coryneform bacterium. J Bacteriol 129:115–123

Leadbetter JR, Schmidt TM, Graber JR, Breznak JA (1999) Acetogenesis from $H_2$ Plus $CO_2$ by Spirochetes from Termite Guts. Science 283:686–689

Lee IH, Fredrickson AG, Tsuchiya HM (1976) Dynamics of mixed cultures of *Lactobacillus plantarum* and *Propionibacterium shermanii*. Biotechnol Bioeng 18:513–526

Lee N, Nielsen PH, Andrasen KH, Juretschko S, Nielsen JL, Schleifer K-H, Wagner M (1999) Combination of fluorescent in situ hybridization and microautoradiography—a new tool for structure: function analyses in microbial ecology. Appl Environ Microbiol 65:1289–1297

Lee KC-Y, Dunfield PF, Morgan XC, Crowe MA, Houghton KM, Vyssotski M, Ryan JLJ, Lagutin K, McDonald IR, Stott MB (2011) *Chthonomonas calidirosea* gen. nov., sp. nov., an aerobic, pigmented, thermophilic

micro-organism of a novel bacterial class, *Chthonomonadetes* classis nov., of the newly described phylum *Armatimonadetes* originally designated candidate division OP10. Int J Syst Evol Microbiol 61:2482–2490

Leedle JAZ, Hespell RB (1980) Differential carbohydrate media and anaerobic replica plating technique in delineating carbohydrate utilizing subgroups in rumen bacterial populations. Appl Environ Microbiol 39:709–719

Legan JD, Owens JD (1988) Bacterial competition for methylamine: computer simulation of a three-strain continuous culture supplied continuously or alternatively with two nutrients. FEMS Microbiol Ecol 53:307–314

Legan JD, Owens JD, Chilvers GA (1987) Competition between specialist and generalist methylotrophic bacteria for an intermittent supply of methylamine. J Gen Microbiol 133:1061–1073

Lengeler JW, Drews G, Schlegel HG (1999) Biology of the prokaryotes. Thieme, New York

Li L, Kato C, Nogi Y, Horikoshi K (1998) Distribution of the pressure-regulated operons in deep-sea bacteria. FEMS Microbiol Lett 159:159–166

Licht TR, Tolker-Nielsen T, Holmstrøm K, Krogfelt KA, Molin S (1999) Inhibition of *Escherichia coli* precursor-16S rRNA processing by mouse intestinal contents. Environ Microbiol 1:23–32

Liesack W, Janssen PH, Rainey FA, Ward-Rainey NL, Stackebrandt E (1997) Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques. In: van Elsas JD, Trevors JT, Wellington EMH (eds) Modern soil microbiology. Marcel Dekker, New York, pp 375–439

Lilburn TG, Kim KS, Ostrom NE, Byzek KR, Leadbetter JR, Breznak JA (2001) Nitrogen fixation by symbiotic and free-living spirochetes. Science 292:2495–2498

Lins U, Farina M (1999) Organization of cells in magnetotactic multicellular aggregates. Microbiol Res 154:9–13

Little B, Gerchakov S, Udey L (1987) A method for sterilization of natural seawater. J Microbiol Methods 7:193–200

Loewen PC, Hengge-Aronis R (1994) The role of the sigma factor $\sigma^6$ (Kat F) in bacterial global regulation. Annu Rev Microbiol 48:53–80

Loewen PC, Hu B, Stutinsky J, Sparling R (1998) Regulation in the rpoS regulon of *Escherichia coli*. Can J Microbiol 44:707–717

Long RA, Azam F (2001) Antagonistic interactions among marine pelagic bacteria. Appl Environ Microbiol 67:4975–4983

Lonhienne T, Gerday C, Feller G (2000) Psychrophilic enzymes: revisiting the thermodynamic parameters of activation may explain local flexibility. Biochim Biophys Acta 1543:1–10

Loomis WD, Durst RW (1992) Chemistry and biology of boron. Biofactors 3:229–239

Loveland-Curtze J, Sheridan PP, Gutshall KR, Brenchley JE (1999) Biochemical and phylogenetic analyses of psychrophilic isolates belonging to the *Arthrobacter* subgroup and description of *Arthrobacter psychrolactophilus*, sp. nov. Arch Microbiol 171:355–363

Lovitt RW, Wimpenny JWT (1981) The gradostat: a bidirectional compound chemostat and its application in microbiological research. J Gen Microbiol 127:261–268

Lovley DR, Blunt-Harris EL (1999) Role of humic-bound iron as an electron transfer agent in dissimilatory Fe(III) reduction. Appl Environ Microbiol 65:4252–4254

Lovley DR, Dwyer DF, Klug MJ (1982) Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. Appl Environ Microbiol 43:1373–1379

Lovley DR, Coates JD, Blunt-Harris EL, Phillips EJP, Woodward JC (1996a) Humic substances as electron acceptors for microbial respiration. Nature 382:445–448

Lovley DR, Woodward JC, Chapelle FH (1996b) Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. Appl Environ Microbiol 62:288–291

Lovley DR, Fraga JL, Coates JD, Blunt-Harris EL (1999) Humics as an electron donor for anaerobic respiration. Environ Microbiol 1:89–98

Ludwig W, Bauer SH, Bauer H, Held I, Kirchhof G, Schulze R, Huber I, Spring S, Hartmann A, Schleifer K-H (1997) Detection and in situ identification of representatives of a widely distributed bacterial phylum. FEMS Microbiol Lett 153:181–190

Lum KT, Meers PD (1989) Boric acid converts urine into an effective bacteriostatic transport medium. J Infect 18:51–58

MacDonald AG (1984) The effects of pressure on the molecular structure and physiological functions of cell membranes. Philos Trans R Soc Lond B Biol Sci 304:47–68

Macy JM, Snellen JE, Hungate RE (1972) Use of syringe methods for anaerobiosis. Am J Clin Nutr 25:1318–1323

Madigan MT (1998) Isolation and characterization of psychrophilic purple bacteria from Antarctica. In: Peschek GA, Löffelhardt W, Schmetterer G (eds) The phototrophic prokaryotes. Kluwer/Plenum, New York, pp 699–706

Madigan MT, Martinko JM, Parker J (2000a) Biology of microorganisms. Prentice-Hall International, Upper Saddle River

Madigan MT, Jung DO, Woese CR, Achenbach LA (2000b) *Rhodoferax antarcticus* sp. nov., a moderately psychrophilic purple nonsulfur bacterium isolated from an Antarctic microbial mat. Arch Microbiol 173:269–277

Malone AS, Shellhammer TH, Courtney PD (2002) Effects of high pressure on the viability, morphology, lysis and cell wall hydrolase activity of *Lactococcus lactis* subsp. *cremoris*. Appl Environ Microbiol 68:4357–4363

Mannisto MK, Schumann P, Rainey FA, Kampfer P, Tsitko I, Tiirola MA, Salkinoja-Salonen MS (2000) *Subtercola boreus* gen. nov., sp. nov., and Subtercola frigoramans sp. nov., two new psychrophilic actinobacteria isolated from boreal groundwater. Int J Syst Evol Microbiol 50:1731–1739

Margesin R, Spröer C, Zhang DC, Busse HJ (2011) *Polaromonas glacialis* sp. nov. and *Polaromonas cryoconiti* sp. nov., two novel bacteria from alpine glacier cryoconite. Int J Syst Evol Microbiol. doi:10.1099/ijs.0.037556-0

Margesin R, Zhang DC, Busse HJ (2012) *Sphingomonas alpina* sp. nov., a psychrophilic bacterium isolated from alpine soil. Int J Syst Evol Microbiol 62:1558–1563

Margulis L (1981) Symbiosis in cell evolution. Freeman, San Francisco

Marquis RE (1976) High pressure microbial physiology. Adv Microb Physiol 14:159–239

Marquis RE, Matsumura P (1978) Microbial life under pressure. In: Kushner DJ (ed) Microbial life in extreme environments. Academic, London, pp 105–158

Marschall E, Jogler M, Henssge U, Overmann J (2010) Large scale distribution and activity patterns of an extremely low-light adapted population of green sulfur bacteria in the Black Sea. Environ Microbiol 12:1348–1362

Martens-Habbena W, Berube PM, Urakawa H, de la Torre JR, Stahl DA (2009) Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature 461:976–979

Martin GA, Hempfling WP (1976) A method for the regulation of microbial population density during continuous culture at high growth rates. Arch Microbiol 107:41–47

Martin SE, Flowers RS, Ordal ZJ (1976) Catalase: its effect on microbial enumeration. Appl Environ Microbiol 32:731–734

Maruyama A, Honda D, Yamamoto H, Kitamura K, Higashihara T (2000) Phylogenetic analysis of psychrophilic bacteria isolated from the Japan Trench, including a description of the deep-sea species *Psychrobacter pacificensis* sp. nov. Int J Syst Evol Microbiol 50:835–846

Mason CA, Egli T (1993) Dynamics of microbial growth in the decelerating and stationary phase of batch culture. In: Kjelleberg S (ed) Starvation in bacteria. Plenum, New York, pp 81–98

Matin A (1981) Regulation of enzyme synthesis as studied in continuous culture. In: Calcott PH (ed) Continuous culture of cells, vol 2. CRC Press, Boca Raton, pp 69–97

Matin A (1990) Keeping a neutral cytoplasm: the bioenergetics of obligate acidophiles. FEMS Microbiol Rev 75:307–318

Matin A, Veldkamp H (1978) Physiological basis of the selective advantage of a *Spirillum* sp. in a carbon-limited environment. J Gen Microbiol 105: 187–197

Mazur P (1980) Limits to life at low temperatures and at reduced water contents and water activities. Orig Life 10:137–159

McDougald D, Kjelleberg S (1999) New perspectives on the viable but nonculturable response. Biologia 54:617–623

McInerney MJ, Bryant MD, Pfennig N (1979) Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch Microbiol 122:129–135

McInerney MJ, Bryant MP, Hespell RB, Costerton JW (1981) *Syntrophomonas wolfei* gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. Appl Environ Microbiol 41:1029–1039

Meers JL (1973) Growth of bacteria in mixed cultures CRC. Crit Rev Microbiol 2:139–184

Megee RD 3rd, Drake JF, Fredrickson AG, Tsuchiya HM (1972) Studies in intermicrobial symbiosis. *Saccharomyces cerevisiae* and *Lactobacillus casei*. Can J Microbiol 18:1733–1742

Meldrum FC, Heywood BR, Mann S, Frankel RB, Bazylinski DA (1993) Electron microscopy study of magnetosomes in a cultured coccoid magnetotactic bacterium. Proc R Soc Lond B Biol Sci 251:231–236

Meyer JS, Tsuchiya HM, Fredrickson AG (1975) Dynamics of mixed populations having complementary metabolism. Biotechnol Bioeng 17:1065–1081

Michels PAM, Michels JPJ, Boonstra J, Konings WN (1979) Generation of an electrochemical proton gradient in bacteria by the excretion of metabolic end-products. FEMS Microbiol Lett 5:357–364

Mikx FJM, van der Hoeven JS (1975) Symbiosis of *Streptococcus mutans* and *Veillonella alcalescens* in mixed continuous cultures. Arch Oral Biol 20: 407–410

Miller TL, Wolin MJ (1974) A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl Microbiol 27:985–987

Miura Y, Tanaka H, Okazaki M (1980) Stability analysis of commensal and mutual relations with competitive assimilation in continuous mixed culture. Biotechnol Bioeng 22:929–948

Miyamoto-Shinohara Y, Sukenobe J, Imaizumi T, Nakahara T (2006) Survival curves for microbial species stored by freeze-drying. Cryobiology 52:27–32

Mizunoe Y, Wai SN, Takade A, Yoshida S (1999) Restoration of culturability of starvation-stressed and low-temperature-stressed *Escherichia coli* O157 cells using $H_2O_2$-degrading compounds. Arch Microbiol 172:63–67

Moench TT, Zeikus JG (1983) An improved preparation method for a titanium (III) media reductant. J Microbiol Methods 1:199–202

Moissl C, Rudolph C, Huber R (2002) Natural communities of novel archaea and bacteria with a string-of-pearls-like morphology: molecular analysis of the bacterial partners. Appl Environ Microbiol 68:933–937

Monod J (1942) Recherches sur la croissance des cultures bacteriennes. Hermann, Paris

Monod J (1950) La technique de culture continue: théorie et applications. Ann Inst Pasteur 79:390–410

Morita RY (1975) Psychrophilic bacteria. Bacteriol Rev 39:144–167

Morita RY (1982) Starvation—survival of heterotrophs in the marine environment. Adv Microbiol Ecol 6:171–198

Morita RY (1986) Pressure as an extreme environment. In: Herbert RA, Codd GA (eds) Microbes in extreme environments. Academic, London, pp 171–185

Morotomi M, Nagai F, Watanabe Y (2011) *Parasutterella secunda* sp. nov., isolated from human faeces and proposal of *Sutterellaceae* fam. nov. in the order *Burkholderiales*. Int J Syst Evol Microbiol 61:637–643

Mossel DAA, Veldman A, Eelderink I (1980) Comparison of the effects of liquid medium repair and the incorporation of catalase in Macconkey type media on the recovery of Enterobacteriaceae sublethally stressed by freezing. J Appl Bacteriol 49:405–419

Mountfort DO, Asher RA (1986) Isolation from a methanogenic ferulate degrading consortium of an anaerobe that converts methoxyl groups of aromatic acids to volatile fatty acids. Arch Microbiol 144:55–61

Mountfort DO, Bryant MP (1985) Isolation and characterization of an anaerobic syntrophic benzoate degrading bacterium from sewage sludge. Arch Microbiol 133:249–256

Mountfort DO, Rainey FA, Burghardt J, Kaspar HF, Stackebrandt E (1997) *Clostridium vincentii* sp. nov., a new anaerobic, saccharolytic, psychrophilic bacterium isolated from low-salinity pond sediment of the McMurdo ice shelf, Antarctica. Arch Microbiol 167:54–60

Mountfort DO, Rainey FA, Burghardt J, Kaspar HF, Stackebrandt E (1998) *Psychromonas antarcticus* gen. nov., sp. nov., a new aerotolerant anaerobic, halophilic psychrophile isolated from pond sediment of the McMurdo ice shelf, Antarctica. Arch Microbiol 169:231–238

Mukamolova GV, Kaprelyants AS, Young DI, Young M, Kell DB (1998) A bacterial cytokine. Proc Natl Acad Sci USA 95:8916–8921

Müller RH, Babel W (1996) Measurement of growth at very low rates ($\mu \geqq 0$), an approach to study the energy requirement for the survival of *Alcaligenes eutrophus* JMP 134. Appl Environ Microbiol 62:147–151

Munro PM, Flatau GN, Clément RL, Gauthier MJ (1995) Influence of the RpoS (KatF) sigma factor on maintenance of viability and culturability of *Escherichia coli* and *Salmonella typhimurium* in seawater. Appl Environ Microbiol 61:1853–1858

Mur LR, Gons HJ, van Liere L (1977) Some experiments on the competition between green algae and blue-green bacteria in light-limited environments. FEMS Microbiol Lett 1:335–338

Murray DR (1989) Biology of food radiation. Wiley, Chichester

Myers J, Clark LB (1944) Culture conditions and the development of the photosynthetic mechanism. II: an apparatus for the continuous culture of *Chlorella*. J Gen Physiol 28:103–112

Nakamura I, Ogimoto K, Izumu H (1995) ATP-dependent calcium release from binding site in *Streptococcus bovis*: bound versus free pools. J Gen Appl Microbiol 41:389–398

Nakasone K, Ikegami A, Kato C, Usami R, Horikoshi K (1998) Mechanisms of gene expression controlled by pressure in deep-sea microorganisms. Extremophiles 2:149–154

National Center for Biotechnology Information (2012) http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_growth.html

Nedwell DB (1984) The input and mineralization of organic carbon in anaerobic aquatic sediments. Adv Microbiol Ecol 7:93–131

Nedwell DB, Rutter M (1994) Influence of temperature on growth rate and competition between two psychrotolerant Antarctic bacteria: low temperature diminishes affinity for substrate uptake. Appl Environ Microbiol 60:1984–1992

Neidhardt FC, Umbarger HE (1996) Chemical composition of *Escherichia coli*. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*, 2nd edn. ASM Press, Washington, DC, pp 13–16

Nelson DC, Jørgensen BB, Revsbech NP (1986) Growth pattern and yield of a chemoautotrophic *Beggiatoa* sp. in oxygen-sulfide microgradients. Appl Environ Microbiol 52:225–233

Neuhard J, Kelln RA (1996) Biosynthesis and conversions of pyrimidines. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*, 2nd edn. ASM Press, Washington, DC, pp 580–599

Nichols DS, Greenhill AR, Shadbolt CT, Ross T, McMeekin TA (1999) Physicochemical parameters for growth of the sea ice bacteria *Glaciecola punicea* ACAM 611T and *Gelidibacter* sp. strain IC158. Appl Environ Microbiol 65:3757–3760

Nichols D, Lewis K, Orjala J, Mo S, Ortenberg R, O'Connor P, Zhao C, Vouros P, Kaeberlein T, Epstein SS (2008) Short peptide induces an "uncultivable" microorganism to grow in vitro. Appl Environ Microbiol 74:4889–4897

Niehaus F, Hantke K, Unden G (1991) Iron content and FNR-dependent gene regulation in *Escherichia coli*. FEMS Microbiol Lett 84:319–324

Nielsen PH, de Muro MA, Nielsen JL (2000) Studies on the in situ physiology of *Thiothrix* spp. present in activated sludge. Environ Microbiol 2:389–398

Nogales B, Guerrero R, Esteve I (1997) A heterotrophic bacterium inhibits growth of several species of the genus *Chlorobium*. Arch Microbiol 167: 396–399

Nogales B, Moore ERB, Abraham WR, Timmis KN (1999) Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. Environ Microbiol 1:199–212

Norland S, Fagerbakke KM, Heldal M (1995) Light element analysis of individual bacteria by X-ray microanalysis. Appl Environ Microbiol 61:1357–1362

Norris JR, Ribbons DW (1970) Methods in microbiology, vol 3. Academic, New York, pp 1–506

Notley L, Ferenci T (1996) Induction of RpoS-dependent functions in glucose-limited continuous culture: what levels of nutrient limitation induces the stationary phase of *Escherichia coli*. J Bacteriol 178:1465–1468

Novick A, Szilard L (1950) Description of the chemostat. Science 112:715–716

Nozhevnikova AN, Simankova MV, Parshina SN, Kotsyurbenko OR (2001) Temperature characteristics of methanogenic archaea and acetogenic bacteria isolated from cold environments. Water Sci Technol 44:41–48

Nurmikko V (1956) Biochemical factors affecting symbiosis among bacteria. Experientia 12:245–249

Oerther DB, Pernthaler J, Schramm A, Amann R, Raskin L (2000) Monitoring precursor 16S rRNAs of *Acinetobacter* spp. in activated sludge wastewater treatment systems. Appl Environ Microbiol 66:2154–2165

Ohmura N, Matsumoto N, Sasaki K, Saiki H (2002) Electrochemical regeneration of Fe(III) to support growth on anaerobic iron respiration. Appl Environ Microbiol 68:405–407

Okada T, Ueyama K, Niya S, Kanazawa H, Futai M, Tsuchiya T (1981) Role of inducer exclusion in preferential utilization of glucose over melibiose in diauxic growth of *Escherichia coli*. J Bacteriol 146:1030–1037

Oliver JD (1995) The viable but non-culturable state in the human pathogen *Vibrio vulnificus*. FEMS Microbiol Lett 133:203–208

Olsen RA, Bakken LR (1987) Viability of soil bacteria: optimization of plate-counting technique and comparison between total counts and plate counts within different size groups. Microb Ecol 13:59–74

Oltmann LF, Schoenmaker GS, Reijnders WNM, Stouthamer AH (1978) Modification of the pH auxostat culture method for the mass cultivation of bacteria. Biotechnol Bioeng 20:921–925

Orcutt KM, Rasmussen U, Webb EA, Waterbury JB, Gundersen K, Bergman B (2002) Characterization of *Trichodesmium* spp. by genetic techniques. Appl Environ Microbiol 68:2236–2245

Oremland RS (1988) Biogeochemistry of methanogenic bacteria. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. Wiley, New York, pp 641–706

Oremland RS, Hoeft SE, Santini JM, Bano N, Hollibough RA (2002) Anaerobic oxidation of arsenite in Mono Lake water and by a facultative, arsenite-oxidizing chemoautotroph, strain MLHE-1. Appl Environ Microbiol 68:4795–4802

Oren A (1986) Intracellular salt concentrations of the halophilic eubacteria *Haloaerobium praevalens* and *Halobacteroides halobius*. Can J Microbiol 32:4–9

Oren A (1999) Bioenergetic aspects of halophilism. Microbiol Mol Biol Rev 63:334–348

Ostrowski M, Cavicchioloi R, Blaauw M, Gottschal JC (2001) Specific growth rate plays a critical role in hydrogen peroxide resistance of the marine oligotrophic ultramicrobacterium *Sphingomonas alaskensis* strain RB2256. Appl Environ Microbiol 67:1292–1299

Ott JA, Novak R, Schiemer F, Hentschel U, Nebelsiek M, Polz M (1991) Tackling the sulfide gradient: a novel strategy involving marine nematodes and chemoautotrophic ectosymbionts. Mar Ecol 12:261–279

Otto R, Hugenholtz J, Konings WN, Veldkamp H (1980) Increase of molar growth yield of *Streptococcus cremoris* for lactose as a consequence of lactate consumption by *Pseudomonas stutzeri* in mixed culture. FEMS Microbiol Lett 9:85–88

Ouverney CC, Fuhrman JA (1999) Combined microautoradiography—16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. Appl Environ Microbiol 65:1746–1752

Ouverney CC, Fuhrman JA (2000) Marine planktonic Archaea take up amino acids. Appl Environ Microbiol 66:4829–4833

Overmann J (2002a) Phototrophic consortia: a tight cooperation between non-related eubacteria. In: Seckbach J (ed) Symbiosis. Mechanisms and model systems. Kluwer, Dordrecht, pp 239–255

Overmann J (2002b) Principles of enrichment, isolation, cultivation, and preservation of bacteria. In: Dworkin M et al (eds) The prokaryotes: an evolving electronic resource for the microbiological community, 3rd edn (latest update release 3.11, September 2002). Springer, New York. http://ep.springer-ny.com:6336/contents/ or http://141.150.157.117:8080/prokPROD/index.htm

Overmann J (2005) Chemotaxis and behavioural physiology of not-yet-cultivated microbes, vol 397, Methods in enzymology. Elsevier, San Diego, pp 133–147, Chap II.8

Overmann J, Pfennig N (1989) *Pelodictyon phaeoclathratiforme* sp. nov., a new brown-colored member of the Chlorobiaceae forming net-like colonies. Arch Microbiol 152:401–406

Overmann J, Pfennig N (1992) Continuous chemotrophic growth and respiration of *Chromatiaceae* species at low oxygen concentrations. Arch Microbiol 158:59–67

Overmann J, Schubert K (2002) Phototrophic consortia: model systems for symbiotic interrelations between prokaryotes. Arch Microbiol 177:201–208

Overmann J, van Gemerden H (2000) Microbial interactions involving sulfur bacteria: implications for the ecology and evolution of bacterial communities. FEMS Microbiol Rev 24:591–599

Overmann J, Lehmann S, Pfennig N (1991) Gas vesicle formation and buoyancy regulation in *Pelodictyon phaeoclathratiforme* (green sulfur bacteria). Arch Microbiol 157:29–37

Overmann J, Beatty JT, Hall KJ (1996) Purple sulfur bacteria control the growth of aerobic heterotrophic bacterioplankton in a meromictic salt lake. Appl Environ Microbiol 62:3251–3258

Overmann J, Tuschak C, Fröstl J, Sass H (1998) The ecological niche of the consortium "*Pelochromatium roseum*". Arch Microbiol 169:120–128

Padan E, Schuldinger S (1986) Intracellular pH regulation in bacterial cells. Methods Enzymol 125:337–352

Paerl H (1978) Role of heterotrophic bacteria in promoting $N_2$-fixation by *Anabaena* in aquatic habitats. Microb Ecol 4:215–231

Paerl HW (1982) Interactions with bacteria. In: Carr NG, Whitton NG (eds) The biology of cyanobacteria. University of California Press, Los Angeles, pp 441–461

Pankratov TA, Dedysh SN (2011) *Granulicella paludicola* gen. nov., sp. nov., *Granulicella pectinivorans* sp. nov., *Granulicella aggregans* sp. nov. and *Granulicella rosea* sp. nov., acidophilic, polymer-degrading acidobacteria from *Sphagnum* peat bogs. Int J Syst Evol Microbiol 60:2951–2959

Parkes RJ, Senior E (1988) Multistage chemostats and other models for studying anoxic ecosystems. In: Wimpenny JWT (ed) Handbook of laboratory model systems for microbial ecosystems, vol 1. CRC Press, Boca Raton, pp 51–71

Pastan I, Perlman R (1969) Repression of β-galactosidase synthesis by glucose in phosphotransferase mutants of *Escherichia coli*. Repression in the absence of glucose phosphorylation. J Biol Chem 244:5836–5842

Pasteur L (1862) Mémoire sur les corpuscules qui existent dans l'atmosphère: examen de la doctrine des générations spontanées. Ann Chém Phys 64:5–110

Pernthaler J, Posch T, Simek K, Vrba J, Pernthaler A, Glöckner FO, Nübel U, Psenner R, Amann R (2001) Predator-specific enrichment of *Actinobacteria* from a cosmopolitan freshwater clade in mixed continuous culture. Appl Environ Microbiol 67:2145–2155

Pfennig N (1980) Syntrophic mixed cultures and symbiotic consortia with phototrophic bacteria: a review. In: Gottschalk G, Pfennig N, Werner HH (eds) Anaerobes and anaerobic infections. Fischer, Stuttgart, pp 127–131

Pfennig N (1993) Reflections of a microbiologist, or how to learn from the microbes. Annu Rev Microbiol 47:1–29

Pfennig N, Trüper HG (1989) Anoxygenic phototrophic bacteria. In: Staley JT, Bryant MP, Pfennig N, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 3. Williams and Wilkins, Baltimore, pp 1635–1709

Pietzsch O (1967) Ein Nährboden zur Schwärmhemmung und gleichzeitigen Unterscheidung der Proteuskeime von Salmonellen. Fleischwirtschaft 1:31–32

Pinhassi J, Berman T (2003) Differential growth response of colony-forming α- and γ-Proteobacteria in dilution culture and nutrient addition experiments from Lake Kinneret (Israel), the Eastern Mediterranean Sea, and the Gulf of Eilat. Appl Environ Microbiol 69:199–211

Pinhassi J, Zweifel UL, Hagström Å (1997) Dominant marine bacterioplankton species found among colony-forming bacteria. Appl Environ Microbiol 63:3359–3366

Pirt SJ (1974) The theory of fed batch culture with reference to the penicillin fermentation. J Appl Chem Biotechnol 24:415–4224

Pirt SJ (1975) Principles of microbe and cell cultivation. Blackwell, Oxford

Ploug H, Grossart HP, Azam F, Jørgensen BB (1999) Photosynthesis, respiration, and carbon turnover in sinking marine snow from surface waters of Southern California Bight: implications for the carbon cycle in the ocean. Mar Ecol Prog Ser 179:1–11

Poindexter JS (1992) Dimorphic protshecate bacteria: the genera Caulobacter, Asticcacaulis, Hyphomicrobium, Pedomicrobium, Hyphomonas, and Thiodendron. In: Balows A, Trüper HG, Dworkin M, Schleifer K-H (eds) The prokaryotes, 2nd edn. Springer, New York, pp 2176–2196

Poindexter JS, Leadbetter ER (1986) Enrichment cultures in bacterial ecology. In: Poindexter JS, Leadbetter ER (eds) Bacteria in nature, vol 2. Plenum, New York, pp 229–260

Porter JR (1976) Antony van Leeuwenhoek: tercentenary of is discovery of bacteria. Microbiol Rev 40:260–269

Pörtner R, Märkl H (1998) Dialysis cultures. Appl Microbiol Biotechnol 50: 403–414

Postgate JR, Hunter JR (1963) Acceleration of bacterial death by growth substrates. Nature 198:273–280

Postgate JR, Hunter JR (1964) Accelerated death of *Aerobacter aerogenes* starved in the presence of growth-limiting substrates. J Gen Microbiol 34:459–473

Powell EO (1958) Criteria for the growth of contaminants and mutants in continuous culture. J Gen Microbiol 18:259–268

Pratuangdejkul J, Dharmsthiti S (2000) Purification and characterization of lipase from psychrophilic *Acinetobacter calcoaceticus* LP009. Microbiol Res 155: 95–100

Price B (2000) A habitat for psychrophiles in deep Antarctic ice. Proc Natl Acad Sci USA 97:1247–1251

Pringault O, de Wit R, Caumette P (1996) A benthic gradient chamber for culturing phototrophic sulfur bacteria on reconstituted sediments. FEMS Microbiol Ecol 20:237–250

Pruesse E, Quast C, Knittel K, Fuchs B, Ludwig W, Peplies J, Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196

Puskas A, Greenberg EP, Kaplan S, Schaefer AL (1997) A quorum-sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides*. J Bacteriol 179:7530–7537

Radajewski S, Ineson P, Parekh NR, Murrell JC (2000) Stable-isotope probing as a tool in microbial ecology. Nature 403:646–649

Rappe MS, Connon SA, Vergin KL, Giovannoni SJ (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature 418:630–633

Ratkowsky PA, Lowry RR, McMeekin TA, Stokes AN, Chandler RE (1983) Model for bacterial culture growth rate throughout the entire biokinetic temperature range. J Bacteriol 154:1222–1226

Reddy GS, Aggarwal RK, Matsumoto GI, Shivaji S (2000) *Arthrobacter flavus* sp. nov., a psychrophilic bacterium isolated from a pond in McMurdo Dry Valley, Antarctica. Int J Syst Evol Microbiol 50:1553–1561

Reed RH, Walsby AE (1985) Changes in turgor pressure in response to increases in external NaCl concentration in the gas-vacuolate cyanobacterium *Microcystis* sp. Arch Microbiol 143:290–296

Reichenbach H (1984) Myxobacteria: a most peculiar group of social prokaryotes. In: Rosenberg E (ed) Myxobacteria: development and cell interactions. Springer, New York, pp 1–50

Reisch CR, Stoudemayer MJ, Varaljay VA, Amster IJ, Moran MA, Whitman WB (2011) Novel pathway for assimilation of dimethylsulphoniopropionate widespread in marine bacteria. Nature 12:208–211

Renesto P, Crapoulet N, Ogata H, La Scola B, Vestris G, Claveri J-M, Raoult D (2003) Genome-based design of a cell-free culture medium for *Tropheryma whipplei*. Lancet 362:447–449

Reusse U, Meyer A (1972) Der "Pril-Mannit-Agar" in der Salmonellen-Diagnostik. Zbl Bakteriol I Abt Orig 219:555–557

Revsbech NP, Jørgensen BB (1986) Microelectrodes: their use in microbial ecology. Adv Microbiol Ecol 9:293–352

Rhee GY (1972) Competition between an algae and an aquatic bacterium for phosphate. Limnol Oceanogr 17:505–514

Ricica J, Dobersky P (1981) Complex systems. In: Calcott PH (ed) Continuous cultures of cells, vol 1. CRC Press, Boca Raton, pp 63–96

Robinson JA, Tiedje JM (1984) Competition between sulfate-reducing and methanogenic bacteria for $H_2$ under resting and growing conditions. Arch Microbiol 137:26–32

Roesch LFW, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, Kent AD, Daroub SH, Camargo FAO, Farmerie WG, Triplett EW (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J 1:283–290

Roeßler M, Müller V (1998) Quantitative and physiological analyses of chloride dependence of growth of *Halobacillus halophilus*. Appl Environ Microbiol 64:3813–3817

Roeßler M, Müller V (2002) Chloride, a new environmental signal molecule involved in gene regulation in a moderately halophilic bacterium, *Halobacillus halophilus*. J Bacteriol 184:6207–6215

Rose AH, Evison LM (1965) Studies on the biochemical basis of the minimum temperature for growth of certain psychrophilic and mesophilic microorganisms. J Gen Microbiol 38:131–141

Rosenberg E, Keller KH, Dworkin M (1977) Cell-density dependent growth of *Myxococcus xanthus* on casein. J Bacteriol 129:770–777

Roslev P, Iversen N (1999) Radioactive fingerprinting of microorganisms that oxidize atmospheric methane in different soils. Appl Environ Microbiol 65:4064–4070

Roszak DB, Colwell RR (1987) Survival strategies of bacteria in the natural environment. Microbiol Rev 51:365–379

Rouf MA (1964) Spectrochemical analysis of inorganic elements in bacteria. J Bacteriol 88:1545–1549

Ruger HJ, Fritze D, Sproer C (2000) New psychrophilic and psychrotolerant *Bacillus marinus* strains from tropical and polar deep-sea sediments and emended description of the species. Int J Syst Evol Microbiol 50:1305–1313

Russel NJ (1984) Mechanisms of thermal adaptation in bacteria: blueprints for survival TIBS. Trends Biol Sci 9:108–112

Russel NJ (2000) Toward a molecular understanding of cold activity of enzymes from psychrophiles. Extremophiles 4:83–90

Russel NJ, Fukunaga N (1990) A comparison of thermal adaptation of membrane lipids in psychrophilic and thermophilic bacteria. FEMS Microbiol Rev 74:171–182

Russell EJ (1923) The micro-organisms of the soil. Longmans, Green, London

Sambanis A, Fredrickson AG (1987) Long-term studies of ciliate-bacterial interactions: use of a chemostat fed with bacteria grown in a separate chemostat. J Gen Microbiol 133:1619–1630

Sánchez O, van Gemerden H, Mas J (1996) Description of a redox-controlled sulfidostat fro the growth of sulfide-oxidizing phototrophs. Appl Environ Microbiol 62:3640–3645

Sandaa R-A, Torsvik V, Enger Ø, Daae FL, Castberg T, Hahn D (1999) Analysis of bacterial communities in heavy metal-contaminated soils at different levels of resolution. FEMS Microbiol Ecol 30:237–251

Sass A, Sass H, Coolen MJL, Cypionka H, Overmann J (2001) Microbial communities in the chemocline of a hypersaline deep-sea basin (Urania Basin, Mediterranean Sea). Appl Environ Microbiol 67:5392–5402

Schauer NL, Brown DP, Ferry JG (1982) Kinetics of formate metabolism in *Methanobacterium formicicum* and *Methanospirillum hungatei*. Appl Environ Microbiol 44:549–554

Schink B (1991) Syntrophism among prokaryotes. In: Balows A, Trüper HG, Dworkin M, Schleifer K-H (eds) The prokaryotes, 2nd edn. Springer, New York, pp 276–299

Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 61:262–280

Schink B, Friedrich M (2000) Phosphite oxidation by sulphate reduction. Nature 406:37

Schink B, Thiemann V, Laue H, Friedrich MW (2002) *Desulfotignum phosphitoxidans* sp. nov., a new marine sulfate reducer that oxidizes phosphite to phosphate. Arch Microbiol 177:381–391

Schlegel HG, Jannasch HW (1967) Enrichment cultures. Annu Rev Microbiol 21:49–70

Schlesner H (1986) *Pirellula marina* sp. nov., a budding peptidoglycanless bacterium from brackish water. Syst Appl Microbiol 8:177–180

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

Schloss PD, Handelsman J (2004) Status of the microbial census. Microbiol Mol Biol Rev 68:686–691

Schmid M, Schmitz-Esser S, Jetten M, Wagner M (2001) 16S–23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. Environ Microbiol 3:450–459

Schmidt GB, Rosano CL, Hurwitz C (1971) Evidence for a magnesium pump in *Bacillus cereus* T. J Bacteriol 105:150–155

Schober A, Günther R, Schwienhorst A, Döring M, Lindemann BF (1993) Accurate high-speed liquid handling of very small biological samples. Biotechniques 15:324–329

Scholten JCM, Conrad R (2000) Energetics of syntrophic propionate oxidation in defined batch and chemostat cultures. Appl Environ Microbiol 66: 2934–2942

Schopfer P, Brennicke A (1999) Pflanzenphysiologie, 5th edn. Springer, New York

Schramm A, de Beer D, van den Heuvel JC, Ottengraf S, Amann R (1999) Microscale distribution of populations and activities of *Nitrosospira* and *Nitrospira* spp. along a macroscale gradient in a nitrifying bioreactor: quantification by in situ hybridization and the use of microsensors. Appl Environ Microbiol 65:3690–3696

Schulten HR, Plage B, Schnitzer M (1991) A chemical structure for humic substances. Naturwissenschaften 78:31–312

Schulz E, Lüdemann H-D, Jaenicke R (1976) High-pressure equilibrium studies on the dissociation-association of *E. coli* ribosomes. FEBS Lett 64:40–43

Schumann P, Zhang DC, Redzic M, Margesin R (2012) *Alpinimonas psychrophila* gen. nov., sp. nov., a novel actinobacterium of the family Microbacteriaceae isolated from alpine lacier cryoconite. Int J Syst Evol Microbiol. doi:10.1099/ijs.0.036160-0

Schut F, de Vries EJ, Gottschal JC, Robertson BR, Harder W, Prins RA, Button DK (1993) Isolation of typical marine bacteria by dilution culture: growth, maintenance, and characteristics of isolates under laboratory conditions. Appl Environ Microbiol 59:2150–2160

Schut F, Jansen M, Pedro Gomes TM, Gottschal JC, Harder W, Prins RA (1995) Substrate uptake and utilization by a marine ultramicrobacterium. Microbiology 141:351–361

Schut F, Prins RA, Gottschal JC (1997) Oligotrophy and pelagic marine bacteria: facts and fiction. Aquat Microb Ecol 12:177–202

Scott DT, McKnight DM, Blunt-Harris EL, Kolesar SE, Lovley DR (1998) Quinone moieties act as electron acceptors in the reduction of humic substances by humic-reducing microorganisms. Environ Sci Technol 32:2984–2989

Seitz AP, Nielsen TH, Overmann J (1993) Physiology of purple sulfur bacteria forming macroscopic aggregates in Great Sippewissett Salt Marsh, Massachusetts. FEMS Microbiol Ecol 12:225–236

Sekiguchi Y, Takahashi H, Kamagata Y, Ohashi A, Harada H (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 non-sulfur bacteria, subdivision I. Appl Environ Microbiol 67:5740–5749

Shilo M (1984) Bdellovibrio as a predator. In: Klug MJ, Reddy CA (eds) Current perspectives in microbial ecology. ASM Press, Washington, DC, pp 334–339

Shindler DB, Wydro RM, Kushner DJ (1977) Cell-bound cations in the moderately halophilic bacterium *Vibrio costicola*. J Bacteriol 130:698–703

Shockey WL, Dehority BA (1989) Comparison of two methods for enumeration of anaerobe numbers on forages and evaluation of ethylene oxide treatment for forage sterilization. Appl Environ Microbiol 55:1766–1768

Siebert J, Hirsch P (1988) Characterization of 15 selected coccal bacteria isolated from Antarctic rock and soil samples from the McMurdo-Dry Valleys (South-Victoria Land). Polar Biol 9:37–44

SILVA (2012) http://www.arb-silva.de/documentation/background/release-108/

Simankova MV, Kotsyurbenko OR, Stackebrandt E, Kostrikina NA, Lysenko AM, Osipov GA, Nozhevnikova AN (2000) *Acetobacterium tundrae* sp. nov., a new psychrophilic acetogenic bacterium from tundra soil. Arch Microbiol 174:440–447

Simek K, Vrba J, Pernthaler J, Posch T, Hartman P, Nedoma J, Psenner R (1997) Morphological and compositional shifts in an experimental bacterial community influenced by protists with contrasting feeding modes. Appl Environ Microbiol 63:587–595

Sitnikov DM, Schineller JB, Baldwin TO (1996) Control of cell division in *Escherichia coli*: regulation of transcription of ftsQA involves both rpoS and SdiA-mediated autoinduction. Proc Natl Acad Sci USA 93:336–341

Skerman VDB (1968) A new type of micromanipulator and microforge. J Gen Microbiol 54:287–297

Skulachev VP (1987) Bacterial sodium transport: bioenergetic functions of sodium ions. In: Rosen BP, Silver S (eds) Ion transport in prokaryotes. Academic, New York, pp 131–164

Slater JH, Bull AT (1978) Interactions between microbial populations. In: Bull AT, Meadow PM (eds) Companion to microbiology. Longman, London, pp 181–206

Slonczewski J, Foster JW (1996) pH-regulated genes and survival at extreme pH. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*, 2nd edn. ASM Press, Washington, DC, pp 1539–1549

Sly LI, Arunpairojana V (1987) Isolation of manganese-oxidizing *Pedomicrobium* cultures from water by micromanipulation. J Microbiol Methods 6:177–182

Soendergaard M, Riemann B, Jörgensen NOG (1985) Extracellular organic carbon (EOC) released by phytoplankton and bacterial production. Oikos 45:323–332

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Herndl GJ (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc Natl Acad Sci USA 103:12115–12120

Somero GN (1992) Adaptations to high hydrostatic pressure. Annu Rev Physiol 54:557–577

Spring S, Amann R, Ludwig W, Schleifer K-H, van Gemerden H, Petersen N (1993) Dominating role of an unusual magnetotactic bacterium in the micraerobic zone of a freshwater sediment. Appl Environ Microbiol 59:2397–2403

Spring S, Schulze R, Overmann J, Schleifer K-H (2000) Identification and characterization of ecologically significant prokaryotes in the sediment of freshwater lakes: molecular and cultivation studies. FEMS Microbiol Rev 24:573–590

Stal LJ, van Gemerden H, Krumbein WE (1985) Structure and development of a benthic marine microbial mat. FEMS Microbiol Ecol 31:111–125

Staley JT, Fuerst JA, Giovannoni SJ, Schlesner H (1992) The order Planctomycetales and the genera Planctomyces, Pirellula, gemmata and Isosphaera. In: Balows A, Dworkin M, Harder W, Schleifer K-H, Trüper HG (eds) The prokaryotes. Springer, New York, pp 3710–3731

Stanton TB, Canale-Parola E (1979) Enumeration and selective isolation of rumen spirochetes. Appl Environ Microbiol 38:965–973

Stetter KO (2001) Genus III: *Pyrolobus*. In: Boone DR, Castenholz RW (eds) Bergey's manual of systematic bacteriology, vol 1. Springer, New York, pp 186–197

Stevens TO (1995) Optimization of media for enumeration and isolation of aerobic heterotrophic bacteria from the deep terrestrial subsurface. J Microbiol Methods 21:293–303

Stewart CS, Bryant MP (1988) The rumen bacteria. In: Hobson PN (ed) The rumen microbial ecosystem. Elsevier Applied Science, London, pp 21–75

Stolp H, Starr MP (1963) *Bdellovibrio bacteriovorus* gen. et sp. n., a predatory ectoparasitic and bacteriolytic microorganism. Antonie Van Leeuwenhoek 29:217–248

Stolp H, Starr MP (1965) Bacteriolysis. Annu Rev Microbiol 19:79–104

Stringfellow WT, Aitken MD (1994) Comparative physiology of phenanthrene degradation by two dissimilar pseudomonads isolated from a creosote-contaminated soil. Can J Microbiol 40:432–438

Sugio T, Kuwano H, Negishi A, Maeda T, Takeuchi F, Kamimura K (2001) Mechanism of growth inhibition by tungsten in *Acidithiobacillus ferrooxidans*. Biosci Biotechnol Biochem 65:555–562

Suh DH, Becker TC, Sands JA, Montenecourt BS (1988) Effects of temperature on xylanase secretion by *Trichoderma reesei*. Biotechnol Bioeng 32:821–825

Suzuki K, Sasaki J, Uramoto M, Nakase T, Komagata K (1997a) *Cryobacterium psychrophilum* gen. nov., sp. nov., nom. rev., comb. nov., an obligately psychophilic actinomycete to accommodate "*Curtobacterium psychrophilum*", Inoue and Komagata 1976. Int J Syst Evol Microbiol 47:474–478

Suzuki MT, Rappé MS, Haimberger ZW, Winfield H, Adair N, Ströbel J, Giovannoni SJ (1997b) Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl Environ Microbiol 63:983–989

Sweerts JPRA, de Beer D (1989) Microelectrode measurements of nitrate gradients in the littoral and profundal sediments of a meso-eutrophic lake (Lake Vechten, The Netherlands). Appl Environ Microbiol 55:754–757

Swift ST, Najita IY, Ohtaguchi K, Fredrickson AG (1982) Continuous culture of the ciliate *Tetrahymena pyriformis* on *Escherichia coli*. Biotechnol Bioeng 24:1953–1964

Szewzyk U, Schink B (1989) Degradation of hydroquinone, gentisate, and benzoate by a fermenting bacterium in pure or defined mixed culture. Arch Microbiol 15:541–545

Talamoto S, Yamada K, Ezura Y (1994) Producing of bacteriolytic enzymes during the growth of a marine bacterium *Alteromonas* sp. no. 8-R. J Gen Appl Microbiol 40:499–508

Tamaki H, Sekiguchi Y, Hanada S, Nakamura K, Nomura N, Matsumara M, Kamagata Y (2005) Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. Appl Environ Microbiol 71:2162–2169

Tamaki H, Tanaka Y, Matsuzawa H, Muramatsu M, Meng X, Hanada S, Mori K, Kamagata Y (2011) *Armatimonas rosea* gen. nov., sp. nov., of a novel bacterial phylum, Armatimonadetes phyl. nov., formally called the candidate phylum OP10. Int J Syst Evol Microbiol 61:1442–1447

Tang W-C, White JC, Alexander M (1998) Utilization of sorbed compounds by microorganisms specifically isolated for that purpose. Appl Microbiol Biotechnol 49:117–121

Tappe W, Tomaschewski C, Rittershaus S, Groeneweg J (1996) Cultivation of nitrifying bacteria in the retentostat, a simple fermenter with internal biomass retention. FEMS Microbiol Ecol 19:47–52

Tappe W, Laverman A, Bohland M, Braster M, Rittershaus S, Groeneweg J, van Versefeld HW (1999) Maintenance energy demand and starvation recovery dynamics of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* cultivated in a retentostat with complete biomass retention. Appl Environ Microbiol 65:2471–2477

Tarin JJ, Trounson AO (1993) Effect of stimulation or inhibition of lipid peroxidation on freezing-thawing of mouse embryos. Biol Reprod 49:1362–1368

Teather RM (1982) Maintenance of laboratory strains of obligately anaerobic rumen bacteria. Appl Environ Microbiol 44:499–501

Tempest DW (1970) The continuous culture in microbial research. Adv Microb Physiol 4:223–250

Tempest DW, Neyssel OM (1978) Eco-physiological aspects of microbial growth in aerobic nutrient limited environments. Adv Microbiol Ecol 2:105–153

Tempest DW, Herbert F, Phipps PJ (1967) Studies on the growth of *Aerobacter aerogenes* at low dilution rates in a chemostat. In: Powell EO, Evans CGT, Strange RE, Tempest DW (eds) Microbial physiology and continuous culture. HMSO, London, pp 240–254

Thauer RK, Käufer B, Fuchs G (1975) The active species of "$CO_2$" utilized by reduced ferredoxin: $CO_2$ oxidoreductase from *Clostridium pasteurianum*. Eur J Biochem 27:282–290

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180

Thiele JH, Zeikus JG (1988) Control of interspecies electron flow during anaerobic digestion: significance of formate transfer versus hydrogen transfer during syntrophic methanogenesis in flocs. Appl Environ Microbiol 54:20–29

Thiele JH, Chartrain M, Zeikus JG (1988) Control of interspecies electron flow during anaerobic digestion: role of floc formation in syntrophic methanogenesis. Appl Environ Microbiol 54:10–19

Thompson LA, Nedwell DB, Balba MT, Banat IM, Senior E (1983) The use of multiple vessel, open flow systems to investigate carbon flow in anaerobic microbial communities. Microb Ecol 9:189–199

Tindall BJ (2007) Vacuum-drying and cryopreservation of prokaryotes. In: Day JG, Stacey GN (eds) Cryopreservation and freeze-drying protocols, vol 368, 2nd edn, Methods in molecular biology. Humana Press, Totowa, pp 73–97

Tomczak MM, Hincha DK, Crowe JH, Harding MM, Haymet ADJ (2003) The effect of hydrophobic analogues of the type I winter flounder antifreeze protein on lipid bilayers. FEBS Lett 551:13–19

Torella F, Morita RY (1981) Microcultural study of bacterial size changes and microcolony and ultramicrocolony formation by heterotrophic bacteria in seawater. Appl Environ Microbiol 41:518–527

Trimbur DE, Gutshall KR, Prema P, Brenchley JE (1994) Characterization of a psychrotrophic *Arthrobacter* gene and its cold-active β-galactosidase. Appl Environ Microbiol 60:4544–4552

Tripp HJ, Kitner JB, Schwalbach MS, Dacey JW, Wilhelm LJ, Giovannoni SJ (2008) SAR11 marine bacteria require exogenous reduced sulphur for growth. Nature 452:741–744

Trüper HG, Pfennig N (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

Tuschak C, Glaeser J, Overmann J (1999) Specific detection of green sulfur bacteria by in situ hybridization with a fluorescent labeled oligonucleotide probe. Arch Microbiol 171:265–272

Uphoff HU, Felske A, Fehr W, Wagner-Döbler I (2001) The microbial diversity in picoplankton enrichment cultures: a molecular screening of marine isolates. FEMS Microbiol Ecol 35:249–258

Urakawa H, Kita-Tsukamoto K, Steven SE, Ohwada K, Colwell RR (1998) A proposal to transfer Vibrio marinus (Russell 1891) to a new genus *Moritella* gen. nov. as *Moritella marina* comb. nov. FEMS Microbiol Lett 165:373–378

Urbach E, Vergin KL, Giovannoni SJ (1999) Immunochemical detection and isolation of DNA from metabolically active bacteria. Appl Environ Microbiol 65:1207–1213

Usui K, Hiraishi T, Kawamoto J, Kurihara T, Nogi Y, Kato C, Abe F (2012) Eicosapentaenoic acid plays a role in stabilizing dynamic membrane structure in the deep-sea piezophile *Shewanella violacea*: a study employing high-pressure time-resolved fluorescence anisotropy measurement. Biochim Biophys Acta 1818:574–583

Van den Ende FPL, Laverman AM, van Gemerden H (1996) Coexistence of aerobic chemotrophic and anaerobic phototrophic sulfur bacteria under oxygen limitations. FEMS Microbiol Ecol 19:141–151

Van den Ende FP, Meier J, van Gemerden H (1997) Syntrophic growth of sulfate-reducing bacteria and colorless sulfur bacteria during oxygen limitation. FEMS Microbiol Ecol 23:65–80

van der Meer MTJ, Schouten S, van Dongen BE, Rijpstra WIC, Fuchs G, Sinninghe Damsté JS, de Leeuw JW, Ward DM (2001) Biosynthetic controls on the $^{13}C$ contents of organic components in the photoautotrophic bacterium *Chloroflexus aurantiacus*. J Biol Chem 276:10971–10976

Van der Wielen PWJJ, Lipman LJA, van Knapen F, Biesterveld S (2002) Competitive exclusion of Salmonella enterica serovar enteritidis by *Lactobacillus crispatus* and *Clostridium lactatifermentans* in a sequencing fed-batch culture. Appl Environ Microbiol 68:555–559

van Gemerden H (1974) Coexistence of organisms competing for the same substrate: an example among purple sulfur bacteria. Microb Ecol 1:104–110

van Gemerden H, Mas J (1995) Ecology of phototrophic sulfur bacteria. In: Blankenship RE, Madigan MT, Bauer CE (eds) Anoxygenic phototrophic bacteria. Kluwer, Dordrecht, pp 49–85

van Gemerden H, Tughan CS, De Witz R, Herbert RA (1989) Laminated microbial ecosystems on sheltered beaches in Scapa Flow, Orkney Islands. FEMS Microbiol Ecol 62:87–102

Van Iterson G Jr, Den Dooren de Jong LE, Kluyver AJ (1940) Martinus Willem Beijerinck, his life and work. Martinus Nijhoff, The Hague

Van Niel CB (1930) Contributions to marine biology. Stanford University Press, Stanford, pp 161–169

Van Niel CB (1967) Prefatory chapter. The education of a microbiologist: some reflections. Annu Rev Microbiol 21:1–30

Van Verseveld HW, Chesbro WR, Braster M, Stouthamer AH (1984) Eubacteria have 3 growth modes keyed to nutrient-flow—consequences for the concept of maintenance and maximal growth yield. Arch Microbiol 137:176–184

Vancanneyt M, Schut F, Snauwaert C, Goris J, Swings J, Gottschal JC (2001) *Sphingomonas alaskensis* sp. nov., a dominant bacterium from a marine oligotrophic environment. Int J Syst Evol Microbiol 51:73–79

Varon M, Shilo M (1980) Ecology of aquatic Bdellovibrios. Adv Aquat Microbiol 2:1–48

Veldkamp H (1965) Enrichment cultures—history and prospects. In: Schlegel HG, Kröger E (eds) Anreicherungskultur und Mutantenauslese: zentralbl. Bakteriol. Parasitenkd. Infektionskrankh. Hygiene I. Abteilung Gustav. Fischer, Stuttgart, Supplement 1, pp 1–13

Veldkamp H (1977) Ecological studies with the chemostat. Adv Microbiol Ecol 1:59–94

Veldkamp H, Jannasch HW (1972) Mixed culture studies with the chemostat. J Appl Chem Biotechnol 22:105–123

Veldkamp H, Kuenen JG (1973) The chemostat as a model system for ecological studies. Bull Ecol Res Commun 17:347–355

Veldkamp H, van Gemerden H, Harder W, Laanbroek HJ (1984) Microbial competition. In: Klug MJ, Reddy CA (eds) Current perspectives in microbial ecology. ASM Press, Washington, DC, pp 279–290

Vester F, Ingvorsen K (1998) Improved most-probable-number method to detect sulfate-reducing bacteria with natural media and a radiotracer. Appl Environ Microbiol 64:1700–1707

Visscher PT, Prins RA, van Gemerden H (1992a) Rates of sulfate reduction and thiosulfate consumption in a marine microbial mat. FEMS Microbiol Ecol 86:283–294

Visscher PT, van den Ende FP, Schaub BEM, van Gemerden H (1992b) Competition between anoxygenic phototrophic bacteria and colorless sulfur bacteria in a microbial mat. FEMS Microbiol Ecol 101:51–58

Vobis G (1992) The genus, *Actinoplanes* and related genera. In: Balows A, Trüper HG, Dworkin M, Schleifer K-H (eds) The prokaryotes, 2nd edn. Springer, New York, pp 1029–1060

Vogl K, Glaeser J, Pfannes KR, Wanner G, Overmann J (2006) *Chlorobium chlorochromatii* sp. nov., a symbiotic green sulfur bacterium isolated from the phototrophic consortium "*Chlorochromatium aggregatum*". Arch Microbiol 185:363–372

Wachenheim DE, Hespell RB (1984) Inhibitory effects of titanium (III) citrate on enumeration of bacteria from rumen contents. Appl Environ Microbiol 48:444–445

Wagner M, Nielsen PH, Loy A, Nielsen JL, Daims H (2006) Linking microbial community structure with function: fluorescence in situ hybridization-microautoradiography and isotope arrays. Curr Opin Biotechnol 17:1–9

Walderhaug MO, Dosch DC, Epstein W (1987) Potassium transport in bacteria. In: Rosen BP, Silver S (eds) Ion transport in prokaryotes. Academic, New York, pp 85–130

Walker GC (1996) The SOS response of *Escherichia coli*. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*, vol 1. ASM Press, Washington, DC, pp 1400–1416

Wang X, de Boer PAJ, Rothfield LI (1991) A factor that positively regulates cell division by activating transcription of the major cluster of essential cell division genes of *Escherichia coli*. EMBO J 10:3363–3372

Ward DM, Bateson MM, Weller R, Ruff-Roberts AL (1992) Ribosomal RNA analysis of microorganisms as they occur in nature. Adv Microbiol Ecol 12:219–286

Warthmann R, Cypionka H, Pfennig N (1992) Photoproduction of $H_2$ from acetate by syntrophic cocultures of green sulfur bacteria and sulfur-reducing bacteria. Arch Microbiol 157:343–348

Waterbury JB (1991) The cyanobacteria—isolation, purification, and identification. In: Balows A, Trüper HG, Dworkin M, Schleifer K-H (eds) The prokaryotes, 2nd edn. Springer, New York, pp 149–196

Waterbury JB, Calloway CB, Turner RD (1983) A cellulolytic nitrogen-fixing bacterium cultured from the gland of Deshayes in shipworms (Bivalvia: Terebrenidae). Science 221:1401–1403

Watson TG (1969) Steady state operation of a continuous culture at maximum growth rate by control of carbon-dioxide production. J Gen Microbiol 59:83–89

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Trüper HG (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37:463–464

Weimer PJ, Zeikus JG (1977) Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. Appl Environ Microbiol 33:289–297

Weise W, Rheinheimer G (1978) Scanning electron microscopy and epifluorescence investigation of bacterial colonization of marine sand sediments. Microb Ecol 4:175–188

Welch TJ, Farewell A, Neidhardt FC, Bartlett D (1993) Stress response of *Escherichia coli* to elevated hydrostatic pressure. J Bacteriol 175:7170–7177

Westermann P, Ahring BK, Mah RA (1989) Temperature compensation in *Methanosarcina barkeri* by modulation of hydrogen and acetate affinity. Appl Environ Microbiol 55:1262–1266

White RH (1984) Hydrolytic stability of biomolecules at high temperatures and its implications for life at 250°C. Nature 310:430–432

Whitesides MD, Oliver JD (1997) Resuscitation of *Vibrio vulnificus* from the viable but non-culturable state. Appl Environ Microbiol 63:1002–1005

Widdel F (1988) Microbiology and ecology of sulfate-and sulfur-reducing bacteria. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. Wiley, New York, pp 469–585

Widdel F, Bak F (1992) Gram-negative mesophilic sulfate-reducing bacteria. In: Balows A, Trüper HG, Dworkin M, Schleifer K-H (eds) The prokaryotes, 2nd edn. Springer, New York, pp 3352–3378

Widdel F, Kohring G-W, Mayer F (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

Wiegel J (1990) Temperature spans for growth: hypothesis and discussion. FEMS Microbiol Rev 75:155–170

Wiegel J, Ljungdahl LG (1986) The importance of thermophilic bacteria in biotechnology CRC. Crit Rev Biotechnol 3:39–107

Wilkinson TG, Topiwala HH, Hamer G (1974) Interactions in a mixed bacterial population growing on methane in continuous culture. Biotechnol Bioeng 16:41–59

Winogradsky S (1949) Microbiologie du sol: oeuvres complèetes. Marson, Paris

Wirsen CO, Jannasch HW (1978) Physiological and morphological observations on *Thiovulum* sp. J Bacteriol 136:765–774

Wirsen CO, Molyneaux SJ (1999) A study of deep-sea natural microbial populations and barophilic pure cultures using a high-pressure chemostat. Appl Environ Microbiol 65:5314–5321

Wirsen CO, Jannasch HW, Wakeham SG, Cannel EA (1987) Membrane lipids of a psychrophilic and barophilic deep-sea bacterium. Curr Microbiol 14:319–322

Wolfe RS, Pfennig N (1977) Reduction of sulfur by *Spirillum* 5175 and syntrophism with *Chlorobium*. Appl Environ Microbiol 33:427–433

Wolin MJ (1982) Hydrogen transfer in microbial communities. In: Bull AT, Slater JH (eds) Microbial interactions and communities, vol 1. Academic, London, pp 323–356

Wolter K (1982) Bacterial incorporation of organic substances released by natural phytoplankton populations. Mar Ecol Prog Ser 7:287–295

Xiao J, Luo Y, Xu J, Xie S, Xu J (2011) *Modestobacter marinus* sp. nov., a psychrotolerant actinobacterium from deep-sea sediment, and emended description of the genus *Modestobacter*. Int J Syst Evol Microbiol 61: 1710–1714

Xu HS, Roberts N, Singleton FL, Attwell RW, Grimes DJ, Colwell RR (1982) Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microbiol Ecol 8:313–323

Xu M, Xin Y, Tian J, Dong K, Yu Y, Zhang J, Liu H, Zhou Y (2011) *Flavobacterium sinopsychrotolerans* sp. nov., isolated from a glacier. Int J Syst Evol Microbiol 61:20–24

Yabe S, Aiba Y, Sakai Y, Hazaka M, Yokota A (2011) *Thermogemmatispora onikobensis* gen. nov., sp. nov. and *Thermogemmatispora foliorum* sp. nov., isolated from fallen leaves on geothermal soils, and description of Thermogemmatisporaceae fam. nov. and Thermogemmatisporales ord. nov. within the class Ktedonobacteria. Int J Syst Evol Microbiol 61:903–910

Yamamoto Y, Fukui K, Koujin N, Ohya H, Kimura K, Kamio Y (2004) Regulation of the intracellular free iron pool by Dpr provides oxygen tolerance to *Streptococcus mutans*. J Bacteriol 186:5997–6002

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science 217:1214–1227

Yarza P, Richter M, Peplies J, Euzeby J, Amann R, Schleifer K-H, Ludwig W, Glöckner FO, Rosselo-Mora R (2008) The all-species living tree project: a 16S rRNA-based phylogenetic tree of all sequenced type strains. Syst Appl Microbiol 31:241–250

Yasumoto-Hirose M, Nishijima M, Ngirchechol MK, Kanoh K, Shizuri Y, Miki W (2006) Isolation of marine bacteria by in situ culture on media-supplemented polyurethane foam. Mar Biotechnol 8:227–237

Yayanos AA (1978) Recovery and maintenance of live amphipods at a pressure of 580 bars from an ocean depth of 5700 meters. Science 200:1056–1059

Yayanos AA (1986) Evolutional and ecological implications of the properties of deep-sea barophilic bacteria. Proc Natl Acad Sci USA 83:9542–9546

Yoshida F, Yamane T, Nakamoto K (1973) Fed-batch hydrocarbon fermentation with colloidal emulsion feed. Biotechnol Bioeng 15:257–270

Yu Y, Li H-R, Zeng Y-Y (2011) *Colwellia chukchiensis* sp. nov., a psychrotolerant bacterium isolated from the Arctic Ocean. Int J Syst Evol Microbiol 61:850–853

Yumoto I, Yamazaki K, Hishinuma M, Nodasaka Y, Nakajima K, Inoue N, Kawasaki K (2001) *Pseudomonas alcaliphila* sp. nov., a novel facultatively psychrophilic alkaliphile isolated from seawater. Int J Syst Evol Microbiol 51:349–355

Yumoto I, Nakamura A, Iwata H, Kusumoto K, Nodasaka Y, Matsuyama H (2002) *Dietzia psychralcaliphila* sp. nov., a novel, facultatively psychrophilic alkaliphile that grows on hydrocarbons. Int J Syst Evol Microbiol 52:85–90

Zehnder AJB, Stumm W (1988) Geochemistry and biogeochemistry of anaerobic habitats. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. Wiley, New York, pp 1–38

Zehnder AJB, Wuhrman K (1976) Titanium (III) citrate as a non-toxic oxidation-reduction buffering system for the culture of obligate anaerobes. Science 194:1165–1166

Deutsche Sammlung für Mikroorganismen und Zellkulturen (2012) Bacterial nomenclature up-to-date. http://www.dsmz.de/bacterial-diversity/bacterial-nomenclature-up-to-date.html

Zengler K, Richnow HH, Rosselló-Mora R, Michaelis W, Widdel F (1999) Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 401:266–269

Zengler K, Toledo G, Rappe M, Elkins J, Mathur EJ, Short JM, Keller M (2002) Cultivating the uncultured. Proc Natl Acad Sci USA 99:15681–15686

Zhang DC, Rezic M, Schinner F, Margesin R (2011a) *Glaciimonas immobilis* gen. nov., sp. nov., a member of the family *Oxalobacteraceae* isolated from alpine glacier cryoconite. Int J Syst Evol Microbiol 61:2186–2190

Zhang DC, Busse H-J, Liu H-C, Zhou Y-G, Schinner F, Margesin R (2011b) *Hymenobacter psychrophilus* sp. nov., a psychrophilic bacterium isolated from soil. Int J Syst Evol Microbiol 61:859–863

Zhang DC, Redzic M, Liu HC, Zhou YG, Schinner F, Margesin R (2012) *Devosia psychrophila* sp. nov. and *Devosia glacialis* sp. nov., two novel bacteria from alpine glacier cryoconite. Int J Syst Evol Microbiol 62:710–715

Zhou Z, Jiang F, Wang S, Peng F, Dai J, Li W, Fang C (2012) *Pedobacter arcticus* sp. nov., a facultative psychrophile isolated from Arctic soil and emended descriptions of the genus *Pedobacter*, *P. heparinus*, *P. daechungensis*, *P. terricola*, *P. glucosidilyticus* and *P. lentus*. Int J Syst Evol Microbiol 62:1963–1969

Zinder SH, Koch M (1984) Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Arch Microbiol 138:263–272

ZoBell CE (1941) Studies on marine bacteria. J Mar Res 4:42–75

Zobell CE, Johnson FH (1949) The influence of hydrostatic pressure on the growth and viability of terrestrial and marine bacteria. J Bacteriol 57:179–189

# 8 Comparative Genomics

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## Prokaryotic Classification

Classification covers the theory and practice of how to order characterized organisms into different groups based on their degree of relatedness. Together with identification and nomenclature, classification is a part of taxonomy, a science that deals with the relatedness of organisms. The goal of many taxonomists is to have a classification system that reflects the natural relationships among organisms. This natural system has been depicted mostly as phylogeny (Doolittle 1999)—or an evolutionary tree—which is a diagram that shows ancestor-descendant relationships of organisms based on their evolutionary history. However, inferring a true phylogeny for prokaryotic organisms is very challenging due to the diversity of these organisms, as well as frequent horizontal transfer of genes.

Prokaryotes, known as unicellular organisms with no nuclear membrane structure, have a history of more than 3.5 billion years on earth, yet humans have been aware of them for only the past few centuries, after first being described by Robert Hooke in the seventeenth century. Louis Pasteur and other scientists of the nineteenth century described microorganisms in detail, and began to categorize them. Their classification was dependent on the development of microbial techniques such as isolating and growing microorganisms in pure cultures, staining and microscope observations. In their early observations, lack of guidelines on naming inevitably led to a vast number of invalid names and synonyms. Ferdinand Cohn made the first classification system of bacteria in 1872; six genera of bacteria were classified based on their shape, cellular structures, pigmentation, and metabolic activities (Cohn 1872).

At the beginning of the twentieth century, besides morphology, the use of physiological and biochemical information could be incorporated. European scientists also proposed physiology, metabolism, pigments, and pathogenicity as new systems for classification. However, some of these methods were then criticized for being not important for assessing taxonomic ranks. Later, advances in biochemistry and molecular biology from the isolation of nucleic acids to elucidation of macromolecular structure of proteins and nucleic acids led to the foundation of genomic sciences. Development of computers in the 1950s was another important step in bacterial taxonomy, where they were first used for analysis of phenotypic and molecular data. Between the years 1960–1980, numerical taxonomy and chemotaxonomy were on the rise (Stackebrandt 2006; Schleifer 2009).

In late 1950s, scientists were able to identify the molecules conserved throughout history of life, such as proteins, DNA, or RNA molecules. The idea of using these molecules as blueprints of the evolutionary history of organisms emerged in the 1960s (Zuckerkandl et al. 1962). Tertiary structure and sequence analysis of molecules, such as cytochrome C, ferrodoxins, and fibrinopeptides, and also immunological approaches were being used afterward. However, the interest in these methods decreased as rapid sequencing techniques for DNA became more significant.

The first genotypic approach that allowed bacteriologists to classify prokaryotes on the basis of their phylogenetic relatedness was DNA-DNA hybridization (DDH) (Wayne et al. 1987). In the following years, more genotypic studies, including comparative analysis of Ribosomal RNA (ribonucleic acid) genes and protein-coding gene sequence, allowed more insight to the relationships of prokaryotes (Schleifer 2009). The small subunit rRNA (16S rRNA in prokaryotes) was shown to be one of the universally conserved molecules became the primary molecule of interest. Being ubiquitous, having functional consistency, genetic stability, appropriate size, and independently evolving domains caused this molecule to be chosen for phylogenetic analysis and this approach became a classical tool for taxonomy (Harayama and Kasai 2006). An important study by Carl Woese

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revolutionized bacterial taxonomy, proposing the new kingdom of *Archaebacteria* (Woese and Fox 1977). His later studies concluded in a phylogenetic scheme of three main branches of life (*Bacteria*, *Archaea*, and *Eukarya*) that he called Domains (Woese et al. 1990).

In other genotypic classifications, many protein-coding genes were used for phylogenetic relatedness, some of which are *recA*, *gyrB*, genes of some chaperonins, RNA polymerase subunits (i.e., *rpoB*) or sigma factors (*rpoD*), elongation factor G (*fus*). The most accepted criteria for selection of these proteins is such that, they should not be subjected to horizontal gene transfer (HGT), should be present in all bacteria, preferably in single copies and at least two highly conserved regions for the design of PCR primers (Yamamoto and Harayama 1996). These properties give them an advantage of being more appropriate for phylogenetic analysis of closely related bacteria than 16S rRNA analysis.

In addition to the single gene based methods, Multi Locus Sequence Typing (MLST) has been widely used for genotypic characterization and classification of prokaryotes by comparing multiple housekeeping gene sequences (Maiden et al. 1998). However, usually a different set of genes is useful for different set of organisms, and some difficulties occur in primer design for amplification of genes in all strains if the analysis is not conducted all in silico. A widely used website and database currently is mlst.net (Aanensen and Spratt 2005).

## Current Taxonomy of Prokaryotes

Classification is done by comparing a newly identified organism with the collection of previously classified organisms and then assigning it with a previously described or new species. If a bacterial species is considered novel, the proper naming for the new or existing taxa are made by nomenclature that is based on the International Code of Nomenclature of Bacteria (Lapage et al. 1992), also named as the *Bacteriological Code*. Nomenclature is, however, subject to changes because classification is a dynamic process. The publication of names for novel prokaryotic taxa is made in the International Journal of Systematic and Evolutionary Microbiology (IJSEM), which is the official journal for this purpose. IJSEM also publishes "Validation Lists" which contain new names published in other journals (Tindall et al. 2006). An updated list of approved names for microorganisms based on the international rules can also be found at The DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures) depository (http://www.dsmz.de).

In taxonomy, groups of organisms that are brought together based on shared properties are called "taxa" or "ranks," and prokaryotic taxonomy makes use of several ranks or levels. The current classification scheme has a hierarchical structure, where the higher taxonomic ranks consist of the lower ranked groups. In other words, higher taxa (e.g., genus) contain lower taxa (e.g., species). In an ideal classification system based on evolutionary history, clear clusters of taxonomic units in a phylogenetic tree are seen such that species that share a common ancestor would form the genus and genera that share a common ancestor form a family and so forth. Major sources for bacterial names and taxonomical order are Bergey's Manual of Systematic Bacteriology (Brenner et al. 2005a), Bergey's Taxonomic Outlines (http://www.bergeys.org/outlines.html), and the comprehensive list available at The Taxonomic Outline of Bacteria and Archaea (TOBA) journal (Garrity et al. 2007).

Taxonomy tools historically have been mainly based on laborious laboratory experiments trying to characterize bacteria based on their phenotypic and biochemical properties until molecular approaches and sequencing technologies were developed. Today, such research can be handled using robotic and computational techniques, where most of the knowledge gained from results rely on the data that is being handled.

## The Explosion of Sequenced Bacterial Genomes

Biological data generated by researchers worldwide has been growing with a tremendous rate, especially with the advances in molecular biology techniques in the past 50 years. Much of this vast information can now be accessed through biological databases that hold records for experimental data, sequence data, classification schemes, literature, and some also provide computational analysis tools.

A part of this huge biological information is the genomic sequences. In modern molecular biology and genetics, a "genome" is the entirety of an organism's hereditary information. Therefore, genomics can be referred to as the science of genome analysis. As such the field of comparative microbial genomics (CMG) work with comparing the entire DNA material of a microbial organism to other organisms. The first two complete bacterial genome sequences were published in 1995. As the technologies advanced and the sequencing cost went down, many more sequences were being published and more databases were established to handle this information. One of the most used databases is based on GenBank, now located as part of the National Center for Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov/genome/browse/). The NCBI GenBank holds the nucleotide sequence data from expression sequence tag (EST), genome survey sequences, other high-throughput sequences such as whole-genome sequences and genome annotations of thousands of organisms. Both prokaryotic and eukaryotic data is available (Benson et al. 2008). GenBank is a part of an international collaboration called International Sequence Database Collaboration, which also consists of DNA Data Base of Japan (DDBJ) and the European Molecular Biology laboratory (EMBL). Another part of NCBI that is highly related to this chapter is NCBI Taxonomy. Although claiming not to be a primary source, NCBI provides taxonomical information that is gathered from various sources.

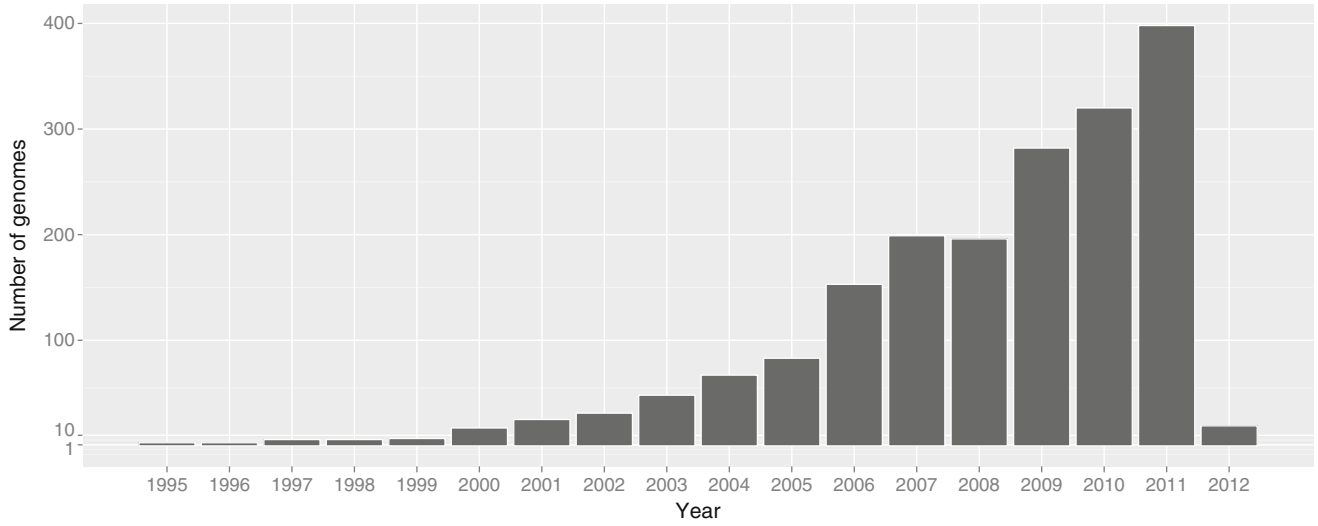


**Fig. 8.1**
**Genomes published and deposited to public NCBI GenBank since 1995 (Data gathered from NCBI (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi, Jan. 2012))**

In May 2011, NCBI GenBank contained around 1,500 genome sequences labeled as "finished." Six months later, at the time of writing (November 2011), this number has gone up to 1,790. Currently (Feb. 2012), the NCBI "Genome Projects" is changing to "BioProjects," in order to relate genomic information to other data types, such as the transcriptome, proteome, and metagenome.

In addition to NCBI (GenBank) and EMBL (Nucleotide Sequence Database), a source for genomic information is the Genomes Online database (GOLD). GOLD aims to provide an accurate and complete set of finished and ongoing genome projects with a broad range of information on each project. The sequence data itself is not stored in the database, however, external links to where the data can be found is given, most of which are to the NCBI Genome Project pages. GOLD also provides taxonomical information, though not the primary source (Bernal et al. 2001; Liolios et al. 2010).

One part of comparative microbial genomics is to monitor the available microbial genomic data. Even though the sequences available may only be a small fraction of the real world, the information gathered is growing every day. It took 14 years to sequence the first thousand bacterial genomes (1995–2009), and already in 2012, less than 3 years later, the two thousandth genome sequence has been deposited to GenBank. Not only has the cost of genome sequencing decrease dramatically but also the time and effort put into the task has also gone down. Also the computational power and software to handle sequencing data is being revolutionized and fast assembly and interpretation is increasing the number of published genomes (Ansorge 2009). The increase in genome data has given rise to a whole new area of problems when it comes to publication and sharing of data. Databases usually have their own formatting of the raw data and though some are more used than others, a clear standard for genome publication has yet to be established (Médigue and Moszer 2007).

## Statistics on Prokaryotic Genomes

With such a large amount of data, it is interesting to see the trends in the basic statistics of the genomic data and comparisons on different taxonomic levels and years of sequence publications. The data presented in this section is taken from the NCBI complete genomes list in Jan. 2012 and GenBank files for 1,500 sequenced genomes were downloaded (November 2011).

### Data Growth over the Years

*Figure 8.1* illustrates how many genomes were published each year, since 1995. The two first complete genomes to be sequenced and deposited was *Mycoplasma genitalium* G37 (Fraser et al. 1995) and *Haemophilus influenzae* Rd KW20 (Fleischmann et al. 1995). From 1995 until 1999, only 25 genomes were published as complete and they covered 14 phyla. Of these the Archaeal genomes constitute a large portion compared to the fraction today (around 31% of the 25 compared to 99 out of 1,500 (6.6%)). The genomes from this first period of genome sequencing cover a large span of the microbial landscape with no obvious medical bias. From 2000 to 2010 the number steadily increased from 26 to 1,423 with the major phyla covered being *Firmicutes*, *Gamma*, and *Alpha* subdivisions of *Proteobacteria*. It is possible that producing a complete genome sequence is becoming less popular due to the improvement in software that can work on draft genomes (Chain et al. 2009).

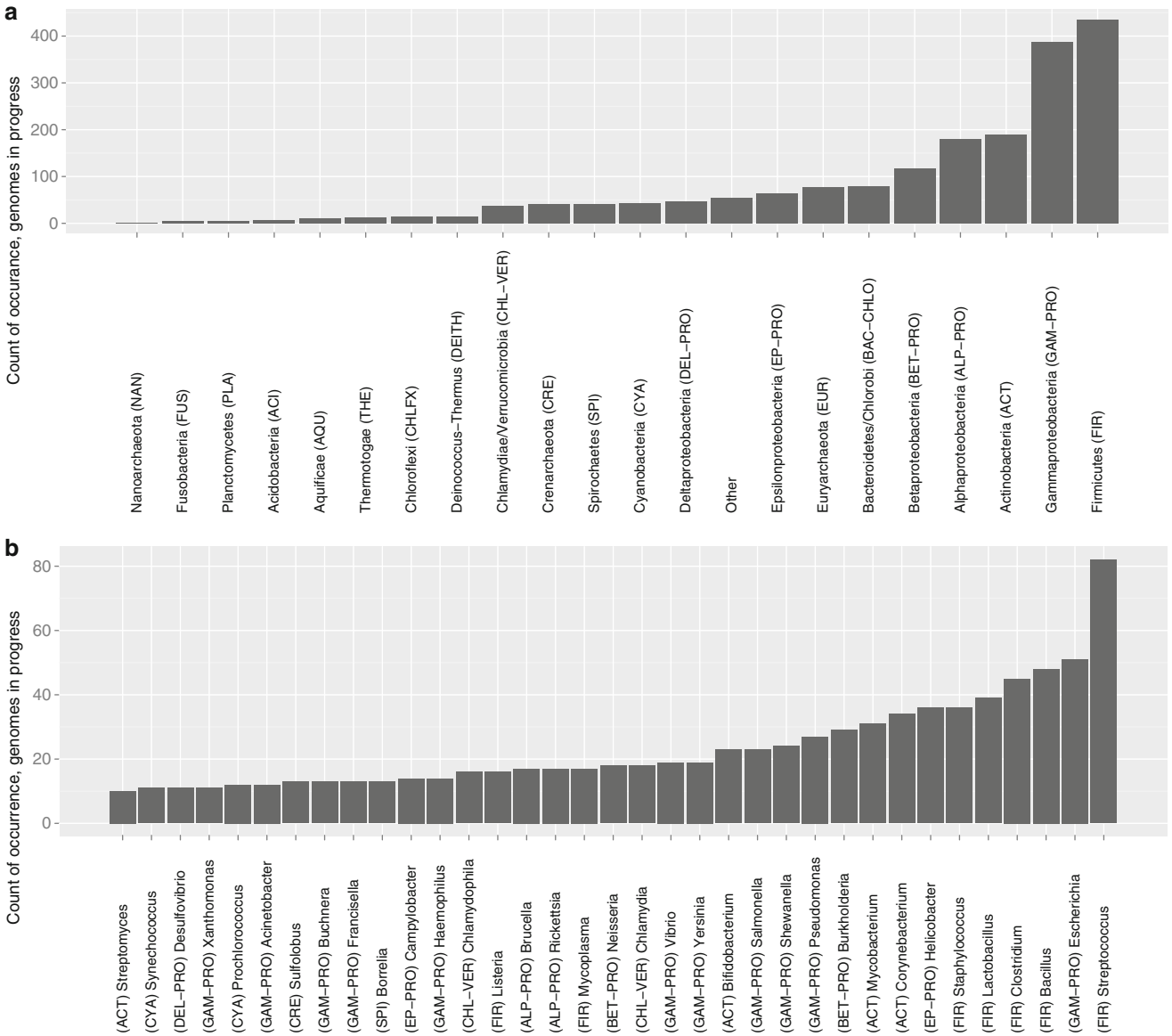


**Fig. 8.2**

**Number of genomes sequences from each phyla and genera. Only genera with more than 10 representative genomes are shown**

The cost of sequencing and the development of cheaper sequencing methods have most definitely had an impact on the rate of sequencing (Sboner et al. 2011).

## Taxonomy Analysis, Most Sequenced Phyla and Genera

*Figure 8.2a* shows the number of genomes within each phyla; *Firmicutes* and *Gamma Proteobacteria* are the most highly represented. The plot in *Fig. 8.2b* shows genera with more than 10 sequenced genomes. The genus of *Streptococcus* is highly overrepresented (63 genomes) while the closest other group is *Escherichia* (45 genomes). According to supporting data from the GOLD database (http://genomesonline.org, March 2011) over 73% of the listed *Streptococcus* and more than 64% of *Escherichia* are labeled as pathogens.

It is likely that some organisms are sequenced because of their medical relevance. Organisms belonging to the *Escherichia* genus have a considerable role within the medical world, with *Escherichia coli* being the cause of serious food poisoning. The tendency of pathogens to be more often sequenced is, however, not as strong overall. The fraction of pathogens within each genus varies from 7% (*Lactobacillus*) to 98% (*Listeria*), for the six different genera belonging to the *Firmicutes*. For all the genera listed in *Fig. 8.2b* the range covers everything from 3% (*Synechococcus*) and 100% (*Borrelia* and *Rickettsia*, supporting data from GOLD). It should be noted that the

annotation of "pathogen" is not always accurate; for example, the first sequenced genome, *Haemophilus influenzae* is listed as a "pathogen," although the strain sequenced (Rd KW20) is a nonpathogenic strain.

In any event, it is clear that there is a strong sequencing bias, making the available data for certain phyla and genera considerably more than others. It could be expected that more pathogens than nonpathogens would be sequenced, as the immediate interest in these organisms is larger. However, the bias is not directly linked to pathogenicity, as some genera are sequenced more often though not being serious pathogens. For example, species like *Escherichia coli* (urinary tract infections, simple diarrhea, dysentery-like conditions) include pathogens but are not as severe as other species like *Borrelia* (Lyme disease) or *Listeria* (Listeriosis in newborn infants, elderly patients, and immunocompromised). *Escherichia coli* is, however, a significant player in the financial aspect of medical relevance. These organisms, though rarely lethal, can occur frequently in the population, and still require treatment; this is a burden on any healthcare system. Another factor could be the economic cost, as some organisms grow less easily or replicate very slowly making experiments long and expensive. Some pathogens require extreme safety procedures when cultured and this consumes time, space, and money. The historic factor could also be partly responsible for sequencing bias. Some organisms became model organisms from the early stage of microbiology and as such, many procedures are optimized for these organisms. Unfortunately, due to the large variety within the microbial world, many organisms will not respond well to procedures developed with *Escherichia coli* as the template. Taxonomical bias in sequencing data is, as stated, a complex and multifaceted discussion that will probably never end. However, these tendencies should be kept in mind when accessing the data available for analysis.

## Basic Genome Statistics

The sequences of 1,500 genomes have been obtained from NCBI GenBank and analyzed according to basic statistical parameters. Here, basic genome statistics refers to certain DNA properties of the genome, such as genome size, frequencies of A and T bases in the DNA, and bias on the third codon positions for the open reading frames.

*Figure 8.3a* is a box and whisker plot showing the variation of genome sizes within each phylum. As seen, several phyla have a wide distribution of sizes. Phyla containing only a few genomes (less than five sequences) show very little size variation that could be the result of sequencing several closely related strains. For most phyla size is not a key feature, although *Chlamydia* and *Nanoarchaeota* are expected to be small genomes. The genomes within the *Firmicutes* are distributed over a broad spectrum (580–8,300 kb), while *Gamma Proteobacteria* size varies between 32−7,215 kb. Large genomes are often seen within *Planctomycetes*, *Beta Proteobacteria*, and *Actinobacteria* while small genomes are found within *Epsilon Proteobacteria*, *Chlamydiae*/*Verrucomicrobia*, and *Nanoarchaeota*. Of the largest genomes (more than 8,000 kb, 32 genomes), members of *Actinobacteria* are prevalent (14 genomes) ranging from 925 kb to 11,937 kb. The largest genome, as of May 2011, at 13,033.779 kb, was *Sorangium cellulosum* So ce 56 (soil-dwelling bacteria) (Schneiker et al. 2007).

An interesting perspective on genome size is the focus on the minimal genome for a free-living organism. Defining the minimal genome is a science in itself and has been heavily discussed in the scientific community (Galperin 2006). In 1995, the genome of *Mycoplasma genitalium* (a parasite) was published, and at that time this was thought to be the smallest genome of any free-living organism (Fraser et al. 1995). Of the 1,500 genomes in this study, *M. genitalium* is the 18th smallest genome. Upon closer inspection, the eight smallest "genomes" are described as phage, Integrating and conjugative elements (ICEs), pathogenicity island, or genomic island, so not free-living organisms. These nongenomic sequences have been reported to GenBank, and since have been deleted from the list of genomes during this work. Other genomes smaller than *M. genitalium* consist of *Buchnera* (an endosymbiant (Pérez-Brocal et al. 2006) and *Nanoarchaeum*) another symbiont (Waters et al. 2003). The remaining seven genomes are *Candidatus* species, from proposed genera, and all of these are described as symbionts (McCutcheon et al. 2009). It is worth mentioning that the smallest genome of a "true" free-living organism (as opposed to parasites like *M. genitalium*) is considerably larger, containing more than a thousand protein-encoding genes. Two proposed "minimal free-living organisms" are *Pelagibacter ubique* (heterotroph, 133th smallest in this study; DeWall and Cheng 2011) and *Prochlorococcus marinus* (autotroph, 209th smallest in this study; Moya et al. 2009). Note that these genomes are still smaller than the largest viral genomes (Arslan et al. 2011).

Another genome statistics commonly used is the percentage of AT (*Fig. 8.3b*), which is calculated as the average AT content of all the DNA sequence. Genomes with high AT content include *Candidatus Zinderia insecticola* CARI (86%, *Beta Proteobacteria*), *Candidatus Carsonella ruddii* PV DNA (83%, *Gamma Proteobacteria*), and *Buchnera aphidicola* str. Cc (*Cinara cedri*; 80%, *Gamma Proteobacteria*). These are all extremely small genomes. They are also all symbiotic organisms living inside insects, the spittlebug *Clastoptera arizonana*, jumping plant lice and plant lice, respectively. Genomes with low AT (high GC) content include *Anaeromyxobacter dehalogenans* (*Delta Proteobacteria;* Sanford et al. 2002) and *Cellulomonas flavigena* (*Actinobacteria*; Abt et al. 2010), both with around 25% AT. These genomes consist of an anaerobic and aerobic soil-bacteria, respectively. The AT content within each phyla shows some specificity with phyla like *Acidobacteria*, *Actinobacteria*, and *Deinococcus*/*Thermus* having a significant skew toward low AT and *Fusobacteria*, *Epsilon Proteobacteria*, and *Aquificae* having a skew toward high AT (*Fig. 8.3b*).

Can the AT content of an organism be related to its size? The answer can be both "yes" and "no." The numbers from 1,500 genomes show that these two properties are not always proportional to each other. However, for very large and small genomes,

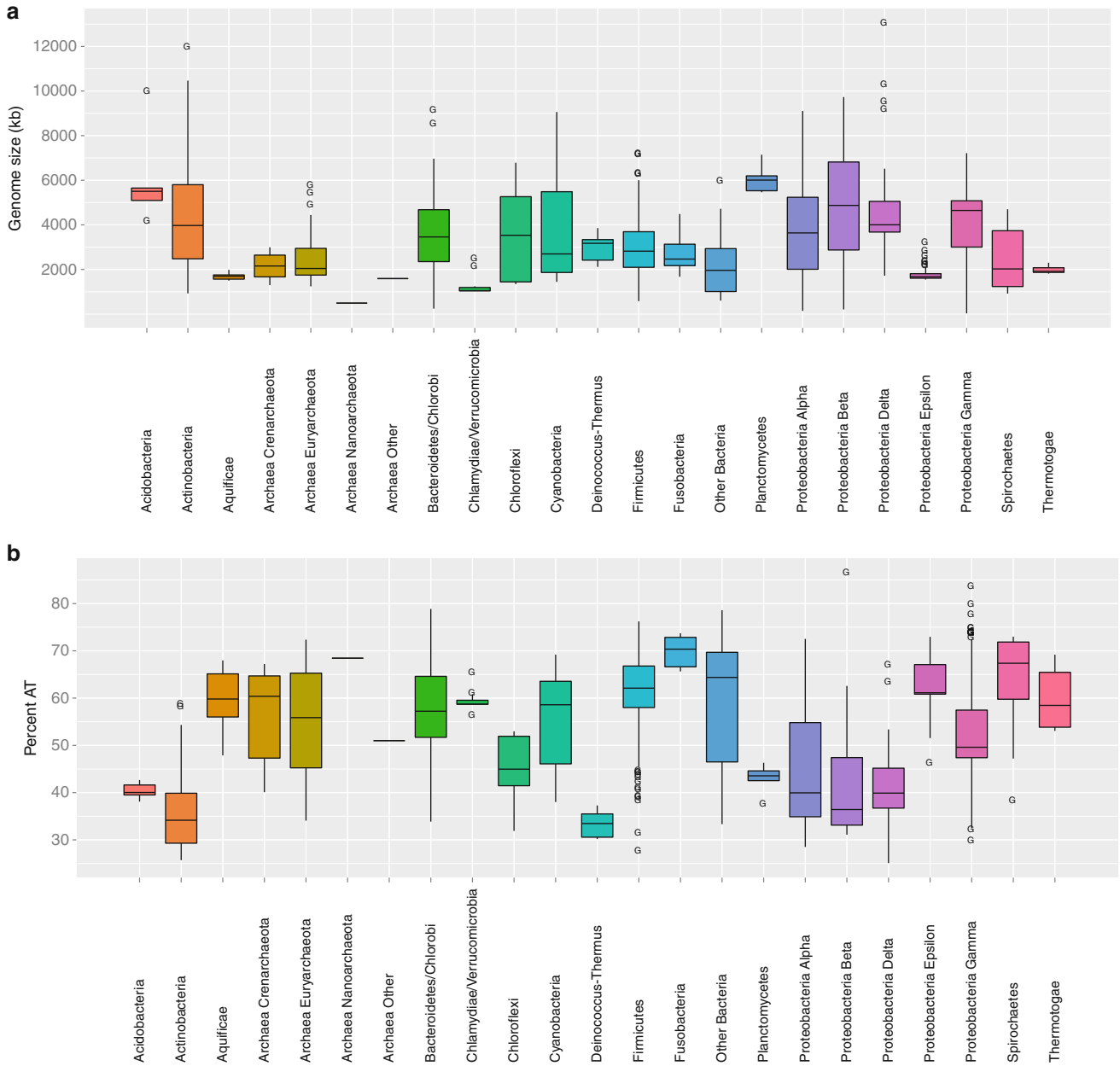


**Fig. 8.3**

**Boxplots showing the distribution of genome size (in kilo base-pairs, *panel a*) and AT content (in percentage, *panel b*) for each phyla (as described by NCBI Taxonomy). The *middle bar* is the 50 % percentile, the *bottom* and *top* of the *box* are the 25 % and 75 % percentiles (Q1 and Q3, respectively). *Whisker bars* extend to the most extreme data point which is no more than +/− 1.58IQR/sqrt (n), where IQR is the interquartile range (IQR = Q3 − Q1). Any data point that exceeds this limit is plotted as an individual data point (outlier). The genome size was calculated as the sum of lengths of all contigs**

the answer can be "yes." *Figure 8.4* shows a scatterplot of genome size and AT content (Pearson correlation coefficient of −0.48), showing that small genomes have high AT content and large genomes have low AT content. The analysis also shows a cloud around the middle values, indicating that average size corresponds to an AT content with high fluctuations.

On the other hand, an interesting relation is seen between AT content and bias in third codon position. *Figure 8.5* illustrates a strong correlation between these two properties of a genome (Pearson correlation coefficient of −0.94). The third codon position is the most variable position for the codon and this is where the largest variation in base use would be expected. The correlation was therefore expected and shows that high AT content in a genome correlates with a bias close to −1 (which is 100% AT in the third codon) and low AT content correlates with a bias close to +1 (which is 100% GC in the third codon).


**Fig. 8.4**

**Scatter plot showing percentage AT compared to total genome size (kb) for 1,500 genome sequences. The Pearson Correlation Coefficient (PCC) for this data is −0.48, which shows a medium correlation. PCC is often used to measure the linear dependence between two variables, and takes a value between +1 and −1, where 0 reflects no linear correlation**

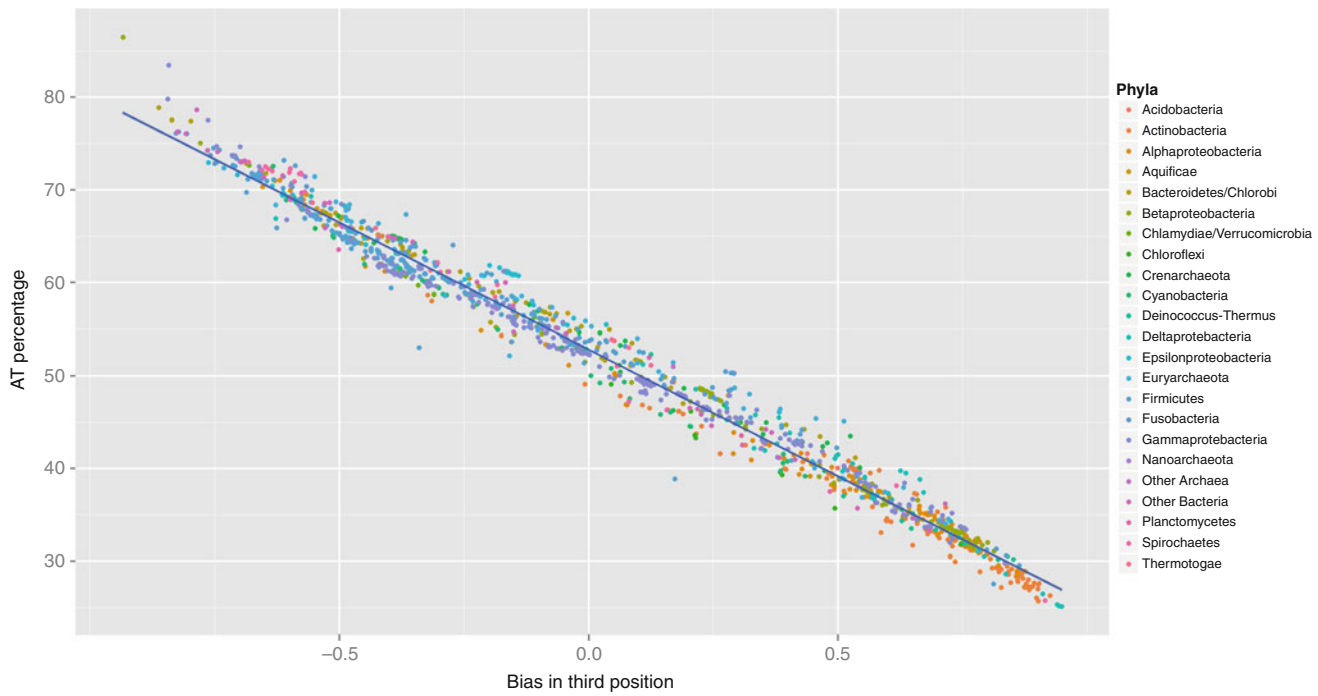


**Fig. 8.5**

**Scatter plot showing percentage AT compared to base bias in third codon position for 1,500 genome sequences. Bias is calculated so that 100 % A or T in third codon position gives a score of −1, 100 % G or C in third position gives a score of +1. The Pearson Correlation Coefficient for this data is −0.94, which shows a strong correlation. PCC is often used to measure the linear dependence between two variables, and takes a value between +1 and −1, where 0 reflects no linear correlation**

## Thousands of Genome Sequences

Availability of thousands of genomes makes it possible to investigate phylogenies based on genomic information and see how current taxonomy is affected. The development of many computational tools and increasing computational power makes it possible to compare whole genomes in a reasonable time, yet comparison of thousands of whole genomes is still a tedious process. Therefore, three data sets were selected that represent different taxonomic levels of prokaryotes. The first data set is chosen to cover a wide coverage of all the prokaryotic organisms (126 genomes and 23 phyla). The second data set is a representative of a well-defined prokaryotic family (*Enterobactericiae* family, 50 genomes). The third one is chosen as an example of a prokaryotic species and close relatives (*Escherichia coli, Escherichia fergusonii, Shigella*). Different computational methods that we have encountered to be fitting in the current taxonomy of prokaryotes were shown for each data set in the following sections.

## Whole-Genome-Based Tools for Taxonomy

The previous section showed the growth in available sequence data as well as the bias and diversity in this data. This large diversity and coverage opens the doors to large-scale phylogenetic analysis of genome sequences. As a result, great insight into bacterial evolution and diversity has come from comparison of many microbial genome sequences in the last decade. The differences, even between strains of a distinct taxonomic cluster, show that bacteria represent a great diversity, which led to the formation of the hypothetical concepts of "pan-genomes" and "core-genomes." The pan-genome contains the total number of genes found in the gene pool of a set of genomes (Ussery et al. 2009) and can be viewed in three separate parts. The part that consists of conserved essential genes common to all genomes compared (core-genome). It has been seen that core-genomes of phylogenetically coherent groups contain genes that are less prone to horizontal gene transfer and are more stable such as housekeeping genes. The genes essential for colonization, survival, or adaptation to a specific environment are thought to form the lifestyle genes, which can also be named as the "shell" for frequently occurring genes. The third part is called "accessory" or "cloud" genes, as these are rarely found, often strain specific and nonessential (Lapierre and Gogarten 2009). Though hypothetical, these terms can serve use for defining and classifying bacteria. These different "genomes" can be used to explain the differences and similarities between species or genera, and visualized by pan-genome trees (Snipen and Ussery 2010). An elaborate work on the comparisons of genomic DNA using oligonucleotide-based methods and proteomes with a pan-genome approach was presented in a study by Bohlin et al., where *Brucella* species were classified using 32 genomes (Bohlin et al. 2010).

Other genome-based methods include measures for replacement of the DDH (DNA-DNA-Hybridization) analysis, such as the Average Nucleotide Identity (ANI) between pairs of genomes or the Average Amino acid Identity (AAI) of the shared genes between two genomes. The study of Goris et al. on pairwise comparison of complete sequenced genomes showed the ANI of the core genes show results similar to analysis of 16S rRNA sequence identity and DDH similarity values, concluding that a 70% DDH value corresponds to 95% ANI. Hence ANI has been shown to be an alternative to the tedious DDH method (Goris et al. 2007). Another genome-based method, AAI, has been shown to result in strong correlation between 16S rRNA gene identity and AAI-based phylogenetic trees congruent with core genome-based trees (Konstantinidis and Tiedje 2005).

There are many methods and types of data used to build evolutionary trees. The results of the whole-genome-based tools are usually values representing similarity between organisms, which can then be converted to distance-based phylogenies. Distance methods constitute a major part of phylogenetic analysis. "Least squares" is one of these methods where the sum of squares of difference between the observed and the predicted distances of a tree should be minimized. Unweighted (Cavalli-Sforza and Edwards 1967) and weighted (Fitch and Margoliash 1967; Beyer et al. 1974) algorithms are suggested for least squares. Minimum Evolution, Neighbor joining, and UPGMA are all distance-based methods. There are also methods that rely on probabilities of evolutionary change. Maximum likelihood is one of them, where different evolutionary rates can be taken into account and several models can be implemented (Felsenstein 2004). The evolutionary models and the distance methods should be chosen carefully when phylogenies are generated, as they might result in different results even for a small set.

## rRNA Phylogenetic Trees

In this section the 16S rRNA and 23S rRNA comparison of 126 various organisms from all bacterial and archaeal phyla is presented (*Fig. 8.6*). This data set represents a collection of distantly related prokaryotic organisms. The first criteria for the selection of organisms for this dataset, was to get the largest and smallest genomes from each phyla (taxonomy reference is Genome metadata from NCBI and GOLD). More organisms from each phyla were selected from different environments or host associations, in order to get a less biased data in total.

Ribosomal RNA sequences of all 126 genomes were predicted using RNAmmer program (Lagesen et al. 2007). Foreach genome one 16S and 23S rRNA sequence was selected based on the highest RNAmmer score and appropriate length (Lagesen et al. 2010). The length requirements were between 1,400 and 1,700 bp for 16s rRNA sequences, 2,500 and 3,800 bp for 23S rRNA sequences. Once the RNA sequences were gathered they were aligned using CLUSTALW with default parameters (10 for gap opening penalty, 0.20 gap extension penalty, 30% Delay divergent sequences, 0.5 for DNA transitions weight, IUB for DNA weight matrix) (Larkin et al. 2007). After obtaining the alignments, the phylogenetic trees were constructed using MEGA5 (Tamura et al. 2011) and

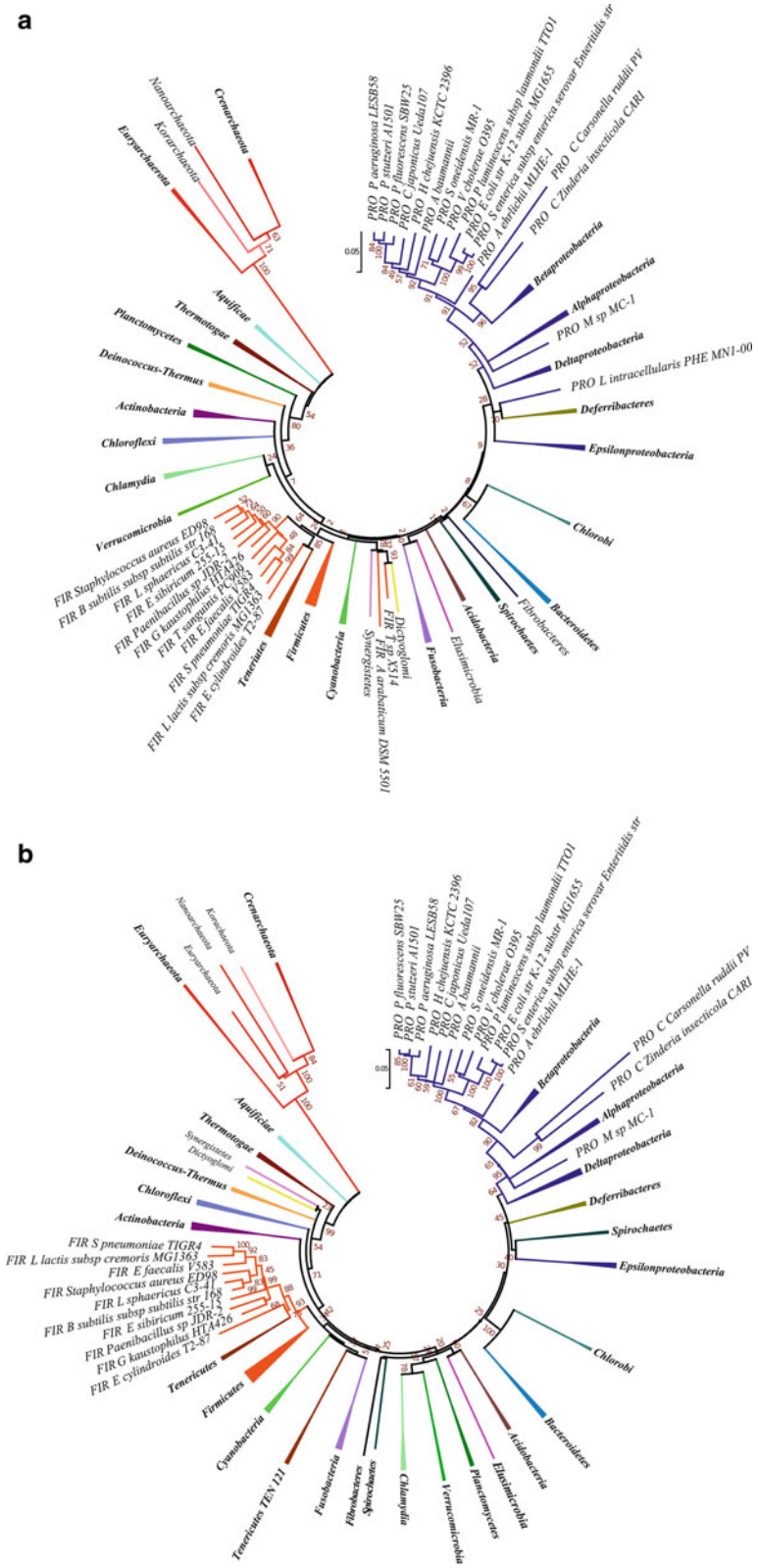


**Fig. 8.6**

**(a)16S rRNA and (b) 23S rRNA tree with NJ method and 1,000 bootstrap resamplings from ClustalW alignment. The trees are viewed and colored with MEGA5. Branch lengths are measured in the number of substitutions per site. Each phylum is collapsed when possible, except classes of *Proteobacteria* were collapsed instead of phyla**

Neighbor-Joining (NJ) with 1,000 bootstrap resamplings. The bootstrap values in these phylogenies were transformed to percentages. They give a statistical measure for how reliable a branch separation is. Therefore, higher percentages support a stronger evidence of grouping, meaning a more prominent common ancestor, whereas lower percentages mean the separation on that branch is statistically insignificant.

Ribosomal RNA phylogenies are usually able to distinguish the domains, phyla, and genera in a given set. The distances on this type of phylogeny show the divergence in the rRNA sequences. According to the bootstrap values in the 16S rRNA phylogenetic tree (*Fig. 8.6a*), the phyla level clusters are significant with higher than 80% bootstrap value on their roots. To better illustrate this, the two different clades were left uncollapsed while the remaining phyla clades were collapsed. The correspondence of the significant clades to phyla in prokaryotes is, however, an expected result. Phylum, as a taxonomic unit is not defined by the official nomenclature (International Code of Nomenclature of Bacteria (Lapage et al. 1992)). The highest rank according to the official nomenclature is a class; however, the rank phylum is also being used in prokaryotic taxonomy quite often and seems to serve practical use for the taxonomists. Historically, phyla were referred as divisions and Prokaryotes, as one of the superkingdoms proposed by Whittaker and Margulis, were divided into three divisions based on cell wall structure or absence (Whittaker and Margulis 1978; Gibbons and Murray 1978). Although the classification largely changed since the division of archaeal phyla (Murray 1989) were discovered, most of these names are still in use today. In the 2nd edition of Bergey's Manual, phylum was defined as the major prokaryotic lineages, based on the 16S rDNA sequence data and used as main organizational unit (Brenner et al. 2005b).

Ribosomal RNA based phylogenies usually involves the 16S rRNA subunit comparisons. Here we show that 23S rRNA phylogeny can also be useful. When the same dataset is analyzed using 23S rRNA genes, the bootstrap values are generally higher than 16S rRNA phylogenies (*Fig. 8.6b*). There are exceptions to this, for example, the bootstrap value on the roots of the *Gamma Proteobacteria* clade that is higher on the 16S rRNA tree. The generally higher values for the 23S rRNA analysis might be due to the size or information content of the sequences and maybe due to the different mutation rates of the genes. The separation of phyla on *Fig. 8.6b* is significant, but the order is different, which might lead to the idea of having different relationships among different phyla. However, since the bootstrap values are very low at that level, it is still not relevant to conclude how close *Firmicutes* is to *Proteobacteria* based on rRNA phylogeny.

## Average Nucleotide Identities (ANI) and Tetra Nucleotide Frequency Calculations

Average nucleotide identity was developed as an alternative to the DDH values, and was initially based on comparison of all shared genes among two genomes. Later on an advance in the method, to make it more similar to DDH, was made by randomly chopping up the genome sequences in 1,020 nucleotide fragments regardless of whether or not they correspond to any ORFs. The fragments from two genomes are aligned using a BLAST (Altschul et al. 1990) algorithm or a fast alignment tool such as MUMmer without fragmentation.

In this section, 50 genomes from different genera of the *Enterobacteriaceae* family (data gathered from NCBI GenBank) are compared based on their ANI values. ANI calculations were performed as explained in the paper by Richter and Rosello-Mora's using Jspecies (Richter and Rosselló-Móra 2009). The genome sequence comparison was based on nucleotide MUMmer (NUCmer) which is a fast DNA alignment tool for large-scale comparisons (Delcher et al. 1999). MUMmer aligns two given genome sequences based on maximal unique matches (MUMs) between the sequences. A "MUM" is an exact string match that occurs once in each genome. Once the MUMs are identified, they are sorted in ascending order according to their positions in the genomes. After the global MUM-alignment, the gaps between them are closed based on the properties of the gaps. A gap can be a single nucleotide polymorphism, an insertion or deletion where a large sequence is found in one but not the other genome, tandem repeats, or polymorphic regions. If gaps are found, they are aligned using the Smith-Waterman algorithm (Smith and Waterman 1981).

Comparisons based on the tetranucleotide frequencies were calculated using Jspecies and the algorithms from Teeling et al. (2004). In this method, all possible combinations of tetranucleotide frequencies (256 frequencies) for each sequence is calculated and their z-scores are computed based on the difference between the observed and the expected frequencies for a genomic fragment. The similarity between the two sequences (or genomic fragments) in terms of having similar patterns of tetranucleotides is addressed by calculating the Pearson correlation coefficient for their z-scores. Similar patterns are expected to correlate and therefore have higher correlation coefficients, whereas the distant patterns would have lower correlation coefficients. Oligonucleotide frequencies are thought to carry species-specific signals, where longer signatures carry more signals. Thus, closely related organisms are expected to show similar distribution of the usage of these signatures.

*Figure 8.7* shows a pairwise genome comparison of ANI value (heatmap). The genomes are manually ordered based on 16S rRNA similarities. It is seen that DNA similarity within a genus is higher compared to the similarity between genera. It is therefore possible to distinguish groups of genus and species based on their DNA similarity. For comparison, Tetra Nucleotide frequencies were calculated for the same data and ordered based on 16S rRNA similarity. The two heatmaps are expected to show similar results, with ANI values above 96% identity would correspond to very high Tetra Nucleotide frequencies correlation coefficients of $\geq$0.99 (Richter and Rosselló-Móra 2009). It is seen that within genera, sequences are highly correlated based on tetranucleotide signature usage. Changing the order of the matrix based on hierarchical clustering might give a better resolution using Tetra Nucleotide frequencies.

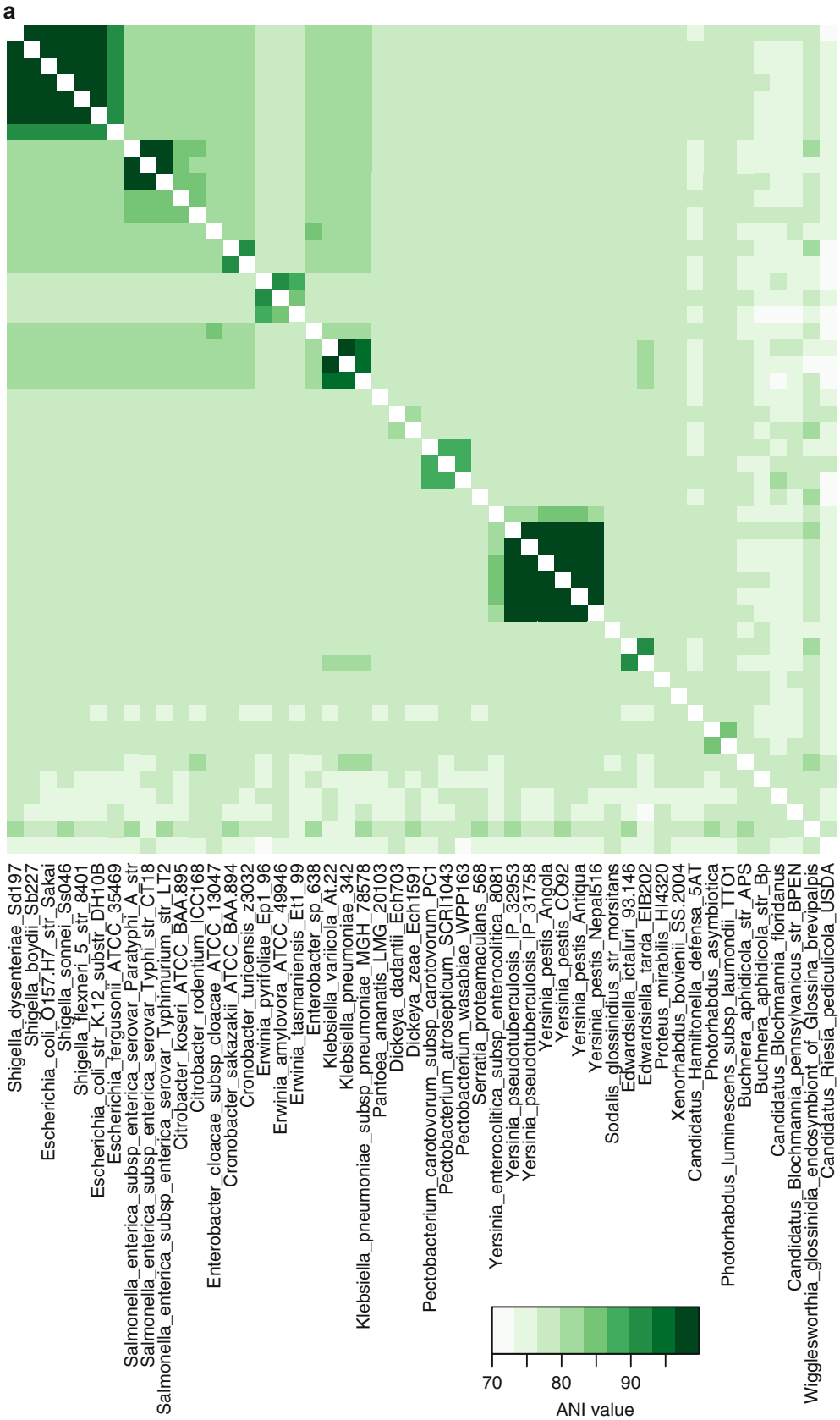


**Fig. 8.7 (continued)**

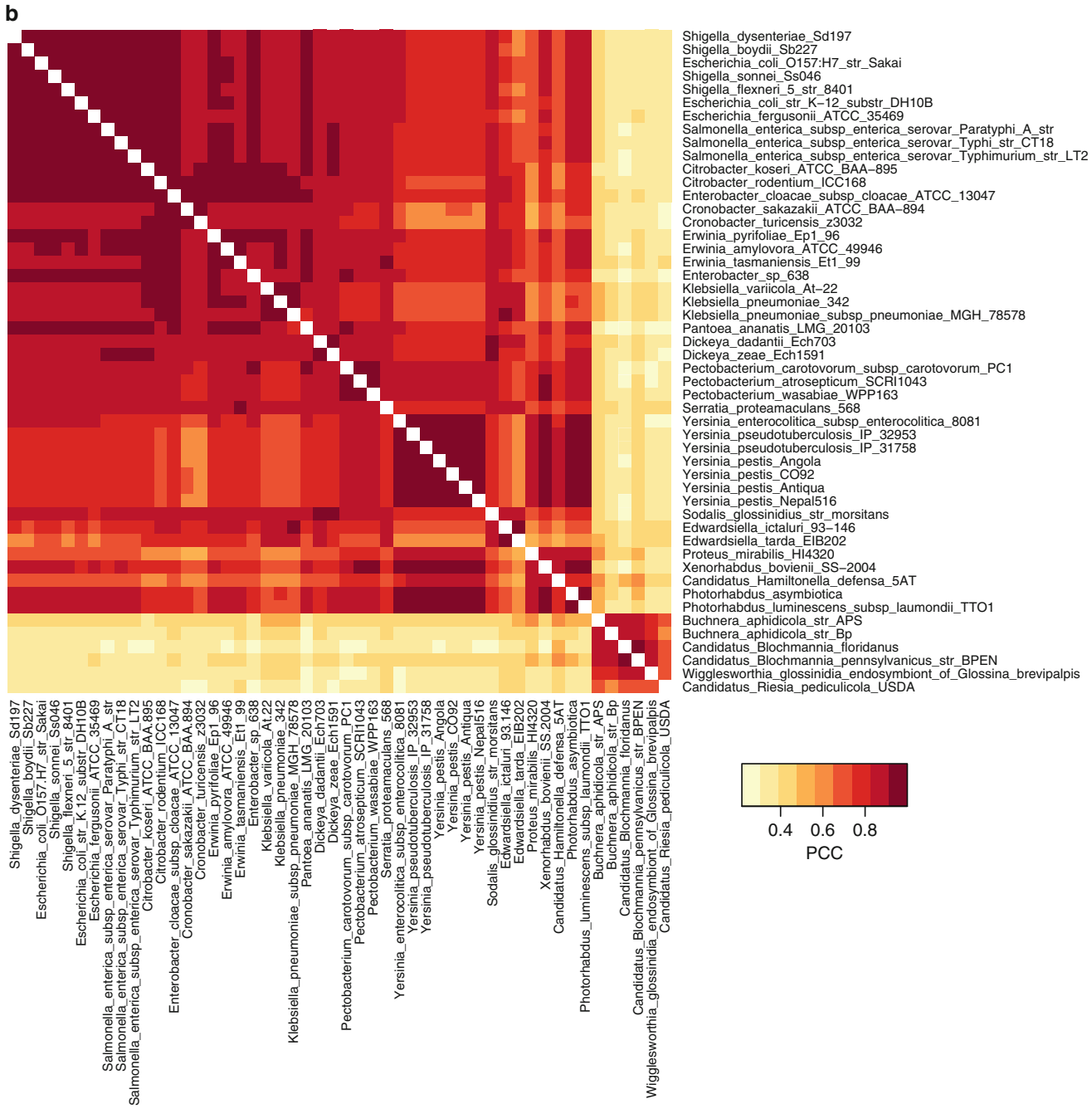


**Fig. 8.7**

**(a) Shows heatmap for ANI values between each genome retrieved by pairwise alignments with MUMmer. Darker colors indicate higher percentages. The columns and rows are ordered based on the clusters in the 16S rRNA NJ tree. (b) Shows Pearson Correlation coefficients yielded by tetranucleotide frequency calculations between genomes of Genera set. The comparison based on Tetra calculations shows higher similarity between organisms as the colors get darker**

## BLASTMatrix Using Reciprocal Best Hits

The dataset of 50 genomes was used to illustrate the BLASTMatrix method. Each proteome was pairwise compared using BLASTP using a reciprocal best-hit criterion. The method is based on an all-against-all BLASTP analysis where all proteins are compared to all other proteins in the dataset. Then a reciprocal best-hit criterion is implemented. According to this, a BLAST hit will be considered significant only if the length of the alignment is at least 95% of the longest protein and has

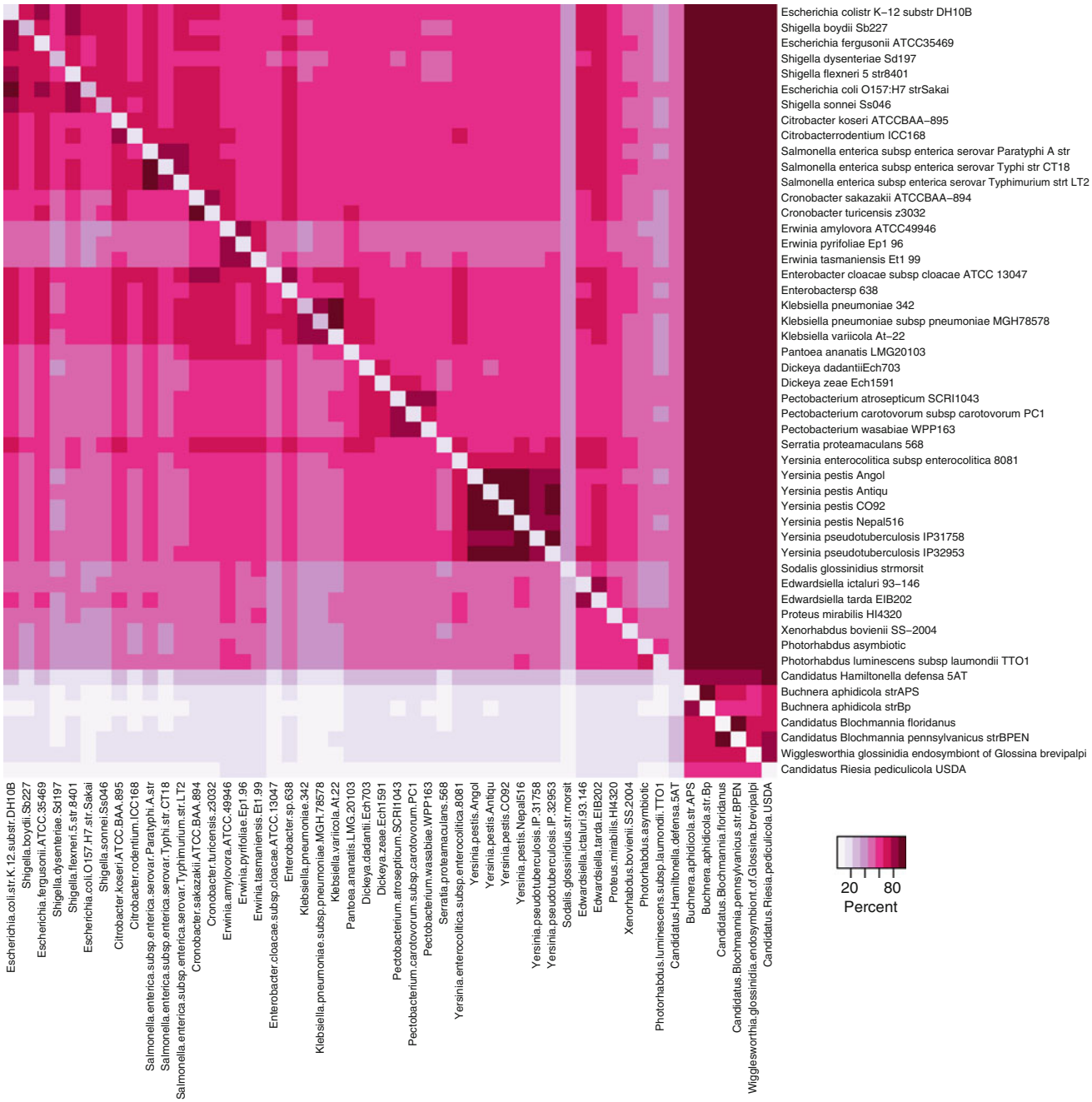


**Fig. 8.8**

**Proteome comparison between the genomes of Enterobacteriaceae based on BLASTP searches**

95% sequence identity. There are three possible outcomes for each protein: (1) The protein does not have any significant hits to any other protein, (2) the protein has one or more hits where the hit with the highest bitscore is chosen as best hit, and (3) the protein has more than one hit where there can be several best hits. Once identified based on this three possibilities, best hits for each protein are stored. For each proteome, a hit will be counted if the best hit of protein X in proteome A is also the best hit of the corresponding protein Y on proteome B. In the case where there are several best hits, the hit is counted if protein Y is one of the best hits for protein X and vice-a-versa. When a proteome is BLAST searched against itself, a protein will have a hit to itself and it might have another hit to another protein in the same genome. The results are then put through homology reduction by the HOBOHM algorithm, in order to reduce the self-hits (Hobohm et al. 1992).

The output of the BLASTMatrix analysis is a matrix of numbers. *Figure 8.8* shows a heatmap of these values though not the actual value. In the discussion below the actual values are presented. In the matrix for the *Enterobacteriaceae* family, the

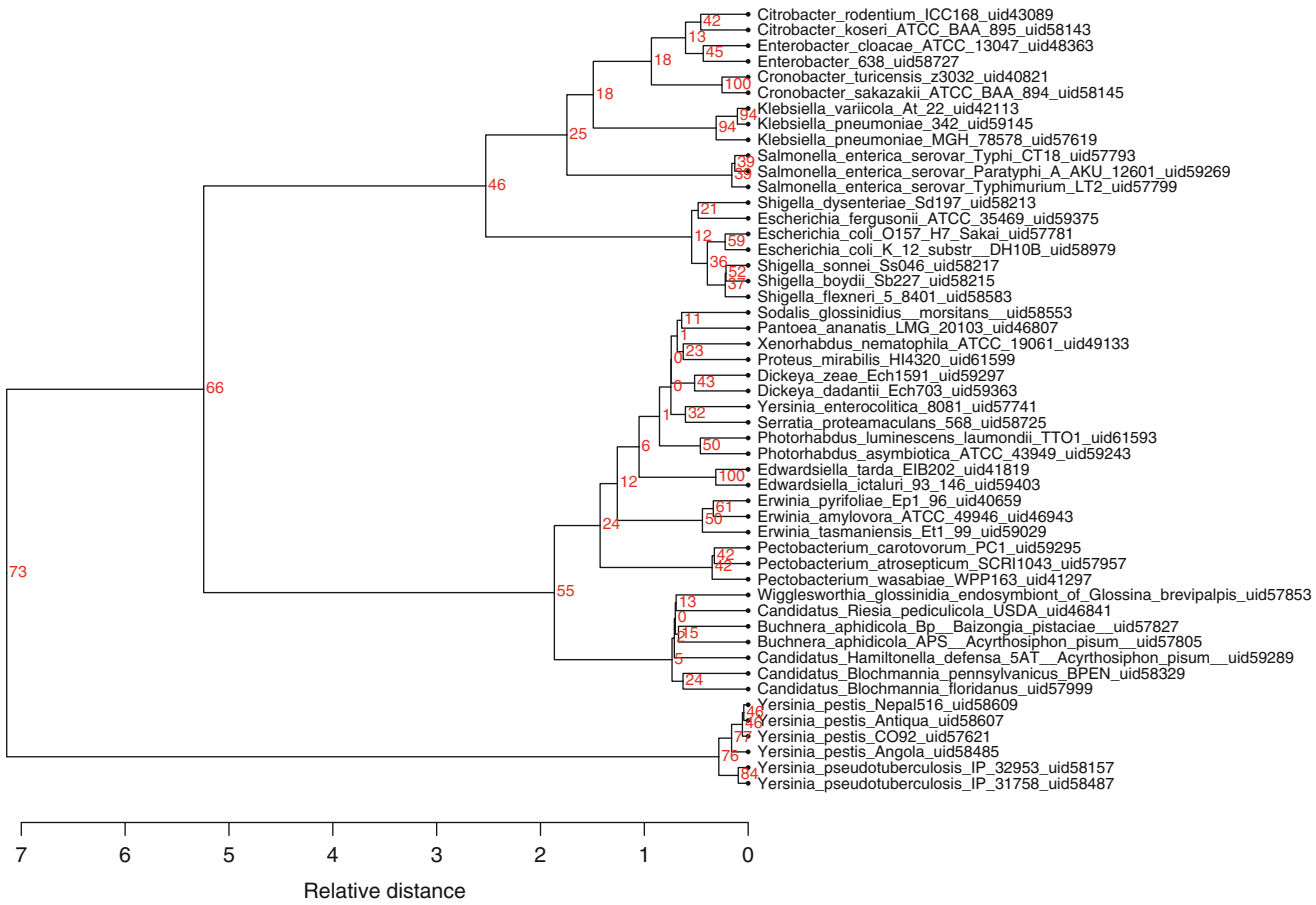


**Fig. 8.9**

**CVTree for the Genera set, generated by using "euclidian" distances with "ward" linkage hierarchical clustering. The values on branches indicate bootstrap values**

order is based on the rRNA phylogenies, because a clear separation of each genus was possible. According to this matrix, proteome similarity within some genera might seem to be higher than others; however, the darkest colors around the diagonal (≥90%) are due to the comparison within a species level, rather than different species in the same genus. Average proteome similarity between *Escherichia* and *Shigella* clusters is 73%. Homology between *Salmonella* strains are 80–96% while *Citrobacter* species show an average of 72% similarity. The similarity values for *Cronobacter* are 88% on average, 80% for *Erwinia*, around 70% for *Enterobacter*. *Klebsiella* species have up to 94% similarity, *Dickeya* species around 74% and *Pectobacterium* species have 75–83% similarity (Fig. 8.8). In the *Yersinia* cluster, similarity values between the strains of same species are 92–98% for *Y. pestis* and 88% for *Y. pseudotuberculosis*. Homology between *Yersinia* species is around 88%. *Edwardsiella* species have 81%, *Buchnera* strains have around 89%, *Candidatus Blochmannia* strains have around 96% similarity.

It is not clear if there should be a proteome similarity cut-off to specify genera, however, the values within a genus are generally between 70% and 80% and within the species it is higher, 80–98%. For example, *Klebsiella variicola* can actually be a *Klebsiella pneumoniae* because it has 94% similarity to the *K. pneumoniae* 342. Another issue is the presence of the reduced genomes in this family. In this plot, the reduced genomes are seen in the upper right corner (Fig. 8.8). The percentage of proteins that these organisms share with the others is very high because of the small size of their proteomes, which creates a dark band on the top of the matrix and a lighter one on the right hand side. This might be due to these genomes containing generally conserved core genes of the whole family, and they have actually very few accessory genes compared to the other genomes. They also have very low internal homology. A *Shigella* genome, on the other hand, has up to 30% internal homology. The largest proteome, *Sodalis*, shares 32–40% with the genomes that are not among the reduced genomes group. Except the reduced and expanded genomes, the similarity levels among different genera range between 40% and 72%.

## Composition Vector Trees (CVTree)

In this section, a CVTree for the 50 *Enterobacteriaceae* genomes is presented. In this method, frequencies of overlapping

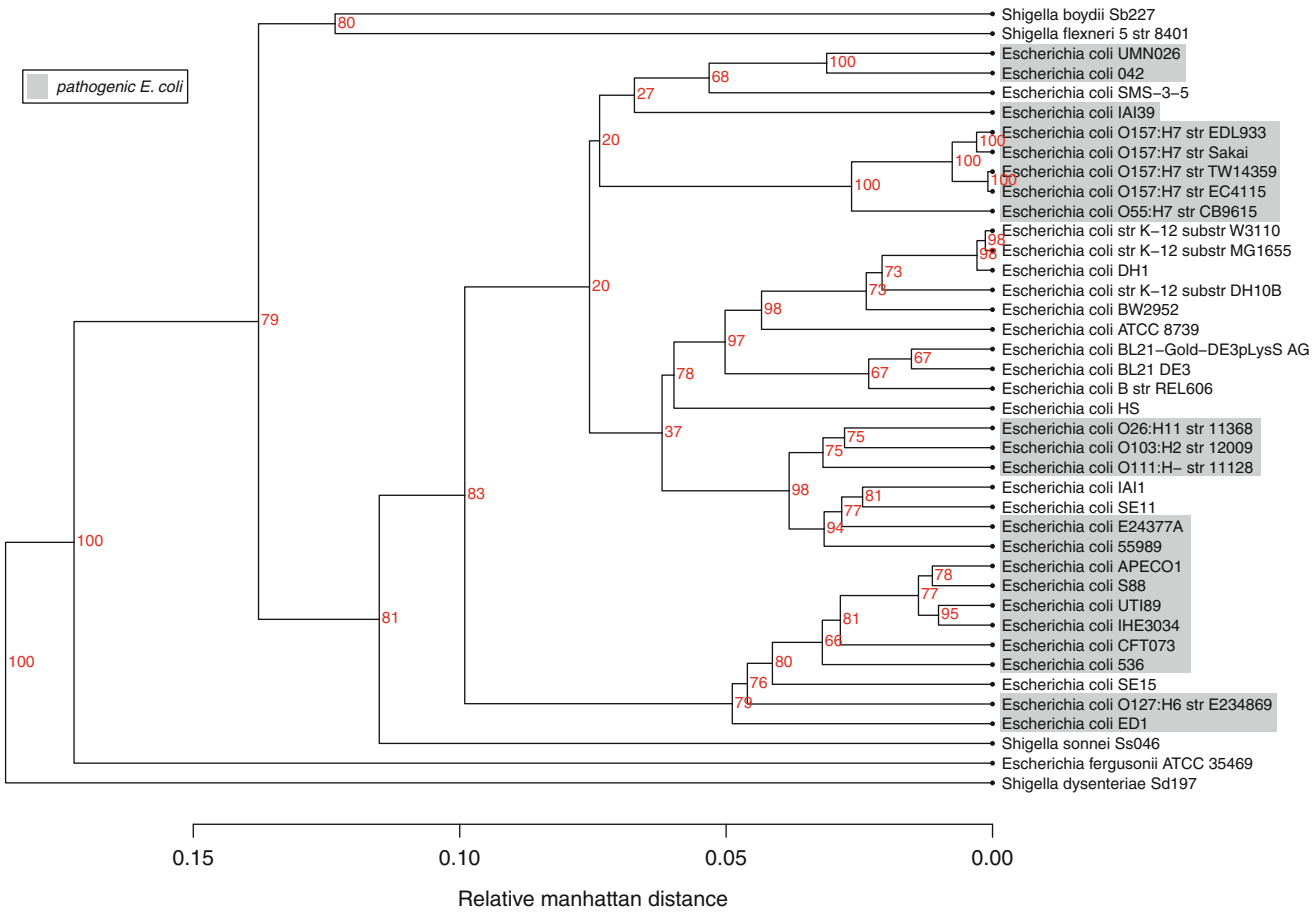


**Fig. 8.10**
**Pan-genome tree for the *Escherichia* and *Shigella* set based on the "shell" genes of the data set. The *gray boxes* indicate pathogenic strains of *E. coli***

oligopeptides of length K are calculated. The random background is subtracted from these frequencies with a (K-2) order Markov model to avoid the bias from neutral mutations, highlighting the selective evolution. After this procedure, the pairwise distance is calculated using the correlation between two organisms. The method describes the resulting distances as $D = (1 - C)/2$, where $C$ is the correlation between genomes and $D$ is the distance (Qi et al. 2004). The updated CVTree method with a faster and more stable web server performance is described in Xu and Hao (2009). The computation was performed on the web server using proteomes for all genomes and a K-parameter set to 6.

The outcome from the analysis is a distance matrix based on amino acid sequence comparison, which is then used to generate phylogenetic trees using neighbor joining. The web server provides an NJtree, made using the PHYLIP package, with a Newick format output. However, the NJtree did not provide bootstrap values so the data was instead analyzed using R, version 2.11. With this approach, the distance matrix from the CVTrees output was used to generate phylogenetic trees with bootstrap value. In *Fig. 8.9*, new distances were calculated from the distance matrix using the "Euclidian" distance function and hierarchical clustering using the "ward" method (Ward 1963). For statistical significance 100 bootstrap samplings were made on these trees, giving values between 0 and 100, showing the reliability for each branches.

The CVTree for the *Enterobactericiae* family can be seen in *Fig. 8.9*. In this tree, *Yersinia pestis* and *pseudotuberculosis* species are clearly separated from the rest of the taxa with 73% bootstrap value, whereas *Yersinia enterocolitica* remains clustered with *Serratia*. Two main clusters are seen after this, where the first consists of *E. coli*, *Shigella*, *Salmonella*, *Klebsiella*, *Cronobacter*, *Citrobacter*, and *Enterobacter*. The branching order is very similar to rRNA phylogeny as well.

To summarize, the CVTree is an alignment-free method, where the sequence similarities are investigated by the use of hexamer frequencies (K = 6); therefore, it is also quite fast. It is seen that the distance matrix generated by the algorithm does not give a clear picture for classification when very diverse organisms are analyzed. On a genera level, all the genera are intact and the clustering is very similar to the rRNA trees. On the species level, *Shigella* and *E. fergusonii ATCC 35469* are separated from *E. coli*.

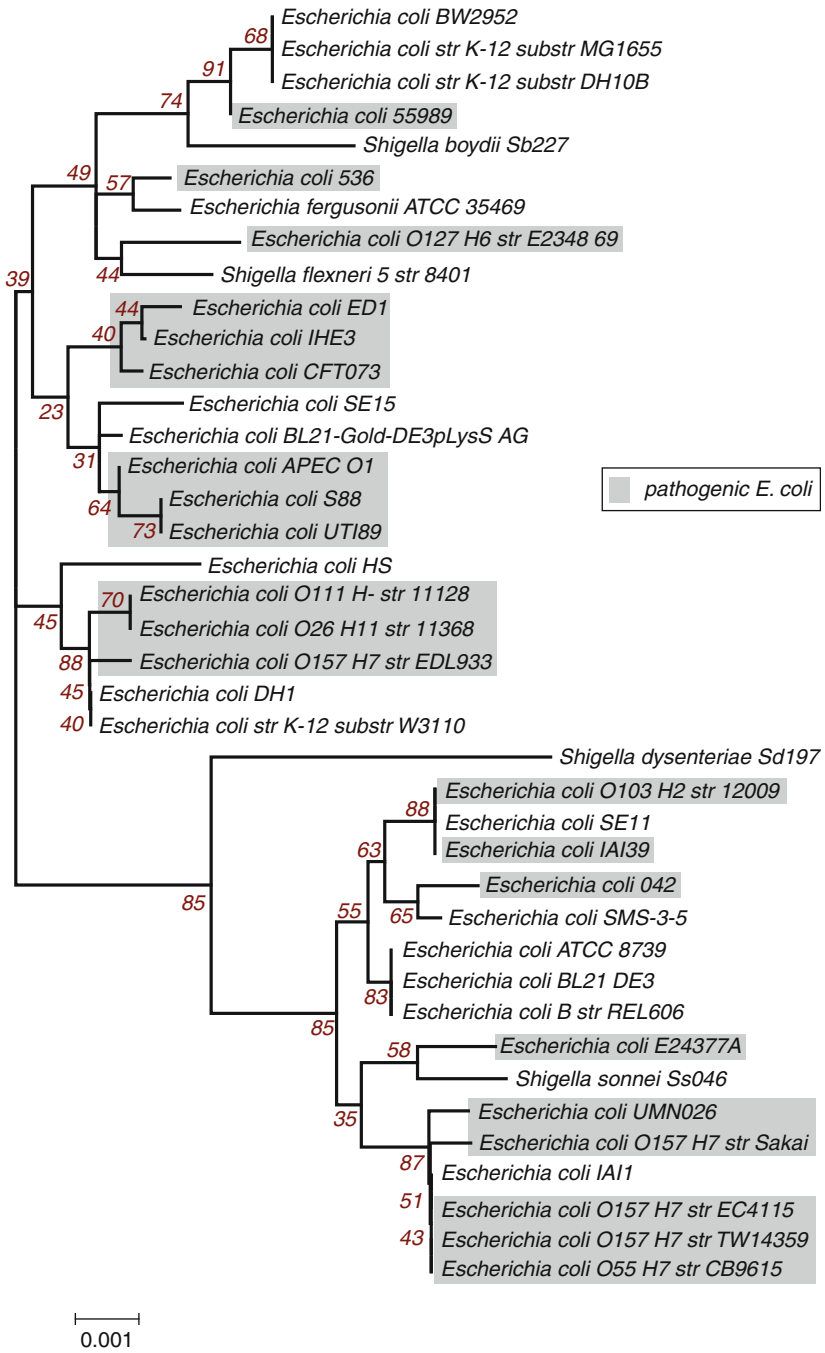


**Fig. 8.11**

**16S rRNA phylogenetic tree for *Escherichia* and *Shigella* genomes. Sequences are obtained as explained in rRNA phylogenies previously. The tree was generated using ClustalW alignments with neighbor-joining method and statistically tested with 1,000 bootstrap resamplings**

## Pan-genome Trees

Pan-genome family trees were generated using BLASTP (Altschul et al. 1990) similarity between each proteome. According to this, genes that have a significant hit to each other are considered to be in one gene family, where the significance cut-off is chosen for each BLAST hit (50% identity over an alignment with a length of at least 50% of the longest gene). Once the gene families are assigned, a matrix is constructed containing the gene families in columns and the genomes in rows, having 1 for the presence of that gene family in the corresponding genome, 0 otherwise. The tree is constructed by calculating Manhattan distances from this matrix and making hierarchical clustering using the Unweighted Pair-Group Method with Arithmetic mean (UPGMA) algorithm. For the stabilome view, the gene families that are represented in only one

genome (ORFans) are weighted down and the tree is based on "shell" genes between the genomes (Snipen and Ussery 2010).

In this section, a pan-genome tree for genomes of 36 *Escherichia coli* and 4 *Shigella* species was constructed (data gathered from NCBI GenBank) (Fig. 8.10). This tree, compared to a 16S rRNA neighbor-joining phylogeny (Fig. 8.11), shows more clear separation on the pathogenicity of the *E. coli* strains. The pan-genome tree shows relationships among different strains of a family with a higher resolution. *Shigella* species and *E. fergusonii ATCC 35469* are also clearly separated from the *E. coli*, showing that they show clear differences in their proteome composition, therefore, they can be separated from *E. coli*.

## Summary

This chapter presents analysis on genomic data that is in public databases and comparative genomics approaches to taxonomy based on rRNA, DNA, and protein molecular sequences.

It is clear that the available genome data is biased which cannot be attributed to any one reason. However, the monoculture approach to genome sequencing is causing a significant skew in sequencing data. From experiments, it is known that the diversity in the microbial world is tremendous, but in the statistical results, this diversity is not covered in the sequenced data. The advances in metagenomic sequencing and the sequencing of noncultivable cells will in time result in a much more realistic view of the bacterial world than is seen now. In the mean time, scientists should be aware of the bias in sequence data and not believe that what is sequenced so far is representative, even with roughly 2,000 genomes finished, and another 3,000 "draft" genomes available; there are currently about 30,000 bacterial genomes available in the "short-read archives," which in principle could be assembled, sometimes into less than 100 pieces—this means that there are ~35,000 genomes available now, and within a year or two, the number is likely to be in the hundreds of thousands. It seems likely that in the near future, draft genomes will become more common; if done properly and assembled well into only a few contigs, these draft genomes can provide useful information for core- and pan-genomes of a given taxa. However, one can hope that in the not too distant future, emerging third-generation sequencing technology will allow for the economical production of high quality full-length genomes for more reliable and robust information.

The amount of available data makes sequence-based taxonomy inevitable. In this chapter, organisms with different levels of taxonomic relations were selected. Since the reference taxonomy used is the current taxonomy, the results shown have been selected to be as close as possible to current taxonomy. rRNA phylogenies were used since it is a classical approach. Although the method is based on a single gene, the more conservative nature of the rRNA genes gives them a unique advantage of identifying more distantly related organisms. Whole-genome approaches, on the other hand, are more precise for understanding relations among closely related organisms. There are also differences between all the methods in the sense of using the sequence information; some use the sequence directly and make use of alignments and some reduces this information content into vectors of numbers. The latter can be thought more as numerical taxonomy, where several properties of organisms are measured and statistical significance tests and clustering methods are used to analyze relationships. As a result of all this analysis, it is suggested that there might not actually be one unified theory on taxonomy of living things, but several which classify well in different taxonomic levels. These methods are shown in Table 8.1, where a method explaining the relationships among different taxa are referred as inter-taxa, and methods that can delineate specific taxa from others regardless of their relations with others are referred as intra-taxa.

**Table 8.1**
**Methods that can be used for investigating inter- and intra-taxa relationships. The highest level is the Three Domains of life, and the lowest level is within the Strains**

| Taxonomic levels | Inter-taxa | Intra-taxa |
|---|---|---|
| *Superkingdom* | | 16S and 23S rRNA phylogeny |
| *Phyla* | None | 16S and 23S rRNA phylogeny |
| *Genus* | 16S and 23S rRNA phylogeny, BLASTMatrix | 16S and 23S rRNA phylogeny ANIm and Tetra, CVTree, BLASTMatrix, Pan-genome tree |
| *Species* | 16S and 23S rRNA phylogeny, BLASTMatrix, Pan-genome tree, CVTtee, ANI and Tetra | 16S and 23S rRNA phylogeny, BLASTMatrix, Pan-genome tree, CVTtee, ANI and Tetra |
| *Strain* | BLASTMatrix, Pan-genome tree, CVTree | Pan-genome tree |

As seen from the results, the largest phylum in terms of having the highest bacterial genome sequence projects, *Proteobacteria*, seems to be a well-defined taxa, where most of the methods catches the clustering, and the classes of *Alpha*, *Beta*, *Gamma*, *Delta*, and *Epsilon Proteobacteria* are usually coherent within themselves based on rRNA base taxonomy. Second largest phyla, Firmicutes are usually clearly separated into two groups of different classes. Another group, *Cyanobacteria* is actually a subdivision, because they do not have any classes or orders defined. Its members usually cluster together in many methods. All the other phyla generally have their members clustered together. This makes sense in classification, if looking for clearly separated groups. However, from an evolutionary point of view, this result is not enough to understand the ancestral relations of different phyla.

In the levels of genera, relationships for *Enterobacteriaceae* family can be seen in many methods. These families include some clinically and industrially important bacteria. Genera inside *Enterobacteriaceae* are clearly separated into different clusters, although sometimes the *Enterobacter* genus clusters separately. The relations of reduced genomes vary in different methods but generally they are separated from the rest of the genera. They share the core genes with the rest of the family and have very few accessory genes.

On a species level it is seen that even though in the same species, bacteria can be very diverse in terms of proteome content. Distinguishing between different types of *E. coli* strains are not possible with classical methods, but with proteome comparisons. It is also clear that although they are historically known to be similar, *Shigella* and *E. coli* can be distinguished from each other with proteome comparison.

In theory, classification simply depends on how one defines the relatedness among the entities of the system. However, in reality the choice of the characters when building a system is not always simple. Whole-genome-based tools do not consistently agree with current taxonomy. In order to make them match the current taxonomical system, different methods should be used for the different levels of taxonomy. The emergence of large amount of molecular and genomic data made it evident that there is not one universal method to naturally classify prokaryotes. Taxonomists should therefore keep the skepticism when using genomic data and using the common methodologies.

## References


Aanensen D, Spratt B (2005) The multilocus sequence typing network: mlst. net. Nucleic Acids Res 33:W728–W733

Abt B, Foster B, Others (2010) Complete genome sequence of cellulomonas flavigena type strain (134). Stand Genomic Sci 3:15–25

Altschul S, Gish W, Miller W, Myers E, Lipman D (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Ansorge WJ (2009) Next-generation DNA sequencing techniques. New Biotechnol 25:195–203

Arslan D, Legendre M, Seltzer V, Abergel C, Claverie J (2011) Distant Mimivirus relative with a larger genome highlights the fundamental features of Megaviridae. Proc Natl Acad Sci 108:17486–17491

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL (2008) GenBank. Nucleic Acids Res 36:D25–D30

Bernal A, Ear U, Kyrpides N (2001) Genomes OnLine Database GOLD: a monitor of genome projects world-wide. Nucleic Acids Res 29:126–127

Beyer WA, Stein M, Smith TF, Ulam S (1974) A molecular sequence metric and evolutionary trees. Math Biosci 19:9–25

Bohlin J, Snipen L, Cloeckaert A, Lagesen K, Ussery D, Kristoffersen AB, Godfroid J (2010) Genomic comparisons of *Brucella* spp and closely related bacteria using base compositional and proteome based methods. BMC Evol Biol 10:249

Brenner DJ, Krieg NR, Staley JT, Garrity GM, Boone DR, Vos P, Goodfellow M, Rainey FA, Schleifer KH (2005a) Bergeys manual® of systematic bacteriology, vol 2. Springer, Boston

Brenner DJ, Staley JT, Krieg NR (2005b) Classification of procaryotic organisms and the concept of bacterial speciation. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM (eds) Bergey's manual® of systematic bacteriology. Springer, Boston, pp 27–32

Cavalli-Sforza LL, Edwards AWF (1967) Phylogenetic analysis: Models and estimation procedures. Evolution 32:550–570

Chain P, Grafham D, Fulton R, Fitzgerald M (2009) Genome project standards in a new era of sequencing. Science 326:4–5

Cohn F (1872) Untersuchungen tiber Bakterien II. Beitr Biol Pflanz 1:127–224

Delcher A, Kasif S, Fleischmann R, Peterson J, White O, Salzberg S (1999) Alignment of whole genomes. Nucleic Acids Res 27:2369–2376

DeWall MT, Cheng DW (2011) The minimal genome–a metabolic and environmental comparison. Brief Funct Genomic Proteomic 105:312–315

Doolittle W (1999) Phylogenetic classification and the universal tree. Science 284:2124–2128

Felsenstein J (2004) Inferring phylogenies. Sinauer Associates, Sunderland

Fitch WM, Margoliash E (1967) Construction of phylogenetic trees. Science 155:279–284

Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM (1995) Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. Science 269:496–512

Fraser CM, Gocayne JD, Others (1995) The minimal gene complement of Mycoplasma genitalium. Science 270:397–403

Galperin MY (2006) The minimal genome keeps growing. Environ Microbiol 84:569–573

Garrity GM, Lilburn GT, Cole JR, Harrison SH, Euzeby J, Tindall BJ (2007) Introduction to the taxonomic outline of bacteria and archaea (TOBA) Release 7.7. The Taxonomic Outline of Bacteria and Archaea. http://www.taxonomicoutline.org/index.php/toba/article/download/190/223. Accessed 23 Feb 2012

Gibbons N, Murray R (1978) Proposals concerning the higher taxa of bacteria. Int J Syst Evol Microbiol 28:1–6

Goris J, Konstantinidis K, Klappenbach J, Coenye T, Vandamme P, Tiedje J (2007) DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57:81–91

Harayama S, Kasai H (2006) Bacterial phylogeny reconstruction from molecular sequences. In: Stackebrandt E (ed) Molecular identification, systematics, and population structure of prokaryotes. Springer, Berlin/New York, pp 105–140

Hobohm U, Scharf M, Schneider R, Sander C (1992) Selection of representative protein data sets. Protein Sci 1:409–417

Konstantinidis K, Tiedje J (2005) Genomic insights that advance the species definition for prokaryotes. Proc Natl Acad Sci USA 102:2567–2572

Lagesen K, Hallin P, Rødland EA, Staerfeldt HH, Rognes T, Ussery DW (2007) RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res 35:3100–3108

Lagesen K, Ussery DW, Wassenaar TM (2010) Genome update: the 1000th genome–a cautionary tale. Microbiology 156:603–608

Lapage S, Sneath P, Lessel E, Skerman V, Seeliger H, Clark W (1992) International code of nomenclature of bacteria: bacteriological code, 1990 revision. American Society of Microbiology, Washington, DC

Lapierre P, Gogarten J (2009) Estimating the size of the bacterial pan-genome. Trends Genet 25:107–110

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948

Liolios K, Chen I-MA, Mavromatis K, Tavernarakis N, Hugenholtz P, Markowitz VM, Kyrpides NC (2010) The genomes on line database GOLD) in 2009: status of genomic and metagenomic projects and their associated metadata. Nucleic Acids Res 38:D346–D354

Maiden M, Bygraves J, Feil E, Morelli G, Russell J, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant D, Others (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA 95:3140–3145

McCutcheon JP, McDonald BR, Moran NA (2009) Origin of an alternative genetic code in the extremely small and GC-rich genome of a bacterial symbiont. PLoS Genet 57:e1000565

Médigue C, Moszer I (2007) Annotation, comparison and databases for hundreds of bacterial genomes. Res Microbiol 158:24–36

Moya A, Gil R, Latorre A, Peretó J, Pilar Garcillán-Barcia M, de la Cruz F (2009) Toward minimal bacterial cells: evolution vs. design. FEMS Microbiol Rev 331:225–235

Murray RGE (1989) The higher taxa, or, a place for everything. . .? In: Williams ST, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 4, 1st edn. Williams & Wilkins, Baltimore, pp 2329–2332

Pérez-Brocal V, Gil R, Ramos S, Lamelas A, Postigo M, Michelena JM, Silva FJ, Moya A, Latorre A (2006) A small microbial genome: the end of a long symbiotic relationship? Science 314:312–313

Qi J, Luo H, Hao B (2004) CVTree: a phylogenetic tree reconstruction tool based on whole genomes. Nucleic Acids Res 32:W45–W47

Richter M, Rosselló-Móra R (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci 106:19126–19131

Sanford RA, Cole JR, Tiedje JM, Al SET, Icrobiol APPLENM (2002) Characterization and description of *Anaeromyxobacter dehalogenans* gen. nov., sp. nov., an aryl-halorespiring facultative anaerobic myxobacterium. Appl Environ Microbiol 68:893–900

Sboner A, Mu XJ, Greenbaum D, Auerbach RK, Gerstein MB (2011) The real cost of sequencing: higher than you think! Genome Biol 12:125

Schleifer KH (2009) Classification of Bacteria and Archaea: past, present and future. Syst Appl Microbiol 32:533–542

Schneiker S, Perlova O, Others (2007) Complete genome sequence of the myxobacterium Sorangium cellulosum. Nat Biotechnol 25:1281–1289

Smith T, Waterman M (1981) Identification of common molecular subsequences. J Mol Biol 147:195–197

Snipen L, Ussery DW (2010) Standard operating procedure for computing pangenome trees. Stand Genomic Sci 2:135–141

Stackebrandt E (2006) Exciting times: the challenge to be a bacterial systematist. Molecular Identification, Systematics, and Population Structure of Prokaryotes 1–21

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739

Teeling H, Meyerdierks A, Bauer M, Amann R, Glöckner FO (2004) Application of tetranucleotide frequencies for the assignment of genomic fragments. Environ Microbiol 6:938–947

Tindall BJ, Kämpfer P, Euzéby JP, Oren A (2001) Valid publication of names of prokaryotes according to the rules of nomenclature: past history and current practice. Int J Syst Evol Microbiol 56:2715–2720

Ussery D, Wassenaar T, Borini S (2009) Computing for comparative microbial genomics: bioinformatics for microbiologists. Springer, London

Ward JH, Jr. (1963) Hierarchical grouping to optimize an objective function. J Am Stat Assoc 48:236–244

Waters E, Hohn MJ, Others (2003) The genome of Nanoarchaeum equitans: insights into early archaeal evolution and derived parasitism. Proc Natl Acad Sci USA 100:12984–12988

Wayne L, Brenner D, Colwell R, Grimont P, Kandler O, Krichevsky M, Moore L, Moore W, Murray R, Stackebrandt E, Others (1987) International committee on systematic bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int J Syst Evol Microbiol 37:463–464

Whittaker R, Margulis L (1978) Protist classification and the kingdoms of organisms. Biosystems 10:3–18

Woese C, Fox G (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci USA 74:5088–5090

Woese C, Kandler O, Wheelis M (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci USA 87:4576–4579

Xu Z, Hao B (2009) CVTree update: a newly designed phylogenetic study platform using composition vectors and whole genomes. Nucleic Acids Res 37:174–178

Yamamoto S, Harayama S (1996) Phylogenetic analysis of acinetobacter strains based on the nucleotide sequences of gyrB genes and on the amino acid sequences of their products. Int J Syst Evol Microbiol 46: 506–511

Zuckerkandl E, Pauling LB, Kasha M, Pullman B (1962) Molecular disease, evolution, and genetic heterogeneity. Academic, New York, pp 189–225

# 9 Defining Taxonomic Ranks

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## Introduction

Those who have chosen systematics, classification, and taxonomy as a research topic have learned to consider this complex topic an exciting and important biological discipline. For others, these topics are mainly dull subjects, which, through changes in names of microbial taxa, may cause confusion in their daily routine. Indeed, the problem of changing names of taxa is an inherent character of classification, which can be explained by the desire of taxonomists to provide the user of taxonomy with a system that, to their opinion, optimally reflects the natural relatedness between the taxa. Looking back in the history of microbiology, the restricted interests in classification can be explained by the enormous problems of past generations of systematists to order the increasing wealth of phenotypic and genotypic properties of the rapidly growing numbers of bacterial isolates. The user of taxonomy was confronted with constantly changing classification concepts and classification systems, taxonomic rearrangements, and synonymy of names. Problems also arose from the terminology: While some regard systematics and taxonomy synonymous, others define taxonomy as the theory and practice of classifying organisms, while systematics cover a broader scope including the evolutionary and phylogenetic components. For many researchers the only aspect of taxonomy they come into contact with is nomenclature and may only notice this component when they are confronted with name changes. However, systematics does not include only the naming of organisms but also includes the following features (Stackebrandt et al. 1999): Classification is the generation of as much data on the properties of a novel isolate as possible in order to decide whether, by the process of identification, e.g., comparison of these data with the database of previously classified organisms, these characters justify the affiliation of the isolate to a described species, or whether a new species need to be described for the isolate. Classification includes the theory and process of ordering the characterized organisms into a single or multiple classification systems. Nomenclature is the naming of the appropriate taxa within the realm of a classification system, and it includes subjective changes in names whenever novel insights into the taxonomic weight of characters stimulate taxonomists to change the rank of taxa. This chapter will not cover the nomenclatural aspects of taxonomy as this is governed and updated in detail by the articles of the Bacteriological Code (Lapage et al. 1975).

As outlined previously (Stackebrandt 1992), several classification systems exist in parallel, and no classification system can claim to be the sole classification. Any two systems do not need to match in the clustering of organisms they represent and as long as a system does not fail to do what it sets out to do, it cannot be described as wrong, or in error.

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**Table 9.1**

**Examples for the classification of prokaryotic species according to risk groups (European Community classification)**

| Genus | Risk group 1 | Risk group 2 | Risk group 3 |
|---|---|---|---|
| *Chlamydia* | Not known | *C. trachomatis* | *C. psittaci* |
| *Bacillus* | *B. circulans* | *None* | *B. anthracis* |
| *Burkholderia* | *Bu. cocovenenans* | *Bu. cepacia* | *Bu. mallei* |
| | *Bu. antropogonis* | *Bu. vietnamensis* | *Bu. pseudomallei* |
| *Francisella* | Not known | *F. tularensis* subsp. *holarctica* | *F. tularensis* subsp. *tularensis* subsp. *tularensis* |
| *Mycobacterium* | *M. asiaticum* | *M. avium* subsp. *avium* | *M. leprae* |
| | *M. fallax* | *M. chelonae* | *M. tuberculosis* |

**Table 9.2**

**Examples for a classification system that is based on phenotypic properties. Members of the family *Bacteroidaceae* are described to be Gram-negative, fermentative anaerobic organisms (Holt et al. 1994)**

| Family | Genera | Main diagnostic differences |
|---|---|---|
| *Bacteroidaceae* | *Bacteroides* | Peritrichous straight rod; produces a mixture of fermentation products from carbohydrate and peptone; butyrate not a major product |
| | *Fusobacterium* | Nonmotile straight rod; butyrate is a major product |
| | *Leptotrichia* | Nonmotile straight rod; lactate is the sole major fermentation product |
| | *Butyrivibrio* | Motile, not peritrichous curved rod; butyric acid is the major fermentation product |
| | *Succinimonas* | Short motile rod or coccobacilli; single polar flagellum; succinate and acetate are major fermentation products |
| | *Succinivibrio* | Motile helical or spiral-shaped cell; single polar flagellum; succinate and acetate are major fermentation products |
| | *Anaerobiospirillum* | Motile helical or spiral-shaped cell; bipolar tufts of flagella; succinate and acetate are major fermentation products |
| | *Wolinella* | Motile helical curved, or straight rod, single polar flagellum; either hydrogen or formate as electron donors for reduction of fumarate to succinate; carbohydrates not fermented |
| | *Selenomonas* | Motile crescent-shaped cell, tufts of flagella on concave side; fermentation products propionate and acetate |
| | *Anaerovibrio* | Motile curved cells, single polar flagellum, lipolytic; fermentation products propionate and acetate |
| | *Pectinatus* | Motile curved cells, lateral flagella aligned on concave side; fermentation products propionate and acetate |

There are systems that group microorganisms on the basis of their increasing degrees of risk to humans, animals, and plants. Here, organisms are artificially, pragmatically classified into risk groups according to their degree of pathogenicity or risk potential, and this system does not serve any other purpose (*Table 9.1*).

Another system focuses on the rapid and reliable identification of bacteria for which knowledge about phylogenetic relatedness is not mandatory (*Table 9.2*). In such diagnostic system, used in the past, affiliation of an isolate to a genus and species includes Gram-stain reaction, oxygen requirement and morphology, chemotaxonomy, numerical phenetic analyses, usage of rapid diagnostic kits (e.g., API and Vitek systems [bioMerieux] and BIOLOG Microbial Identification Systems [Biolog Inc/]), and combinations of selected physiological tests.

Yet another trend and the one favored today, takes into consideration the similarities in homologous molecules which leads to the grouping of organisms according to their evolutionary relatedness. The classification process then circumscribes the emerging clusters by a wide range of characteristics originating from the genomic and epigenetic level. This genealogy-based classification system is the most comprehensive one in terms of overall understanding of the biology of the organisms, including the evolution of core processes of genetics, biochemistry, and physiology. This approach, which was outlined two decades ago (Wayne et al. 1987), is now applied by the vast majority of microbiologists. In the following text, the term "phylogeny" is used when evolutionary relatedness among members of taxa or natural populations has been explored, via means of molecular sequence data or DNA-DNA hybridization approaches.

**Table 9.3**

**Examples of changes in higher classification following phylogenetic-polyphasic taxonomic analyses as exemplified by the fate of some species of *Bacteroides* (Table 9.2)**

| Traditional classification | Reclassification following phylogenetic assessment | Affiliation to higher taxon 2000 | Affiliation to higher taxon 2010 |
|---|---|---|---|
| *Bacteroides amylophilus* | *Ruminobacter amylophilus* | Phylum Proteobacteria | Family |
| | | Class *Gammaproteobacteria* | *Succinivibrionaceae* |
| *Bacteroides bivius* | *Prevotella bivia* | Phylum Bacteroidetes | Family *Prevotellaceae* |
| | | Family *Bacteroidaceae* | |
| *Bacteroides cappilosus* | *Pseudoflavonifractor cappilosus* | Not described | Phylum Firmicutes |
| | | | Family *Clostridiaceae* |
| *Bacteroides endodontalis* | *Porphyromonas endodontalis* | Phylum Bacteroidetes | Family *Porphyromonadaceae* |
| | | Family *Bacteroidaceae* | |
| *Bacteroides distasonis* | *Parabacteroides distasonis* | Not described | Family *Porphyromonadaceae* |
| *Bacteroides forsythus* | *Tannerella forsythus* | Not described | Phylum Bacteroidetes |
| | | | Family *Porphyromonadaceae* |
| *Bacteroides furcosus* | *Anaerorhabdus furcosa* | Phylum Bacteroidetes | Unchanged |
| | | Family *Bacteroidaceae* | |
| *Bacteroides gracilis* | *Campylobacter gracilis* | Phylum Proteobacteria Family *Campylobacteraceae* | Unchanged |
| *Bacteroides hypermegas* | *Megamonas hypermegale* | Phylum Firmicutes | Phylum Firmicutes |
| | | Family *Veillonellaceae* | Family "Acidamniococcaceae" |
| *Bacteroides microfuscus* | *Rikenella microfuscus* | Phylum Bacteroidetes | Family *Rikenellaceae* |
| | | Family *Bacteroidaceae* | |
| *Bacteroides multiacidus* | *Mitsuokella multiacida* | Phylum Bacteroidetes | Phylum Firmicutes |
| | | | Family *Veillonellaceae* |
| *Bacteroides nodosus* | *Dichelobacter nodosus* | Phylum Proteobacteria | Unchanged |
| | | Class *Gammaproteobacteria* | |
| | | Family *Cardiobacteriaceae* | |
| *Bacteroides ochraceus* | *Capnocytophaga ochraceae* | Phylum Bacteroidetes | Phylum Bacteroidetes |
| | | | Family *Flavobacteriaceae* |
| *Bacteroides splanchnicus* | *Odoribacter splanchnicus* | Phylum Bacteroidetes | Phylum Bacteroidetes |
| | | | Family *Porphyromonadaceae* |
| *Bacteroides praeacutus* | *Tisseriella praeacuta* | Phylum Firmicutes | Family *Veillonellaceae* |
| *Bacteroides pseudosintes* | *Dialister pneumosintes* | Phylum Firmicutes | Phylum Firmicutes |
| | | | Family "Acidamniococcaceae" |
| *Bacteroides putredinis* | *Alistipes putredinis* | Phylum Bacteroidetes | Phylum Bacteroidetes |
| | | | Family *Rikenellaceae* |
| *Bacteroides succinogenes* | *Fibrobacter succinogenes* | Unassigned | Phylum *Fibrobacteres* |
| | | | Family *Fibrobacteraceae* |
| *Bacteroides termitidis* | *Sebaldella termitidis* | Phylum Fusobacterium | Unchanged |
| | | Family *Leptotrichiaceae* | |

The fact that the genealogy is derived from gene sequence similarities leads to the advantage of working with a reliable, objective, and stable basis for identification and classification. The dramatic changes that occurred in the classification of species of *Bacteroides*, lumped together in the past on the basis of a few superficial properties, is an excellent example of the shift toward a system that is primarily based on phylogenetic relatedness as the most reliable means for classification (Table 9.3). The reclassification of a species makes it necessary to redefine its properties. Above all, analysis of the genomic relatedness of a species will provide information on its phylogenetic position, i.e., who its nearest neighbor(s) is. However, in many cases, the position will not provide information on other properties needed to make decisions whether this species can be

considered a species of a known genus or whether this species forms the nucleus of a novel genus. These conclusions depend upon the results of a targeted search for a wide array of phenotypic and genomic properties.

This chapter introduces the importance of working with sequences of homologous genes and gene products as the basis for providing an objective framework of the order at which lineages of prokaryotic organisms evolved. It then describes the (subjective) decision-making strategy of how bacteriologists on the basis of this phylogenetic framework define the ranks of species and genera. Special emphasis is placed here on the pragmatic definition of the species. It also deals with problems of delineating ranks above the genus level for which only a few common characters are available, and it discusses recent findings that higher taxa of the same rank are often nonequivalent taxa.

## Past Classification Attempts

Ranks or taxa have been introduced in the classification of biological specimens to facilitate communication among scientists and to order the living matter in a hierarchic way according to morphological, physiological, ecological, and genomic features present in one rank but absent in the others. The basis of any system is the species and the genus, and according to the binomial system (Linnaeus 1753), the description of a type species is not possible without describing a genus and vice versa a genus cannot be described without a species. This is a clear and simple recommendation, and the simplicity explains why the binomial system is still in use for the naming of organisms within the three primary domains of life (Woese 1987), the Archaea, the Bacteria, and the Eukarya (Woese et al. 1990). Considering the way description of biological material was done prior to Linnaeus, Moreno (1997) states, "The wisdom of Linnaeus was not only to create a comprehensive classification system, but more importantly, a useful one." The definition of a species has been debated extensively since the publication of the key work *On the Origin of Species* (Darwin 1859). The debate concentrated on animals and plants but excluded the prokaryotes mainly because of the lack of an evolutionary record.

Bacterial classification as a science began with the contribution of the botanist Cohn (Cohan 2002, 2006) in the second half of the nineteenth century. He was the first to raise the fundamental question whether bacteria, like plants and animals, can be arranged in species and genera, and he presented a classification scheme, composed of six genera, which was based on morphological criteria. However, he clearly pointed out that the restriction to morphological properties is insufficient as he noticed that similarity in shape does not exclude the bacteria to differ from each other in physiological characters. Cohn regarded genera as natural entities, but he described species as largely provisional. As judged from today's view, early microbial systematists, having to rely on superficial characters which were easy to observe and to describe, were not in a position to fully acknowledge the complexity of the bacterial cell. It took almost 100 years for bacteriologists to gather enough information to recognize that most of the characters used for systematics in the past were inappropriate for unraveling a phylogenetic framework.

During the beginning of the twentieth century, the number of determinable properties expanded dramatically, and consequently, the number of species increased. Early taxonomists usually worked in a clinical environment, leading to the description and identification of mainly pathogenic species. At the beginning of the twentieth century, new systems were proposed in which the accent shifted from morphology to physiology, metabolism, pigments, and pathogenicity (Migula 1900; Orla-Jensen 1909; Pringsheim 1923; Prévot 1938; Kluyver and van Niel 1936; Stanier and van Niel 1941). In order to get a grasp on the wealth of information and to harmonize the different systems, a single unifying formal system of bacterial classification was established by Buchanan (1916, 1918) in accordance with the systems of higher animals and plants. This system provided the basis for *Bergey's Manual of Determinative Bacteriology*, which, in the many editions that followed over the years, presented better than any other source the most useful references for identification but, following the tradition, retained a nomenclature that was believed to reflect phylogenetic relationships. Attempts to either construct a single formal classification system or to work with several systems in parallel were both criticized by Kluyver and van Niel (1936). These scientists suggested that rather than searching for a natural system taxonomists should develop determinative keys to provide the easiest possible identification of species and genera. However, as the characters used for the establishment of the system were chosen according to the subjectivity of the taxonomist, it was admitted that an empirical system was largely unmodifiable. Consequently, the whole system was disrupted when novel characters were taken as the basis for the establishment of a new classification system. The main advantage of the empirical system was seen in its immediate practical utility, but it became obvious that even this advantage disappeared when the differential characters were not mutually exclusive. Scientists of this era, lasting to the end of the third quarter of last century, recognized the importance of developing a natural classification system (Stanier and van Niel 1941) but attempts for doing so were not considered achievable. The question then remains why it was impossible for past generations of microbiologists to develop a phylogenetic framework of prokaryotes? In hindsight, the answer is quite easy: Without information about fundamental genetic information and without understanding the mechanisms of heredity and the technical ability to unravel the structure of these units, early attempts were prone to failure.

## Phylogeny Is Based on Homology

Phylogenetic systematics is seeking congruency between the lines of descents evolved through time and the supraspecific taxa described by taxonomists. Prerequisite for the description of a taxon of any rank in a phylogenetic system is the recognition that all members to be included originate from one ancestral form and that homologous traits evolved in the ancestral form are

found in their descendants. The question then remained which of the several thousand semantides present in a prokaryotic cell to use for phylogenetic studies. The establishment of a system which is set up to include all species requires the presence of phylogenetic markers that should be ubiquitously distributed, functionally equivalent, and homologous. These markers should be homologous apomorphic characters that evolved only once (synapomorphy) but not by convergence. Homology is the sharing by two taxa of a property that is derived from the same or equivalent property of the nearest common ancestor. Furthermore, the fossil record, morphological complexity, and comparative anatomy, extremely useful properties of eukaryotes for determining homologies, are lacking in the morphological and developmental simple prokaryotes, which lead to the consequence that a phylogenetic classification system became only available after the theoretical and methodological basis had been laid some 30 years ago.

One of the main intellectual breakthroughs concerning the potential of unraveling the phylogeny of life was provided by Zuckerkandl and Pauling (1965). They recognized that contemporary organisms are the products of historical events and that all cellular structures reflect their evolutionary history. These scientists also commented that in the case of microorganisms, historical documents of early evolutionary events can only be found at the primary structure level of homologous informative molecules. Number and composition of sequence differences that exist among proteins and genes coding for rRNA and proteins reflect their phylogenies and consequently allow recognizing pairs or groups of organisms which originated from a common ancestor. The basis for unraveling relatedness is provided by sequence analysis of certain biological molecules, the semantides. Based on their information content, three categories can be identified:

1. Semantides, i.e., genes or their transcripts (DNA [primary semantides], RNA [secondary semantides], and proteins [tertiary semantides]). Sequences of these molecules are molecular chronometers, records of evolution, as they indirectly measure the time elapsed since their origin, and the comparative analysis of the primary structure provide a powerful approach to measure evolutionary relationships.
2. Episemantic molecules to be used in comparative studies (e.g., peptidoglycan, isoprenoid quinones, polar lipids, membrane constituents) are synthesized under the control of tertiary semantides, and above all, it is the chemical composition of cell constituents that have received considerable attention (Schleifer and Kandler 1972). Episemantic molecules were not considered useful for deriving evolutionary conclusions because enzymes with different primary structures can lead to the synthesis of identical episemantic or similar molecules in different organisms as long as the active enzymatic sites are similar.
3. Asemantic molecules are not produced by the organisms themselves and do not express any of the historic information that organisms contain (e.g., exogeneously supplied vitamins, phosphate ions, oxygen, viruses).

Zuckerkandl and Pauling state that "at any level of integration, the amount of history preserved will be the greater, the greater the complexity of the elements at that level and the smaller the parts of elements that have to be affected to bring about a significant change. Under favorable conditions of this kind, recognition of many differences between two elements does not preclude the recognition of their similarity." The correctness of this hypothesis was justified by the impressive phylogenetic trees of gene and proteins sequences.

## A Word About Horizontal Gene Transfer (HGT)

Genomic studies during the past decade have revealed that bacterial (and archaeal) genomes are much more dynamic and diverse than previously anticipated. For instance, it is not uncommon that strains of the same species would differ in up to 30 % of the genes in their genomes (Konstantinidis and Tiedje 2005b). Horizontal (as opposed to vertical descent) gene transfer (HGT) and gene loss account for the majority of this immense genome plasticity and diversity (Lawrence 2002). It has been argued that HGT might be so pervasive in the prokaryotic world that the prokaryotic tree of life is actually a web of life and that the bacterial phylogeny cannot be reconstructed faithfully based on the sequence analysis of genes (Doolittle and Bapteste 2007). HGT (mediated by homologous recombination) might also be so frequent and unbiased (neutral) that it can serve as the force of population cohesion (Gevers et al. 2005), similar to the role of sex in sexual organisms (discuss later in this chapter). Nonetheless, several other studies have shown that genes involved in central cellular processes such as DNA replication and protein translation machinery are subjected to HGT much less frequent than metabolic genes because the transfer of the former genes typically confers no selective advantage to the recipient cell (Gogarten and Townsend 2005; Beiko et al. 2005). Indeed, HGT of 16S rRNA genes has been documented for only a few cases (e.g., Miller et al. 2005). As pointed out by van Berkum et al. (2003), deciding taxonomic relationship based solely on 16S rRNA gene sequence divergence to reflect phylogenetic relatedness may be misleading; tree topologies based upon 16S rRNA gene sequences of members of rhizobia differ from those based upon 23S rRNA genes and internally transcribed space region sequences, and the authors suggested gene conversion of certain stretches within the 16S rRNA gene to be responsible for the discordant phylogenies. Accordingly, although the real phylogeny of organisms is probably impossible to be accurately reconstructed, even with complete genome sequences available, an average "consensus" phylogeny may have been attained.

## Main Phylogenetic Parameters for Classification of Ranks Above the Genus Level

The two main components in determining phylogenetic relationships among the prokaryotes – sequence analysis of the semantides DNA, RNA, and proteins (Zuckerkandl and

Pauling 1965) and DNA-DNA hybridization techniques—were developed approximately at the same time, around the mid-1960s. Historically, the molecular approaches to evolution involved sequence analyses of proteins, such as cytochrome C, fibrinopeptides, and ferrodoxins, as well as immunological approaches, such as immunodiffusion and microcomplement fixation. However, the latter methods as well as protein sequencing have lost significance when rapid sequencing techniques for DNA became available. Above all, the 16S rRNA gene is the most widely analyzed molecule as it has certain advantages, not provided by other rRNA molecules (5S rRNA is too short and 23S rRNA is too large to be sequenced routinely) or genes coding for proteins (e.g., degeneration of the code makes it difficult to design universal PCR primers). The primary structure of the 16S rRNA gene is apt to cover an enormous geological time span, most likely covering almost the whole time span since the origin of prokaryotes (3.8 Gy ago). The molecule has been selected as suitable phylogenetic marker mainly because it is ubiquitously distributed and orthologous among all forms of life, functionally constant, genetically stable, of an appropriate size, and possesses independently evolving domains within the molecule.

Sequence analysis of rRNA genes and certain other genes has become a rapid standard technique, and the sequences generated have a very low error rate. The restrictions in the use as a phylogenetic marker are due to certain intrinsic properties of the molecule: In comparison to the billions of years that have passed since its origin, the number of informative positions, i.e., positions that changed over time in proportion to the time elapsed, within the molecular sequences is limited. As a consequence, the majority of evolutionary change that did occur at the organism level will remain undetected at the sequence level of rRNA. Another restriction is that in most prokaryotic organisms, the multigenic rRNA operon is present in several copies in the genome ranging from 2 to 14 (Farelly et al. 1995). PCR amplification will mask possibly occurring intracistronic microheterogeneities which consequently may obscure elucidation of the fine differences of relatedness of closely related organisms. Sequence analysis of individually cloned operons can unravel these heterogeneities, but these few changes are typically regarded "noise," hence without phylogenetic implications in most instances. Higher order structures of rRNA molecules facilitate sequence alignment, which today can be analyzed by a wide range of publicly available databases (e.g., RDP II, ARB, Greengenes) and algorithms (e.g., maximum likelihood, parsimony, neighbor-joining, Bayesian methods), facilitating checks the robustness of resulting branching patterns. Numerous factors have been shown to influence the branching pattern (topology), which is a dynamic construct and will change with any new sequence included or region selected for analysis. Nevertheless, the topology of trees which are generated on the basis of genes subjected to the same fate in evolution is rather stable and robust constructs as demonstrated by results of comparative analyses of other conservative molecules responsible for central cellular functions. Thus, trees based on rRNAs (early studies) and rRNA genes not only reflect the evolution of these molecules but most likely the evolution of a major portion of the genome. In principle, the primary structure of the most widely analyzed 16S rRNA gene can be regarded as representing a miniaturized approximation of the core genome's history, though, due to its size of only 1,540 bases, with much less resolution power. It should be noted that *rrn* operons, consisting of genes coding for rRNAs and the intrageneric spacers, are in most organisms present in multiple copies per genome. While a single copy is often found in slow growing organisms, copy numbers of up to 14 may be found in some organisms, e.g., Firmicutes. Nucleotide variations (microheterogeneity) often occur among the multiple gene copies, in most cases in variable helices, which may cause problems in deciphering sequences generated by the PCR technique. If only a low percentage of copies are subjected to microheterogeneity, a PCR-based analysis run will detect such events as noise. Thus, the selection of cloned genes may have an influence on the positioning of an organisms, especially in those taxa in which species share high sequence similarities. With the advent of fully sequenced genomes, the extent of microheterogeneity will be unraveled, allowing even the elucidation of possible HGT events of rRNA genes.

The branching patterns based upon 16S rRNA and 16S rDNA sequences have sometimes surprised biologists in the past mainly because it suggested that the characters traditionally used to cluster organisms show restricted phylogenetic significance. Prominent early examples of phenotypic characteristics shown to have failed to circumscribe higher taxa in the past are the chemical composition of peptidoglycan (Kandler and H. König 1985; Stackebrandt 1985; Schleifer et al. 1990), aerobic metabolism (Fox et al. 1980; Seewaldt et al. 1982), spore formation (Ash et al. 1991; Collins et al. 1994; Stackebrandt and Rainey 1997), biosynthetic pathways (Balch et al. 1979; Fowler et al. 1986; Stackebrandt et al. 1988), and photosynthesis (Gibson et al. 1985; Woese et al. 1985; Stackebrandt et al. 1988; Imhoff et al. 1998a, b). Today, the use of 16S rRNA gene sequences in bacteriology is so widely accepted that students consider sequencing of this molecule a classical approach. The sequencing and analysis strategies and the main results for the evolution, phylogeny, classification, and identification are now textbook knowledge. The need to include into phylogenetic studies genes with a higher resolution power than that of the 16S rRNA gene arose once the conserved primary structure of *rrn* genes excluded the fine resolution of closely related species and genera (e.g., see certain members of the genus *Aeromonas* [Alperi et al. 2010]). On the other hand, protein sequences were evaluate to provide better resolution of deep branches, were 16S rRNA genes sequence analysis resulted in a forklike separation of taxa, implying a sudden evolutionary burst of lineages (e.g., Fox et al. 1980). Protein genes analyzed for these purposes were, among others, the β-subunit of ATP synthases (Amann et al. 1988), HSP70 (dnak) genes (Gupta and Golding 1993), DNA-dependent RNA polymerase (Klenk and Zillig 1994), or groEL (chaparonin) genes (Douglas 1998). These sequences, however, did not always agree in their phylogenetic assessment: While one set of genes strongly supported the results of 16S rRNA phylogenies, other genes represented a different picture of their own

evolution. The increasing availability of draft and complete genome sequences will facilitate the search for appropriate genes, and especially in the absence of a wide range of universal markers, for sets of taxon-specific genes. Lately, with the availability of thousands of genomes of Archaea, Bacteria, and Eukarya, relatedness among organisms is elevated from the level of individual genes to sets of core genes. By and large, independent approaches for tree construction from completely sequenced genomes (Wolf et al. 2001; Cicarelli et al. 2006) support the main 16S rDNA gene-based phylogenetic lineages of prokaryotes, though the order of branches may differ in some cases.

The criteria for describing higher ranks do not follow a coherent strategy as neither rules nor minimal requirements for their description are laid down. Ranks governed by the Code of Nomenclature are regularly validated by listing them in notification tables published in the *International Journal of Systematic and Evolutionary Microbiology* (http://ijs.sgmjournals.org/). It should also be noted that taxonomic categories above the rank of class (classis) are not covered by the rules of the Bacteriological Code though the names of several phyla/divisions have been included in validations lists. While the phylum (division) Firmicutes (Gibbons and Murray 1978) was created in the pre-nucleic acid sequencing era, almost all other phyla were described later, once the outline of the phylogenetic tree became visible. Higher ranks are mainly created on the basis of phylogenetic coherency and distinctness from neighboring taxa based, primarily, on the 16S rRNA gene phylogeny. Recent overall views of major higher ranks were provided by Gibbons and Murray (1978) and by Cavalier-Smith (2002), but, though validly published, the names introduced did not always find their way into the vocabulary of taxonomists. While authors usually concentrate on the description of species, genera, and families, higher ranks have been considered less important. This changed by the release of the so far four published volumes of the 2nd edition of *Bergey's Manual of Systematic Bacteriology* (from 2001 to 2012). The increase of the number of phyla, class, and orders over the past decade (*Table 9.4*, *Fig. 9.1*) is mainly due to the desire of the editors to provide a most complete hierarchic system. As a consequence of the availability of such framework, authors are encouraged to include higher ranks for novel taxa whenever appropriate.

Many ranks above the order were mainly defined for organisms having 16S rRNA gene sequences related to those of members of the type order. This strategy has been outlined in depth by the editors of Bergey's Manual (http://www.taxonomicoutline.org/). The attempt to support these ranks by phenotypic properties, often result in very broad descriptions, which would not allow proper classification without the aid of molecular sequences. As phenotypic properties are deduced from shared properties of most or all taxa defined by molecular analyses to belong to the same lineage, the circumscription is a reflection of the phylogenetic depth and phenotypic heterogeneity of its members. *Table 9.5* gives some examples for phyla of the domain Bacteria. In most of these cases, the phylum description is based on that of the order. In the case of Proteobacteria, the authors refrain from indicating any phenotypic traits because of the enormous phenotypic heterogeneity of its members.

To summarize, the ranks above the species rank are defined mainly on the basis of the 16S rRNA gene phylogeny, but no definite standards exist for the genetic relatedness among the organisms grouped at the same rank. Konstantinidis and Tiedje (2005a) published an independent assessment of the higher taxa by comparing the shared gene content and genetic relatedness among 175 fully sequenced bacterial genomes. Genetic relatedness was assessed using the average amino acid identity (AAI) of

**Table 9.4**

**Number of taxonomic ranks of prokaryotes each compiled at the end of four decades[a]**

| Rank | 1980 | 1990 | 2000 | 2010 |
|---|---|---|---|---|
| Domain | – | 2 | 2 | 2 |
| Phylum/division | – | – | 14 | 34 |
| Class | 7 | 12 | 14 | 70 |
| Order | 21 | 30 | 39 | 106 |
| Family | 68 | 96 | 151 | 220 |
| Genus | 300 | 545 | 988 | 1,676 |
| Species | 1,841 | 3,233 | 5,315 | 8,659 |
| Subspecies | 132 | 138 | 116 | 115 |

[a]According to Euzeby (http://www.bacterio.cict.fr/)

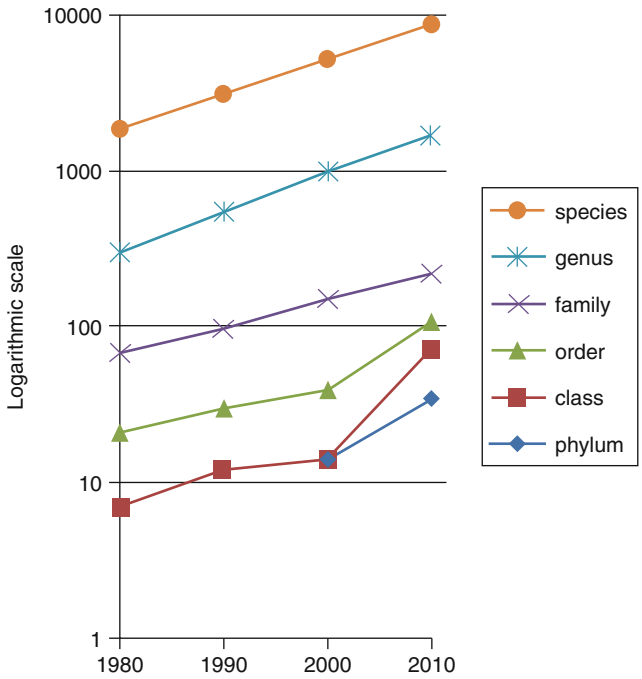


**Fig. 9.1**

**Logarithmic display of the evolution of descriptions of higher ranks over the past 30 years**

**Table 9.5**
**Definition of some higher ranks, according to volumes 1–3 of *Bergey's Manual of Systematic Bacteriology*, 2nd ed (2001–2009)**

| Phylum | Key properties as indicated in the phylum description |
|---|---|
| Aquificae/ Aquificales Reysenbach (2001) | Rod-shaped, moderate thermophilic to hyperthermophilic. Includes microaerophilic chemolithotrophic hydrogen oxidizers; most members are motile |
| Deinococci Garrity and Holt (2001a) | A phylogenetic lineage that contains species of the genus *Deinococcus* as a coherent group. A set of 16SRNA-rDNA signature nucleotides defines the order |
| Nitrospirae Garrity and Holt (2001b) | Defined mainly on phylogenetic grounds. Gram-negative, curved, vibroid, or spiral shaped. Metabolically diverse, most members are aerobic chemolithotrophs including nitrifiers, dissimilatory sulfate reducers, and magnetotactic forms |
| Chloroflexi Garrity and Holt (2001c) | Gram-negative, filamentous, exhibiting gliding motility. Peptidoglycan contains L-ornithine as the diamino acid. Lipopolysacchride-containing outer membrane not present |
| Chlorobi lino et al. (2010) | Defined on the basis of phylogenetic analyis of 16S rRNA gene sequences. Gram-negative bacteria that grow under strictly anaerobic conditions |
| Proteobacteria Garrity et al. (2005) | Defined on the basis of phylogenetic analyis of 16S rRNA gene sequences. Gram-negative bacteria classified in six classes |
| Firmicutes/ Bacillales Gibbons and Murray (1978) | Rigid, muramic acid-containing cell wall. Most members Gram-positive, some Gram-negative staining. Phenotypically diverse: spherical, straight curved, helical rods or filaments, flagella may be present. With or without heat-resistant endospores. Aerobes, facultative or strict anaerobe. Thermophiles and holophiles present. Most of them are chemoorganotrophs, a few are anoxygenic phothohetrotrophs. The mol% G + C content of DNA is generally >50 |

the shared genes between two strains (orthologs and paralogs), a genome-derived robust measure of genetic relatedness. Plotting 16S rRNA gene identity against AAI showed that, in general, there is a gradient of genetic cohesiveness, i.e., higher ranks tend to group more diverse genomes than lower ranks. However, the analysis also revealed that adjacent ranks (e.g., phylum vs. class) show, on average, 30.7 % overlap, meaning that 30.7 % of the pairs of organisms showing the exact same genetic and gene-content relatedness to each other belong to different ranks and hence, the current system is of limited predictive power in this respect. The overlap between nonadjacent ranks (e.g., phylum vs. family) is generally limited and attributable to clear inconsistencies of the taxonomy. For instance, the genetic relatedness among the *Prochlorococcus marinus* or the *Buchnera aphidicola* genomes is far too low, compared with the remaining data set, to justify their inclusion in the same species. Similarly, the *Treponema* and *Leptospira* genomes are assigned to the same order due to their common spirochete-like morphology, even though they are as divergent from each other as some organisms of different classes, even phyla, are. Such cases are apparently due to historic reasons and old taxonomic assignments, which need to be adjusted in order for a system that is (more) predictive of the genetic relatedness of the grouped taxa can emerge (Konstantinidis and Tiedje 2007). Taxonomic changes will also be necessary in the future in order to adjust the single-gene 16S rRNA gene-based hierarchy of ranks to the emerging genome-based taxonomy.

## Main Phylogenetic Parameters for Classification of Genera and Species

Until the late 1960s of the last century, methods for describing species and genera were based upon phenotypic, epigenetic properties (*Table 9.6*). This is exemplified for the characterization of *Vibrio cholerae* over the past 120 years. Looking at the description given by Lehmann and Neumann (1896), morphological properties of cells and colonies, growth conditions, simple physiological and chemical reactions, as well as range of susceptible animals dominated. This description remained more or less unchanged during seven editions of *Bergey's Manual of Determinative Bacteriology* but was supplemented with the characterization of antigens and typing of proteins and cell wall components, which were introduced in the 1950s. The major increase in knowledge occurred between 1960 and 2000, by the introduction of numerical taxonomic methods among other methods, providing huge databases of substrate utilization and reactions toward changing physicochemical conditions (manual version of today's BIOLOG Phenotype MicroArrays™), and by chemotaxonomic analyses of cellular constituents such as peptidoglycan, cellular fatty acids, phospholipids, polyamins, and isoprenoid quinones. Ecological parameters such as dormant stages and epidemiological traits were likewise considered as were results of morphological and genomic makeup and lytic specificity of bacteriophages. Although MLST and MLSA studies on vibrios were already available at the release of Bergey's Manual in 2005, these data were not included in the description of *V. cholerae*. The main progress concerning the elucidation of relationships between strains and species and the delineation of species and genera started with the introduction of DNA-DNA (DDH) and DNA-rRNA hybridization techniques (see below). While the latter method was soon replaced by sequence analyses of the 16S rRNA gene, the DDH approach is still considered a fundamental element of any species description. Today the advanced MLST and MLSA approaches allow a much deeper insight into the population structure of species with far-reaching conclusions on the evolution, epidemiology, systematics, and ecology.

**Table 9.6**

**Transfer of increased taxonomic knowledge into the description of the species *Vibrio cholerae* (*V. comma*)**

| *Vibrio cholerae* | Bakteriologische Diagnostik, Lehmann and Neumann (1896), *V. cholerae* (Koch) Buchner | *Bergey's Manual of Determinative Bacteriology*, 7. Aufl. (1957), *V. comma* (Schroeter 1886) (Winslow et al. 1920) | *Bergey's Manual of Systematic Bacteriology*, 2. Aufl. (2005), *V. cholerae* Pacini 1854, 411$^{AL}$ (Farmer et al. 2005) |
|---|---|---|---|
| Culture description | Morphology, luminescence, size, colony form and color, motility; life span in patient material; resistance | As 1899, plus tolerance against acidic and alkaline conditions | As 1899 |
| Growth requirements | Gelatin liquification, growth in agar, bouillon serum, blood, milk, potato; relation toward $O_2$ | As 1866, plus starch hydrolysis, Litmus milk reaction | As 1899, plus $Na^+$-requirement |
| Chemical reactions | Pigments, gas and acid production, enzymes, formation of $H_2S$, indol; toxins | As 1899, plus formation of $NO_2$ from $NO_3$ | As 1899, plus broad substrate utilization spectrum; fermentation end products, transport systems; degradation pathways |
| Pathogenicity, immunity | Mouse, rat, guinea pig, rabbit | Not covered | Antibiotic resistance; population analyses; pandemic analysis |
| Habitat | Patient, healthy person, environment | As 1899 | Life cycle, resting stages ("viable but nonculturable state"); ecology |
| Chemical structures | – | Typing of proteins, phospholipids- and polysaccharides, O- and H-antigens | As 1957, plus cytochrome and siderophore analyses; antigen structures; mol% DNA G + C |
| Genomic properties | – | – | DNA restriction, genome size chromosome number, plasmids, genome analyses, gene transfer, bacteriophages, phage typing; bacteriocins |
| Relationships | – | – | DNA-DNA- and rRNA-DNA-hybridization, 16S rRNA-sequences, immunological relationships of enzymes and other proteins |

## Classification Is a Dynamic Process (Revised from Stackebrandt 1992)

Microbiologists are aware that the available phylogenetic-branching patterns reflect the actual situation in nature only very incompletely. Phylogenetic reconstructions are based on inferred homologies but, unless witnessed by the evolutionary history of taxa, i.e., by fossil record data, the latter cannot be considered definitive (Rothschild et al. 1986). The isolated position of certain taxa, well defined by genotype and phenotype today, may disappear tomorrow with more organisms investigated. Thus, whenever new information requires corrections, either within established taxa or in neighboring groups, flexibility is called for and changes have to be made for the benefit of a better agreement between phylogeny and taxonomy. The main advantages of the phylogenetic system lie in its stability: only the rank (either vertical or horizontal) within the hierarchic structure, but not its place, will be changed—as happened in past systems (a comparison of Bergeys Manual of Determinative Bacteriology from the first through eighth edition is instructive).

Even the most convincing tree is always in a dynamic state; this forces taxonomists to stay flexible in order to adjust not only established ranks but also to modify nomenclature according to new insights. One problem still remains: The original advantage of the tree—its objectivity (insofar as this is possible)—is weakened by subjective clustering of organisms due to the differences in the emphasis taxonomists place on phenotypic characters. As in previous decades, the one system (or parts thereof) with highest practicability will succeed against competing systems with less persuasive arguments (see, e.g., the system devised by Cavalier Smith (2002) vs. the one used by the editors of Bergey's Manual). The ultimate goal is to establish a hierarchic system where all taxa show phylogenetic coherency and, at least for ranks below the family level, a great deal of phenotypic coherency as well. On the other hand, sufficient differences need to be known to distinguish taxa from each other by stable and easily determinable characters. While phylogenetic coherency is easy to define, the term "phenotypic coherency" varies according to the taxonomist. Again, practical considerations must come before petty splitting or lumping.

Prerequisite is a profound knowledge about the phylogenetic clustering of members of the higher taxon in question. An optimal survey would work with coded, unnamed organisms to judge the resulting branching pattern without prejudice. The study should include the type strain of all type species. The resulting pattern depicts the relative branching order that,

depending on the size of the database and the selection of reference organisms, will immediately yield information about the phylogenetic homogeneity of a group of isolates. In the second step, the branching pattern is superimposed on phenotypic data with the goal to delineate clusters of organisms that are phylogenetic coherent and easy to recognize by phenotypic characters. This is not only prerequisite for identification, but also to decide which of the possibly several available branching patterns is the most likely one to reflect phylogeny most closely. It should be mentioned in this context that in the presence of varying evolutionary rates, species with the highest nucleic acid sequence similarity are not necessarily the most closely related ones; while programs that optimize branch length take care of this problem, numerical phenetic analyses would in fact cluster the respective species as neighbors. The combination of taxon-describing characters will, in most cases, not be predictable, and the search will have to be extended to features not considered of taxonomic significance previously. Still many phylogenetic coherent taxa exist for which appropriate characters have not been found yet.

## DNA-DNA Hybridization

The DNA-DNA hybridization (DDH) method has received much attention in the literature because of its outstanding role in the classification of lower ranks. While several publications highlight the need, but also the restrictions, of this method as an integral part of the polyphasic taxonomy (e.g., Stackebrandt and Goebel 1994; Rosselló-Mora and Amann 2001; Rosselló-Móra 2006; Stackebrandt and Ebers 2006), others challenge the polyphasic approach including DDH as it is not encompassed by a theoretical "species concept" (Cohan 2002; Gevers et al. 2005; Achtman and Wagner 2008; Ward 1998). Despite the shortcomings, one should, however, not forget the enormous influence DDH had on stabilizing bacterial classification of species and genera. This method was the first molecular approach used routinely for measuring degrees of relatedness and the first phylogenetic one to be generally accepted for improving bacterial classification. Despite the rapid deposition of draft or fully sequenced genomes, DDH is still the most rapid and inexpensive of all phylogenetic methods for measuring the degree of nucleotide similarity of the entire genome. The main drawback of this approach, though, is the lack of information on which genes of the two strains compared contribute to the similarity and which genes do not, because of not being shared between the strains or showing lower sequence similarities. DNA concentration, fragment size, temperature, DNA mol% G + C content, concentration of salt, and denaturing agent influence the hybridization process. Though unsatisfactory in the lack of transparency, the simple similarity values obtained offer an advantage over those techniques that involve the comparison of individual genes or gene products only. Several hybridization techniques exist that have been thoroughly tested to determine the influence of various experimental parameters, and all have been compared to each other with respect to reproducibility and limitations (De Ley 1970; Grimont et al. 1980; Huss et al. 1983; Baumann et al. 1983; De Ley 1970; Schleifer and Stackebrandt 1983). Comparative DNA-DNA hybridization studies on the same strains using different techniques were in good congruence (see Schleifer and Stackebrandt 1983 for examples). Some novel techniques and variations of established methods have been introduced such as hybridization in microdilution wells (Ezaki et al. 1989; Hara et al. 1991; Kaznowski 1995), the random-primed labeling and signal amplification system (Amersham Life Science), or detection of double-stranded, DIG-labeled DNA with anti-DIG antibodies conjugated with alkaline phosphatase (Lind and Ursing 1986; Ziemke et al. 1998). Evaluation of these novel methods with the established ones (S1-, renaturation- and filter methods) has not been performed except for the microplate technique (Ezaki et al. 1989) and the renaturation technique, where good correlations were observed (Goris et al. 1999).

Relationships are usually expressed in terms of DNA similarities. It should be noted that due to the failure to unravel the underlying processes of renaturation, the expression "DNA similarity" but not "DNA homology" should be used in connection with DNA reassociation techniques. Wayne and colleagues (Wayne et al. 1987) recommended the use of a second parameter, the $Tm_{(e)}$ (e for eluted) value, especially in those cases where, under optimal hybridization conditions, fine resolution in DNA similarities is needed. The lower the degree of reassociation specificity (relaxed conditions), the higher the degree of mismatches. When the reassociated DNA strands are remelted, the melting temperature Tm indicates the degree of thermal instability caused by incomplete reassociation between heterologous DNA fragments. As compared to values of homologous reactions, the $Tm_{(e)}$ value of the heterologous reaction is thus an indication of the degree of mispaired bases in the hybrid formed after reassociation. However, as an inverse linear correlation exists between $Tm_{(e)}$ and DNA similarity, which makes determination of both parameters somewhat redundant (Grimont et al. 1980; Baumann et al. 1983), determination of $Tm_{(e)}$ values is usually not included in DNA-DNA reassociation studies.

Reproducibility, together with small sampling error (Sneath 1989), is an obvious advantage of DDH. Its limitation is the accessibility of equipment, i.e., a thermo-controlled spectrophotometer, the accessibility of DNA of sufficient quantity and purity, e.g., for many archaea and lithoautotrophic bacteria and uncultivated organisms. The limited resolution power has been recognized from the very first experiments. It has been calculated that for reassociation under optimal hybridization conditions (25 °C below the Tm of the DNA), the two DNA strands must exhibit at least 80 % sequence complementarity. Depending on the sequence similarity of the reassociating single strands, a difference of about 20 % is then spread between 0 % (no hybridization) and 100 % (as defined by maximal reassociation obtained with homologous DNA strands). It is therefore obvious that a given DNA similarity value does not reflect the actual degree of sequence similarity at the level of the primary structure of DNA. As measured with experimentally introduced mispairings, thermal stabilities have been estimated to decrease from 1 % to 2.2 % for each percent mispairing (Bautz and Bautz 1964;

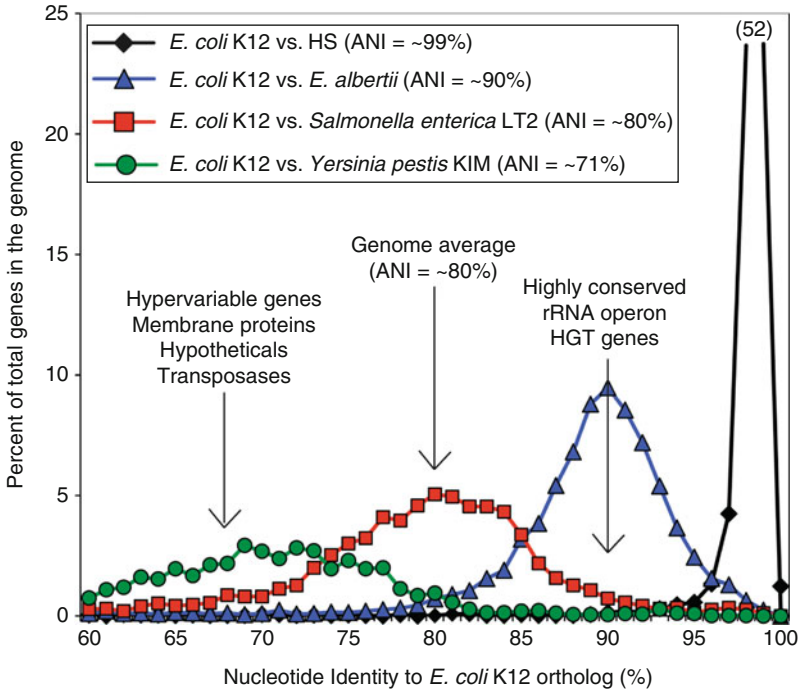


**Fig. 9.2**

***ANI values among selected Enterobacteriaceae genomes*. The figure shows the distribution of the nucleotide identities (x-axis) among orthologous genes shared between *E. coli* K12 and four other *Enterobacteriaceae* genomes (y-axis), which show increasing genetic relatedness to strain K12 (see figure key). Note that the gene sequence identities represent a, more or less, normal distribution around the genome average value (ANI) for each pair of genomes, the distribution has a smaller standard deviation for more closely related genomes, and the distribution starts deviating from normal for divergent genomes (*Y. pestis*), which is consistent with the fact that the ANI approach is not robust for genomes showing less than about 80 % ANI. The genes that typically deviate the most from the genome average in terms of their degree of sequence conservation (outliers) are denoted in the *E. coli* K12 versus *Salmonella enterica* LT2 comparison (*solid squares*). The patterns shown apply to other prokaryotic families and genera**

Britten and Kohne 1968; Ullman and McCarthy 1973). Although these experiments have been performed on short stretches and not on complete genomes, one can nevertheless argue that organisms that share 70 % DNA similarities share at least 96 % DNA sequence identity (Johnson 1973).

## Translating Traditional Standards to Portable Sequence Information: The ANI Approach

Several attempts to replace traditional cumbersome classification techniques such as the DNA-DNA hybridization (DDH) with more user-friendly approaches have been made recently. In perhaps the most promising of these studies, Goris and colleagues proposed the genome-aggregate average nucleotide identity (ANI) as a means of replacing DDH and translating DDH values to sequence identity information. ANI is the average nucleotide identity of all homologous genes shared between two genomes, and the work of Goris and colleagues showed that it is the genome-derived parameter that best correlates with DDH values, among several parameters evaluated (e.g., G + C % content, number of shared genes). In particular, it was found that the 70 % DDH standard corresponds tightly to 95 % ANI, independently of the genome size or morphological properties (e.g., Gram-positive vs. Gram-negative) of the organisms compared. These results are also comparable to those obtained earlier based on short stretches of DNA (Johnson 1973). Thus, organisms that share higher than 95 % ANI should be expected to belong to the same species accordingly to current recommendations for species delineation. Similarly, it was found that the 95 % ANI value corresponds to 98.5 % 16S rRNA gene sequence identity; hence, organisms that show less than 98.5 % 16S rRNA gene sequence identity to all characterized type strains should be expected to represent a new species (Konstantinidis and Tiedje 2007). The latter results are also consistent with those reported by Stackebrandt and Ebers (2006) based on comparisons between DDH and 16S rRNA gene sequence identity.

Similar to DDH, the resolution level of ANI is restricted to closely related organisms, showing between 80 % and 100 % ANI among each other. *Figure 9.2* represents an example for some members of the family *Enterobacteriaceae* showing different degrees of relationships. This is primarily due to the fact that multiple substitutions have frequently occurred in homologous nucleotide positions that differ between genomes related at about the 80 % ANI level or lower. Such substitutions are not taken into account in the ANI calculation; thus, lower

than 80 % ANI values correspond to increasingly longer divergent times between the genomes compared (Konstantinidis et al. 2006). Further, no DNA fragments that showed lower than 80 % nucleotide identity were found to cross-hybridize during DDH experiments (Goris et al. 2007); thus, this level of sequence identity appears to represent the low boundary of robust resolution for both the ANI and the DDH methods. Below the 80 % nucleotide level, the average amino acid identity (AAI) should be used instead. To date, no cases have been reported where ANI fails to precisely represent the genetic relatedness among strains of the same or closely related species. Hence, this approach holds great potential for prokaryotic taxonomy [see for instance (Rossello-Mora 2005; Lilburn et al. 2006; Staley 2009)] especially since obtaining the genomic sequence of a microorganism becomes an increasingly easier task nowadays.

A simple way to calculate ANI between any two complete genome sequences is to identify, using reciprocal best match blast searches (nucleotide level; blastn), the conserved gene sequences between the genomes and calculate their average nucleotide identity based on the identities reported by the blast algorithm. It is recommended that users run blastn with default settings (tested in blast versions 2.2.17 to 2.2.25) as long as the genomes compared show between 80 % and 100 % ANI. If instead of gene sequences, one uses 1-Kb long nonoverlapping consecutive fragments of the genome sequence, which simulate well the sheered DNA fragments produced during the most popular DDH protocols, the same ANI values are essentially obtained. The ANI values can also be robustly estimated based on draft genomes, i.e., genomes sequenced at 10X coverage or better, as this has been implemented, for instance, in the freely available software JSpecies (Richter and Rossello-Mora 2009).

## The Use of Typing Methods and MALDI TOF

Typing methods are routinely used for the characterization of strains at the species and subspecies level. The literature on these approaches is vast, and key publications were compiled by Gürtler and Mayall (2001). The use of comparative one-dimensional polyacrylamide gel electrophoresis of whole-organism protein pattern has been used widely in species descriptions in the 1990s, but nowadays, after the emergence of a wide array of molecular DNA- and rRNA-based techniques, protein patterns are rarely included in descriptions. The methods for generating one-dimensional patterns of proteins and nucleic acids need to be highly standardized to allow intra- and interlaboratory comparisons. Among the methods, it is especially the macrorestriction of DNA after pulsed field gel electrophoresis (PFGE) that gives highly standardized patterns. Though not commonly used in bacterial classification, PFGE was commonly considered a gold standard in epidemiological studies of pathogenic organisms before the introduction of MLST and MLSA techniques. Two other methods with similar highly standardized protocols are AFLP (amplified fragment-length polymorphism PCR) and ribotyping. The latter method is predominantly used in the medical environment and by the food industry as the availability of an automated system (RiboPrinter® System, Dupont) and an extensive database of relevant strains facilitates handling and analyses. Other methods, such as analyses of multiple locus VNTR (variable number of tandem repeat) (MLVA), random amplified polymorphic DNA (RAPD), repetitive extragenic palindromic-PCR (REP-PCR), enterobacterial repetitive intergenic consensus sequences (ERIC-PCR), amplified ribosomal DNA-restriction analysis (ARDRA), and rRNA intergenic spacer analysis (RISA), are hardly portable and are mainly used as screening tools to narrow down a large strain collection to subgroups with different genotypes. As described by Christensen et al. (2007), "...however, all methods [the ones just mentioned] might potentially be of use in testing the diversity of isolates. REP-PCR, ERIC-PCR and BOX-PCR are only suitable for assignment to a certain species based on obvious similarities in the banding patterns. On the other hand, banding patterns without any similarity do not necessarily demonstrate that two strains are members of different species." This notion correlates with the possibility that discrete centers of genetic diversity exist within strains defining a species. Comparison of methods have been published by, e.g., Scheldeman et al. (2004), Rademaker et al. (2000), and Rodas et al. (2005). As compared to the more than 600 annually named species, only a few authors included in their description studies more than a single typing method, e.g., subspecies of *Aeromonas hydrophila* (Huys et al. 2002). This is perhaps not surprising, as more than 70 % of novel species descriptions embrace a single strain, the type strain, only (Stackebrandt 2010).

The introduction of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) into the routine microbiology laboratory at the end of the 1990s has been a breakthrough in the rapid characterization of bacteria at the strain, species, and genus levels (van Baar 2000; Lay 2001; Shah et al. 2002). As little as about $5 \times 10^3$ cells are necessary for reliable MALDI-TOF analysis (Hsieh et al. 2008). The remarkable reproducibility of the MALDI-TOF approach is due to the fact that many of the individual single charged proteins of size 2,000–20,000 m/z (mass/charge; daltons) present in high abundance in the cell include many ribosomal proteins. Being part of the cellular translational machinery, MALDI protein fingerprints are therefore not significantly influenced by variability in environmental or growth conditions (there are some notable exceptions such as polymers formed during prolonged cold storage or presence of spores).

As cell extraction procedures and machine-specific settings will have an influence on the recorded mass spectral profiles, their direct comparison with existing databases (e.g., BioTyper™ [Bruker Daltonics] or Saramis [bioMérieux]) is not immediately straightforward. However, by using the same type of mass spectrometer and keeping growth and extraction procedures constant, this method is unchallenged for the purpose of identification and authentication in terms of preparation effort required, speed (30 min), and identification accuracy. The identification of strain-specific protein sequences by subsequent mass spectroscopic methods is only a question of time.

Firstly, the spectrum and number of fully sequences genomes are expanding enormously, and secondly, the necessary analytical equipment will be easier to access through increased -omics studies.

The question remains, however, how the spectra can be used in the classification process. The presence/absence of peaks of a defined mass between any pair of organisms is conventionally transformed into a similarity matrix and then into a graphic display of relationship, such as a dendrogram (Stackebrandt et al. 2005; Cousin et al. 2008). The identification scores given by the Biotyper software defines 3 categories such as "not valid" for a score below 1.7, "valid at the genus level" for scores between 1.7 and 2, and "valid for the species level" for values above 2.0. Several studies have used these threshold values for the identification of medical strains. With a minor failure rate, all studies, including 158 staphylococcal strains of six species from prosthetic joint infection (Harris et al. 2010), 602 *Staphylococcus aureus* strains, and 412 strains of 20 different non-*S. aureus* species of a medical culture collection (Szabados et al. 2010), as well as 304 g-negative stool isolates (He et al. 2010), were correctly classified to the species level. Strains of *Shigella* species and EHEC isolates could not be distinguished from typical *E. coli* strains, which is not surprising considering the genomic closeness of these organisms. In the study of Prod'hom et al. (2011), including 122 strains from blood culture pellets, 79 strains were correctly identified, while 21 % of the strains gave scores below 1.7; half of the latter were phenotypically identified as *Staphylococcus epidermidis*. The inability of the BioTyper to correctly identify strains points toward one of the main problems of any identification databases, which can identify only as correctly as the deposited patterns allow.

## Correlation of Individual Phylogenetic Parameters

It is generally accepted that if two organisms have highly similar DNA, they are closely related genetically. With increasing similarities between strain X and a type strain of a defined species A in either one or a combination of taxonomic parameters, e.g., DDH, gene sequences, DNA and MALDI-TOF protein patterns, ANI parameters, or even full genome sequences, corroborated by phenotypic similarities, the rational is also increased to classify strain X as a member of species A. This strategy has been successfully applied during the history of microbiology. However, advances in genomic studies clearly indicated that vast genomic differences may occur even among strains assigned to the same species, such as those expressed by differences in genome size, e.g., *Escherichia coli* O157:H7 with a genome size of 5.44 Mb possesses 1,346 genes not found in *E. coli* K-12 with a genome size of 4,64 Mb. Also, the MLST/MLSA approach sees discrete centers of variance in most of the species investigated (Lester et al. (2008) and Maiden and Dingle (2008) to name only two). Interestingly, even species of the same genus may show a different tempo and/or mode of evolution as exemplified in species of the genus *Campylobacter*, where *Campylobacter fetis* appears to be genetically much more homogeneous than other species of the genus (Maiden and Dingle 2008).

Comparison of different DDH methods used in the determination of relatedness has shown excellent agreement when closely related organisms are compared. However, when parameters from more distantly related organisms are used, it has been shown difficult to merge data from different techniques (Huss et al. 1983; Grimont et al. 1980). The borderline of 70 % similarity obtained under optimal hybridization conditions is recommended for species differentiation (Wayne et al. 1987) because a close agreement between phenotypic and genetic similarities was demonstrated once the DNA homology reached the 70 % level in numerous studies. Values from 30 % to 70 % reflect a moderate degree of relatedness while values become increasingly unreliable once they fall below the 30 % level, under which conditions taxonomic conclusions should be avoided. One has, however, to consider that the original recommendation for species delineation was derived mainly from the experience made with numerous strains of enterobacterial species (Steigerwalt et al. 1976; Brenner 1991). Thus, transferring the results found for a phylogenetically very shallow group of mainly eukaryote-associated organisms to the ancient and enormously diverse structure of the two prokaryotic domains is grossly underestimating the different mechanisms as well as the mode and tempo at which organisms develop. One has to remember that the delineation value is an artificial value used to structure the bacterial world at the level of species in a coherent way.

Correlation blots determined in the early 1990s for the parameters of the two most widely used approaches in prokaryotic phylogeny, the DDH and 16S rRNA gene sequence identity, justified the continuous use of the DNA-DNA reassociation technique. In case these plots would have shown a linear relationship between intraspecies DNA similarities above 70 % and 16S rDNA sequence similarities above 97.0 %, the DNA hybridization method would have disappeared over night. However, the situation was different: As the 16S rRNA gene is a miniaturized mirror image of the genome though too constrained in its function to follow immediately the changes that occur in less conserved molecules, the relationship between these two parameters was curvilinear, with the rrn sequences showing almost no divergence at DDH values above 70 % (Amann et al. 1992; Fox et al. 1992; Stackebrandt and Goebel 1994). Each approach is strong in those areas of relationships in which the other method fails to reliably depict relationships. Sequence analysis has proven to be a reliable marker for the phylogeny of organisms between the levels of domains (around 55–60 % 16S rDNA gene sequence similarities) to moderately related species (around 97 % similarities). Above this value, DNA values can be as low as 55 % or as high as 100 %. Several organisms are known to share 99.8 % or even 100 % rRNA gene sequence similarity that belong to different species because the DNA reassociation values were below the 70 % threshold value. Even if one considers the noise of DNA reassociation values originating from different laboratories using different reassociation methods, the evidence is strong enough to state that the resolution power of DNA-DNA reassociation is

significantly higher than that of 16S rDNA sequences. The finding that below a 16S rDNA gene sequence similarity value of 97 % the corresponding DNA-DNA reassociation value will not be higher than 60 % has led to the recommendation (Stackebrandt and Goebel 1994) that, at this and lower level of sequence similarity, no DNA pairing studies need to be performed as the strain concerned will be highly unlikely to be a member of a described species. Above 97 % rRNA similarities, however, taxonomists were encouraged to perform DDH to verify the species status of isolates. Using a significantly broader data set, the 97 % threshold value defined in 1994 was corrected to 98.5 % in 2007 (Stackebrandt and Ebers 2006).

Konstantinidis and colleagues (2006) also showed that even a few genes in the genome, such as the 6–10 genes sequenced in typical MLSA applications, could provide reliable estimates of the genome-aggregate ANI values. The correlation between the average nucleotide identity of 10 randomly selected genes in the genome and the genomic ANI was always significant and ranged from 0.4 to 0.9 (Kendall $\tau$), depending on the genes used. In particular, if selected genes do not represent fast evolving (e.g., surface proteins, transposases) or slowly evolving (e.g., rRNA operon genes) genes (see *Fig. 9.2*) then the ANI value of a small subset of genes approximates well the genome-aggregate ANI. This study also revealed that ANI values correlate strongly ($r^2 > 0.9$, p-value $<0.001$) with the genetic relatedness values estimated using state of the art phylogenetic approaches, such as maximum likelihood analysis of concatenated gene sequences. Yet, ANI is a much simpler parameter to estimate, and the ANI calculation is less computationally intensive compared to the alternative phylogenetic approaches because it employs the fast blast algorithm and is performed in a pair-wise fashion similar to DDH.

### Comparison of Phylogenetic Patterns Derived from 16S rRNA Gene Sequences

A previous comment (Stackebrandt 1992) that many trees are not comparable as they were generated on the basis of partial sequences and different treeing methods is of historic interest only, as today mostly nearly complete sequences are compared using two or three different treeing algorithms of proven resolution power and statistical significance. The former inability of computers programs to handle the enormous amount of data in a reasonable time without omitting either the number of reference organisms or sequence information became obsolete with increasing computing power and more sophisticated algorithms. Some problems still remain which are firstly the subjective omission of parts of the sequence judged to be of less phylogenetic importance than other parts and secondly the sometimes arbitrary omission of sequences available for members of the taxon under investigation. However, one must differentiate between different goals that determine the selection of sequences to use. For the elucidation of intrageneric relationships, the number of sequences is mostly restricted to those of type strains. In this case the complete sequence information, including that of the variable regions, can be compared. The branching pattern obtained will change when this small dataset is embedded in a larger one composed of sequences of members of families, orders, classes, and so on. At each level of ranks, information will be lost, either by removal of variable regions or trimming of stem and loop structures to the minimum length common to all members of the data set and by omission of those regions for which ambiguous sequence information is provided. Each of these steps will most likely lead to changes in the branching pattern of any lineage. Thus, the picture unraveled from the inclusion of thousands of sequences in a single data is not more than an approximation of the phylogeny. The literature is full of examples that demonstrate the changes of the phylogenetic relatedness within genera and families through the influence of new entries in the database. Branching patterns supported by high bootstrap values indicate that for a given branching point the statistical analysis supports the order of lineages—but this statistical analysis is per se no indication that the pattern reflects the natural relationship with a similar degree of confidence.

Assuming that no algorithm exists that provides a tree reflecting precisely the evolution of prokaryotes we must then accept the pattern(s) that appears to be the most plausible one. It may be the one showing the highest degree of topographic similarity to patterns derived from different informative molecules. With the availability of a wide range of MLSA-based and full genome-based trees, the branching order and intrataxon coherency of 16S rRNA gene trees could be scrutinized. By and large the delineation of the major clusters, especially at the domain, phylum, and class level could be confirmed, though the branching order often showed significant deviations.

## Delineation of Genera: Pragmatic Approach

The definition of a genus, given by Cowan in 1968 (Cowan 1968), has not been changed with the input of molecular data. Cowan states that the genus is "one of the basic ranks in the hierarchical systems used in biology, and probably the highest rank with any significance in microbiology. In position between 'FAMILY' and 'SPECIES', it is best considered as a collection of species with many characters in common; unfortunately no one has indicated the extent of this sharing of characters, and it is purely a matter of personal judgement (....) as to what constitutes a genus. Like the SPECIES, the genus is a subjective concept without any foundation in fact."

A significant finding of the analysis of rRNA, rDNA, and DNA-DNA reassociation studies was to point out the genetic heterogeneity of many phenotypically defined genera. The new results were often in discord with the classification of species (Stackebrandt and Woese 1984): The working basis, i.e., the genus, had to be redefined or dissected or the type species was found to actually be a member of a different genus. Examples have been described for *Methanobacterium, Azospirillum, Pseudomonas, Bacillus*, Clostridium, *Streptococcus, Flavobacterium, Bacteroides, Arthrobacter, Micrococcus, Brevibacterium, Nocardia*,

and several genera of phototrophic organisms, but it should be stressed that almost each genus was involved in the reclassification process to varying degree. Some prominent examples are listed in Stackebrandt (2000). Now, after 30 years of reclassification the situation is much more stable and reclassifications are rare and mostly happen when intrafamily relationships change with the description of novel genera belonging to the family.

Scientists became aware of the high degree of horizontal gene transfer among prokaryotes (see above) during genome evolution, which challenges the use of universal genes in determining the whole range of phylogenetic relationships. A second wave of analyses of protein-coding genes was initiated by multilocus sequence analysis (MLSA) of housekeeping genes, a method derived from multilocus sequence typing (Maiden et al. 1996) to follow the epidemiology of pathogens and to unravel relationships among closely related species (as opposed to the whole prokaryotic tree covered by universal genes). Usually a set of three to ten sequences of orthologous protein genes (e.g., lepA, gyrB, fusA, pheS, rpoA/B, or atpA) are analyzed, using highly conserved primers for PCR amplification and sequencing. Either concatenated or individually, phylogenetic analyses contribute significantly to the clarification of strains and species to species and genus, respectively. Results often display chromosomal crossover (HTG) events among species, warning against the practice of single gene-based identification. An ad hoc committee for the re-evaluation of the species definition in bacteriology (Stackebrandt et al. 2002) strongly encouraged taxonomists to include this method in future species descriptions and taxonomic studies, a suggestion which has been widely accepted (e.g., Naser et al. 2005; Thompson et al. 2008; Rong and Huang 2010). Criteria for the use of gene sequences for the characterization of prokaryotic strains, including criteria for quality check of sequences and alignment, use of alternative treeing methods and helpful hints for the interpretation of 16S rRNA gene data have been compiled by Tindall et al. (2010).

A new genus has to be described when a strain or a strain cluster is shown to branch outside the radiation of a validly named genus and the isolated phylogenetic position is accompanied by the presence of distinct phenotypic properties not found among the neighboring genera. On the other hand, the placement of a new taxon with a novel pattern of phenetic properties within the radiation of a genus may point toward its taxonomic heterogeneity, which consequently may lead to the dissection of the genus. The decision about which phenotypic properties to use for the circumscription of a novel genus is up to the taxonomist but depends to some extend upon the description of the neighboring genera. The genus-specific characteristics must be present in each species of the genus or the genus description needs amendment. The following gives two examples for the usefulness of phenotypic properties to corroborate phylogeny-based delineations:

Chemotaxonomic properties: In the order *Actinomycetales*, the high degree of chemical diversity in the peptidoglycan, fatty acids, polar lipids, menaquinones, whole cell sugars, or teichoic acid offers superb diversity at the epigenetic level to delineate genera. The correlation with phylogenetic analysis is so high that the finding of a new combination of such patterns is indicative of the presence of a new genus (Embley and Stackebrandt 1994).

Morphological, chemotaxonomic, and growth properties: The main basis for dissection of the former genus *Bacillus* (today the family *Bacillaceae* embraces more than 40 genera [http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/]) has been the extensive phylogenetic analysis of its species (originally by Ash et al. 1991 and Rainey and Stackebrandt 1993). The separation into genera is mainly based upon the chemical structure of the peptidoglycan, cellular fatty acids, polyamins, polar lipids, cell shape, spore shape, anaerobic growth, optimum pH, and growth in 10 % NaCl. As compared to the actinomycete genera, the phylogenetic significance of chemotaxonomic properties is much lower. The most species-rich genus, *Bacillus* (>165 species), is heterogeneous with respect to amino acid composition of peptidoglycan, spore shape, anaerobic growth, presence of swollen sporangium, and other features, and it can be expected that this genus will be subject of even further dissection.

## The Prokaryotic Species: A Historical View

The rank "species" as defined today is an artificial, a pragmatic construct that serves the main goal of identifying new isolates and, if appropriate, providing sufficient properties to allow their classification. The previous paragraphs have already indicated the scientific and conceptional limitations of this construct, which become increasingly obvious in the light of the insights from recent comparative MLST and MLSA as well as whole-genome studies. One hundred and thirty years of history of bacterial classification is now open to scrutiny, and, as in the past, the majority of taxonomists will welcome the input of population geneticists, molecular biologists, and ecologists to either improve the definition or to suggest a concept that better fits the evolutionary processes. However, a transition will not be easy to achieve as the pragmatic definition of a prokaryotic species is well accepted among bacteriologists. During a process of revision and constant adaptation, new insights into cell structure and cell function are incorporated, taking into account the microbiologists' perception. As Staley and Krieg (1984) indicated, "a classification that is of little use to the microbiologist, no matter how fine a scheme or who devised, it will be ignored or significantly modified." This is true for each level within a hierarchic system and the history of microbiology has witnessed ample examples for the description and rejection of such systems. Even more prone to the microbiologists' idea about the concept of a species are the higher ranks which are almost completely defined by subjective arguments—to a level where the importance of working with taxa above the rank of genera is considered trivial and unimportant (O'Hara 1994) (see also above). The importance of any hierarchic system goes beyond the main function of classification and identification. Higher ranks are developed to explain and to understand the evolution of organisms and parts thereof, based upon the knowledge that has been available at the time of their establishment.

In bacteriology, time has seen various hierarchic systems and proposed phylogenetic paths which in hindsight failed because they were not based upon the natural relationship of the organisms concerned but on properties that were believed to express natural relationships: morphology (Cohn 1872; Stanier and van Niel 1941), pigmentation, physiology (Orla-Jensen 1909; Margulis 1981), and cell constituents (Schleifer and Kandler 1972). Some of these attempts represented important contributions in their time because the classification system based upon them and some of their constituent does actually reflect phylogenetic divergence (e.g., peptidoglycan structure, lipids, fatty acids). The order of phylogenetic lineages guides the bacteriologists to the two basic units, the genus and species, without the need for a superimposed system. Actually, there is no immediate need to work with a hierarchic system but is tempting to do so in order to comprehensively classify according to similarities and differences and evolutionary traits. Today, we see the emergence of higher taxa along the phylogenetic structure, and, as in systems of plants and animals, taxa of the same rank are not necessarily comparable units and described in a coherent way. Also, we must be aware that only a small fraction of prokaryotic species are described, and new entries will not only change the description of the higher ranks but may change the composition of taxa as well. However, the advantage of basing the hierarchic structure on a rational basis, i.e., on semantides reflecting the organisms' evolutionary history, makes it highly likely that changes within the system will occur mainly within ranks of a common genealogical lineage and not, as in the past, affect and possibly change the units of remotely related taxa.

Surprising perhaps to microbiologists, there are some zoologists (Hull 1976) and botanists (Bachmann 1998) who discuss the possibility that the species as an objective basic unit of taxonomy as defined in the past does not exist. The nonexistence, to be more precise the inability of microbiologists to define a species as an objective category, a product of natural selection that after sufficient studies identifies itself to the taxonomist, has been recognized more than 30 years ago. Bacteriologists in particular follow guidelines and recommendations in order to provide stability, reproducibility, and coherency in taxonomy—although the final decision about species description is still based upon his or her subjective judgment. This concept does not include the role of reproductive isolation, i.e., the barriers to horizontal gene transfer over large phylogenetic distances; it does not even try to explain the mode of speciation. One may be amused upon such a naive approach to handle the "species" matter—but once you decide that a species cannot be recognized as a natural entity, it is only consequent to work with the compromise of a working definition. This strategy has facilitated the practice of taxonomy—a way that may also be gone by protozoologists, mycologists, and algologists. As Bachmann (1998) points out, the most useful general species definition would be the one that allows "the largest number of individual organisms be unequivocally be assigned to species so that some basic conditions are satisfied." These conditions are (1) membership of a strain to only one species and never to none, (2) all descents of a species are member of that species, (3) members of a species should be phylogenetically related, and (4) the species so defined should apply to taxa that coincide more or less with the intuitively recognized species. Most obviously, points (1) to (3) are already matched in bacteriology while point (4) has failed significantly in the past because of inability to classify a prokaryotic species by intuition.

The species definition applied today does not incorporate the concept of how strains may develop into entities named "natural" species. Several factors have been identified through intensive multilocus sequence typing of housekeeping genes (Maiden et al. 1996), RAPDs and multilocus enzyme electrophoreses (Selander et al. 1994; Istock et al. 1996), to contribute to the evolution of the genome. Some organisms, e.g., *Neisseria* and *Rhizobium* species, as well as enterobacterial species (Guttman and Dykhuizen 1994), are subjected to reticulate events or panmixis (Maynart-Smith et al. 1993; Istock et al. 1996) in which clonal relationships, due to mutational events and vertically transmitted accessory genetic elements, are perturbated by horizontal genetic transfer, e.g., conjugation, and phage transduction DNA transformation (Achtman 1998). Other strains, which are mostly endosymbionts and obligate pathogenic organisms, such as members of the genera *Bartonella, Brucella*, and *Rickettsia*, are mainly clonal because they are only rarely subjected to horizontal gene transfer. In some species, the recombination among strains of the same species is more frequent among strains of different species (e.g., the enterobacteria), which may lead to the homogenization of the gene pool of the interacting organisms (Guttman and Dykhuizen 1994). In an attempt to come to a biological species definition for bacteria, it has been proposed (Dykhuizen and Green 1991) to base the species definition on the following observations: (1) Phylogenetic trees from different genes from members of a single species should be the same. (2) Phylogenetic trees from different genes from members of different species should be the different (as shown for two genes from seven species of *Neisseria*). Without questioning the validity of this approach, it is obvious that this strategy is far beyond a routine method at the present status of sequence analysis, especially as several strains of a single species must be investigated, and although worth discussing, this approach cannot replace the present pragmatic species definition.

The current pragmatic approach to the definition of species is also not taking into account the ecological niche, although the source of isolation should be part of a species description. However, the isolation site can be considered to reflect the actual place or the role in the ecosystem that the organism occupies only in those instances in which the organism has a close dependence to its environment, e.g., the rhizoplane, rhizosphere, in endosymbiotic, and pathogenic relationships. The description marine water, fresh water, mud, sediment, soil, rumen, skin, and so on, is too superficial to describe the exact niche in complex environmental samples. Knowledge about the site of speciation and the environmental selection of members of a clonal

population may be helpful in explaining the path of evolution and the mode of speciation—but it provides no clue in the definition at what level subpopulations may be regarded as individual species.

The evolutionary record of molecular sequences provides a basis for phylogenetic studies, which has allowed incorporating the bacterial world within the constraints of genealogical studies. But this information alone does not per se conclude on the decision about what a species is and how a species has to be defined—it just puts the prokaryotes on the same level with animals and the higher evolved plants in the discussion about the concept of the species category as a general unit for biodiversity, evolution, and taxonomy. The mere availability of molecular data does not allow biologists to define the category species as a comparable biological entity throughout the diversity of organisms. The dilemma must be seen in the fact that biologists themselves are not clear about the definition "species," for which a theoretical basis is lacking (Bachmann 1998). The phylogenetic species concept (Cracraft 1983), the taxonomic species concept (Staley and Krieg 1984), the biological species concept (Dobzhansky 1937; Istock et al. 1996) (disregarding asexual reproduction entirely), and the ecological species concept (Istock et al. 1996), and more concepts, all have their strengths and weaknesses, and each of them stresses different aspects of biology and evolution. This topic has been discussed by Gevers et al. (2005) in the light of multilocus nucleotide-sequence-based approaches and has recently been in the focus of intense discussions. Modes of horizontal gene transfer and homologous recombination (Lawrence and Retchless 2009), as well as population genetic and macroevolutionary theories, are considered necessary for the evaluation of interspecific and intraspecific variation. Barraclough et al. (2009), Cohan and Koeppel (2008), and Fraser et al. (2009) added the ecological theory to the genetic theory of speciation. Doolittle and Zhaxybayeva (2009), reviewing existing theories, argue that "there is no principled way in which questions about prokaryotic species, such as how many there are, how large their populations are, or how globally they are distributed, can be answered." Metagenomic data in combination with alternative theories, however, will allow meaningful questions about the biological processes of speciation to be addressed and one day one may develop alternatives to the present pragmatic approach. Thane (2009) come to a similar conclusion arguing that "species as being discrete clusters or monophyletic lineages are at odds with most of the data, suggesting that taxon circumscription can only proceed by informed compromise, pragmatism, and subjectivity." In this respect, the conclusion is similar to the perspective of 40 years ago, when Cowan (1968) already bluntly stated in the Dictionary of Microbial Taxonomic Usage that the category species does not exist and does not represent a natural entity.

The aspect, theories, and evolving methods reflected upon in the past 10 years on the "species" concept will sooner or later influence the today's pragmatic species definition and the guardians of taxonomy will have to adapt to the changing wind. This will not be an easy goal to achieve as the current scaffold of the hierarchic structure represents a solid foundation, emerging from 100 years of changes in nomenclature and relatedness of taxa at all levels of ranks. In order to maintain and to safeguard prokaryotic systematics, taxonomists developed a conservative attitude, and they will await a concept of proof that the emerging broad range of molecular methods and theories derived there from will be of benefit and manageable as a framework to either substitute or to add to the present approach.

## The Pragmatic Species Definition

The definition of a prokaryotic species embraces the phylogenetic component given by Cracraft (1983) for a phylogenetic species as "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descendent" and the taxonomic component given by Colwell (1970) as "a group of related organisms that is distinguished from similar groups by a constellation of significant genotypic, phenotypic, and ecological characteristics." The definition used today includes components of a geno-, or genomo-species, a taxo-species, and a pheno-species, which reflect the different concepts of species descriptions in the past decades. From a pragmatic point of view, all these facets are incorporated into a single definition, though the terms are still in use. An "optimal" species would be the one that, at the same time, is not only representing a phylogenetically and phenotypically coherent unit but also one that naturally exists. However, except for some strains of pathogenic species, the ecological niche is either not known or the number of isolated strains is too small to conclude on their original habitat (e.g., airborne isolates; aquatic isolates that entered rivers, lakes, and the ocean by terrestrial runoffs; or sediment isolates). It should also be remembered that for the description of the type strain, a single strain is selected out of a broad diversity of naturally occurring prokaryotic organisms, which may reflect a continuum of genetic and epigenetic diversity. Strains that are sufficiently similar in those aspects used today in prokaryotic taxonomy, i.e., mainly DNA-DNA reassociation, are considered members of this species. This concept of selecting species has been described as the arbitrary species concept (Staley 1997). The combination of arbitrary selection and artificial species delineation is admittedly arguable and open to discussion, which has recently been intensified with the advent of genomic information. The entity species circumscribed by bacteriologists cannot be compared to that of zoology and botany. For example, *Homo sapiens* and its closest relatives, the higher evolved apes, comprising about 200 species, are related by higher than 75 % DNA reassociation (Sibley et al. 1990); transferring this concept to bacteriology, all these species would fall into a single bacterial species delineated by the 70 % threshold value. In the absence of knowledge of (an) underlying speciation process(es), the pragmatic approach to the species definition in bacteriology has nevertheless been extremely useful and the success of the definition is measured by its widespread acceptance.

## Delineating a Species in the Frame of the Pragmatic Definition

In the daily routine, a new isolate is subjected to an identification process, which may be different from laboratory to laboratory and from taxon to taxon. Many scientists, however, are not interested in a fine resolution of relatedness or they are not in a position to go through the laborious identification process. Some initial superficial tests are performed, like determination of colony morphology and pigmentation, shape, spore formation, Gram-stain, and relationship to oxygen. The aim of the study defines the identification procedures to follow, but even with the availability of a broad range of molecular tools, the phylogenetic diversity is rarely fully explored for superficially similar strains.

Often, the classification process starts when the isolate does not match the description of 1 of the about 8,750 species validly described today (http://www.bacterio.cict.fr/number.html). In most cases today, this is performed by examining the phylogenetic position of a novel isolate determined by sequence analysis of its 16S rRNA genes, as the database of prokaryotic strains is enormous, covering more than 95 % of the described species. This approach may change with the advent of inexpensive and fast generation of draft genome sequences, foreseen as early as 1999 (Relman and Strauss 1999) and further elaborated by Buckley and Roberts (2007) and Riley and Buckley (2008). In order to search for the closest relative for which 16S rDNA gene sequence data are publicly available, taxonomists make use of electronic tools such as, to name the most widely used ones, the BLAST system (blast.help@ncbi.nlm.hih.gov; http://www.ncbi.nih.gov/cgi-bin/BLAST/nph-newblast), the Ribosomal Database Project (rdp@phylo.life.uiuc.edu; http://www.cme.msu.edu/RDP), the ARB program (arb@mikro.biologie.tu-muenchen.de; http://www.mikro.biologie.tu-muenchen.de), the GreenGenes database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi), or the living tree project embracing over 8,000 curated sequences, each of which represents a single type of strain of a classified species (http://www.arb-silva.de/projects/living-tree/). The search in the public databases will show the phylogenetic distance to the isolates' nearest neighbor(s), but the quality of the search depends upon the completeness of the 16S rDNA gene database, both, in terms of available sequences and species analyzed as well as the completeness or not of the sequences obtained. In this respect, we recommend the use of the living tree project database. Once the approximate nearest phylogenetic neighbors have been identified, it is recommended to search the public databases in order to not miss any recent entries. The recent publication of Tindall et al. (2010) includes guidelines for proper analyses of 16S rRNA gene sequences, including the description of novel species.

The result of the phylogenetic analysis will have consequences on the strategy to continue. Let us assume that the 16S rDNA gene similarity values will be higher than 98.5 % to its nearest neighbor. Many scientists will be satisfied knowing the approximate phylogenetic position and will not continue with the identification process. Others, however, will be interested to determine the more precise affiliation of the isolate and will continue with the classification process. The way to proceed depends upon the number of species in the phylogenetic vicinity of the isolate.

1. In case the isolate falls within the boundaries of a genus, the description of this genus will guide the few key properties to analyze, which will demonstrate that the isolate actually should be considered as a member of this genus. If the species within this genus are separated by distinct phenotypic properties (which one should assume but which is not always the case), these should be searched for in the isolate, and, if present, the isolate has been identified. If not, it is recommended to perform DDH or ANI studies to determine whether the isolate is the nucleus of a new species. DDH similarities of lower than about 70 % are indicative of a new species and properties that distinguish the new species from the established ones should be sought to identify. In those cases where the DDH similarity between an isolate and a described species is 70 %, this information will usually not be recorded unless the new strain leads to the description of a subspecies or to an emendation of the species description.

   The recommendation (Wayne et al. 1987) to delineate species in genomic terms at a threshold value of around 70 % DDH similarity are guidelines but should not be applied as fixed rules. Though the majority of species are actually described as suggested, there are a few exceptions, which are indicated by Stackebrandt (2000):

   Despite the recommended value of 70 % DNA similarity, taxonomists working with some defined prokaryotic groups have altered this value to come to a better correlation between phenotypic and genotypic similarities. Within the family *Pasteurellaceae*, a DNA reassociation value of at least 85 % describes a species. Similar values have been found for the interspecies relatedness of *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica* (Kloos et al. 1981) and between members of the spotted fever group of *Rickettsia* (Walker 1989).
2. In case the 16S rDNA gene sequence similarity values indicate an approximately equidistant relationship to members of different genera, the diagnostic properties given for these genera must be tested for the isolate. Such highly related genera have been described, e.g., in the *Actinomycetales*, *Flavobacteriaceae*, Alphaproteobacteria, Gammaproteobacteria, and Firmicutes. If the properties match those of one of the genera, the identification process will be restricted to members of this genus, and one has to proceed as indicated above. If the analysis of the genus-specific properties will reveal no unambiguous match with any of the neighboring genera, it is likely that the isolate represents yet another closely related genus of this genus cluster, and the description of the new species and the new genus will go hand in hand.
3. In case the new isolate shares less than 98.5 % sequence similarity to the nearest phylogenetic neighbor, DHH studies are unnecessary to perform as similarity values will range

clearly below the 70 % reassociation borderline value recommended for the present species definition.

In contrast to the rather stringent genomic definition of a "species," the phenotypic characterization of a new species is very variable. Information about the phylogenetic position of a putative new type strain facilitates the selection of such features and guides the search for taxonomically relevant properties. The properties to be investigated depend upon firstly on those indicated as being specific for the genus and secondly on the set of characters already indicated to be of discriminatory value for species described for the genus.

Extensive morphological and ultrastructural characterization must be presented especially for species of novel genera. Records on enrichment and isolation, motility, colony characterization, optimal growth conditions, growth requirement and substrates, and base composition of DNA are part of a set of characterizing features (Tindall et al. 2010). Analysis of special features, such as antigenic characterization, is required for certain taxa. Many of the properties to be provided for the description of a species are listed either in the descriptions of minimal standards, which are available for species of some genera, or they are compiled in *Bergey's Manual of Systematic Bacteriology.*

## Definition of a Subspecies

The rank subspecies is the lowest rank covered by the Code of Nomenclature. Its definition is somewhat vague as, unlike in the definition of the species, no molecular or phenetic threshold values are indicated at which intrageneric clusters are separated from each other. According to Staley and Krieg (1984), a subspecies is "based on minor but consistent phenotypic variations within the species or on genetically determined clusters of strains within the species," while Wayne et al. (1987) argue that "Subspecies designations can be used for genetically close organisms that diverge in phenotype. . . .. . . There is a need for further guidelines for designation of subspecies." The definition used in botany and zoology, i.e., "a geographically defined grouping of local populations which differs taxonomically from similar subdivisions of species" (Gorilla gorilla subsp. gorilla is the western lowland gorilla, and Gorilla gorilla subsp. graueri is the eastern lowland gorilla), has not influenced the definition used for prokaryotes yet. This may change with the advent of high-resolution molecular analysis, apt to reveal restricted geographical distribution among strains. Multispacer typing [MST], MLST, and MLSA analyses did already influence recent descriptions of subspecies, e.g., *Rickettsia conorii* (Zhu et al. 2005), while comparative genome sequence analyses supported a polyphasic approach to describe two subspecies of *Bacillus amyloliquefaciens* (Borris et al. 2010). MLSA have been used to investigate the molecular coherence and relatedness of subspecies, e.g., in *Mycobacterium avium* (Turenne et al. 2008), and even used to combine several species and subspecies of the *Streptomyces griseus* clade (Rong and Huang 2010) and *Streptomyces albidoflavus* clade (Rong et al. 2009).

The previous difficulties to determine intraspecies variations consistent with a specific function explain the rather low and over the years rather constant number of subspecies descriptions (see *Table 9.4*), which are mainly found in species of medical species (e.g., *M. avium, M. tuberculosis, Campylobacter lari, Francisella tularensis*) and technological importance (e.g., *Bacillus subtilis, Acetobacter xylinus, Photorhadus luminescens, Xylella fastidiosa*, to name a few recent descriptions). The criteria to delineate subspecies are at the discretion of the taxonomist and generally no rationale for the subspecies description is given, other than the indication of discrete genomic centers of variance: For example, the description of the three subspecies of *Pseudomonas chlororaphis*, *P. chlororaphis* subsp. *chlororaphis*, *P. chlororaphis* subsp. *aureofaciens*, and *P. chlororaphis* subsp. *aurantiaca*, are based on strains of previously separate species (Peix et al. 2007) which showed higher than 75 % DDH similarities, quantitative differences in fatty acids, phylogenetic placement of strains based upon 16S rRNA, atpD, recA, and carA gene sequences and differences in five phenotypic properties. The presence of different DDH groups, electrophoretic motility of certain enzymes, and 16S rRNA gene typing led to the description of three subspecies of *Fusobacterium nucleatum* (Dzink et al. 1990; Gharbia and Shah 1992). In yet another case (Yanagida et al. 1997), the two subspecies of *Sporolactobacillus nakayama* share lower than 65 % DDH similarities, which, in the presence of distinct phenotypic differences, would have allowed the description of two separate species.

It is desirable to base descriptions of taxonomic ranks on easily identifiable properties—a criterion that is not always met at the subspecies rank. In the absence of chemical or metabolic differences, recognized molecular intraspecific clusters can be described on the basis of reproducible typing patterns, including MALDI TOF profiles, signature nucleotides of genes coding for proteins and rRNA, presence/absence of genes, chromosome topology and the like. These centers of intraspecific variations may be the origin of future species and thus, are of scientific value to be named. The question whether a formal naming is indicated for subspecies with no recognized metabolic function, host affiliation, or pathogenicity remains open, requiring a formal decision by an internally recognized body.

## Species Concept in the Era of Metagenomics

The species concept issue has been discussed extensively earlier in this chapter and elsewhere (e.g., Rosselló-Mora and Amann 2001). Here, we focus on the recent developments and the challenges remaining, giving first a brief introduction into metagenomics, which is necessary for the remaining of this section.

Metagenomics represents a new field, where the power of genomic tools such as cloning and sequencing of small pieces from a DNA sample is applied to entire communities of microbes, bypassing the need to isolate and culture community members (Handelsman et al. 2007). Thus, metagenomics can reveal the total naturally occurring diversity and provide novel insights into

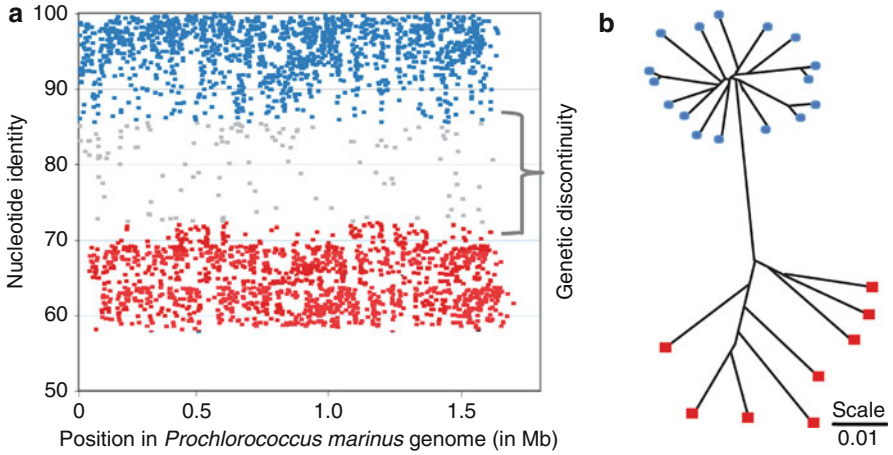


**Fig. 9.3**

***Sequence-discrete populations of photosynthetic Prochlorococcus marinus recovered in metagenomic datasets from the Sargasso Sea*. The whole metagenomic dataset from the Sargasso Sea reported by Venter and colleagues (2004) was searched against the genome sequence of *P. marinus* strain AS9601, which represents the high-light surface *Prochlorococcus*, as described in Konstantinidis and DeLong (2008). The graph represents the nucleotide identity of each metagenomic sequence that shows at least ~60 % nucleotide identity to the reference genome sequence (y-axis) plotted against the position of the genome that the metagenomic sequence maps on (x-axis) (*panel A*). The analysis reveals that *Prochlorococcus* forms a sequence-discrete population, which is composed of genotypes that show at least 90 % nucleotide identity among themselves (*blue dots*; each dot represents a different metagenomic sequence; each sequence is about 1 kb long) and are clearly divergent from their co-occurring genotypes in the sample (*red dots*). The few reads of intermediate genetic relatedness (*gray dots*) represent primarily sequence errors or artifacts and, secondarily, ecologically divergent genotypes, since under the same environmental conditions (i.e., they are co-occurring in the sample) they show substantially lower abundance based on the number of reads recovered and as explained in Konstantinidis and DeLong (2008). The sequence-discrete population is also obvious based on a phylogentic approach, using a tree built from randomly drawn fully overlapping metagenomic reads (*panel B*). Similar patterns of diversity were observed for numerous other marine microbial groups**

the gene content and function of microbial populations, including those that are resistant to laboratory cultivation. Numerous examples of successful application of metagenomics to the study of both low-diversity (e.g., biofilms) and high-diversity (e.g., complex) natural microbial communities have been conducted recently, e.g., (Tyson et al. 2004; Rusch et al. 2007; Konstantinidis and DeLong 2008). Therefore, metagenomics hold great potential to provide new insights into the most fundamental question in taxonomy, namely, whether or not the prokaryotic diversity is organized in discrete units, species.

In all metagenomic studies performed today, the microbial populations comprising the communities sampled were found to represent discrete, sequence-defined populations, with intrapopulation genomic relatedness ranging from ~94 % to ~100 % ANI depending on the population considered, at least for the abundant populations that were adequately sampled by the sequencing coverage achieved in the studies (Tyson et al. 2004; Rusch et al. 2007; Konstantinidis and DeLong 2008). The abundant populations sampled included both autotrophic and heterotrophic populations of several prokaryotic phyla, including archaeal ones, indicating that the patterns observed may be universal. *Figure 9.3* gives such an example for members of the Sargasso Sea population of *P. marinus*. Additional analyses showed that the genotypes of a population were clearly distinguishable from their closest co-occurring relatives in the community, if any, based on their relative abundance in situ and lower interpopulation ANI values (typically <85 % ANI) relative to the intrapopulation ANI. These genotypes also showed significantly smaller gene-content differences compared to the differences seen within many commonly defined bacterial species and exhibited substantial levels of genetic exchange among themselves, mediated by homologous recombination (Konstantinidis and DeLong 2008). Although more habitats (e.g., soil, sediment) and samples over time need to be analyzed before more robust interpretations can emerge, it is intriguing to hypothesize that the sequence-discrete populations identified may correspond to genuine species and represent the important units of microbial diversity. The genotypes of these populations meet several of the desirable properties expected for strains of the same species such as the genetic distinctiveness and similar ecophysiological properties. It is also interesting to point out that the areas of genetic discontinuities usually observed between sequence-discrete populations in the previous metagenomics studies (85–95 % ANI) correspond roughly to the 70 % DDH threshold, indicating that there might be an important underlying mechanism(s) that accounts for the patterns of species diversity described by taxonomists over the past few decades (see also discussion about recombination below).

In contrast, when genotypes from different populations and habitats characterized by dissimilar physicochemical properties

were compared, a genetic continuum, as opposed to sequence-discrete clusters, was frequently observed. For instance, when Konstantinidis and DeLong compared populations of ammonia oxidizing *Crenarchaea* group I from different depths in the Pacific Ocean, they recovered organisms that showed the complete range of genetic relatedness among themselves from 70 % to 100 % ANI. However, the *Crenarchaea* populations were found to be sequence-discrete within a specific depth (intrapopulation ANI values >95 %), which is consistent with independent studies suggesting that microbial life is stratified with depth in the ocean's interior (e.g., DeLong et al. 2006), resulting from the unique physicochemical properties characterizing different depths (e.g., light intensity, nutrient availability, temperature, salinity, hydrostatic pressure). A subsequent study identified several genomic adaptations that presumably account for the spatial (depth) isolation of the sequence-discrete populations such as the preferential use of specific amino acids in the protein sequences of organisms from deeper waters that maintain protein tertiary structure under high hydrostatic pressure (Konstantinidis et al. 2009). These findings confirm that in order to better understand the drivers of genetic variation in bacterial populations, within-community genomic variation should be analyzed, focusing ideally on abundant populations that are less likely to represent transient and/or allochthonous members of the community, as opposed to genotypes recovered from different populations and habitats. The findings also imply that several previous studies that have recovered continuous genetic gradient as opposed to discrete populations might have been biased by the samples analyzed and the limitation of the culture-based approaches to not distinguish between transient and abundant community members. Interestingly, Konstantinidis and DeLong (2008) also found that populations of *Crenarchaea* and photosynthetic *Prochlorococcus* from similar depths in the Pacific and Atlantic Oceans are indistinguishable from each other at the ANI level, indicating panmictic populations across the World's two largest oceans (see also Konstantinidis et al. 2009).

Several species concepts based on recombination frequency (Fraser et al. 2007) or population sweeps caused by periodic natural selection (Acinas et al. 2004; Cohan 2006) have been advanced to explain the maintenance of sequence clusters such as the sequence-discrete populations discussed above. Alternative explanations such as population bottlenecks and random birth/extinction are less favorable and probably applicable to more restricted microbial populations and habitats, such as the vertically transmitted microbial pathogens (Moran 2007), compared to the populations in open environments and complex communities discussed above. Recent studies have provided evidence in support of both of the previous two major species concepts; however, the number of populations and samples per population analyzed to date are simply too small to allow for universal conclusions to emerge. We discuss the emerging new insights and highlight the remaining open questions below.

HGT can introduce novel DNA into a genome through a homologous (the sequences are sufficiently similar to allow one displacing the other) or nonhomologous recombination mechanism. The former can potentially serve as a population cohesion mechanism if it is rampant and unbiased, similar to the role of sex in higher eukaryotes. If, on the other hand, homologous recombination is driven by selection for a few gene functions important in the environment then it is unlikely to lead to genome homogenization (except for the genes under selection) and population cohesion. Several cases of selection-driven recombination have been documented in recent high-resolution genomic studies such as for pathogenic *Campylobacter* and *Streptococcus* populations, which preferentially exchange only genes related to antibiotic resistance and evasion of host immune system (Caro-Quintero et al. 2009; Beres et al. 2009). Nonetheless, substantial levels of unbiased intrapopulation homologous recombination were detected for several sequence-discrete marine populations and populations of biofilms related to acid mine drainage, using various methods (Tyson et al. 2004; Konstantinidis and DeLong 2008). Homologous recombination frequency was also shown to drop logarithmically with increasing evolutionary divergence of the recombining sequences, particularly in the range of 80–90 % sequence nucleotide identity (Zawadzki et al. 1995; Eppley et al. 2007) that roughly corresponded to the areas of genetic discontinuities identified between sequence-discrete populations. Although these studies were based on short, error-prone metagenomic sequences, recent whole-genome-based analyses of *Shewanella baltica* strains, which co-occur in the Baltic Sea, revealed that homologous recombination was affecting (purging) more nucleotide substitution positions than those created by point mutations (sexual evolution) (Caro-Quintero et al. 2011). These findings make homologous recombination an intriguing candidate mechanism responsible for population cohesion and for maintaining the sequence-discrete populations identified in metagenomic studies. However, more quantitative data on the role of homologous recombination is needed to support this hypothesis and exclude alternatives. For instance, to what the extent the patterns seen in the *S. baltica* case represent the norm as opposed to a rare event remains currently unclear. Related to this, contemporary methods (McVean et al. 2002; Kosakovsky Pond et al. 2006) frequently vary by an order of magnitude in their estimates of recombination rates (Eppley et al. 2007; Konstantinidis and DeLong 2008). This is primarily due to the low accuracy in detecting recombination among very closely related organisms and to the fact that several important parameters such as the effective population size remain speculative for natural populations (Fraser et al. 2007).

On the other hand, the genotypes of a population could cohere together via means of a shared ecological niche, as this has been shown to be the case for several microbial populations and extensively discussed theoretically in various versions of the ecological species concept (e.g., Acinas et al. 2004; Cohan 2006; Ward 2006). It is also important to realize that values of a few percent nucleotide sequence divergence (e.g., 97–98 % ANI) correspond to long periods of time elapsed since the last common ancestor of the genomes related at this level, in the order of thousands of years (Lawrence and Ochman 1998). During such long evolutionary time, it is likely that genotypes that are more

divergent compared to the genotypes comprising the population have become extinct (e.g., by accumulating deleterious mutations), differentiated ecologically (speciated), or simply been transported to other habitats. Thus, the ecological species concept can explain the sequence-discrete populations observed in metagenomic studies; albeit direct experimental evidence in support of the previous interpretations is difficult to obtain.

Ecological niche differentiation could also lead to subpopulations, also called ecotypes (Cohan 2006), and eventually to speciation. Although the ecotype is not recognized as an official rank of the bacterial taxonomy, ecotypes are commonly defined as collections of strains that preserve the full phenotypic and ecological potential that characterizes the species and are able to exploit only a slightly different ecological niche compared to closely related species, such as growth on a new carbon substrate. Therefore, an ecotype should be expected to have only a small gene-content (or expression) difference compared with other ecotypes of the same species, or if larger differences exist, they would be encoded by unstable parts of the genome such as plasmids. The major challenge in identifying and characterizing ecotypes, and, in general, species based on the ecological species concept, is to define the ecological niche. Niche is a multidimensional space that cannot be measured precisely with current technologies; for some researchers, defining the ecological niche of microorganisms within most natural habitats on Earth represents a futile exercise. Accordingly, ecotypes are typically detected indirectly, through the phylogenetic analysis of a single or a few genes from the population(s) under scrutiny, assuming that the appearance of a distinct clade in the phylogeny (equated to ecotype) is accompanied by ecological (ecotype) differentiation. However, the genes frequently employed for such purposes are not responsible for the distinctive ecophysiological trait of the ecotype (e.g., they are housekeeping genes); thus, when the distinct clade becomes detectable in the phylogeny, it has probably diverged enough to be recognized as a distinct species rather than an ecotype. Due to the inability to define the ecological niche and the ambiguity associated with deciding when a clade should be defined as a species versus ecotype, the ecotype concept remains of limited practical use in taxonomy. With the recent developments in genome sequencing technologies, however, which allow the inexpensive identification of all genes encoded by the genome of ecotypes, and the availability of techniques such as nanoSIMS (Wagner 2009), which allow detailed measurements of many cell activities at the individual cell level in situ, it might be possible to robustly detect and characterize ecotypes in the not-too-distant future.

The recent technological developments in genomics and metagenomics have propelled the microbial systematic and phylogeny studies in a new era. We believe that it is within reach that new robust conclusions about what is the relative importance of the genetic mechanisms generating and sustaining population diversity and whether or not there is a continuum of diversity as opposed to discrete taxa will emerge soon. Until then, the current pragmatic species definition and the 16S rRNA gene-based phylogeny remain reliable and useful means to guide prokaryotic systematics.

## References


Achtman M (1998) Microevolution during epidemic spread of *Neisseria meningitidis*. Electrophoresis 19:593–596

Achtman M, Wagner M (2008) Microbial diversity and the genetic nature of microbial species. Nat Rev Microbiol 6:431–440

Acinas SG, Klepac-Ceraj V, Hunt DE, Pharino C, Ceraj I, Distel DL, Polz MF (2004) Fine-scale phylogenetic architecture of a complex bacterial community. Nature 430:551–554

Alperi A, Martínez-Murcia AJ, Monera A, Saavedra MJ, Figueras MJ (2010) *Aeromonas fluvialis* sp. nov., isolated from a Spanish river. Int J Syst Evol Microbiol 60:72–77

Amann R, Ludwig W, Schleifer KH (1988) Beta-subunit of ATP-synthase: a useful marker for studying the phylogenetic relationship of eubacteria. J Gen Microbiol 134:2815–2821

Amann RI, Lin C, Key R, Montgomery L, Stahl DA (1992) Diversity among *Fibrobacter* strains: towards a phylogenetic classification. Syst Appl Microbiol 15:23–31

Ash C, Farrow JAE, Wallbanks S, Collins MD (1991) Phylogenetic heterogeneity of the genus Bacillus revealed by comparative analysis of small-subunit ribosomal RNA sequences. Lett Appl Microbiol 13:202–206

Bachmann K (1998) Species as units of diversity: an outdated concept. Theor Biosci 117:213–230

Balch WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. Micribiol Rev 43:260–296

Barraclough TG, Hughes M, Ashford-Hodges N, Fujisawa T (2009) Inferring evolutionarily significant units of bacterial diversity from broad environmental surveys of single-locus data. Biol Lett 23:425–428

Baumann P, Baumann L, Woolkalis MJ, Bang SS (1983) Evolutionary relationships in *Vibrio* and *Photobacterium* a basis for a natural classification. Annu Rev Microbiol 37:369–398

Bautz EKF, Bautz FA (1964) The influence of non-complementary bases on the stability of ordered polynucleotides. Proc Natl Acad Sci USA 52:1476–1481

Beiko RG, Harlow TJ, Ragan MA (2005) Highways of gene sharing in prokaryotes. Proc Natl Acad Sci USA 102:14332–14337

Beres SB, Carroll RK, Shea PR, Sitkiewicz I, Martinez-Gutierrez JC et al (2009) Molecular complexity of successive bacterial epidemics deconvoluted by comparative pathogenomics. Proc Natl Acad Sci USA 107:4371–4376

Borriss R, Chen X, Rueckert C, Blom J, Becker A, Baumgarth B, Fan B, Pukall R, Schumann P, Spröer C, Junge H, Vater J, Pühler A, Klenk H-P (2010) Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM7T and FZB42: a proposal for *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* subsp. nov. and *Bacillus amyloli*quefaciens subsp. *plantarum* subsp. nov. based on their discriminating complete genome sequences. Int J Syst Evol Microbiol. First published on Sep 3, 2010 as doi: 10.1099/ijs.0.023267-0

Brenner DJ (1991) Additional genera of the *Enterobacteriaceae*. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, applications. Springer, New York, pp 2922–2937

Britten RJ, Kohne DE (1968) Repeated sequences in DNA. Science 161:529–540

Buchanan RE (1916) Studies in the nomenclature and classification of the bacteria I. The problem of bacterial nomenclature. J Bacteriol 1:591–596

Buchanan RE (1918) Studies in the nomenclature and classification of the bacteria V. Subgroups and genera of the *Bacteriaceae*. J Bacteriol 3:27–61

Buckley M, Roberts RJ (2007) Reconciling microbial systematics and genomics. Colloquium Report. The American Society for Microbiology, Washington, DC

Caro-Quintero A, Rodriguez-Castano GP, Konstantinidis KT (2009) Genomic insights into the convergence and pathogenicity factors of *Campylobacter jejuni* and *Campylobacter coli* species. J Bacteriol 191:5824–5831

Caro-Quintero A, Deng J, Auchtung J, Brettar I, Höfle MG, Klappenbach J, Konstantinidis KT (2011) Unprecedented levels of horizontal gene transfer among spatially co-occurring *Shewanella* bacteria from the Baltic Sea. ISME J 5:131–140

Cavalier-Smith T (2002) The neomuran origin of archaebacteria, the negibacterial root of the universal tree, and bacterial mega classification. Int J Syst Evol Microbiol 52:7–76

Christensen H, Kuhnert P, Busse H-J, Frederiksen WC, Bisgaard M (2007) Proposed minimal standards for the description of genera, species and subspecies of the *Pasteurellaceae*. Int J Syst Evol Microbiol 57:166–178

Cicarelli FD, Doerks T, von Mering C, Creevey CJ, Snel B, Bork P (2006) Toward automatic reconstruction of a highly resolved tree of life. Science 311: 1283–1287

Cohan FM (2002) What are bacterial species? Annu Rev Microbiol 56:457–487

Cohan FM (2006) Towards a conceptual and operational union of bacterial systematics, ecology, and evolution. Philos Trans R Soc Lond B Biol Sci 361:1985–1996

Cohan FM, Koeppel AF (2008) The origins of ecological diversity in prokaryotes. Curr Biol 18R:1024–1034

Cohn F (1872) Untersuchungen über Bacterien. Beitr Biol Pfl 2:127–224

Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P, Cai J, Hippe H, Farrow JAE (1994) The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int J Syst Bacteriol 44:812–826

Colwell RR (1970) Polyphasic taxonomy of the genus *Vibrio*: numerica taxonomy of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and related *Vibrio* species. J Bacteriol 104:410–433

Cousin S, Brambilla E, Yang J, Stackebrandt E (2008) Culturable aerobic bacteria from the upstream region of a karst water rivulet. Int Microbiol 11:91–100

Cowan ST (1968) A dictionary of microbial taxonomic usage. Oliver & Boyd, Edinburgh

Cracraft J (1983) Species concept and speciation analysis. Current ornithology. Plenum Press, New York, pp 159–187

Darwin C (1859) On the origin of species. Murray, London

De Ley J (1970) Reexamination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. J Bacteriol 101:738–754

DeLong EF, Preston CM, Mincer T, Rich V, Hallam SJ, Frigaard NU, Martinez A, Sullivan MB, Edwards R, Brito BR, Chisholm SW, Karl DM (2006) Community genomics among stratified microbial assemblages in the ocean's interior. Science 311:496–503

Dobzhansky T (1937) Genetics and the origin of species. Columbia University Press, New York

Doolittle WF, Bapteste E (2007) Pattern pluralism and the tree of life hypothesis. Proc Natl Acad Sci USA 104:2043–2049

Doolittle WF, Zhaxybayeva O (2009) On the origin of prokaryotic species. Genome Res 19:744–756

Douglas AE (1998) Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu Rev Entomol 43:17–37

Dykhuizen DE, Green L (1991) Recombination in *Escherichia coli* and the definition of biological species. J Bacteriol 173:7257–7268

Dzink JL, Sheehan MT, Socransky SS (1990) Proposal of three subspecies of *Fusobacterium nucleatum* Knorr 1922: *Fusobacterium nucleatum* subsp. *nucleatum* subsp. nov., comb. nov.; *Fusobacterium nucleatum* subsp. *polymorphum* subsp. nov., nom. rev., comb. nov.; and *Fusobacterium nucleatum* subsp. *vincentii* subsp. nov., nom. rev., comb. nov. Int J Syst Bacteriol 40:74–78

Embley MT, Stackebrandt E (1994) The molecular phylogeny and systematics of the Actinomycetes. Annu Rev Microbiol 48:257–289

Eppley JM, Tyson GW, Getz WM, Banfield JF (2007) Genetic exchange across a species boundary in the archaeal genus *Ferroplasma*. Genetics 177:407–416

Ezaki T, Hashimoto Y, Yabuuchi E (1989) Fluorimetric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol 39: 224–229

Farelly V, Rainey FA, Stackebrandt E (1995) Effect of genomic size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl Environ Microbiol 61:2798–2801

Farmer JJ III, Janda JM, Brenner FW, Cameron DN, Birkhead KM (2005) Genus 1. *Vibrio* Pacini 1854, 411AL. In: Brenner DJ, Krieg NR, Staley JR (eds) Bergey's manual systematic bacteriology: the proteobacteria, 2nd edn. Springer, New York, pp 494–546

Fowler VJ, Widdel F, Pfennig N, Stackebrandt E (1986) Phylogenetic relationships of sulfate- and sulfur-reducing eubacteria. Syst Appl Microbiol 8:32–41

Fox GE, Stackebrandt E, Hespell RB, Gibson J, Maniloff J, Dyer TA, Wolfe RS, Balch WE, Tanner R, Magrum L, Zablen L-B, Blakemore R, Gupta R, Lewis BJ, Stahl DA, Luehrsen R, Chen KN, Woese CR (1980) The phylogeny of prokaryotes. Science 209:457–463

Fox GE, Wisotzkey JD, Jurtshuk P Jr (1992) How close is close: 16S RNA sequence identity may not be sufficient to guarantee species identity. Int J Syst Bacteriol 42:166–170

Fraser C, Hanage WP, Spratt BG (2007) Recombination and the nature of bacterial speciation. Science 315:476–480

Fraser C, Alm EJ, Polz MF, Spratt BG, Hanage WP (2009) The bacterial species challenge: making sense of genetic and ecological diversity. Science 323:741–746

Garrity GM, Holt JG (2001a) Class I. *Deinococci* class. nov. In: Boone DR, Castenholz RW, Garrity GM (eds) Bergey's manual of systematic bacteriology, vol 1, 2nd edn, The *Archaea* and the deeply branching and phototrophic bacteria. Springer, New York, p 395

Garrity GM, Holt JG (2001b) Phylum BVIII. Nitrospirae phy. nov. In: Boone DR, Castenholz RW, Garrity GM (eds) Bergey's manual of systematic bacteriology. Springer, New York, pp 451–464

Garrity GM, Holt JG (2001c) Phylum BVI. Chloroflexi phy. nov. In: Boone DR, Castenholz RW, Garrity GM (eds) Bergey's manual of systematic bacteriology, vol 1, 2nd edn, The *Archaea* and the deeply branching and phototrophic bacteria. Springer, New York, pp 427–446

Garrity GM, Garrity GM, Bell JA, Lilburn T (2005) Phylum XIV. Proteobacteria phyl. nov. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM (eds) Bergey's manual of systematic bacteriology, vol 2, 2nd edn, The proteobacteria, part B: the gammaproteobacteria. Springer, New York, p 1

Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, Stackebrandt E, Van de Peer Y, Vandamme P, Thompson FL, Swings J (2005) Re-evaluating prokaryotic species. Nat Rev Microbiol 3:733–739

Gharbia SE, Shah HN (1992) *Fusobacterium nucleatum* subsp. *fusiforme* subsp. nov. and *Fusobacterium nucleatum* subsp. *animalis* subsp. nov. as additional subspecies within *Fusobacterium nucleatum*. Int J Syst Bacteriol 42:296–298

Gibbons NE, Murray RGE (1978) Proposals concerning the higher taxa of bacteria. Int J Syst Bacteriol 28:1–6

Gibson J, Ludwig W, Stackebrandt E, Woese CR (1985) The phylogeny of the green photosynthetic bacteria: absence of a close relationship between *Chlorobium* and *Chloroflexus*. Syst Appl Microbiol 6:152–156

Gogarten JP, Townsend JP (2005) Horizontal gene transfer, genome innovation and evolution. Nat Rev Microbiol 3:679–687

Goris J, Suzuki K, De Vos P, Nakase T, Kersters K (1999) Evaluation of a microplate DNA-DNA hybridization method compared to the initial renaturation method. Can J Microbiol 44:1148–1153

Goris J, Konstantinidis KT, Coenye T, Vandamme P, Tiedje JM (2007) DNA-DNA hybridization values and their relation to whole genome sequence. Int J Syst Evol Microbiol 57:81–91

Grimont PAD, Popoff MY, Grimont F, Coynault C, Lemelin M (1980) Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. Curr Microbiol 4:325–330

Gupta RS, Golding GB (1993) Evolution of HSP70 gene and its implications regarding relationships between Archaebacteria, Eubacteria and Eukaryotes. J Mol Evol 37:573–582

Gürtler V, Mayall BC (2001) Genomic approaches to typing, taxonomy and evolution of bacterial isolates. Int J Syst Evol Microbiol 51:3–16

Guttman DS, Dykhuizen DE (1994) Clonal divergence in *Escherichia coli* as a result of recombination, not mutation. Science 266:1380–1383

Handelsman J, Tiedje J, Alvarez-Cohen L, Ashburner M, Cann I, Delong E, Doolittle W, Fraser-Ligget C, Godzik A, Gordon J, Riley M, Schmidt T (2007) The news of metagenomics: revealing the secrets of our microbial planet. The National Academies Press, Washington, DC

Hara T, Shimoda T, Nonaka K, Ogata S (1991) Colorimetric detection of DNA-DNA hybridization in microdilution wells for taxonomic application on bacteria strains. J Ferment Bioeng 72:122–124

Harris LG, El-Bouri K, Johnston S, Rees E, Frommelt L, Siemssen N, Christner M, Davies AP, Rohde H, Mack D (2010) Rapid identification of staphylococci from prosthetic joint infections using MALDI-TOF mass-spectrometry. Int J Artif Organs 33:568–574

He Y, Li H, Lu X, Stratton CW, Tang YW (2010) Mass spectrometry biotyper system identifies enteric bacterial pathogens directly from colonies grown on selective stool culture media. J Clin Microbiol 48:3888–3892

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th edn. Williams & Wilkins, Baltimore

Hsieh S-Y, Tseng C-L, Lee Y-S, Kuo A-J, Sun C-F, Lin Y-H, Chen J-K (2008) Highly efficient classification and identification of human pathogenic bacteria by MALDI-TOF MS. Mol Cell Proteomics 7:448–456

Hull D (1976) Are species really individuals? Syst Zool 25:174–191

Huss VAR, Festl H, Schleifer KH (1983) Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. Syst Appl Microbiol 4:184–192

Huys G, Kämpfer P, Albert MJ, Kuhn I, Denys R, Swings J (2002) *Aeromonas hydrophila* subsp. *dhakensis* subsp. nov., isolated from children with diarrhoea in Bangladesh, and extended description of *Aeromonas hydrophila* subsp. *hydrophila* Chester 1901 Stanier 1943 Approved Lists 1980. Int J Syst Evol Microbiol 52:705–712

Iino T, Mori K, Uchino Y, Nakagawa T, Harayama S, Suzuki KI (2010) *Ignavibacterium album* gen. nov., sp. nov., a moderately thermophilic anaerobic bacterium isolated from microbial mats at a terrestrial hot spring and proposal of Ignavibacteria classis nov., for a novel lineage at the periphery of green sulfur bacteria. Int J Syst Evol Microbiol 60: 1376–1382

Imhoff JF, Süling J, Petri R (1998a) Phylogenetic relationships among the *Chromatiaceae*, their taxomonic reclassification and description of the new genera *Allochromatium*, *Halochromatium*, *Isochromatium*, *Marichromatium*, *Thiococcus*, *Thiohalocapsa* and *Thermochromatium*. Int J Syst Bacteriol 48:1129–1143

Imhoff JF, Petri R, Süling J (1998b) Reclassification of species of the spiral-shaped phototrophic purple non-sulfur bacteria of the γ-Proteobacteria: description of the genera *Phaeospirillum* gen. nov., *Rhodovibrio* gen. nov., *Rhodothalassium* gen. nov. and *Roseospiragen* nov., as well as transfer of *Rhodospirillum fulvum* to *Phaeospirillum fulvum* comb. nov., of *Rhodospirillum molischianum* to *Phaeospirillum molischianum* comb. nov., of *Rhodospirillum salinarum* to *Rhodovibrion salinarum* comb. nov., of *Rhodospirillum sodomense* to *Rhodovibrio sodomensis* comb. nov., of *Rhodospirillum salexigens* to *Rhodothalassium salexigens* comb. nov. and of *Rhodospirillum medosalinum* to *Roseospira mediosalina*. Int J Syst Bacteriol 48:793–798

Istock CA, Bell JA, Ferguson N, Istock NL (1996) Bacterial species and evolution: theoretical and practical perspectives. J Ind Microbiol 17:137–150

Johnson JL (1973) The use of nucleic acid homologies in the taxonomy of anaerobic bacteria. Int J Syst Bacteriol 23:308–315

Kandler O, König H (1985) Cell envelopes of archaebacteria. In: Woese CR, Wolfe RS (eds) Archaebacteria (The bacteria. A treatise on structure and function), vol VIII. Academic, New York, pp 413–457

Kaznowski A (1995) A method of colorimetric DNA-DNA hybridization in microplates with covalently immobilized DNA for identification of *Aeromonas* sp. Med Microbiol Lett 4:362–369

Klenk HP, Zillig W (1994) DNA-dependent RNA polymerase subunit B as a tool for phylogenetic reconstructions: branching topology of the archaeal domain. J Mol Evol 38:4420–4432

Kloos WE, Mohapatra N, Dobrogosz J, Ezell JW, Manclark CR (1981) Deoxyribonucleotide sequence relatioships among Bordetella species. Int J Syst Bacteriol 31:173–176

Kluyver AJ, van Niel CB (1936) Prospects for a natural system of classification of bacteria. Zbl Bakt Abt 2(94):369–403

Konstantinidis KT, DeLong EF (2008) Genomic patterns of recombination, clonal divergence and environment in marine microbial populations. ISME J 2:1052–1065

Konstantinidis KT, Tiedje JM (2005a) Towards a genome-based taxonomy for prokaryotes. J Bacteriol 187:6258–6264

Konstantinidis KT, Tiedje JM (2005b) Genomic insights that advance the species concept for prokaryotes. Proc Natl Acad Sci USA 102:2567–2572

Konstantinidis KT, Tiedje JM (2007) Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. Curr Opin Microbiol 105:504–509

Konstantinidis KT, Ramette A, Tiedje JM (2006) Toward a more robust assessment of intra-species diversity using fewer genetic markers. Appl Environ Microbiol 72:7286–7293

Konstantinidis KT, Braff J, Karl DM, DeLong EF (2009) Comparative metagenomic analysis of a microbial community residing at a depth of 4,000 meters at station ALOHA in the North Pacific subtropical gyre. Appl Environ Microbiol 75:5345–5355

Kosakovsky Pond SL, Posada D, Gravenor MB, Woelk CH, Frost SD (2006) Automated phylogenetic detection of recombination using a genetic algorithm. Mol Biol Evol 23:1891–1901

Lapage SP, Sneath PHA, Lessel EF, Skerman VBD, Seeliger HPR, Clark WA (eds) (1975) International code of nomenclature of bacteria (1975 revision). American Society for Microbiology, Washington, DC

Lawrence JG (2002) Gene transfer in bacteria: speciation without species? Theor Popul Biol 61:449–460

Lawrence JG, Ochman H (1998) Molecular archaeology of the *Escherichia coli* genome. Proc Natl Acad Sci USA 95:9413–9417

Lawrence JG, Retchless AC (2009) The interplay of homologous recombination and horizontal gene transfer in bacterial speciation. Methods Mol Biol 532:29–53

Lay JO Jr (2001) MALDI-TOF mass spectrometry of bacteria. Mass Spectrom Rev 20(4):172–194

Lehmann KB, Neumann R (1896) Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen Bakteriologischen Diagnostik, 1st edn. Munich J.F, Lehmann

Lester CH, Sandvang D, Olsen SS, Schønheyder HC, Jarløv JO, Bangsborg J, Hansen DS, Jensen TG, Frimodt-Møller N, Hammerum AM, Lester CH, Sandvang D, Olsen SS, Schønheyder HC, Jarløv JO, Bangsborg J, Hansen DS, Jensen TG, Frimodt-Møller N, Hammerum AM, DANRES Study Group (2008) Emergence of ampicillin-resistant *Enterococcus faecium* in Danish hospitals. J Antimicrob Chemother 62:1203–1206

Lilburn TG, Harrison SH, Cole JR, Garrity GM (2006) Computational aspects of systematic biology. Brief Bioinform 7:186–195

Lind E, Ursing J (1986) Clinical strains *of Enterobacter aggomeruns* synonyms: *Erwinia herbicola,Envinia milletiae* identified by DNA-DNA-hybridization. Acta Pathol Microbiol Scand B Microbiol Immunol 94:205–213

Linnaeus C (1753) Species plantarum. Holmiae

Maiden CJM, Dingle KC (2008) Campylobacter. In: Nachamkin I, Szymanski CM, Blaser MJ (eds) Population biology of *Campylobacter jejuni* and related organisms, 3rd edn. American Society for Microbiology Press, Washington, DC, pp 27–40

Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG (1996) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA 95:3140–3145

Margulis L (1981) Symbiosis in cell evolution. W.H. Freeman, San Francisco

Maynart-Smith J, Smith NH, O'Rourke M, Spratt BG (1993) How clonal are bacteria? Proc Natl Acad Sci USA 90:4384–4388

McVean G, Awadalla P, Fearnhead P (2002) A coalescent-based method for detecting and estimating recombination from gene sequences. Genetics 160:1231–1241

Migula W (1900) System der Bakterien. Gustav Fischer, Jena

Miller SR, Augustine S, Olson TL, Blankenship RE, Selker J, Wood AM (2005) Discovery of a free-living chlorophyll d-producing cyanobacterium with a hybrid proteobacterial/cyanobacterial small-subunit rRNA gene. Proc Natl Acad Sci USA 102:850–855

Moran NA (2007) Symbiosis as an adaptive process and source of phenotypic complexity. Proc Natl Acad Sci USA 104:8627–8633

Moreno E (1997) In search of a bacterial species definition. Rev Biol Trop 45:753–771

Naser SM, Thompson FL, Hoste B, Gevers D, Dawyndt P, Vancanneyt M, Swings J (2005) Application of multilocus sequence analysis MLSA for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. Microbiol (Reading) 151:2141–2150

O'Hara RJ (1994) Evolutionary history and the species problem. Amer Zool 34:12–22

Orla-Jensen S (1909) Die Hauptlinien des natürlichen Bakteriensystems. Zbl Bakt Abt 2(22):305–346

Peix A, Valverde A, Rivas R, Igual JM, Ramrez-Bahena M-H, Mateos PF, Santa-Regina I, Rodrguez-Barrueco C, Martnez-Molina E, Velazquez C (2007) Reclassification of *Pseudomonas aurantiaca* as a synonym of *Pseudomonas chlororaphis* and proposal of three subspecies, *P. chlororaphis* subsp. *chlororaphis* subsp. nov., *P. chlororaphis* subsp. *aureofaciens* subsp. nov., comb. nov. and *P. chlororaphis* subsp. *aurantiaca* subsp. nov., comb. nov. Int J Syst Evol Microbiol 57:1286–1290

Prévot AR (1938) Études de systématique bactérienne. Ann Inst Pasteur 60:285–307

Pringsheim EG (1923) Zur Kritik der Bakteriensystematik. Lotus 71:357–377

Prod'hom G, Bizzini A, Durussel C, Bille J, Gilbert Greub G (2011) MALDI-TOF mass spectrometry for direct bacterial identification from positive ammonium chloride lysed blood culture pellets. J Clin Microbiol. doi:10.1128/JCM.01780-09

Rademaker JLW, Hoste B, Louws FJ, Kersters K, Swings J, Vauterin L, Vauterin P, de Bruijn FJ (2000) Comparison of AFLP and rep-PCR genomic fingerprinting with DNA–DNA homology studies: Xanthomonas as a model system. Int J Syst Evol Microbiol 50:665–677

Rainey FA, Stackebrandt E (1993) Phylogenetic evidence for the relationship of *Saccharococcus thermophilus* to *Bacillus thermoglucosidasius*, *Bacillus kaustophilus* and *Bacillus stearothermophilus*. Syst Appl Microbiol 16:224–226

Relman DA, Strauss E (1999) Microbial genomes: blueprint for life. American Society for Microbiology Press, Washington, DC

Reysenbach A-L (2001) Phylum BI. Aquificae phy. nov. In: Boone DR, Castenholz RW, Garrity GM (eds) Bergey's manual of systematic bacteriology, vol 1, 2nd edn, The *Archaea* and the deeply branching and phototrophic bacteria. Springer, New York, pp 359–367

Richter M, Rossello-Mora R (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci USA 106: 19126–19131

Riley M, Buckley M (2008) Large-scale sequencing: the future of genomic sciences? American Society for Microbiology Press, Washington, DC

Rodas AM, Ferrer S, Pardo I (2005) Polyphasic study of wine *Lactobacillus* strains: taxonomic implications. Int J Syst Evol Microbiol 55:197–207

Rong X, Huang Y (2010) Taxonomic evaluation of the *Streptomyces griseus* clade using multilocus sequence analysis and DNA-DNA hybridization, with proposal to combine 29 species and three subspecies as 11 genomic species. Int J Syst Evol Microbiol 60:696–703

Rong X, Guop Y, Huag Y (2009) Proposal to reclassify the *Streptomyces albidoflavus* clade on the basis of multilocus sequence analysis and DNA-DNA hybridization, and taxonomic elucidation of *Streptomyces griseus* subsp. *solvifaciens*. Syst Appl Microbiol 32:314–322

Rossello-Mora R (2005) Updating prokaryotic taxonomy. J Bacteriol 187: 6255–6257

Rosselló-Móra R (2006) DNA-DNA reassociation methods applied to microbial taxonomy and their critical evaluation. In: Stackebrandt E (ed) Molecular identification, systematics, and population structure of prokaryotes. Springer, Berlin, pp 23–50

Rosselló-Mora R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25:39–67

Rothschild LJ, Ragan A, Coleman AW, Heywood P, Gerbi SA (1986) Are rRNA sequence comparisons the Rosetta stone of phylogeny? Cell 47(5):640

Rusch DB, Halpern AL, Sutton G, Heidelberg KB, Williamson S, Yooseph S, Wu D, Eisen JA, Hoffman JM, Remington K et al (2007) The sorcerer II global ocean sampling expedition: Northwest Atlantic through Eastern Tropical Pacific. PLoS Biol 5:e77

Scheldeman P, Goossens K, Rodriguez-Diaz M, Pil A, Goris J, Herman L, De Vos P, Logan NA, Heyndrickx M (2004) *Paenibacillus lactis* sp. nov. isolated from raw and heat-treated milk. Int J Syst Evol Microbiol 54:885–891

Schleifer K-H, Kandler O (1972) The peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36:407–477

Schleifer K-H, Stackebrandt E (1983) Molecular systematics of prokaryotes. Annu Rev Microbiol 37:143–187

Schleifer K-H, Leuteritz M, Weiss N, Ludwig W, Kirchhof G, Seidel-Rüfer H (1990) Taxonomic study of anaerobic, Gram-negative, rod-shaped bacteria from breweries: emended description of *Pectinatus cerevisiiphilus* and description of *Pectinatus frisingensis* sp. nov., *Selenomonas lacticifex* sp. nov., *Zymophilus raffinosivorans* gen. nov., sp. nov., and *Zymophilus paucivorans* sp. nov. Int J Syst Bacteriol 40:19–27

Schroeter J (1886) Microspira comma. In: Cohn F (ed) Kryprogamen-Flora von Schlesien 3:168

Seewaldt E, Schleifer K-H, Bock E, Stackebrandt E (1982) The close phylogenetic relationship of *Nitrobacter* and *Rhodopseudomonas palustris*. Arch Microbiol 131:287–290

Selander RK, Li J, Boyd F, Wang F-S, Nelson K (1994) DNA sequence analysis of the genetic structure of populations of *Salmonella enterica* and *Escherichia coli*. In: Priest FG, Ramos-Cormenzana A, Tindall R (eds) Bacterial systematics and diversity. Plenum Press, New York, pp 17–50

Shah HN, Keys CJ, Schmid O, Gharbia SE (2002) Matrix-assisted laser desorption/ionisation time of flight mass spectrometry and proteomics; a new era in anaerobic microbiology. Clin Infect Dis 35:58–64

Sibley CG, Comstock JA, Ahlquist JE (1990) DNA hybridization evidence of homoid phylogeny: a reanalysis of the data. J Mol Evol 30:202–236

Sneath PAH (1989) Analysis and interpretation of sequence data for bacterial systematics: the view of a numerical taxonomist. Syst Appl Microbiol 12:15–31

Stackebrandt E (1992) The prokaryotes. a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) Unifying phylogeny and phenotypic properties. Springer, New York, pp 19–47

Stackebrandt E (2000) The prokaryotes: an evolving electronic resource for the microbiological community. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (eds) Defining taxonomic ranks. Springer, New York, pp 29–57

Stackebrandt E (2010) Diversification and focusing: strategies of microbial culture collections. Trends Microbiol 18:283–287

Stackebrandt E, Ebers J (2006) Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 33:152–155

Stackebrandt E, Goebel BM (1994) 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, Rainey FA (1997) Phylogenetic relationships. In: Rood IJ, McClane BA, Songer JB, Titball RW (eds) The clostridia: molecular biology and pathogenesis. Academic, New York, pp 3–19

Stackebrandt E, Woese CR (1984) The phylogeny of prokaryotes. Microbiol Sci 1:117–122

Stackebrandt E (1985) The significance of "wall types" in phylogenetically based taxonomic studies on actinomycetes. In: Szabó G, Biró S. Goodfellow M (eds) Sixth Int Symp on Actinomycete Biology. Akademiai Kiado, Budapest, pp 497–606

Stackebrandt E, Embley M, Weckesser J (1988) Phylogenetic, evolutionary, and taxonomic aspects of phototrophic eubacteria. In: Olson JM, Ormerod JG, Amesz J, Stackebrandt E, Trüper HG (eds) Green photosynthetic bacteria. Plenum Press, New York, pp 201–215

Stackebrandt E, Tindall B, Ludwig W, Goodfellow M (1999) Prokaryotic diversity and systematics. In: Lengeler J, Drews G, Schlegel HG (eds) Biology of the prokaryotes. Thieme, Stuttgart, pp 675–720

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PAD, Kämpfer P, Maiden MC, Nesme X, Rossello-Mora R, Swings J, Trüper HG, Vauterin L, Ward AC, Whitman WB (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52:1043–1052

Stackebrandt E, Päuker O, Erhard M (2005) Grouping myxococci (*Corallococcus*) strains by matrix-assisted laser desorption ionization time-of-flight MALDI TOF mass spectrometry: comparison with gene sequence phylogenies. Curr Microbiol 50:71–77

Staley JT (1997) Biodiversity: are microbial species threatened? Curr Opin Biotechnol 8:340–345

Staley J (2009) The phylogenomics species concept. Microbiol Today 36:82–83

Staley JT, Krieg NR (1984) Bacterial classification I. Classification of prokaryotic organisms: an overview. In: Williams ST, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, pp 1–4

Stanier RY, van Niel CB (1941) The main outlines of bacterial classification. J Bacteriol 42:437–466

Steigerwalt AG, Fanning GR, Fife-Ashbury MA, Brenner DJ (1976) DNA relatedness among species of *Enterobacter* and *Serratia*. Can J Microbiol 22:121–137

Szabados F, Woloszyn J, Richter C, Kaase M, Gatermann SG (2010) A Biotyper 2.0 Bruker Daltonics-based score cut-off of actually 2 is superior to molecular *Staphylococcus aureus* identification. J Med Microbiol 59:787–790

Thane PR (2009) A critique of prokaryotic species concepts. Methods Mol Biol 532:379–395

Thompson CC, Thompson FL, Vicente ACP (2008) Identification of *Vibrio cholerae* and *Vibrio mimicus* by multilocus sequence analysis MLSA. Int J Syst Evol Microbiol 58:617–621

Tindall BJ, Rosselló-Móra R, Busse H-J, Ludwig W, Kämpfer P (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. Int J Syst Evol Microbiol 60:249–266

Turenne CY, Collins DM, Alexander DC, Behr MA (2008) *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* are independently evolved pathogenic clones of a much broader group of *M. avium* organisms. J Bacteriol 190:2479–2487

Tyson GW, Chapman J, Hugenholtz P, Allen EE, Ram RJ, Richardson PM, Solovyev VV, Rubin EM, Rokhsar DS, Banfield JF (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 428:37–43

Ullman JS, McCarthy BJ (1973) The relationship between mismatched base pairs and the thermal stability of DNA duplexes. Biochim Biophys Acta 294:416–424

van Baar BL (2000) Characterisation of bacteria by matrix-assisted laser desorption/ionisation and electrospray mass spectrometry. FEMS Microbiol Rev 24:193–219

van Berkum P, Terefework Z, Paulin L, Suomalainen S, Lindström K, Eardly BD (2003) Discordant phylogenies within the *rrn*l of rhizobia. J Bacteriol 185:2988–2998

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D et al (2004) Environmental genome shotgun sequencing of the Sargasso sea. Science 304:66–74

Wagner M (2009) Single-cell ecophysiology of microbes as revealed by Raman microspectroscopy or secondary ion mass spectrometry imaging. Annu Rev Microbiol 63:411–429

Ward DM (1998) A natural species concept for prokaryotes. Curr Opin Microbiol 1:271–277

Ward DM (2006) A macrobiological perspective on microbial species. Microbe 1:269–278

Walker DH (1989) Rocky Mountain spotted fever: a new disease in need of microbiological concern. Clin Microbiol Rev 2:227–240

Wayne L, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Trüper HG (1987) International committee on systematic bacteriology: report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37:463–464

Winslow C-EA, Broadhurst J, Buchanan RE (1920) The families and genera of the bacteria. J Bacteriol 3:191–229

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271

Woese CR, Debrunner-Vossbrinck B, Oyaizu H, Stackebrandt E, Ludwig W (1985) Gram-positive bacteria: possible photosynthetic ancestry. Science 229:762–765

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains archaea, bacteria and eucaryas. Proc Natl Acad Sci USA 87:4576–4579

Wolf YI, Rogozin IB, Grishin NV, Tatusov RL, Koonin EV (2001) Genome trees constructed using five different approaches suggest new major bacterial clades. BMC Evol Biol 1:8–28

Yanagida F, Suzuki K-I, Kozaki M, Komagata K (1997) Proposal of *Sporolactobacillus nakayamae* subsp. *nakayamae* sp. nov., subsp. nov., *Sporolactobacillus nakayamae* subsp. *racemicus* subsp. nov., *Sporolactobacillus terrae* sp. nov., *Sporolactobacillus kofuensis* sp. nov., and *Sporolactobacillus lactosus* sp. nov. Int J Syst Bacteriol 47:499–504

Zawadzki P, Roberts MS, Cohan FM (1995) The log-linear relationship between sexual isolation and sequence divergence in *Bacillus* transformation is robust. Genetics 140:917–932

Zhu Y, Fournier PE, Eremeeva M, Raoult D (2005) Proposal to create subspecies of *Rickettsia conorii* based on multi-locus sequence typing and an emended description of *Rickettsia conorii*. BMC Microbiol 5:11. doi:10.1186/1471-2180-5-11

Ziemke F, Höfle MG, Lalucat J, Rosello-Mora R (1998) Reclassification of Owen's genomic group II as *Shewanella baltica* sp. nov. Int J Syst Bacteriol 48:179–186

Zuckerkandl E, Pauling L (1965) Molecules as documents of evolutionary history. J Theor Biol 8:357–366

# 10 Population Genetics

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## Introduction and Background

Molecular data come in waves. The first nucleotide-sequence-based population studies on bacteria were published in the late 1980s and early 1990s and typically focused on one or two gene sequences from around a dozen bacterial isolates (DuBose et al. 1988; Milkman and Bridges 1990; Nelson et al. 1991; Nelson and Selander 1992, 1994; Boyd et al. 1994; ). Despite the modest size of these datasets by contemporary standards, these early works laid the foundation for a number of interrelated debates: (1) the extent to which bacterial evolution is tree-like or network-like, (2) the search for a meaningful "species" concept, (3) biogeography and the extent to which "everything is everywhere," and (4) the roles of selection (ecological adaptation) and neutrality (drift) in shaping population structure. More specifically, data generated during the late 1980s and early 1990s provided unequivocal evidence that at least some species experience high rates of genome-wide homologous recombination. These early data also brought into sharp relief the importance (and difficulties) in assembling representative population samples in bacteria (Smith et al. 1993; Fleischmann et al. 1995).

The generation of complete genome sequences for major human pathogens from 1995 onward (Fleischmann et al. 1995) was of immediate practical benefit for population biology and epidemiological studies, in that it enabled primer design for any gene present on the sequenced strain. The contemporaneous advances of capillary-based automated sequencers and the ability to maintain and readily access databases on the Internet provided the means not only to sequence several gene loci for large population samples (100s of isolates), but also to instantly interrogate these data from anywhere in the world. The development of Multilocus Sequence Typing (MLST) in 1998 aimed to exploit these advances for pathogenic bacteria (Maiden et al. 1998; Maiden 2006). Although clear sampling biases have meant that the dual questions of epidemiological surveillance and population biology have, at times, been uncomfortable bedfellows (Maiden 2006), these datasets have significantly impacted on both fronts.

Microarray technology represented another important advance. Combined with the rapidly increasing genomics datasets, this approach confirmed a fundamental duality within bacterial chromosomes, the stable "core genome" versus the dynamic "accessory" or "non-core" genome (Feil 2004). For any given taxon (e.g., named species), the core genome is defined simply as all those genes universally present in all strains. In contrast, the non-core genome is composed of genes variably present or absent. Microarray data revealed that the non-core genome is not randomly interspersed throughout the chromosome, but is largely confined to regions of high gene content variability, and that the locations of these variable regions are the same in all strains (Alm and Trust 1999; Dorrell et al. 2001; Fitzgerald et al. 2001). Complete genome sequencing revealed a second key feature of the non-core genome, that of atypical base composition (GC content), GC skew, and codon bias, which implicates extrachromosomal origins and the role of mobile genetic elements (Ochman et al. 2000). A useful analogy is to consider the core genome as the cell's "operating system" (encoding housekeeping metabolism and DNA processing), and the non-core genome as software modules providing specialized functionality (*Fig. 10.1*). Thus, the non-core genome can play a major phenotypic role (e.g., by conferring antibiotic resistance or virulence traits) (Holden et al. 2004a), but the rapidity by which such genes can be gained or lost may make these accessory genes poor markers for reconstructing evolutionary history (Turner and Feil 2007).

Molecular typing protocols such as MLST represent pragmatic trade-offs between inter-strain discrimination, the amenability of the data to evolutionary analyses, and the time and expense required to characterize large population samples. The

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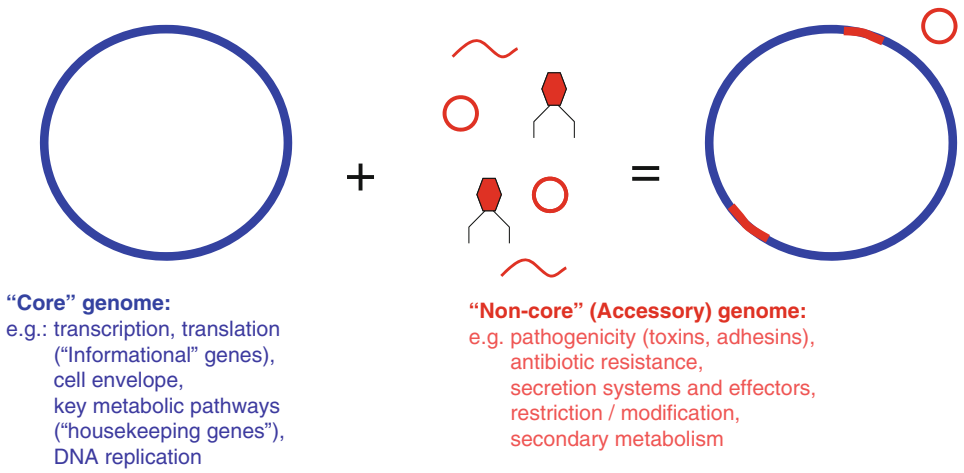


**Fig. 10.1**

**The duality of the bacterial genome into core and accessory genes. The "accessory" genes are acquired by horizontal transfer, a process which usually involves a vector such as a plasmid or phage. Such genes can help the recipient bacterium to exploit new niches; thus this model supports a saltational view of bacterial evolution**

optimal solution depends both on the taxon being considered, and on the aims of the study; there is no universally "correct" typing method. For MLST a small sample of core genes (typically seven) are sequenced for each isolate, and each strain is defined on the basis of the combined alleles observed over all loci. Freely available databases house MLST data for thousands of isolates for many major pathogens (Chan et al. 2001). While this approach has been very successful for large-scale epidemiological surveillance and for understanding migration rates and evolutionary dynamics, it has two serious drawbacks. First, as MLST is based exclusively on a small sample of core genes, it is blind to large-scale genome rearrangements and changes in the non-core genome. A striking example of this is provided by a comparison between two related species: *Burkholderia pseudomallei* (an opportunistic pathogen of man) and *B. mallei* (an equine pathogen). While phylogenetic analysis based on the MLST data suggested that *B. mallei* represents a specialized "clone" nested within the *B. pseudomallei* population (Gevers et al. 2005), comparative genomics revealed that the former had undergone extensive genome degradation (the deletion of around 1 Mb of DNA) and revealed a complete loss of synteny with *B. pseudomallei* (Holden et al. 2004b). These dramatic changes reveal much about the ecological and molecular dynamics underpinning the emergence of *B. mallei*, but would have been completely missed on the basis of MLST data alone.

The second drawback of MLST is that a small sample of seven (or so) core genes provides insufficient discrimination for highly monomorphic species (recently reviewed in (Achtman 2008)) or, similarly, for single clonal complexes within more diverse species. Cases where a single "type" predominates on a local scale present a pertinent problem for epidemiological surveillance, as the majority of isolates recovered are indistinguishable by standard procedures. Alternative techniques based on hypervariable repeat regions have been established for some monomorphic species such as *Mycobacterium* sp.; it is not always clear to what extent the patterns of divergence in the repeat loci are homoplasious, or conflict in some other way with the rare single nucleotide polymorphisms (SNPs) present elsewhere in the genome (Smith et al. 2003, 2006).

Full genome sequencing will, of course, provide ultimate discriminatory power, but until recently it has not been possible to generate genome-wide datasets for large population samples. Latest advances in next-generation sequencing technology are set to close this technology gap. Platforms, such as the Illumina Genome Analyzer (IGA), Roche 454, and SOLiD, provide the means to identify genome-wide SNPs for large population samples. Below we discuss how this technology has been recently applied to the important pathogen *Staphylococcus aureus*, and how the high discriminatory power of the data can be used in an epidemiological setting. We will go on to emphasize how these data might also provide information on more fundamental questions like how ecological (or epidemiological) specialisms of specific clones might impact on short-term molecular evolution.

## The Population Structure of *Staphylococcus aureus*

*S. aureus* is a low-GC Gram-positive bacterium which asymptomatically colonizes the skin and anterior nares (nostrils) of approximately one third of the human population at any given point in time (Peacock et al. 2001; Nulens et al. 2005; van Belkum 2006). The species is also commonly recovered from domesticated animals, particularly cows, pigs, and chickens (Vanderhaeghen et al. 2010), though the true ecological range

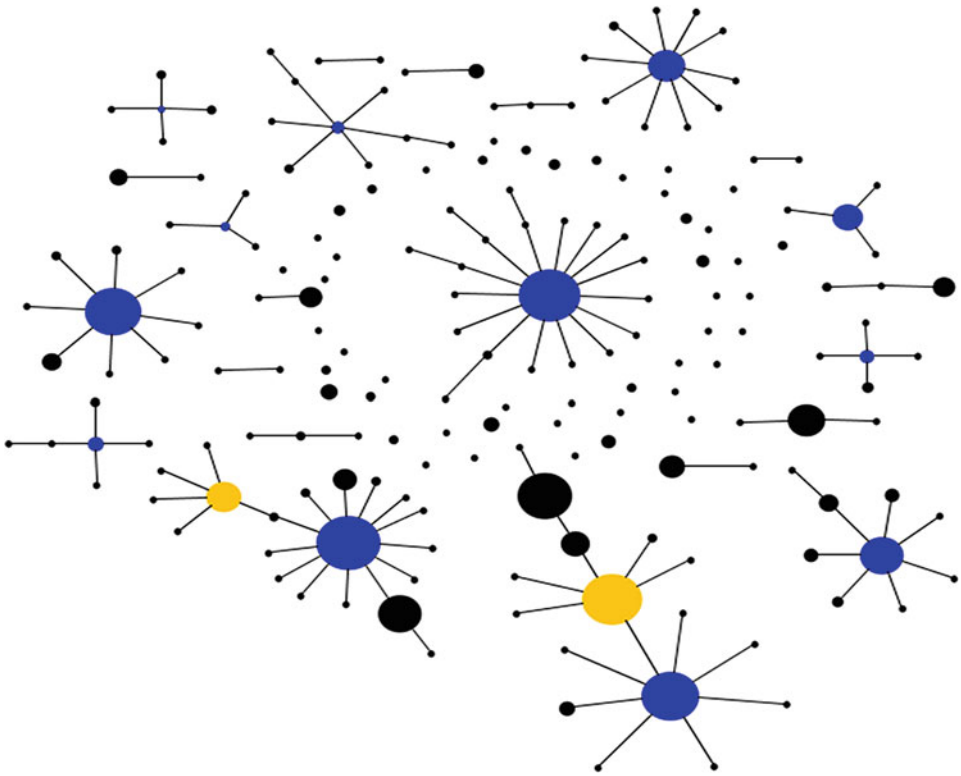

**Fig. 10.2**
**Simplified clonal population structure of *S. aureus* visualized using MLST/eBURST (www.mlst.net/eburst.mlst.net). Each *circle* represents a unique MLST haplotype. The size of the *circle* represents the frequency of the haplotype. Haplotypes differing by only one of the seven loci are linked. The vast majority of isolates correspond to a small number of haplotypes and clusters of haplotypes. Distances, angles, and positions have no meaning**

in wild animals and the environment is not known. Infection by *S. aureus* can cause a number of conditions ranging in severity from boils to life-threatening endocarditis. Serious infections are much more common within health-care settings, such as hospitals and nursing homes, where disease management is substantially hampered by the spread of resistance to β-lactam antibiotics (initially penicillin, then methicillin-resistant *S. aureus*; MRSA). Resistance to methicillin is conferred via the horizontal acquisition of a large (20–60 Kb) chromosomal cassette (*SCC*mec), which is thought to have been introduced from naturally resistance commensal staphylococcal species on multiple occasions (Enright et al. 2002). In recent years, sporadic infection by MRSA has become more common in the community, outside of health-care settings (Deleo et al. 2010).

Several typing methods have been used for epidemiological surveillance of this species, including pulse-field gel electrophoresis (PFGE), MLST (Cookson et al. 2007), and MLVA (multiple loci VNTR analysis, where VNTR stands for variable number of tandem repeats) (Melles et al. 2009; Schouls et al. 2009). VNTR loci are hypervariable microsatellites, the most notable example in *S. aureus* being the *spa* gene which has been used extensively for typing studies in this species (Mellmann et al. 2008; Basset et al. 2009). While these methods present a range of utility for more detailed evolutionary analyses, they (more or less) consistently delimit the *S. aureus* population in to the same discrete clusters, or clonal complexes. These can be visualized using UPGMA dendrograms or a simple clustering algorithm called BURST, implemented as the freely available eBURST (Feil et al. 2004) (*Fig. 10.2*) or goeBURST (Francisco et al. 2009). As it is based on nucleotide sequence data, MLST data is also amenable to phylogenetic analysis. These analyses have also revealed that hospital-acquired (HA-) MRSA isolates are particularly clonal, with a very small number of clusters accounting for almost all the cases of infection worldwide(Crisostomo et al. 2001; Aires de Sousa et al. 2005; Gomes et al. 2006; Conceicao et al. 2007). Methicillin-sensitive *S. aureus* (MSSA) are more diverse, as are community-acquired (CA-) MRSA isolates. This has led to the view that HA-MRSA strains, which are more likely to be multiply resistant than CA-MRSA strains and are relatively rare outside of health-care settings, are specifically adapted to the hospital environment.

*S. aureus* experiences homologous recombination at relatively low frequency. This is thought to explain in part why discrete sub-clusters have emerged and been maintained in the population (Feil et al. 2003), and why it is possible to reconstruct relatively robust phylogenies (Cooper and Feil 2006), at least in comparison to the naturally transformable and frequently recombining species, such as *Neisseria*

*meningitidis* (Maiden 2008). Although there is some evidence for phage specificity in different clusters (Waldron and Lindsay 2006), the evidence that these clusters present barriers to gene flow, and hence equate to biological species, remains equivocal. Discounting the recently emerged (and massively over-sampled) HA-MRSA subgroups, the adaptive relevance of clusters within the broader population remains unclear. Nevertheless, the consistent delineation of the same clonal complexes, even from gene content data generated using microarrays, underline that they are real biological entities, and as such are both of evolutionary interest and of epidemiological utility (Turner and Feil 2007).

Taking the species as a whole, MLST, MLVA, and PFGE all do an excellent job in assigning isolates to one or other of these groups, and in revealing the changing frequencies of the clusters over time and space (over years and decades, and on both national and international scales). Considering HA-MRSA, typically a very small number of clones tend to predominate at a given location at any given point in time, a result of sequential waves of infection (de Lencastre et al. 2007). This epidemiological pattern lessens the utility of MLST and other typing schemes to track strain transmission patterns on local scales (within and between hospitals) over short time scales (weeks and months) because isolates belonging to a single clonal group are often very difficult to distinguish. However, the typing data clearly reveals minor variation within clonal complexes, usually manifest as single point mutation on the basis of MLST, occasionally different *spa* types, and minor banding pattern changes by PFGE. Thus, more powerful sequencing studies should be able to dissect out sufficient variation within clonal complexes in order to provide clues as to the emergence, spread, and demise of these clusters, and to understand evolutionary processes in bacteria over very short time periods. Although next-generation sequencing platforms might not currently be available for routine epidemiological surveillance, such data will greatly facilitate the development of rapid and cheap typing protocols based on PCR assays tailored for specific lineages which may provide a useful stop-gap until such a time (say, between 5 and 10 years) when next-generation sequencing is commonplace.

## Zooming in on Single *S. aureus* Clones: ST5

Initial studies aimed at increasing the resolution of MLST by supplementing the data with hypervariable loci (Robinson et al. 2005; Kuhn et al. 2006) provided further evidence in support of low rates of recombination, and facilitated the reconstruction of more robust phylogenetic trees, but shed little extra light on the patterns of diversification within clonal complexes over time and space. A leap forward was taken by Nubel et al. (2008), who used a mutation discovery procedure based on denaturing high-performance liquid chromatography (dHPLC) to identify SNPs in ~45.5 Kb (1.6% of the genome) in 135 *S. aureus* isolates from 22 countries. All the isolates were identical by MLST (corresponding to the multilocus sequence haplotype ST5). The authors detected sufficient variation to reveal striking geographical structuring, indicating the presence of local variants. This study, therefore, points to the potential of using high-resolution sequence data to reconstruct local transmission dynamics, and to reveal microevolutionary changes as clones expand locally. An important caveat is that the rate of immigration of variants from other locales should be sufficiently low so as not to obscure local patterns of migration and diversification. As "local" might mean anything from a single hospital ward to a whole country, and rates of diversification and short- and long-range migration patterns may vary widely between taxa, so it is unlikely that there will be any general rules concerning the utility of high-resolution data for understanding transmission routes, or how this is likely to change with epidemiological scale.

The data of Nubel et al. also pointed to multiple independent acquisitions of the *SCC*mec element responsible for methicillin resistance, even within ST5. This argued against the prevailing model of the rapid global dissemination of a few MRSA strains in favor of a model of frequent emergence of "home-grown" locally restricted MRSA clones, many of which belong to the same MLST-defined haplotype owing to local clonal expansion. This has important implications concerning the high mobility of the *SCC*mec element, and its wide distribution within staphylococcal reservoirs. This observation also has a practical relevance, in that genome-wide SNP data should resolve cohesive subtypes within ST5, each corresponding to independent acquisitions of *SCC*mec, which could potentially be distinguished by tailor-made typing protocols based on PCR. A more recent paper using similar methodology focused on one such sub-cluster within the broader ST5 group, called ST225 (Nubel et al. 2010). The universal presence of a defining deletion within *SCC*mec showed that this cluster corresponds to a single acquisition of this element, and the authors were able to posit that this sub-group was introduced into central Europe from the USA approximately a decade ago, and has subsequently spread rapidly between hospitals.

## Zooming in on Single *S. aureus* Clones: ST239

One of the earliest HA-MRSA clones, and currently the most dominant globally is called ST239. This haplotype evolved via a homologous recombination event between unrelated *S. aureus* strains (i.e., belonging to different clonal complexes) which resulted in the replacement of ~20% of the recipient genome (>600 Kb) (Robinson and Enright 2004). This event remains the largest single homologous replacement in bacteria described in the literature and arose only once via a completely unknown mechanism (*Fig. 10.3*). This makes this hybrid clade unusual in that it can be defined by a single and unambiguous marker. Any strain exhibiting this replacement can be confidently assumed to have descended from the original recipient. Further, as "reversals" are implausible, any isolate that does not exhibit this replacement can be excluded (Feil et al. 2008). Although the occasional de novo point mutation means that not all members of this clade are identical by MLST, for ease of terminology we will use the term ST239 to include all

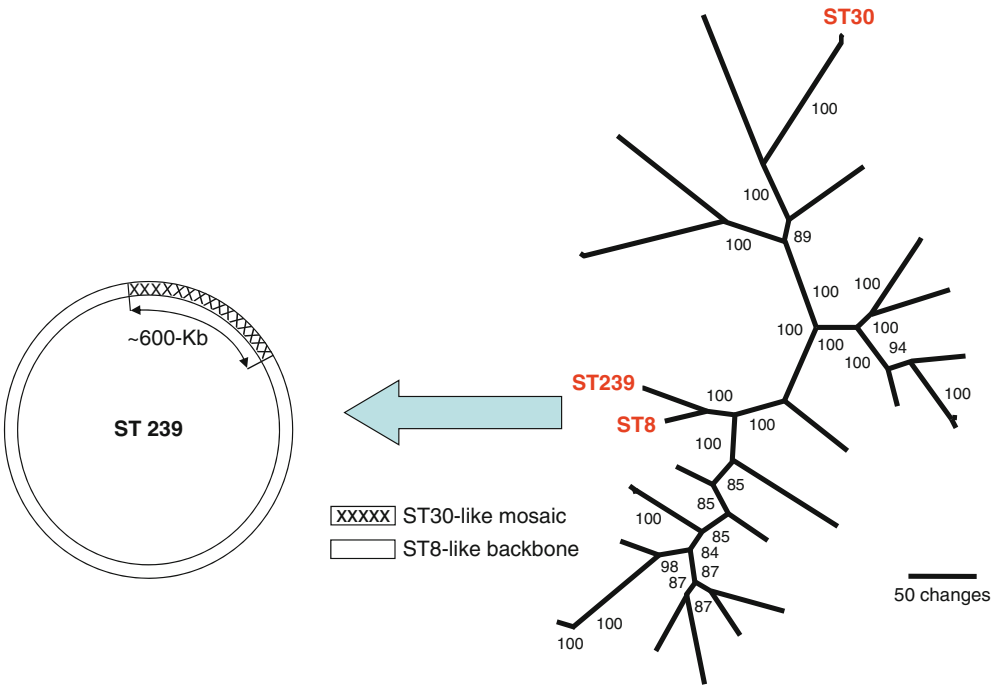


**Fig. 10.3**

**A representative phylogeny of *S. aureus* generated using concatenated data from 40 gene loci (17.8 Kb), reconstructed using MrBayes. Values at the nodes are posterior probabilities (see Cooper and Feil 2006). The hybrid ST239 genome is the result of a large-scale recombination event between unrelated lineages ST8 and ST30 (marked on the tree). ~600 Kb of the ST8 genome has been replaced by the homologous region from an ST30-like donor (see Robinson and Enright 2004). Only one example of each of the major clonal complexes depicted in *Fig. 10.2* is included**

members of this clade as defined by the presence of the large replacement.

All ST239 isolates are resistant to methicillin (MRSA) and all isolates possess the same "type" (variant) of *SCC*mec cassette (although minor *SCC*mec variation is detected). This again points to common ancestry, and is not consistent with multiple independent acquisitions of *SCC*mec within this clade. The specific type common to all ST239 isolates is known as type III which, at over 60-Kb, is the largest *SCC*mec cassette yet described in *S. aureus*. Type III *SCC*mec elements confer multiple resistance, and have only been observed in ST239 isolates. ST239 is responsible for ~90% of hospital-acquired MRSA infection throughout most of mainland Asia (from the Middle East to China), and much of South America (including Brazil) (Diekema et al. 2001; Chongtrakool et al. 2006; Xu et al. 2009). However, similar to ST225, ST239 is almost exclusively observed within hospital environments. This is thought to be because the large *SCC*mec cassette confers a fitness cost which renders it uncompetitive in the community. This implicates direct hospital-to-hospital transmission as playing a key role in its global dissemination.

ST239 is also unusual in that there is both epidemiological and experimental evidence pointing to increased virulence (Amaral et al. 2005; Edgeworth et al. 2007). ST239 was also the predominant MRSA clone in Western Europe during the 1980s and 1990s but has subsequently been replaced by other strains (although it is still common in Eastern Europe) (Conceicao et al. 2007). Following an outbreak in a London hospital (Edgeworth et al. 2007), a variant of ST239 called TW20 was sequenced (Holden et al. 2010). The clinical significance, widespread dissemination, availability of a reference sequence, and unusual evolution and epidemiology of ST239 makes it an ideal candidate for high-resolution analysis. Smyth et al. identified all the mutations within ~15 Kb of sequence of 111 ST239 isolates representing 34 years and 29 countries (Smyth et al. 2010). Again, these authors noted geographical structuring on a continental scale, and identified European, South American, and Asian sub-clades. These data also pointed to repeated and independent deletions within the *SCC*mec element during diversification of the ST239 clade, but (unlike ST5) there was no evidence for multiple acquisitions of completely different SCCmec types.

## A New Dawn for Next-Generation Sequencing

The ST239 clade was also chosen as the focus for the first population study which utilized the Illumina Genome Analyzer (IGA) platform to identify genome-wide SNPs and INDELs compared to TW20 as a reference sequence. Harris and

colleagues used index adapters to create individually tagged genomic libraries in order to rapidly generate whole-genome DNA sequence data for a large number of isolates (Harris et al. 2010). The authors characterized 62 ST239 isolates, 42 of which were globally representative, while the remaining 20 were isolated from a single hospital in northeast Thailand over a 7-month period. The reference TW20 strain was also re-sequenced as a control. The study was thus designed to simultaneously address both extremes of epidemiological scale: the global diversity of this clone and the utility of next-generation sequencing for highly localized epidemiology within a single health-care setting.

The study identified 6,714 high-quality SNPs, but these were not equally distributed throughout the genome. Regions of high density SNPs were clearly identified, and these also tended to correspond to regions with relatively low coverage. These diverse regions represented the non-core genome, principally consisting of mobile genetic elements (MGEs), such as *SCC*mec, genomic islands, conjugative elements, and prophage. Harris et al. defined core regions simply and conservatively as all regions >1 Kb which were mapped to a high quality in all isolates. This definition provides a subtly different set of genes assigned as "accessory" than previous microarray and comparative genomic studies on the broader *S. aureus* population. For example, any accessory elements present in the founding ST239 genome which had been stably inherited were assigned as core, whereas in contrast any small INDELs which have arisen since the emergence of ST239 will mean that the corresponding region will be assigned as non-core, even though they may be "native" to the genome.

The vast majority of those SNPs thus assigned as corresponding to non-core regions are likely to have been acquired by horizontal transfer, as has been confirmed subsequently (Castillo-Ramirez et al. 2011). For this reason, they were excluded from the phylogenetic analysis, leaving 4,310 variable sites in the core genome.

Similar to the studies mentioned above, Harris et al. noted striking geographical structuring in the data from their tree, which was rooted using ST8, the original recipient of the large replacement (Harris et al. 2010). The increased resolution provided by IGA also allowed a number of specific inferences concerning the emergence and spread of ST239. First, it is apparent that European isolates are more diverse than Asian or South American isolates. This observation points to a European origin of ST239, which is consistent with the fact that it was first recorded in this continent, but has subsequently been largely replaced, at least in Western Europe. Second, isolates from S. America are extremely homogenous, despite the fact that they represent Brazil, Chile, Argentina, and Uruguay and were recovered from 1993 to 1998. This strongly points to a single introduction of ST239 into South America followed by dramatic spread. Third, no two isolates were identical even when excluding the non-core genome, and this includes isolates recovered days apart from the same ward in Sappasithiprasong hospital in northeast Thailand. Furthermore, five isolates were differentiated by only 14 SNPs, and these were isolated within a few weeks and from adjacent wards in the same block in the hospital. This observation supports the possibility that this approach could provide information on transmission chains even at the scale of a single hospital, which has clear implications for infection control.

More large-scale transmission events were clearly evident in the data of Harris et al. Portugal experienced two waves of infection due to ST239 during the 1990s. Isolates recovered from the second wave showed subtle band differences by PFGE from the first wave and were more similar to isolates from Brazil than those previously isolated from Portugal. The latter clone from Portugal had been previously dubbed the "Brazillian clone" on the basis of the PFGE profiles (Sanches et al. 1998), and the three Portuguese isolates from this second wave included in the study of Harris et al. clearly cluster with the South American isolates. This then confirmed that this second wave of infection in Portugal was seeded from South America. More surprisingly, the TW20 reference genome, which originated from a recent outbreak in an Intensive Care Unit in London (Edgeworth et al. 2007), clustered with the Thai isolates, thus implicating a hitherto unexpected Southeast Asian origin of this outbreak strain.

## A Paucity of Homoplasies

A common feature of all the datasets discussed thus far, and also for those of some monomorphic species, such as *Salmonella* Typhi (Roumagnac et al. 2006; Holt et al. 2008), is the lack of phylogenetic conflict in the data due to homoplasy (at least, once the non-core regions have been excluded). While this is of obvious benefit in enabling easy and robust tree construction, does it betray a deeper significance? Homoplasies are character states (in this case SNPs) that are observed in unrelated lineages, and there are three possible means by which they might be generated. The first is mutational reversal in other sequences, such that homoplasies are in fact identical by descent but have been lost in intervening sequences. In the context of the current discussion, this scenario is so unlikely it can be effectively discounted. The second possibility is that identical mutational events may arise de novo independently in different lineages. The third possibility is recombination, in this case SNPs emerge de novo by mutation in one lineage and are then horizontally transferred to other lineages.

Harris et al. noted only 38 homoplasies among the 4310 SNPs in the core genome (<0.1%) (Harris et al. 2010). Although recombination is known to be rare in *S. aureus*, it does occasionally happen, the large replacement in ST239 being a notable example. Given the unlikely alternatives, it is, therefore, reasonable to assume that at least some of the observed homoplasies have arisen through recombination, and this is supported simply by the physical clustering of these SNPs. Harris et al. also considered the selective consequences of the observed homoplasies, and noted that almost a third of them correspond to mutations known to confer antibiotic resistance. This means they will confer a strong selective advantage and be much

more likely to be observed than neutral or slightly deleterious changes, which are more likely to be quickly lost through drift. Indeed, an important message from the study of Harris et al. is that examining homoplasies is likely to be a powerful means to identify changes which confer an adaptive advantage, and in particular those conferring drug resistance. Homoplasies can, therefore, be explained either by occasional recombination or by positive selection. Occasionally, these forces may work in concert through hitch-hiking. For example, in the data of Harries et al. four synonymous homoplasies clustering within approximately 1 Kb are noted. These are likely to have hitch-hiked with two mutations conferring trimethoprim resistance which were located 200–300 bp away.

## Dating with Confidence

The discussion above hints at a discrepancy in the rate of molecular evolution over extremely short time scales even when compared to more modestly related lineages within the named species. The relative paucity of homoplasies within the ST239 clone suggests that recombination rates in *S. aureus* might be lower within ST239 than in the broader population. However, an equivalent interpretation is that recombination rates are similar, or even slightly higher (as might be expected given the high sequence identity), but that mutation rates are much higher. The estimation of mutation rates (hence dating the emergence of clades) represents a central theme in the high-resolution studies of bacterial populations currently being published, and (at least for *S. aureus*) a clear consensus is emerging. The three intraclonal *S. aureus* studies discussed above propose strikingly similar figures: $3.3–4.6 \times 10^{-6}$ per site per year (Smyth et al. 2010), $2.5–4.0 \times 10^{-6}$ (Harris et al. 2010), and $1.2 \times 10^{-6}$ to $2.9 \times 10^{-6}$ (Nubel et al. 2010). Harris et al. dated the emergence of ST239 to the mid-to-late 1960s, whereas the date estimate of Smyth et al. was approximately a decade earlier. Harris et al. also noted that this rate equates to approximately 1 SNP every 6 weeks (in the core genome), and Smyth et al. similarly opined that this clone is "measurably evolving."

The date estimates given above are approximately 100-fold higher than the standard estimate of $3 \times 10^{-8}$ for *E. coli* (Achtman et al. 1999), but approximately 10-fold slower than estimates for *Campylobacter jejuni* (Wilson et al. 2009), *Helicobacter pylori* (Falush et al. 2001), and *Neisseria gonorrhoeae* (Perez-Losada et al. 2007). If we cancel out generation times by assuming they are similar between species (although they are in fact notoriously difficult to measure for natural populations) then the faster estimates for these latter species are still easily explained by the fact that they all recombine at a much higher frequency than *S. aureus*. This means that the majority of the mutations observed in these studies will not have arisen de novo but will have been horizontally acquired. However, this does not explain why all these estimates are so fast relative to the canonical estimate for *E. coli* reflects. The key to understanding this is the fact that these estimates were based on extremely closely related isolates, and there is an apparent acceleration of mutation rate as one moves toward the very tip of the trees (Balbi and Feil 2007). This effect has recently been discussed in terms of two categories: a mutation rate and a substitution rate (Achtman 2008; Nubel et al. 2010). The mutation rate is simply the rate at which mutations are generated de novo. The high rate of change within the groups and subgroups described is assumed to approximate this rate. The substitution rate refers to all the mutations which have subsequently become fixed within the population; this is necessarily much slower than the mutation rate, as most mutations will be lost by selection or drift.

Although classically correct, this interpretation remains something of an oversimplification. Prokaryotes do not conform to idealized sexual (eukaryotic) populations, and what is true for elephants is not always true for *E. coli*.

Difficulties arise because it is not always obvious where the boundary of a "population," as defined by the limits of drift, should lie. One is inclined to use the (often pragmatically) named species to define such a population, but the assumption that these two boundaries actually coincide is rarely, if ever, explicitly examined. Indeed, despite the recent soul searching on the nature of bacterial "species" (Hanage et al. 2006), the less loaded term "population" continues to be deployed rather casually. To put the problem succinctly, it is currently not clear whether the term "fixation" (or "substitution") should apply only to those mutations which are present in all strains in the named species, or whether the concept may also apply on the level of clonal complex. In the highly monomorphic species, such as *Bacillus anthracis* or *Mycobacterium tuberculosis*, the question is simple as named species equates to a single clonal lineage, and likely a single "ecotype." In such cases, there is little justification for further "splitting." However, in more diverse species, such as *S. aureus*, *E. coli*, or *N. meningitidis*, there is substantial substructuring, that is to say, a number of different clonal lineages which may represent different adaptive peaks, and hence different populations. One might then consider a type of "hierarchical fixation," encompassing different phylogenetic levels from clonal complex, major clades of related complexes, and whole species. It follows that every level should also correspond to a different rate of change, getting progressively slower as one moves back in the tree, and representing intermediate rates between "mutation" and "substitution." This is an important question, as dating estimates form a central plank of the analyses in re-sequencing studies. Although the relationship between "mutation" rate and divergence has not yet been examined at the intraspecies level for bacteria, one might predict (based on the studies discussed below) a log-linear relationship, such that there is a rapid drop of rate moving back from the most fine-scale clusters.

## The Purging of Deleterious Mutations Over Time

The decrease in the rate of change moving back from the tips of trees is due to the accumulative purging of slightly deleterious mutations. Purifying selection acts as a sieve in removing these

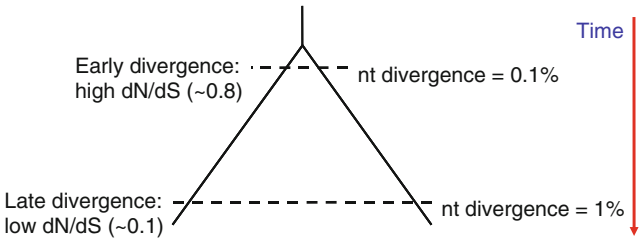


**Fig. 10.4**

**The selective purging of slightly deleterious non-synonymous mutations over time results in a decrease in the dN/dS ratio (see Rocha et al. 2006). The figures of divergence and dN/dS given are approximate for *S. aureus*. Early divergence corresponds to comparisons within a clonal complex, and late divergence corresponds to comparisons between unrelated clonal complexes**

mutations from the population, but this purging does not occur instantaneously; the excess mutations observed at the very tips of the tree represent those slightly deleterious changes destined for subsequent loss. According to the nearly neutral model, the efficiency of purifying selection is determined by the selection coefficient($s$) and the effective population size ($N_e$). If $N_e s < 1$, then the chances of the mutation becoming fixed are predicted to be as if the mutation were neutral, otherwise they are predicted to be slightly deleterious and stand a lower chance of becoming fixed. The progressive purging of slightly deleterious mutations can be easily plotted over time. As de novo non-synonymous mutations are on average more likely to be slightly deleterious than synonymous mutations, they should be enriched between very closely related genomes but preferentially removed over time (*Fig. 10.4*). Rocha et al. confirmed this effect by demonstrating that dN/dS ratio between pairs of genomes decreases with increasing divergence (Rocha et al. 2006), and this effect has subsequently been confirmed by other studies (Hughes et al. 2008; Kryazhimskiy and Plotkin 2008; Garcia Pelayo et al. 2009; Novichkov et al. 2009). Furthermore, Rocha et al. showed by simulation that the trajectory of this decline is dependent upon both $N_e$ and $s$, as predicted under the nearly neutral theory.

It is important to note that there is no reason to suppose that this effect is reserved for non-synonymous changes. Although the dN/dS ratio is a very convenient metric, the same principle should apply to any kind of slightly deleterious change relative to a more neutral counterpart. A curious, yet striking, example may be the maintenance of GC content in bacterial genomes. Comparative genomics analyses of a large range of bacterial taxa have revealed that GC → AT mutations are far more common than the reverse (Hershberg and Petrov 2010) (Hildebrand et al. 2010). This bias is so strong that at mutational equilibrium the base composition of most bacterial genomes would rest at ~20% GC. As base composition is typically much higher than this, there must be some force which acts to favor GC over AT. As recently discussed, a number of selective explanations have been proposed, as well as the purely mechanistic explanation of biased gene transfer (a phenomenon yet to be described for bacteria) (Rocha and Feil 2010).

Whatever force is responsible for the maintenance of GC content in bacterial genomes, in certain taxa this force appears to be operating very weakly, if at all. Insect endosymbionts, such as *Buchnera aphidicola*, are characterized by very high AT contents and very high dN/dS ratios, both of which may be consistent with weak purging of slightly deleterious mutations (Moran et al. 2009). Such species are transmitted from mother to offspring transovarially down the generations (vertically), a lifestyle that imposes repeated bottlenecking, thus lowering the $N_e$. However, as such a specialization also results in ecological isolation and hence an absence of recombination, the high AT content would also be predicted by a lack of biased gene conversion. For this reason, high AT endosymbiont genomes do not currently constitute equivocal evidence for a selective maintenance of high GC content in other, more typical, genomes.

The fact that the GC content in most bacterial genomes resides at a point higher than predicted by mutation pressure alone suggests that GC → AT mutations must be lost over time more frequently than AT → GC mutations, in much the same way that non-synonymous mutations are lost preferentially to synonymous mutations. Balbi et al. examined the selective purging over time of both non-synonymous mutations and GC → AT mutations (relative to their counterparts) through a comparative genomic analysis of *Escherichia coli* and *Shigella* (Balbi et al. 2009). The $N_e$ of *E. coli* can be assumed to be very large, as this is a very generalist species, which occupies a wide range of animal guts (usually without harm to the host), and is able to survive in soil and water (Hartl et al. 1994). The four *Shigella* named "species" are the causative agents of bacillary dysentery and are essentially specialized clones of *E. coli* that have independently acquired a large "invasion" plasmid which confers on them the ability to invade host cells and replicate intracellularly (Lan and Reeves 2002). This adaptation to a specialized lifestyle might be accompanied by a reduction in $N_e$, in a way somewhat analogous to "island" populations of eukaryotes (Johnson and Seger 2001; Balbi and Feil 2007).

Balbi et al. examined the molecular evolutionary consequences of this niche restriction by comparing both the proportions of GC → AT polymorphisms over the reverse and dN/dS in the *E. coli* and *Shigella* genomes. They confirmed two key predictions: (1) that slightly deleterious mutations (synonymous or GC → AT) are selectively purged over time, and (2) slightly deleterious mutations are enriched in *Shigella* relative to *E. coli* (when divergence time is considered). *Figure 10.5* reveals a log-linear purging of two types of slightly deleterious mutations over time A: GC- > AT mutations; B: Non-synonymous mutations. In both cases, this purging is less marked in *Shigella* than it is in *E. coli*. Note that only fourfold degenerate sites are used for the GC → AT analysis, hence these two effects are independent. This also means that if a selective force is responsible for the maintenance of GC content, it operates on synonymous (and intergenic) sites, so is not directly related to amino-acid sequence. These observations, therefore, challenge the widely held view that synonymous changes can be assumed to be

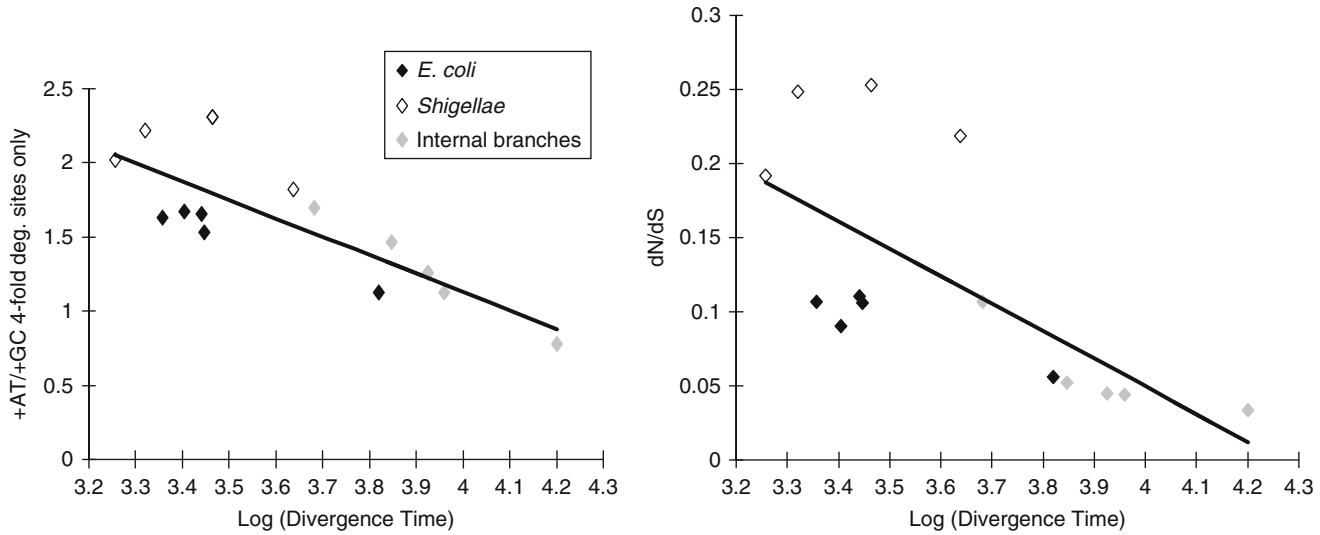


**Fig. 10.5**

**The selective purging of GC → AT mutations and non-synonymous mutations (relative to their counterparts) over time in *Shigella* and *E. coli*. The effect is weaker in *Shigella* than in *E. coli* because the former has a smaller effective population size. "Divergence time" is measured as the total number of SNPs, and the relationships are log-linear (see Balbi et al. 2009)**

neutral. Transversions behave in the same way (relative to transitions), but this is because transversions are more likely to be non-synonymous (Balbi et al. 2009).

## Synthesizing Selection and Ecology Using Next-Generation Sequence Data

The relative preponderance of non-synonymous changes between very closely related genomes, with subsequent purification of these SNPs over time, has been repeatedly confirmed for *S. aureus* and a number of other species (Larsson et al. 2009), (Holt et al. 2008), (He et al. 2010). However, the increased resolution afforded by next-generation sequencing will reveal the dynamics of this process in greater detail. Revisiting the data of Harris et al., Castillo-Ramirez et al. plotted the dN/dS of ST239 isolates against divergence for the core and non-core genomes separately (Castillo-Ramirez et al. 2011). They noted that non-synonymous SNPs were proportionately far more common in the core genome than the non-core genome. Furthermore, although the dN/dS ratio declined against divergence when non-core SNPs were considered, no obvious purging of non-synonymous changes over time was noted in the core SNPs, apart from a steep initial decrease owing to the rapid purging of a highly deleterious class. The authors suggest that the relative enrichment of non-synonymous changes in the core, relative to the non-core, reflects the fact that many of the SNPs in the non-core genome have been acquired via horizontal transfer, while those in the core genome have arisen by de novo mutation. This means that the core SNPs are, on average, younger and (by the argument outlined above) should, therefore, contain a higher proportion of non-synonymous mutations. This effect is apparent from ❯ *Fig. 10.6* where regions of high SNP density (corresponding to various mobile non-core elements) show a relative enrichment of synonymous changes relative to the core genome. Essentially, those SNPs acquired by horizontal transfer are more likely to be synonymous because they have already passed through a selective filter in the wider population. An analysis of the large replacement present in ST239 supports this argument, and a similar enrichment of synonymous changes within recombined blocks is noted in *Clostridium difficile* as noted using the data of He et al. (Castillo-Ramirez et al. 2011) (He et al. 2010).

In the study by Balbi et al. on *Shigella* genomes described above, it was noted that the decline in dN/dS over time was moderated in *Shigella* relative to *E. coli* because ecological specialization has resulted in a decrease in the effective population size and hence weaker purifying selection (Balbi et al. 2009). Such a model provides a direct bridge between ecology and molecular evolution, which may also have a bearing on the analysis of the ST239 *S. aureus* clone. As discussed, this clone is highly specialized to the hospital environment, and competes very poorly in the community, probably as a consequence of the high fitness cost associated with multiple drug resistance and a very large *SCC*mec element. This then raises the possibility that this ecological specialism will similarly reduce the power of purifying selection in this clone, leading to an accumulation of slightly deleterious mutations. In other words, it is possible that hospitals may be acting as ecological islands. Such a process could be of key importance in understanding the change in clonal composition of a bacterial population over time. Most attempts to understand clonal replacement, where one clone within a given locale rapidly disappears and another takes its place, begin from the perspective of trying to understand what it is that makes a new clone successful. In this context, it may also be fruitful to consider what makes old clones less successful; that

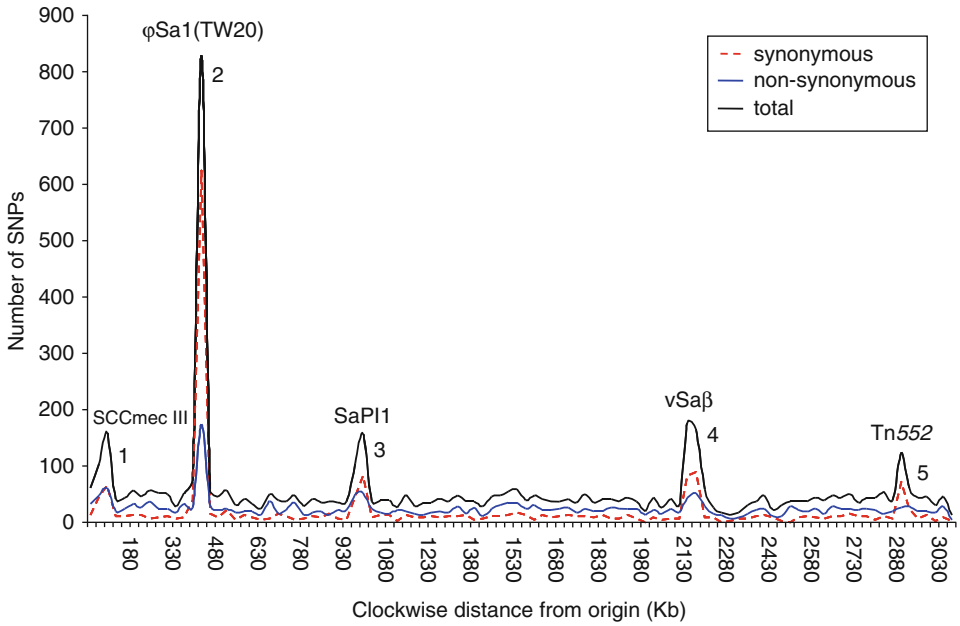


**Fig. 10.6**

**The distribution of SNPs across the TW20 (reference) genome. The five peaks of high SNP density correspond to well-characterized components of the accessory genome; SCCmec III (a resistance casette), *φ*Sa1(TW20) (a prophage), SaPI1 (a pathogenicity island), *ν*Saβ (a genomic island), and Tn*552* (a transposon). The figure illustrates that for peaks 2–5 synonymous SNPs (*dashed line*) are more common than non-synonymous SNPs, whereas the reverse is true for regions between the peaks. Other well-characterized elements known to be present in the TW20 genome do not correspond to a clear peak of SNP density because they are less variable between ST239 isolates**

is, to what extent clones can consolidate their advantage in a population over time in the face of deleterious mutations, and whether the rapid spread of new "successful" clones is inevitably self-limiting. This may have some bearing on the ongoing decline and replacement of *S. aureus* ST239 in many parts of Europe and Asia, and such a model is consistent with the apparently weak purifying selection acting on this clone evidence by the poor purging of non-synonymous mutations.

## Concluding Remarks

Next-generation sequencing clearly heralds a new dawn in bacterial microevolution. Similar to previous developments, such as MLST, initial studies using the new technology have (for obvious reasons) focused on pathogenic bacteria, with a view to increased power of epidemiological surveillance and understanding patterns of transmission. However, here we have considered how these data might also provide information on basic evolutionary and population-level processes and the molecular consequences of lifestyle changes. These future directions are certain to impact on our understanding of the ecology of environmental species, as well as the epidemiology of important pathogens. A consideration of the strength of purifying selection, and how this relates to the ecology of the clone//species, may even help to define the limits of drift, thus finally providing a conceptual basis for identifying population (and hence "species") boundaries. As the trajectories depend both on $N_e$ and $s$, so comparisons may also be drawn between different mutation types, or between different classes of genes. Many other analytical approaches can be taken with these data, for example to understand the dynamics of gene loss and gain, the size of the accessory gene "library" from which any strain of a given population may access, and how evolutionary processes vary according to genomic position. A comprehensive recent study on multiple *E. coli* genomes provides an excellent template for such studies (Touchon et al. 2009).

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## References

Achtman M (2008) Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. Annu Rev Microbiol 62:53–70

Achtman M, Zurth K, Morelli G, Torrea G, Guiyoule A, Carniel E (1999) Yersinia pestis, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. Proc Natl Acad Sci USA 96:14043–14048

Aires de Sousa M, Conceicao T, Simas C, de Lencastre H (2005) Comparison of genetic backgrounds of methicillin-resistant and -susceptible *Staphylococcus aureus* isolates from Portuguese hospitals and the community. J Clin Microbiol 43:5150–5157

Alm RA, Trust TJ (1999) Analysis of the genetic diversity of *Helicobacter pylori*:the tale of two genomes. J Mol Med 77:834–846

Amaral MM, Coelho LR, Flores RP, Souza RR, Silva-Carvalho MC, Teixeira LA, Ferreira-Carvalho BT, Figueiredo AM (2005) The predominant variant of the Brazilian epidemic clonal complex of methicillin-resistant Staphylococcus aureus has an enhanced ability to produce biofilm and to adhere to and invade airway epithelial cells. J Infect Dis 192(5):801–810

Balbi KJ, Feil EJ (2007) The rise and fall of deleterious mutation. Res Microbiol 158:779–786

Balbi KJ, Rocha EP, Feil EJ (2009) The temporal dynamics of slightly deleterious mutations in *Escherichia coli* and *Shigella* spp. Mol Biol Evol 26:345–355

Basset P, Hammer NB, Kuhn G, Vogel V, Sakwinska O, Blanc DS (2009) *Staphylococcus aureus* clfB and spa alleles of the repeat regions are segregated into major phylogenetic lineages. Infect Genet Evol 9:941–947

Boyd EF, Nelson K, Wang FS, Whittam TS, Selander RK (1994) Molecular genetic basis of allelic polymorphism in malate dehydrogenase (mdh) in natural populations of *Escherichia coli* and *Salmonella enterica*. Proc Natl Acad Sci USA 91:1280–1284

Castillo-Ramirez S, Harris SR, Holden MT, He M, Parkhill J, Bentley SD, Feil EJ (2011) The impact of recombination on dN/dS within recently emerged bacterial clones. PLoS Pathog 7:e1002129

Chan MS, Maiden MC, Spratt BG (2001) Database-driven multi locus sequence typing (MLST) of bacterial pathogens. Bioinformatics 17:1077–1083

Chongtrakool P, Ito T, Ma XX, Kondo Y, Trakulsomboon S, Tiensasitorn C, Jamklang M, Chavalit T, Song JH, Hiramatsu K (2006) Staphylococcal cassette chromosome mec (SCCmec) typing of methicillin-resistant Staphylococcus aureus strains isolated in 11 Asian countries: a proposal for a new nomenclature for SCCmec elements. Antimicrob Agents Chemother 50 (3):1001–1012

Conceicao T, Aires-de-Sousa M, Fuzi M, Toth A, Paszti J, Ungvari E, van Leeuwen WB, van Belkum A, Grundmann H, de Lencastre H (2007) Replacement of methicillin-resistant *Staphylococcus aureus* clones in Hungary over time: a 10-year surveillance study. Clin Microbiol Infect 13:971–979

Cooper JE, Feil EJ (2006) The phylogeny of *Staphylococcus aureus*—Which genes make the best intra-species markers? Microbiology 152:1297–1305

Cookson BD, Robinson DA, Monk AB, Murchan S, Deplano A, de Ryck R, Struelens MJ, Scheel C, Fussing V, Salmenlinna S et al (2007) Evaluation of molecular typing methods in characterizing a European collection of epidemic methicillin-resistant Staphylococcus aureus strains: the HARMONY collection. J Clin Microbiol 45(6):1830–1837

Crisostomo MI, Westh H, Tomasz A, Chung M, Oliveira DC, de Lencastre H (2001) The evolution of methicillin resistance in *Staphylococcus aureus*: similarity of genetic backgrounds in historically early methicillin-susceptible and -resistant isolates and contemporary epidemic clones. Proc Natl Acad Sci USA 98:9865–9870

de Lencastre H, Oliveira D, Tomasz A (2007) Antibiotic resistant *Staphylococcus aureus*: a paradigm of adaptive power. Curr Opin Microbiol 10:428–435

Deleo FR, Otto M, Kreiswirth BN, Chambers HF (2010) Community-associated methicillin-resistant *Staphylococcus aureus*. Lancet 375:1557–1568

Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN, Beach M (2001) Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. Clin Infect Dis 32(Suppl 2):S114–132

Dorrell N, Mangan JA, Laing KG, Hinds J, Linton D, Al-Ghusein H, Barrell BG, Parkhill J, Stoker NG, Karlyshev AV et al (2001) Whole genome comparison of Campylobacter jejuni human isolates using a low-cost microarray reveals extensive genetic diversity. Genome Res 11(10):1706–1715

DuBose RF, Dykhuizen DE, Hartl DL (1988) Genetic exchange among natural isolates of bacteria: recombination within the phoA gene of *Escherichia coli*. Proc Natl Acad Sci USA 85:7036–7040

Edgeworth JD, Yadegarfar G, Pathak S, Batra R, Cockfield JD, Wyncoll D, Beale R, Lindsay JA (2007) An outbreak in an intensive care unit of a strain of methicillin-resistant *Staphylococcus aureus* sequence type 239 associated with an increased rate of vascular access device-related bacteremia. Clin Infect Dis 44:493–501

Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG (2002) The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc Natl Acad Sci USA 99:7687–7692

Falush D, Kraft C, Taylor NS, Correa P, Fox JG, Achtman M, Suerbaum S (2001) Recombination and mutation during long-term gastric colonization by *Helicobacter pylori*: estimates of clock rates, recombination size, and minimal age. Proc Natl Acad Sci USA 98:15056–15061

Feil EJ (2004) Small change: keeping pace with microevolution. Nat Rev Microbiol 2:483–495

Feil EJ, Cooper JE, Grundmann H et al (2003) How clonal is *Staphylococcus aureus*? J Bacteriol 185:3307–3316

Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol 186:1518–1530

Feil EJ, Nickerson EK, Chantratita N et al (2008) Rapid detection of the pandemic methicillin-resistant *Staphylococcus aureus* clone ST 239, a dominant strain in Asian hospitals. J Clin Microbiol 46:1520–1522

Fitzgerald JR, Monday SR, Foster TJ, Bohach GA, Hartigan PJ, Meaney WJ, Smyth CJ (2001) Characterization of a putative pathogenicity island from bovine Staphylococcus aureus encoding multiple superantigens. J Bacteriol 183(1): 63–70

Fleischmann RD, Adams MD, White O et al (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 269:496–512

Francisco AP, Bugalho M, Ramirez M, Carrico JA (2009) Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. BMC Bioinformatics 10:152

Garcia Pelayo MC, Uplekar S, Keniry A et al (2009) A comprehensive survey of single nucleotide polymorphisms (SNPs) across *Mycobacterium bovis* strains and *M. bovis* BCG vaccine strains refines the genealogy and defines a minimal set of SNPs that separate virulent *M. bovis* strains and *M. bovis* BCG strains. Infect Immun 77:2230–2238

Gevers D, Cohan FM, Lawrence JG et al (2005) Opinion: re-evaluating prokaryotic species. Nat Rev Microbiol 3:733–739

Gomes AR, Westh H, de Lencastre H (2006) Origins and evolution of methicillin-resistant *Staphylococcus aureus* clonal lineages. Antimicrob Agents Chemother 50:3237–3244

Hanage WP, Fraser C, Spratt BG (2006) Sequences, sequence clusters and bacterial species. Philos Trans R Soc Lond B Biol Sci 361:1917–1927

Harris SR, Feil EJ, Holden MT et al (2010) Evolution of MRSA during hospital transmission and intercontinental spread. Science 327:469–474

Hartl DL, Moriyama EN, Sawyer SA (1994) Selection intensity for codon bias. Genetics 138:227–234

He M, Sebaihia M, Lawley TD et al (2010) Evolutionary dynamics of *Clostridium difficile* over short and long time scales. Proc Natl Acad Sci USA 107:7527–7532

Hershberg R, Petrov DA (2010) Evidence that mutation is universally biased towards AT in bacteria. PLoS Genet 6:e1001115

Hildebrand F, Meyer A, Eyre-Walker A (2010) Evidence of selection upon genomic GC-content in bacteria. PLoS Genet 6:e1001107

Holden MT, Feil EJ, Lindsay JA et al (2004a) Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci USA 101:9786–9791

Holden MT, Titball RW, Peacock SJ et al (2004b) Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. Proc Natl Acad Sci USA 101:14240–14245

Holden MT, Lindsay JA, Corton C, Quail MA, Cockfield JD, Pathak S, Batra R, Parkhill J, Bentley SD, Edgeworth JD (2010) Genome sequence of a recently emerged, highly transmissible, multi-antibiotic- and antiseptic-resistant variant of methicillin-resistant *Staphylococcus aureus*, sequence type 239 (TW). J Bacteriol 192:888–892

Holt KE, Parkhill J, Mazzoni CJ et al (2008) High-throughput sequencing provides insights into genome variation and evolution in Salmonella Typhi. Nat Genet 40:987–993

Hughes AL, Friedman R, Rivailler P, French JO (2008) Synonymous and nonsynonymous polymorphisms versus divergences in bacterial genomes. Mol Biol Evol 25:2199–2209

Johnson KP, Seger J (2001) Elevated rates of nonsynonymous substitution in island birds. Mol Biol Evol 18:874–881

Kryazhimskiy S, Plotkin JB (2008) The population genetics of dN/dS. PLoS Genet 4:e1000304

Kuhn G, Francioli P, Blanc DS (2006) Evidence for clonal evolution among highly polymorphic genes in methicillin-resistant *Staphylococcus aureus*. J Bacteriol 188:169–178

Lan R, Reeves PR (2002) *Escherichia coli* in disguise: molecular origins of Shigella. Microbes Infect 4:1125–1132

Larsson P, Elfsmark D, Svensson K, Wikstrom P, Forsman M, Brettin T, Keim P, Johansson A (2009) Molecular evolutionary consequences of niche restriction in *Francisella tularensis*, a facultative intracellular pathogen. PLoS Pathog 5:e1000472

Maiden MC (2006) Multilocus sequence typing of bacteria. Annu Rev Microbiol 60:561–588

Maiden MC (2008) Population genomics: diversity and virulence in the neisseria. Curr Opin Microbiol 11:467–471

Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA et al (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA 95(6):3140–3145

Melles DC, Schouls L, Francois P, Herzig S, Verbrugh HA, van Belkum A, Schrenzel J (2009) High-throughput typing of *Staphylococcus aureus* by amplified fragment length polymorphism (AFLP) or multi-locus variable number of tandem repeat analysis (MLVA) reveals consistent strain relatedness. Eur J Clin Microbiol Infect Dis 28:39–45

Mellmann A, Weniger T, Berssenbrugge C, Keckevoet U, Friedrich AW, Harmsen D, Grundmann H (2008) Characterization of clonal relatedness among the natural population of *Staphylococcus aureus* strains by using spa sequence typing and the BURP (based upon repeat patterns) algorithm. J Clin Microbiol 46:2805–2808

Milkman R, Bridges MM (1990) Molecular evolution of the *Escherichia coli* chromosome. III. Clonal frames. Genetics 126:505–517

Moran NA, McLaughlin HJ, Sorek R (2009) The dynamics and time scale of ongoing genomic erosion in symbiotic bacteria. Science 323:379–382

Nelson K, Selander RK (1992) Evolutionary genetics of the proline permease gene (putP) and the control region of the proline utilization operon in populations of *Salmonella* and *Escherichia coli*. J Bacteriol 174:6886–6895

Nelson K, Selander RK (1994) Intergeneric transfer and recombination of the 6-phosphogluconate dehydrogenase gene (gnd) in enteric bacteria. Proc Natl Acad Sci USA 91:10227–10231

Nelson K, Whittam TS, Selander RK (1991) Nucleotide polymorphism and evolution in the glyceraldehyde-3-phosphate dehydrogenase gene (gapA) in natural populations of *Salmonella* and *Escherichia coli*. Proc Natl Acad Sci USA 88:6667–6671

Novichkov PS, Wolf YI, Dubchak I, Koonin EV (2009) Trends in prokaryotic evolution revealed by comparison of closely related bacterial and archaeal genomes. J Bacteriol 191:65–73

Nubel U, Roumagnac P, Feldkamp M et al (2008) Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*. Proc Natl Acad Sci USA 105:14130–14135

Nubel U, Dordel J, Kurt K et al (2010) A timescale for evolution, population expansion, and spatial spread of an emerging clone of methicillin-resistant *Staphylococcus aureus*. PLoS Pathog 6:e1000855

Nulens E, Gould I, MacKenzie F, Deplano A, Cookson B, Alp E, Bouza E, Voss A (2005) Staphylococcus aureus carriage among participants at the 13th European Congress of Clinical Microbiology and Infectious Diseases. Eur J Clin Microbiol Infect Dis 24(2):145–148

Ochman H, Lawrence JG, Groisman EA (2000) Lateral gene transfer and the nature of bacterial innovation. Nature 405:299–304

Peacock SJ, de Silva I, Lowy FD (2001) What determines nasal carriage of Staphylococcus aureus? Trends Microbiol 9(12):605–610

Perez-Losada M, Crandall KA, Zenilman J, Viscidi RP (2007) Temporal trends in gonococcal population genetics in a high prevalence urban community. Infect Genet Evol 7:271–278

Robinson DA, Enright MC (2004) Evolution of *Staphylococcus aureus* by large chromosomal replacements. J Bacteriol 186:1060–1064

Robinson DA, Monk AB, Cooper JE, Feil EJ, Enright MC (2005) Evolutionary genetics of the accessory gene regulator (agr) locus in *Staphylococcus aureus*. J Bacteriol 187:8312–8321

Rocha EP, Feil EJ (2010) Mutational patterns cannot explain genome composition: are there any neutral sites in the genomes of bacteria? PLoS Genet 6: e1001104

Rocha EP, Smith JM, Hurst LD, Holden MT, Cooper JE, Smith NH, Feil EJ (2006) Comparisons of dN/dS are time dependent for closely related bacterial genomes. J Theor Biol 239:226–235

Roumagnac P, Weill FX, Dolecek C et al (2006) Evolutionary history of *Salmonella typhi*. Science 314:1301–1304

Sanches IS, Saraiva ZC, Tendeiro TC, Serra JM, Dias DC, de Lencastre H (1998) Extensive intra-hospital spread of a methicillin-resistant staphylococcal clone. Int J Infect Dis 3:26–31

Schouls LM, Spalburg EC, van Luit M, Huijsdens XW, Pluister GN, van Santen-Verheuvel MG, van der Heide HG, Grundmann H, Heck ME, de Neeling AJ (2009) Multiple-locus variable number tandem repeat analysis of *Staphylococcus aureus*: comparison with pulsed-field gel electrophoresis and spa-typing. PLoS One 4:e5082

Smith JM, Smith NH, O'Rourke M, Spratt BG (1993) How clonal are bacteria? Proc Natl Acad Sci USA 90:4384–4388

Smith NH, Dale J, Inwald J, Palmer S, Gordon SV, Hewinson RG, Smith JM (2003) The population structure of *Mycobacterium bovis* in Great Britain: clonal expansion. Proc Natl Acad Sci USA 100:15271–15275

Smith NH, Gordon SV, de la Rua-Domenech R, Clifton-Hadley RS, Hewinson RG (2006) Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis*. Nat Rev Microbiol 4:670–681

Smyth DS, McDougal LK, Gran FW, Manoharan A, Enright MC, Song JH, de Lencastre H, Robinson DA (2010) Population structure of a hybrid clonal group of methicillin-resistant *Staphylococcus aureus*, ST239-MRSA-III. PLoS One 5:e8582

Touchon M, Hoede C, Tenaillon O et al (2009) Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. PLoS Genet 5:e1000344

Turner KM, Feil EJ (2007) The secret life of the multilocus sequence type. Int J Antimicrob Agents 29:129–135

van Belkum A (2006) Staphylococcal colonization and infection: homeostasis versus disbalance of human (innate) immunity and bacterial virulence. Curr Opin Infect Dis 19(4):339–344

Vanderhaeghen W, Hermans K, Haesebrouck F, Butaye P (2010) Methicillin-resistant *Staphylococcus aureus* (MRSA) in food production animals. Epidemiol Infect 138:606–625

Waldron DE, Lindsay JA (2006) Sau1: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. J Bacteriol 188:5578–5585

Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, Bolton E, Fox A, Hart CA, Diggle PJ, Fearnhead P (2009) Rapid evolution and the importance of recombination to the gastroenteric pathogen *Campylobacter jejuni*. Mol Biol Evol 26:385–397

Xu BL, Zhang G, Ye HF, Feil EJ, Chen GR, Zhou XM, Zhan XM, Chen SM, Pan WB (2009) Predominance of the Hungarian clone (ST 239-III) among hospital-acquired meticillin-resistant Staphylococcus aureus isolates recovered throughout mainland China. J Hosp Infect 71(3): 245–255

# 11 Public Service Collections and Biological Resource Centers of Microorganisms

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**Abbreviations** *ACM,* The Asian Consortium for the Conservation and Sustainable Use of Microbial Resources (http://www.nbrc.nite.go.jp/e/project01-e.html), a network of Asian culture collections and BRCs (see *Box 11.5*).; *ATCC™,* American Type Culture Collection; Manassas; VA, USA.; *BCC,* Biotech culture collection, Bangkok, Thailand.; *BCCM™/LMG,* Belgian Co-Ordinated Collections of Micro-Organisms, Laboratorium voor Microbiologie, University Gent, Belgium.; *BCCUSP,* Brazilian Cyanobacteria Collection, University Sao Paulo, Brazil.; *BRC,* In the context of this chapter defined as a microbial Biological Resource Center (sensu OECD), a CC running under a defined quality management system which yet needs to be agreed upon by the stakeholders.; *CABI,* CAB International, Egham, UK; *CABRI,* Common Access to Biological Resources and Information (www.cabri.org), a EU-funded network of eight European Collections (1996–1999), (http://www.cabri.org).; *CBD,* Convention on Biological Diversity (http://www.cbd.int/), a global agreement addressing all aspects of biological diversity: genetic resources, species, and ecosystems. Their protection, sustainable use and access to including benefit sharing of the advantages arising from their use.; *CBS,* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.; *CC,* In the context of this chapter defined as a microbial Culture Collection, a general term of a facility accessioning and maintaining microbial resources (prokaryotes, fungi, yeast), DNA, plasmids, phages, and material derived therefrom. Public Culture Collections provide this material to users. For a comprehensive list of abbreviations see WDCM and http://www.bacterio.cict.fr/collections.html.; *CCAP,* Culture Collection of Algae and Protozoa, Scottish Marine Institute, Oban, Argyll, UK.; *CCMM,* Moroccan Coordinated Collections of Micro-organisms, Morocco.; *CCMP,* Culture Collection of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine USA.; *CCTCC,* Chinese Center for Type Cultures Collections, Wuhan University, Wuhan, Hubei, China.; *CCUG,* Culture Collection of the University of Göteborg, Institute of Clinical Bacteriology, Immunology, and Virology, Göteborg, Sweden.; *CECT,* Colección Española de Cultivos Tipo, Valencia, Spain.; *CGMCC,* China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, PR China.; *CIP,* Collection of the Institut Pasteur, Paris, France; *CPCC,* Canadian Phycological Culture Center (formerly known as UTCC), University of Waterloo, Waterloo, ON, Canada.; *DSMZ,* Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.; *EBRCN,* European Biological Resource Centers Network (http://www.ebrcn.net), a EU-funded network of 15 European culture collections of microorganisms and cell cultures (2001–2004).; *ECCO,* European Culture Collection's Organisation (http://www.eccosite.org), a network of European Culture Collections and BRCs (see *Box 11.4*).; *EMbaRC,* European Consortium of Microbial Resource Centers (http://www.embarc.eu/) networking the BCCM™/LMG, Belgium; *CECT,* Spain; *CIP,* France; *DSMZ,* Germany and two French research collections INRA-CIRM-BP in Tours and CIRM-BIA, Rennes), aiming to improve, coordinate and validate microbial resource center (MRC) delivery to European and International researchers from both public and private sectors (*Box 11.4*).; *ENBI,* European Network of Biodiversity Information (www.enbi.org), an EU funded project established to include all European national nodes of the Global Biodiversity Information Facility (GBIF). (2003–2006).; *ESFRI,* European Strategy Forum for Research Infrastructures a strategic instrument to develop the scientific integration of Europe and to strengthen its international outreach (http://ec.europa.eu/research/infrastructures/index_en.cfm?pg=esfri); *FEMS,* Federation of European Microbiological Societies (http://www.fems-microbiology.org).; *GBIF,* Global Biodiversity Information Facility (http://www.gbif.org/), an international government-initiated and funded initiative focused on making biodiversity data free and openly available online.; *GBRCN,* Global Biological Resource Center Network (http://www.gbrcn.org), a project following work in the OECD to improve access to high quality biological resources and information to support research and biotechnology as a platform for a knowledge-based bio-economy.; *INRA CIRM-BIA,* Center International de Ressources Microbiennes - Bacteries d'Interet Alimentaire, Institut National de la Recherche Agronomique, Rennes, France.; *INRA CIRM-BP,* Center International de Ressources Microbiennes – Bacteries Pathogenes, Institut National de la Recherche Agronomique, Nouzilly, France.; *KACC,* Korean Agricultural Culture Collection, National Institute of Agricultural Science and Technology, Suwon, Republic of Korea.; *KTCT,* Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Taejon, Republic of Korea.; *LMG,* The Belgian Consortium of Collections of Microorganisms (BCCM™), represented by the Universiteit Gent, Belgium.; *MINE,* Microbial Information Network Europe, an EU-funded network of European culture collections, running between 1986–1989 and 1990–1993.; *MIRRI,* Microbial Resource Research Infrastructure (http://www.mirri.org/), a pan- European distributed research infrastructure established on the European Strategy Forum for Research Infrastructures (ESFRI) road map with the goal to improve access to the microbial resources and services that are needed to accelerate research and discovery processes.; *MOSAICC,* Micro-Organisms Sustainable use and Access regulation International Code of Conduct, an EU-funded project (1997–1999), a tool to support the implementation of CBD at the microbial level, in accordance with other relevant rules of international and national laws.; *MUM,* Microtheca do Universidade do Minho, Braga, Portugal.; *NBRC,* Biological Resource Center, National Institute of Technology and Evaluation, Chiba Pref., Japan.; *NCAIM,* National

Collection of Agricultural and Industrial Microorganisms, Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary.; *NCCB,* Netherlands Culture Collection of Bacteria, Utrecht, The Netherlands.; *NCIMB,* National Collection of Industrial and Marine Bacteria, National Collections of Industrial, Food and Marine Bacteria, Aberdeen, UK.; *NCTC,* National Collection of Type Cultures, Central Public Health Laboratory, London, UK.; *NRRL,* Northern Regional Research Center, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, US Department of Agriculture, Peoria, Illinois, USA.; *OECD,* Organisation for Economic Cooperation and Development (http://www.oecd.org/).; *SAG,* Culture Collection of Algae Sammlung von Algenkulturen, University Göttingen, Göttingen, Germany.; *UCL,* The Belgian Consortium of Collections of Microorganisms (BCCM™), Université Catholique de Louvain, Belgium.; *WDCM,* World Data Center of Microorganisms, an activity of the WFCC, providing an electronic gateway to databases on microbes and cell lines and resources on biodiversity, molecular biology and genomes (see *Box 11.3*).; *WFCC,* World Federation for Culture Collections (http://www.wfcc.info/), a Federation within the International Union of Microbiological Societies (IUMS, http://www.iums.org) (see *Box 11.2*).

## Introduction

Culture collections have been preserving organisms and supplying them for research and development for over a century. The term "culture collection" actually does not reflect a common standard, however, since tasks, holdings, size, funding system, affiliation, mandate, and other parameters differ widely. Though the basic principles of operation are the same, namely, accessioning, maintenance, and provision of microorganisms, collections may significantly differ from each other, and hardly any two collections operate under the same system. To name a few extremes, printed or electronic catalogues may be missing completely, while other collections display their holdings electronically in a most professional way, while collections may maintain a very regional selection of microbial strains of a narrow taxonomic or physiological range; others try to accession the complete range of validly named type strains, and yet others access their strains from geographically diverse regions; some collections are affiliated and funded as part of an academic institute; others receive strong governmental support, while others are in charge of nonpublic strains used by the biotech industry. Though the range of collection types is vast and many are not even visible to the public, information is available for those 592 collections (status May 2011, including those with non-microorganism holdings) which are registered at the MIRCEN-World Data Center for Microorganisms (WDCM) (http://www.wfcc.info/index.php/wdcmdb/) which is overseen by the World Federation for Culture Collections (WFCC). Statistics on these collections and summaries of kind and number of holdings, number of staff, funding system, and services offered are compiled in the WDCM.

Before the globalization of information by the internet, printed catalogues were the only means by which the users could have an insight into collection holdings. Thus, collections usually served the national market, and only a few highly visible collections operated on an international level. The common goals of collections and their same basic operations for collection functioning triggered the need for better cooperation which started after the mid-1980s. National networks were created, some of which, such as the Belgian Network and more recently the Chinese and Brazilian networks, proved successful, while others, such the British and the US networks of culture collections, passed through some stormy times. Regional networks have operated since the early 1980s, first in Europe but now also in East Asia. The need for better harmonization among those collections which provide users in academia and bio-industry with biological material was recognized by Organization for Economic Cooperation and Development *(OECD)*. From 1998, this organization, together with collection managers and representatives of governments and industry from member and associated states, developed a strategic plan to improve the quality of management and operation of collections for their own benefit and for the benefit of the user, specifically to play a key role in underpinning the knowledge-based bio-economy. The growing market in Biotech R&D in the USA, China, India, Brazil, and Europe required the improvement of the operation of providers of biological material as their authenticity, purity, and the availability of associated database resources were considered indispensible for the success of downstream processes.

As a result, the role of those collections that agreed to follow the route of higher quality has changed dramatically, culminating in the introduction of the term Biological Resource Center (BRC) to reflect the delivery of services and products compliant with a standard agreed by national authorities. BRCs focus on the following quality criteria:

- *Achieving the primary objective* to maintain strains in a viable state without morphological, physiological, or genetic change
- *Implementing best practice in the provision of services by ensuring:*
  - Authentication of biological materials
  - Validity of data
  - Continued availability and reproducibility of materials
  - Safe and legitimate shipping
  - Legitimate acquisition of biological material
  - Compliance with biosafety and biosecurity guidance
  - Protection of intellectual property rights, particularly for patents
- *Applying long-term methods of preservation essential to ensure availability of biological materials for the long-term*
  - Selection of most suitable method
  - Optimization
  - Viability, purity, and stability

Meeting the requirements of BRC status requires investment and change. While it will be feasible for some of the well-funded public service collections to implement international accepted

guidelines, others will need more support, both strategically as well as financially. The mandate to develop a framework for the evolution of collections to BRC status is being undertaken by the Global Biological Resource Center Network, which is supported by regional activities (e.g., MIRRI in Europe). Additional expertise in various areas of collection-related issues, such as the CBD, intellectual property issues, material transfer agreement, biosecurity, and the like, is being harnessed to deliver common policy and strategy for implementation. An overview of the legal and regulatory environment in which microbiologists and in particular microbial service collections dwell and their reactions is given in Fritze (2010).

This chapter will highlight some of the recent developments, focusing on the core activities of any type of collection of microorganisms, and will describe in detail the way forward to achieve the goal of their global networking.

## Prokaryotic Holdings in Public Service Collections

On 30 May 2011, a total of 1,751,439 microbial resources were listed in the 592 culture collections in 68 countries registered in the WDCM: about 761,000 of them were bacteria and 506,500 of them were fungi. Other holdings embrace bacteriophages, plant and animal viruses, microscopic algae, protozoa, dedifferentiated plant cells, and immortalized human and animal cell lines. Collections of microorganisms maintain two categories of strains with relevance to taxonomy, type strains, and non-type strains. For prokaryotes, the deposition of type strains, the nomenclatural type to which the binominal species designation is linked, is mandatory (Tindall et al. 2006). This means that no name will be valid without the written confirmation of at least two public collections in two different countries that the respective strain has been deposited without restrictions and checked for authenticity by the original depositor (for descriptions before 2006, the type may be available in a single collection only). This procedure was internationally accepted in order to make the type available as reference for scientific studies. Non-type strains can be either authenticated strains of a described species or be any taxonomically less well-identified strain found worthwhile maintaining by scientists and collection managers for their specific properties. A 2011 survey on holdings of prokaryotes in several of the major and some of the smaller public collections (see legend to *Fig. 11.1*) clearly indicated the higher number of deposits of non-type strains over type strains. Over the past 11 years, about 136,000 strains were accessioned by these collections, about 80 % of which were non-type strains. In the same period, 5,412 type strains were validly named and described, meaning that type strains were, on average, distributed to five public collections. Duplication in collections at a reasonable level is considered good practice as backup. The decision to maintain copies of strains depends upon a wide range of scientific and user-related interests: it guarantees the long-term maintenance of the prime reference strain in bacteriology, while the rapid provision of these strains to users at the regional/national level facilitates

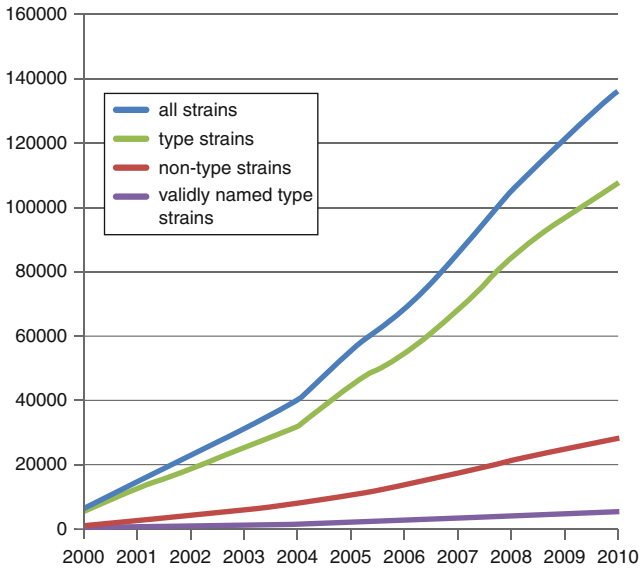


**Fig. 11.1**
**Cumulative display of accessions of type and non-type strains by selected public collections over a period of 11 years. Collections included are ATCC™, USA; BCC, Thailand; BCCM™/LMG, Belgium; CCM, Czech Republic; CCMM, Morocco; CCUG, Sweden; CCTCC and CGMCC, China; CECT, Spain; CIP, France; DSMZ, Germany; KACC and KCTC, Korea; NBRC, Japan (starting 2002); NCAIM, Hungary; NCCB, the Netherlands; and NCIMB, the UK**

access and overcomes costly shipping and lengthy documentation procedures. The graph in *Fig. 11.1*, showing the cumulative accessions, clearly displays the significant uptake of non-type strains from 2005 on which may be a reflection of increased interest in biodiversity studies and the emergence of the bioeconomy. The same trend is visible in the duplication of numbers of new type strains around 2005, a direct reflection of the isolation of novel pheno- and genotypes. It must be noted that not all public collections follow the same accessioning strategy: while some (e.g., BCC, CCUG, CGMCC, CCMM, CCTCC, or NCCB; see Abbreviations) concentrate on the deposition of non-type strains (≥75 %), others (e.g., ATCC™, BCCM™/LMG, CCM, CECT, DSMZ, CIP, KACC, NCAIM, or NBRC) accession about as many type strains as non-type strains (35–60 %), while among the collections surveyed, only NCIMB and KCTC concentrate on the deposition of type strains (70–75 %).

While deposition of type strains is free of charge, the increasing number of newly described species (*Fig. 11.1*) challenges collections by increasing the necessary manpower and maintenance costs. These costs include not only the administrative responsibilities according to the CBD and other rules and regulations (Smith et al. 2008) but also identification, authentication, maintenance, and long-term storage, as well as the generation of accompanying bio-information and regular technological updates and training. Some revenues are generated by providing strains to users, but this income does not cover the costs involved in state-of-the-art maintenance of resources. This indicates that even with the descriptions reaching a plateau in the next years (due to the lack of systematists, not of novel

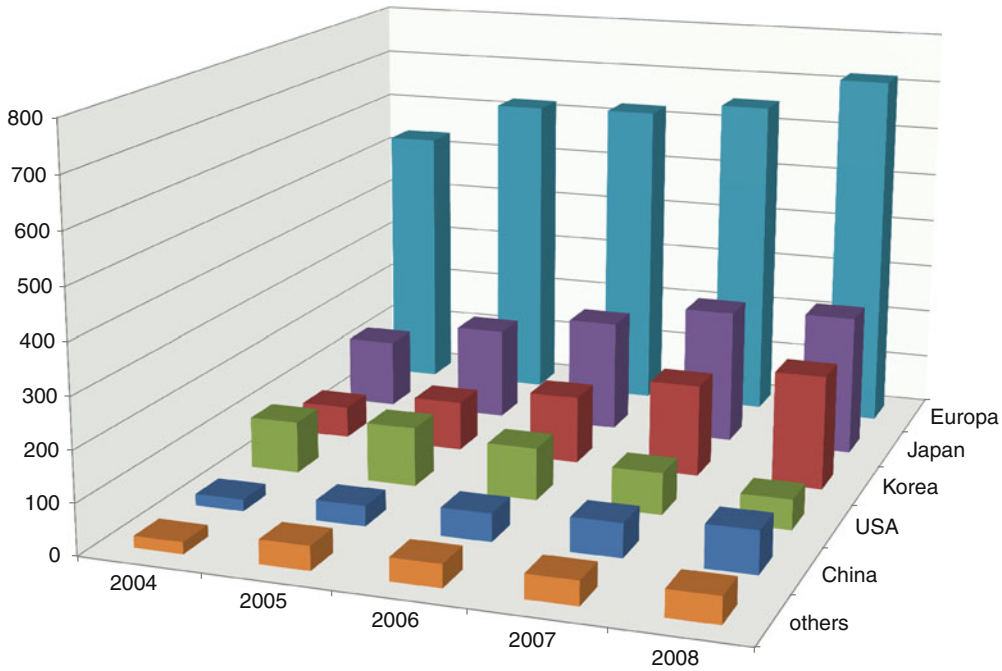


**Fig. 11.2**
**Deposition of new strains in culture collections per country/region between 2004 and 2008 (Courtesy of Ken Suzuki, NBRC, Japan)**

organisms), the workload of maintaining and providing novel biodiversity under high quality standards remains a huge task for accessioning collections.

As shown in *Fig. 11.2*, European collections were used most frequently as repositories for new strains over the last years (data available from 2004 to 2008). The decline of the USA collections as primary source for deposition is worth noting as is the increased deposition in Korea, Japan, and China, countries that today contribute most to the description of novel species. It is to the advantage of new public collections, mainly in East Asia and Brazil, that their planning fell in the times of increasing awareness of microbial diversity and the genomic revolution, resulting in the proper provision of infrastructure for their future tasks.

## Gaps and Strengths

Public collections do not only differ in numbers and types of material deposited, they also differ significantly in the range of taxa maintained. A worldwide comparative analysis of holdings of prokaryotes in WFCC/WDCM member collections, as displayed individually in their respective strain catalogues, is not available; a 2009 survey of some West European collections, members of the EMbaRC project (see Abbreviations), most likely mirrors the situation at a more regional level, such as those existing in North America and East Asia. The range of gaps at the generic level is rather small as the majority of phyla are covered at least by some strains. At the genus level, most phyla are covered above 80 % (*Table 11.1*); only the monogeneric phyla Fibrobacteres and Lentisphaera are not covered in any of these collections, and some of the "rare" (rare in the sense of under-sampled or low diversity) phyla are represented by a few type strains only. The policy of mandatory deposition, however, guarantees that the type strain is available from at least one public collection (e.g., the respective type strains of species of *Fibrobacter* and *Lentisphaera* are held in the ATCC™ and ATCC™ and KCTC, respectively). Obvious gaps detected are within the Tenericutes (formerly Mollicutes) and within Cyanobacteria. Mollicutes embrace primarily parasites of various animals and plants, living within the host's cells. Their maintenance often requires host tissues which are out of range for most resource centers. Collections of Cyanobacteria exist in several countries (e.g., ATCC™ and CCMP, USA; PCC, France; UTCC, HAMBI, Finland; Canada; CCAP, UK; SAG, Germany; BCCUSP, Brazil) and often in conjunction with collections of eukaryotic algae.

At the species level gaps are obvious in the so-called rare species, here, the more specialized collections show their strength, especially in holdings of the extremophiles. These species are usually less frequently requested than species of medical and biotechnological interest, and their maintenance is more demanding than those of the majority of aerobic and heterotrophic species in the phyla Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes. Other gaps are also seen among some of the genera of obligate endosymbionts and pathogens, as well as among the obligate chemolithotrophs.

Only 12 % of all genera described are not covered by any of the six EMbaRC collections surveyed. These are either those recently described, embracing obligate endosymbionts or fastidious pathogens. More telling than genus numbers, however, is the availability of type strains and range of diversity covered at the strain level. In this respect, too, collections differ widely from each other (for EMbaRC collections, see *Table 11.2*). Certainly, the history of

**Table 11.1**
**In percentage coverage of bacterial diversity at the genus level by six EMbaRC collections (Survey from 2009)**

| Taxon | Number of described genera | Number of genera covered | Percent coverage |
|---|---|---|---|
| Archaea | 91 | 86 | **95** |
| Bacteria | | | |
| Aquificae | 12 | 12 | **100** |
| Thermotogae | 6 | 5 | **83** |
| Thermodesulfobacteria | 4 | 4 | **100** |
| Deinococcus/Thermus | 6 | 6 | **100** |
| Chrysiogenetes | 1 | 1 | **100** |
| Chlorobi | 3 | 3 | **100** |
| Chloroflexi | 13 | 10 | **77** |
| Thermomicrobia | 1 | 1 | **100** |
| Nitrospirae | 3 | 2 | **67** |
| Deferribacteres | 6 | 5 | **83** |
| Synergistetes | 5 | 3 | **60** |
| Planctomycetes | 9 | 7 | **78** |
| Fusobacteria | 9 | 7 | **78** |
| Chlamydiae | 5 | 1 | **20** |
| Spirochaetes | 14 | 7 | **50** |
| Fibrobacteres | 2 | 0 | 0 |
| Acidobacteria | 6 | 5 | **83** |
| Verrucomicrobia | 13 | 8 | **62** |
| Dictyoglomi | 1 | 1 | **100** |
| Gemmatimonadetes | 1 | 1 | **100** |
| Lentisphaerae | 1 | 0 | 0 |
| Bacteroidetes | 181 | 148 | **82** |
| Firmicutes | 312 | 289 | **93** |
| Actinobacteria | 238 | 228 | **96** |
| Proteobacteria | | | |
| *Alphaproteobacteria* | 235 | 188 | **80** |
| *Betaproteobacteria* | 143 | 131 | **92** |
| *Gammaproteobacteria* | 269 | 232 | **86** |
| *Deltaproteobacteria* | 84 | 76 | **90** |
| *Epsilonproteobacteria* | 15 | 12 | **80** |

a collection significantly determines the size and phylogenetic affiliations of individual holdings, and the expertise of curators is determined to a great extent by the history and tradition of collections (to name a few, the actinobacterial collection in the DSMZ and NBRC, Japan; the *Bacillus* and mycelium-forming actinomycetes holdings in the NRRL, USA; the *Lactobacillus* holdings in the CIP, or the *Vibrio*, *Pseudomonas*, or *Enterococcus* collections in the BCCM™/LMG). The history, including the research emphasis of the collection founders, also explains the strength in methods used in-house for authentication and characterization and the range of skills offered to the public, for example, providing identification service and training courses.

Based upon the range of genera and species covered, collections fall into one of several types. One type, represented by, for example, DSMZ, ATCC™, or NBRC, covers in-depth genera and type strains. Members of a second type do not, or do not only, concentrate on phylogenetic diversity, habitat, metabolism, or ecology but are also specifically strong in holdings of intra-generic and often intraspecies diversity, representing either a pathogenic potential (to humans, animals or plants), or organisms relevant to biotechnology (food, agriculture, pharmacy). The CIP, ATCC™, NCTC, or CCUG are well known for their holdings of pathogens, while the BCCM™/LMG collection, NRRL, and especially collections in subtropical and tropical regions maintain in-depth diversity of nitrogen-fixing bacteria and plant pathogens. The collections maintain a higher number of strains per taxon than those of the first category. The research collections usually cover a very narrow spectrum of genus diversity but in-depth coverage of intraspecies diversity. This is clearly shown by the example of the two INRA collections in which holdings are strains involved in either milk or cheese processing (CIRM-BIA) or in pathogenicity (CIRM-BP).

## Non-type Strains as an Important Source of Biodiversity

The increasing deposition of non-type strains is due to several factors. Firstly, the strains can originate from a collection's own research, enlarging the holdings of those taxa which are in the prime focus of individual curators. Though often the number of isolates originating from environmental studies is too high to maintain the complete set of the strains isolated, the short intra-collection distances and the in-house availability of maintenance procedures favor rapid and competent deposition.

A second source of strains originates from research laboratories. These facilities may work independently or in a network of collaboration, including public collections. With the advent of molecular ecology and the growing awareness of the huge, yet unexplored biodiversity of microorganisms from the early 1990s on, increased funding has strongly supported research in genomics, metagenomic, and functional diversity. Microbiological research also benefitted from increased funding for sampling due to the awareness that linking sequences to function requires research on the organism itself and due to the inquisitiveness of scientists to isolate the organism for which the molecular data signaled phylogenetic uniqueness. The heydays of the recovery of extremophiles and the discovery and description of novel phyla and classes fall into the last two decades. While many of the more recently described novel taxa rarely embrace more than the type strain of the type species only, it can be assumed that more strains of these taxa are hidden in research collections. Normally, the result of the lack of proper identification and demanding circumscription protocols, not to mention the lack of funding for taxonomic research, which is not matched at all to the support for expensive sampling expeditions and isolation regimes. It is mostly up to the individual scientist to identify non-type strains that are worthwhile depositing, and this is done

**Table 11.2**

**Comparison of genera and type strains described per phylum and holdings in EMbaRC collections, representing a regional network (Survey from 2009)**

| Phyla | Genera/species described[a] | DSMZ Germany | CIP France | BCCM™/LMG Belgium | CECT Spain | INRA CIRM-BP France | INRA CIRM-BIA France |
|---|---|---|---|---|---|---|---|
| Archaea | 91/*379* | | | | | | |
| Crenarchaeota | 26/*56* | 24/*52* | – | – | – | – | – |
| Euryarchaeota | 65/*322* | 58/*276* | – | – | 12/*20* | – | – |
| Bacteria | 1,595/*9,056* | | | | | | |
| Aquificae | 12/*27* | 12/*25* | 1/*1* | – | – | – | – |
| Thermotogae | 6/*31* | 5/*28* | 2/*3* | – | – | – | – |
| Thermodesulfobacteria | 4/*8* | 4/*8* | 1/*1* | 1/*1* | – | – | – |
| Deinococcus/Thermus | 6/*61* | 6/*52* | 2/*15* | 3/*26* | 1/*2* | – | – |
| Chrysiogenetes | 1/*1* | 1/1 | – | – | – | – | – |
| Chlorobi | 3/*15* | 3/9 | 3/8 | – | – | – | – |
| Chloroflexi | 13/*20* | 9/12 | – | – | – | – | – |
| Thermomicrobia | 1/*2* | 1/1 | – | – | – | – | – |
| Nitrospirae | 3/10 | 2/6 | 1/1 | – | – | – | – |
| Deferribacteres | 6/9 | 4/6 | 2/2 | – | – | – | – |
| Synergistetes | 5/5 | 2/2 | 1/1 | – | – | – | – |
| Planctomycetes | 9/14 | 6/9 | – | – | – | – | – |
| Fusobacteria | 9/45 | 5/33 | 4/15 | 1/1 | – | – | – |
| Chlamydiae | 5/10 | 1/1 | – | – | – | – | – |
| Spirochaetes | 14/112 | 7/42 | 4/26 | – | – | – | – |
| Fibrobacteres | 1/4 | – | – | – | – | – | – |
| Acidobacteria | 6/7 | 5/6 | – | – | – | – | – |
| Verrucomicrobia | 13/31 | 5/7 | 3/3 | – | – | – | – |
| Dictyoglomi | 1/3 | 1/3 | – | – | – | – | – |
| Gemmatimonadetes | 1/1 | 1/1 | – | – | – | – | – |
| Lentisphaera | 1/1 | – | – | – | – | – | – |
| Bacteroidetes | 181/711 | 105/453 | 107/340 | 76/249 | 13//18 | 5/2 | – |
| Firmicutes, Tenericutes | 312/1,962 | 269/1.752 | 146/936 | 76/730 | 42/247 | 1/2 | 7/73 |
| Actinobacteria | 238/2,381 | 226/2.293 | 147/1.069 | 79/569 | 46/191 | 2/3 | 3/40 |
| Proteobacteria | 746/3,582 | 572/2.618 | 367/1.645 | 277/1.334 | 126/351 | 50/157 | – |
| Total number of strains | | 19.735 | 22.452 | 16.505 | 2.658 | 2.239 | 3.082 |

[a]It should be noted that the total number of genera and species includes synonyms (see http://www.bacterio.cict.fr/number.html). This is due to the fact that the name of some species appears in two or more genera (synonyms), depending on the number of reclassifications (names once validly published or notified will remain valid irrespective of its present classification). The deviations from the actual numbers will remain uncorrected as the visualization of holdings in the individual collections will only be slightly affected

in an environment of underfunding of public collections. Generally, the number and kind of non-type strains to be deposited is a matter of negotiation between scientist and curator. Rarely are entire research collections transferred to public collections; in most cases, such collections consist of unique holdings which are at high risk of being discarded or transferred to a new facility with unknown long-term perspective. The WFCC established a specific task group in order to provide a focal point of call for any collection (industrial/private/academic) which itself considers to be endangered or in need of help or advice with respect to its future sustainability. One example for a successful rescue has been the post-emeritus transfer of the collection containing myxobacteria and cytophagas from Hans Reichenbach to the DSMZ or the Seeliger collection of *Listeria* strains transferred to Mark Achtman, Cork, Ireland.

Surprisingly, it is only recently that funding bodies have developed an interest in microbial collections established in the course of projects funded with taxpayer's money. Starting with the long-term and secure availability of taxon-affiliated data of mainly eukaryotes, such as the barcode of life sequences, environmental observatory data, remote sensing, or geographic atlas of plants and animals (to name only a few), microbiological

information was restricted to gene and genome sequences. But it is the living culture that is needed in order to verify data and to explore new scientific horizons on the basis of the deposited information. This situation is slowly changing as funding agencies place more emphasis on measures for appropriate maintenance of research collections and their evaluation; they foster collaboration with the expertise of curators working in acknowledged collections; they even financially support basic taxonomic groundwork in order to allow collection curators to objectively judge the novelty of strains which could complement their holdings with the goal to broaden the biodiversity of taxa for research in general. Only a small fraction of strains maintained in research collections will be deposited in public collections, unless a completely novel long-term storage strategy is developed. Here, the same criteria could be applied to those listed in ❯ *Box 11.1* for post-publication deposits. It should also be stressed that researchers should communicate specific experience on growth and maintenance for fastidious strains to collection curators prior to deposition in public collections. Usually, curators lack experience needed for members of mainly higher and novel taxa for which no strain had been deposited before, and they need to be informed before the arrival of such strains in order to optimally preserve them long-term. The number of isolates in research collections worldwide cannot be estimated, and, likewise, the percentage of novel strains worthwhile depositing cannot be assessed. The transfer of research isolates to the safe environment of a public collection will only be decided through an intense dialog between curators and researchers in academia.

The third source of strains is currently almost unavailable to the scientific public: namely, those strains which are included in the scientific literature. The instructions to authors of almost all peer-reviewed journals state that the editors expect that new and variant organisms, viruses, and vectors described in journals will be made available to all qualified members of the scientific community. Some journals even explicitly encourage authors to deposit important strains in publicly accessible culture collections and to refer to the collections and strain numbers in the text (e.g., FEMS journals). The Guide to Authors in Nature publications states that resources should be made available in order to allow others to "replicate and build upon the authors' published claims" (http://www.nature.com/nature/authors/gta/#a1.3), to check when aberrant results are discovered or to reevaluate the strains when new technologies are available.

Though the number of deposits of non-type strains indicated in ❯ *Fig. 11.1* over a period of 11 years sounds impressive, it is only a minute fraction of strains annually included in scientific studies. To give only two examples, in the first two issues of Volume 46 (2008) of the Journal of Clinical Microbiology, around 32,000 strains of mostly clinical origin were included, while about 20,000 strains were included in the publications of the 2008 volumes of ten European microbiology journals covering mostly applied and ecological topics (Stackebrandt 2010). However, hardly any of them were deposited in public collections for long-term availability. In the first example, only 0.03 % of strains investigated were deposited which is perhaps not surprising considering the taxonomic affiliation of these strains (mainly staphylococci, mycobacteria, Clostridia, enterobacteria, *Acinetobacter*, *Burkholderia*, *Chlamydia*) which accumulate rapidly in daily hospital routine and represent in almost every case, isolates of described species and few exhibiting new properties. In the second survey, only 0.94 % found their way into public collections. Release of material and/or deposition in public collections is left to the authors' discretion; although some journals may have a stricter implementation policy than others, enforcement mechanisms do not exist for those frequent cases where authors deny sharing the requested material.

During a recent EMbaRC meeting of editors, collection managers, and authors, it was confirmed that, though access to published material may work smoothly among scientists in certain disciplines and tightly knit scientific communities, access overall is dismal. Discussion on a strategy to enhance and facilitate access to microbial resources was done with awareness that deposition of *all* microbial strains is neither necessary nor achievable under the present funding system of public repositories. The rationale for recommending deposition in public collections was not based on the concern that authors are incapable of short-term handling of research strains; it was based on the fact that microbial resource centers have decades of experience in handling, safeguarding, and shipping a wide range of diverse materials that is otherwise prone to involuntary extinction by negligence or deliberate clearing of laboratory holdings. Against this background, a set of selection criteria were recommended that would allow all stakeholders to prioritize material for deposition (❯ *Box 11.1*).

**Box 11.1 Post-publication Deposit of Microbial Strains to Underpin Good Practice in Science**

Despite recommendations to release to the community microbial resources post-publication, the reality is far from satisfying. A recent workshop discussed the need for a coordinated and effective deposition policy and proposed a set of criteria to facilitate deposition into public service collections (Biological Resource Centers) of "key" prokaryotic strains.

The workshop participants decided against a mandatory post-publication deposition of microbial strains but agreed on a set of criteria based on the phylogenetic, metabolic, and genomic uniqueness of "key" strains worthy of deposit. The checklist would also contain the contact addresses of a range of public service collections together with their taxonomic priorities to facilitate contact between authors and curators. Completion of this checklist would be mandatory prior to manuscript submission. The definition of "key" strains should be seen as a first but not exclusive step to initiate the strain sharing strategy; environmental samples, including as-yet-uncultured microorganisms, metagenome libraries, and other material should also be considered medium-term. The following criteria were agreed upon:

- Uniqueness, based on a cutoff point of $\leq$98% of 16S rRNA gene sequence similarity to the most closely related species with a validated name. This sequence is currently the gold standard for phylogenetic affiliation of an isolate at the genus level.

- Metabolic uniqueness, based on the presence of a new pathway, modification of an existing pathway, metabolic differences compared to the type strain or the production of novel products.
- Genomic uniqueness, such as significant differences (≥20%) in genome size, genome architecture, or new regulatory mechanisms.
- Resources and parts thereof with fully sequenced genomes (prokaryotes, phages, plasmids).
- A second strain of those species or subspecies for which only the type strain has been deposited. For 79% of new species described in 2009, only the type strain is available.

A survey among scientists was carried out to determine the reasons for the lack of materials in public service collections and whether they felt improved access was needed. Of the 3,950 scientists in 49 countries who were asked to participate, 517 responded (13.1%). When asked if they had encountered problems in accessing strains, 76.8% indicated that they had encountered problems, frequently to always, when asking for strains. Around 50% indicated that they received no response at all, others were requested to pay for the strain and some were denied access because of patent issues. Almost 87% agreed there was a need to improve access to microbial resources and 79% agreed that journal publication guidelines should request that strains with particular properties, such as those listed above, must be deposited in public culture collections to maintain them for further research.

This response suggests that the responders believe that a behavioral change is necessary and that journals should request that strains associated with publications be deposited. The reasons given for lack of response or failure to receive strains were specifically indicated by about 100 scientists but are subject to conjecture, being a mixture of guesses and author citations. In approximately 39% of cases it is feared that researchers simply want to protect their research from exploitation by others. This appears to be the very opposite of the philosophy behind publication and dissemination of results and conflicts with accepted scientific principles and morals. About 31% referred to the authors response that strains were lost or were unavailable for nonspecified reasons; 25% referred to quarantine, customs, and biosafety regulations as severe obstacles for releasing strains, problems that would be better solved by international, experienced BRCs than by individual scientists. Additionally, to protect the investment made using public funds, the research funders must also consider whether they make similar deposit and availability requirements on material subject to their funded research. The workshop participants stressed that authors should make every reasonable effort to make material available, if they do not deposit material in public collections, it should be because their strains do not meet the above criteria; it was also considered important that journals and funding agencies police their policies and have a mechanism for accepting complaints where access to material is denied. Journals were recommended to introduce a mechanism for active agreement by authors to make material available when they submit an article.

The workshop did not address the financial consequences of enhanced deposition but, considering the urgency to act now, funding agencies need to reevaluate their responsibilities by providing long-term and increasing support for public repositories to allow these tasks to be performed (Emerson and Wilson 2009; Stackebrandt 2010, 2011)

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## The Paradigm Shift: From Collections to Biological Resource Centers (BRCs)

Culture collections need to adopt new technologies and work toward providing what today's research community needs. BRCs need to work together to address these needs through coordinated and harmonized approaches and sharing tasks in a cost-effective and appropriate manner.

It is absolutely essential that any service industry moves with the times and the needs of its users. Culture collections are no different and have been adapting to change and increasing challenges. They have been providing a public service for over a century essentially collecting and distributing organisms. The core function of providing an authentically named strain has remained but broadened to characterization of their holdings to a greater extent. Such change is most often an independent decision dependent on the sector in which the collection or its host institution focuses with the consequence that public service collections have deviated in collection focus and service provided. Naturally, collections have learnt from one another in introducing new approaches and products that have worked for others as well as introducing their own innovations. More recently, certainly over the last four decades, change has been driven by consensus through communities such as the WFCC. The WFCC introduced guidelines for the establishment and operation of culture collections (http://www.wfcc.info/guideline.htm) to help collections to

provide the best service to the scientific community. A coordinated approach to microbial and cell culture resource has been fostered. New collections are created while others disappear. Over 980 WDCM registration numbers have been issued, but only 592 collections remain (Box 11.3). This has been attributed to several causes, the retirement of individuals who maintained them, a change of focus of the scientist or institution or the loss of funding. In the late 1980s, the Japanese government listened to their scientific community and challenged the OECD to address sustainability and development of culture collections. The OECD responded with the Biological Resource Center Initiative which now describes the modern-day culture collection as a Biological Resource Center and defines them as follows:

> Biological Resource Centers are an essential part of the infrastructure underpinning biotechnology. They consist of service providers and repositories of the living cells, genomes of organisms, and information relating to heredity and the functions of biological systems. BRCs contain collections of culturable organisms (e.g., microorganisms, plant, animal and human cells), replicable parts of these (e.g., genomes, plasmids, viruses, cDNAs), viable but not yet culturable organisms cells and tissues, as well as data bases containing molecular, physiological and structural information relevant to these collections and related bioinformatics" (Definition based on the one adopted at the 1999 Tokyo Workshop on Biological Resource Centers, where the concept of BRCs as an outgrowth of conventional pre-genomics ex situ collections of biological materials was developed – and incorporating scientific developments since 1999.) BRCs must meet the high standards of quality and expertise demanded by the international community of scientists and industry for the delivery of biological information and materials. They should provide access to biological resources on which R&D in the life sciences and the advancement of biotechnology depends. (http://oecdpublications.gfi-nb.com/cgi-bin/oecdbookshop.storefront)

## Meeting the Challenges

The OECD report (2001) on BRCs stresses that to cope with the massive expansion of biological resources, including living biological materials and data on genomics, BRCs need to

- Contribute to the coordination of efforts to conserve biodiversity and to provide access to natural and engineered biological resources
- Assist in the development of a coordinated international system for decision-making to guide appropriate acquisition, maintenance, and distribution of biological resources so as to avoid unnecessary duplication of effort while preserving critical levels of biodiversity
- Modernize to incorporate the latest developments in web-based electronic communication, bio-informational science, and informatics technologies
- Coordinate and unify catalogues and databases to meet the requirements of science in the developing post-genomics era
- Develop new systems and technologies for the long-term maintenance and distribution of large numbers of diverse biological resources
- Coordinate curation, as well as development and networking of informatics tools for data analysis, comparison, and visualization
- Ensure that the scientific community has access to affordable products and services

The development of BRCs to make available high-quality biological materials for research and development was considered necessary to underpin biotechnology. BRCs must focus on adding value to their biological materials and link more intimately to the life sciences and bio-industry to help deliver the developing bio-economy. The OECD report "The bio-economy to 2030: designing a policy agenda" (2011) emphasizes that the biological sciences are adding value to a host of products and services, supporting what some have labeled the "bio-economy." The report explains that from a broad economic perspective, the bio-economy refers to the set of economic activities relating to the invention, development, production, and use of biological products and processes. If it continues on course, the bio-economy could make major socioeconomic contributions in OECD and non-OECD countries. These benefits are expected to improve health outcomes, boost the productivity of agriculture and industrial processes, and enhance environmental sustainability. The bio-economy's success is not, however, guaranteed: harnessing its potential will require coordinated policy action by governments to reap the benefits of the biotechnology revolution. The plethora of uses of microorganisms, not least their use as reference strains in taxonomy or use in standards, demands access to expertise, technologies, and data analysis.

The change in how science research is conducted today, utilizing new technologies and information, requires culture collections to adapt in order to provide the resources in a way that will facilitate their use and enable an accelerated discovery chain. The transition to the OECD envisaged BRC is the first step. The modern-day BRC can support countries by establishing a means to release the potential of their microbial resources to provide solutions to national economic, environmental, food, and healthcare problems and consequently contribute to achieving the United Nations Millennium Development Goals. This ambitious agenda for reducing poverty and improving lives can be partially delivered by better management and utilization of biological resources:

- Improve livelihoods (Millennium Goal – MG 1).
- Provide new sources of food and reduce agricultural losses (MG1).
- Lead to discovery of new drugs and treatments of disease to reduce child mortality and improve maternal health (MG 4, 5, and 6).
- Understand and contribute to environmental stability (MG7).
- Develop a global partnership in the conservation and utilization of microbial resources for development (MG8).

- Resources created from the above can be mobilized to promote gender equality and empower women (MG3) and achieve universal primary education.

The OECD BRC Initiative took into consideration that the growing worldwide demand for biological resources provides good reasons for greatly increasing the number and quality of culture collections. Only a very few large national centers are able to perform a comprehensive role. A higher number and a broader geographic coverage of high-quality public service collections are needed to reach this goal. The development, expansion, and survival of these face many challenges. These include those posed by the molecular revolution (genomics and the information revealed by DNA sequencing), accelerating efforts to conserve biodiversity, funding uncertainties that threaten stability, the need for adequate quality assurance and constraints on access to biological resources within countries and across international borders resulting from private industry's protection of investments and industrial secrecy, import/export regulations, intellectual property rights, safety issues, and ethical concerns about the uses of genes and other biological resources (OECD 2001).

## Introduction of Quality Management Systems, Accreditation, OECD Best Practice Guidelines

The modern-day collection or BRC is an entity compliant with appropriate national law, regulations and policies, and operates to internationally validated criteria. The impact of legislation on the collection, handling and distribution is enormous. Keeping pace with new and changing legislation is absolutely essential and places an additional burden on the culture collection. To demonstrate that a BRC is implementing best practice, a third-party independent assessment process is needed. The OECD agreed best practice guidance to enable the delivery of high-quality materials ensuring they are authentic, preserved by "state-of-the-art" technology and that all associated information is validated. The OECD best practice guidelines for BRCs (OECD 2007) outline a process for certification or accreditation of BRCs. The BRC must apply for accreditation through a process approved by national governments *but* either through an accreditation body recognized by government *or* through a transparent accreditation procedure recognized by government *or* directly by government. There are a number of ways this might be achieved, but it is considered that the process should be based upon existing systems. Many collections although implementing best practices may not wish to go this far. The most appropriate model is one that sets the baseline for authentic, well-preserved, and validated strains and requires development in excellence (see below). The BRC seeks to add value to its holdings by further research on the characterization of the strains held to enable improvement in their public service role. It is envisaged that not all culture collections will become BRCs, but best practice should be implemented nevertheless.

The OECD BRC Task Force considered that the establishment of a common quality standard was a key issue in the development of BRCs. There are several examples of existing guidelines for microbial and cell culture collections available:

- The WFCC *Guidelines for the establishment and operation of collections of microorganisms* (http://www.cabri.org)
- The Microbial Information Network for Europe (MINE) project standards for the member collections (Hawksworth and Schipper 1989; Stalpers et al. 1990)
- Common Access to Biological Resources and Information (CABRI) guidelines (http://www.cabri.org)

There are also a number of general nonspecific standards and norms that can be applied to microbial laboratories, such as

- Good Laboratory Practice (GLP)
- ISO 9001 quality management systems
- ISO 17025 general requirements for the competence of testing and calibration laboratories
- ISO Guide 34 general requirements for the competence of reference material producers

Industry is expressing the need for quality control and standards within collections. Although publications on collection management and methodology give information on protocols and procedures, a quality management system must go further and set minimum standards (Smith et al. 2001). The CABRI electronic catalogue project made available a set of guidelines to aid collections to put in place best practice (CABRI 2002). These cover critical elements in the handling, storage, characterization, and distribution of microorganisms and cell cultures and the handling of associated information. The EU project (QLRT-2000-00221) European Biological Resource Centers Network (EBRCN) ran in parallel to the OECD Task Force. The EBRCN consortium supported the work of the OECD Task Force by drawing together the key elements of the above-mentioned guidelines and standards to form the basis of the OECD best practice guidelines for BRCs (OECD 2007). This was formulated at two levels, the general criteria that can be applied to all BRCs and secondly, organism domain specific criteria that are applied to BRCs based on the biological materials they hold. Currently, two sets of general guidelines ("general best practice guidelines for all BRCs" and "Best Practice Guidelines on Biosecurity for BRCs") exist in parallel to two sets of domain-specific guidelines ("best practice guidelines for the microorganism domain" and "best practice guidelines for human-derived material").

- *The general best practice guidelines for all BRCs cover*
  - Organizational requirements
  - Equipment use, calibration, testing, and maintenance records
  - Documentation management
  - Data management, processing, and publication
  - Preparation of media and reagents
  - Accession of deposits to the BRC
  - Preservation and maintenance
  - Supply
  - Quality audit and quality review

- *The best practice guidelines for the microorganism domain cover*
  - Staff qualifications and training
  - Hygiene and biosafety
  - Equipment use, calibration, testing, and maintenance records
  - Preparation of samples
  - Information provided with the biological material supplied

  Additionally, specific guidance was prepared to cover potential dual-use organisms and to ensure BRCs implemented practice to ensure biosecurity. Dual-use is a term to refer to any technology or material which can be used for peaceful and military aims. Thus, the exploitation of biological material and biotechnology is an essential part of the international dual-use regulations with regard to bioterrorism and bioweapons.
- *The best practice guidelines on biosecurity for BRCs cover*
  - Assessing biosecurity risks of biological material
  - New acquisitions/reassessment of inventory
  - Biosecurity risk management practices
  - Physical security of BRCs
  - Security management of personnel and visitors
  - Incident response plan
  - Material control and accountability
  - Supply and transport security

Over 20 WDCM registered collections have some form of certification or accreditation to demonstrate the provision of quality services and material. The OECD best practice guidelines extend such certification criteria to address BRC operations more specifically setting a benchmark for culture collections worldwide. Mechanisms to ensure collections adopt these standards to deliver high quality should be put in place (OECD 2001). Although the OECD BRC Task Force wished to see high quality proven through an independent third-party auditing process, for example, certification, it wishes to base it on existing systems and internationally accepted scientifically based quality criteria.

The actual system of international standards is focusing the complementary aspects of management, technical skills, product conformity, and process stability. The OECD best practice guidelines added the aspect of regulatory affairs to this complementary system. Each standard is specialized to enhance the compliance of an organization to a single aspect (e.g., ISO 9001 –> management). Are the OECD guidelines really a new approach going further than the already existing standards, establishing a complementary system with an integrative character covering all aspects? Can the OECD guidelines in addition answer both the Task Force and the OECD demands? These main questions can be answered by identifying the issues, which impact significantly on the implementation and maintenance of quality management in culture collections and BRCs. These issues reflect a variety of managerial operations and perspectives including continuous improvement, organizational behavior, human resources management, customer relations, and the core processes in the laboratories. But in addition to these traditional key issues of quality management, mainly covered and endorsed by the global standards ISO 9001 or ISO 17025, modern culture collections are faced with far-reaching issues in their shift toward the modern BRC as defined by the OECD. These issues include social and socioeconomic tasks, sustainable financial management, balancing of commercial and scientific interests as equitable stakeholders, linkage to innovative information technologies, and realization of governmental and cross-national legislation in the fields of biotechnology and security interests. The transformational change from a national though networked repository for biological material toward a multitask facility being part of a global infrastructure for the emerging knowledge-based bio-economy requires not only an enlargement of managerial requirements but also a new mutual standard in quality management. Taking up the necessity to standardize and systemize the core activities of a BRC within a special tailored guideline covering most of the key issues, the OECD best practice guidelines have not only a high coverage of all requirements in one single standard, but in addition, they cover a broad spectrum of the requirements delivered by other standards. In fact, the new guidelines for BRCs offer an integrated approach to support a culture collection in their own development as well as in reaching a high compliance level in many normative aspects.

But, having the new set of guidelines for BRCs demands a new approach for third-party assessment. Especially in consideration that the transition of the guidelines into one's own organization, the handling of increasing requirements coeval to diminishing financial support and the exposure to regulatory compliance is an unsolved problem left to the individual BRC and their quality managers. Thus, the internationally organized, German Federal Ministry of Education and Research funded project, "GBRCN – Global Biological Resource Center Network" – is working on an assessment model based on the excellence principle. Implementing the OECD guidelines brings a multidimensional capability into an organization; the principle of the GBRCN of sharing and continuously improving these capabilities among all BRCs and culture collections is opening the way toward an excellence model in performance and in the delivery of the OECD requirements. The excellence approach would resolve the restrictive regime of a standard and its full compliance assessment by offering a stepwise development in accordance to the available resources and demands. Self-evaluation and third-party audits will still be the important instruments to gain confidence in the system and recognition of the delivered results. However, the new assessment model is not propagating the golden way; it shows that many approaches will lead to excellent quality in services, material, and science.

Currently, the discussion is ongoing, whether the OECD best practice guidelines will remain a part of the so-called GxP world, for example, Good Laboratory Practice or if they should become an ISO standard, thus broadening the existing set of standards with the special requirements for culture collections and their living biological material.

## Quality and Certification: Costs and Benefits

Whatever system is selected, there will be costs associated with the achievement and implementation of the standards, and it is therefore important that the benefits to users and the BRCs themselves are clear. Some of these needs and benefits are outlined in the OECD report (OECD 2001). If the user benefits from the certification or accreditation of BRCs through better access to authentic and reproducible materials in a transparent and traceable way, how does the BRC benefit? There is an ever-increasing demand for authentic materials as more and more industries are adopting certification or accreditation as a means to demonstrate quality and competence. This may be the driving force for the business elements of a BRCs strategy for long-term sustainability, but it is also an increasing requirement to satisfy the funders of research who seek high-quality science and solutions.

It is imperative that organisms utilized in biotechnology are maintained in a way that will ensure that they retain their full capacity. BRCs must ensure a high-quality product that will give reproducible results. To achieve this, BRCs must apply quality control and assurance measures to maintain these standards, taking into account the needs of users and of the facilities and resources available. The need for common standards is evident as the task of maintaining representative samples of microbial diversity cannot be achieved by one collection alone. Therefore, it is essential that a worldwide network of collections interacts to provide the coverage required by the user. In order that a customer of such a network would get a consistent level of service and quality, it is necessary to set standards for all collections to attain.

Standards also provide a useful target for new collections to achieve, but it must be remembered that standards should become part of the operations and not be a set of rules implemented separately. Their aim is to ensure good quality and traceability and encourage improvement and further development. Standards must fit the operation and not add excessive unnecessary burden. Implementing standards for operation allows collections to convince investors to establish the facilities, skills, and mechanisms needed to participate in international activities. However, it is not sufficient to set the standard and then forget about it. A process for review and update must be put in place to ensure that new technologies can be brought in to improve the standard.

The advantages of certified or accredited BRCs forming a network can be split into two groups, those that give benefits to the users and those that benefit the BRC itself although several could fall in both categories.

### User Benefits

- A one-stop shop where both high-quality biological materials and the information associated with them can be found
- Conformity of both quality and authenticity of biological materials but also of processes and procedures to access them
- Confidence that the materials are fit for purpose
- Assurance that national law, policies, and procedures have been followed

### BRC Benefits

- Recognition that they operate to international scientifically based quality criteria
- An international mark of quality
- Raised profile
- Sharing of tasks
- Common policies and procedures
- Competitive edge
- Level playing field
- Common access to data enabling links to be made to other international initiatives without duplication of effort
- Common approach to data access, sharing, and interoperability
- Improved data usage
- Collaborative research and development

Inevitably, introducing the requirements of the standard and accreditation or certification procedures to the collections to achieve the status of a BRC will be costly. However, used correctly, it can attract investment in the development of BRCs, and the outcome will be beneficial to all concerned.

## Financial Sustainability of Public Service Collections and BRCs and Networks in Both Developed and Developing Nations

Implementing common standards and improving operations have additional costs. Despite there not being one model for the operational and financial sustainability of a collection, we can learn from the experience of existing culture collections. Studies by the OECD BRC Task Force and the EMbaRC consortium provide working models for BRCs. Although culture collections or BRCs have similar activities and objectives, they can be quite different in size, scope, and function. They may be described as either specialist or generalist collections, be small, based around an individual researcher or research team, or be large public service collections and a multitude of structures in between with differing financial models supporting each. Culture collection revenues traditionally come from supply, preservation, and various services associated with these, such as identification, characterization, or specialist consultancies. Culture collections also participate in research or service contracts, but most rely on some form of governmental or host institutional funding. A variety of activities relate directly to quality control, collection development, and operation that may include opportunities for some additional cost-recovery activities. Among several potential new sources of revenue is the generation of genomics and proteomics data that complement and add value to the biological materials themselves. The degree to which such activities may

actually provide support, sufficient to ensure financial sustainability of a BRC, is unproven. There are a few centers only that purport to declare themselves self financially sustainable.

The OECD model of BRCs includes considerable diversity of funding mechanisms for individual centers. However, it is to be expected that most BRCs will require some degree of commitment to core funding by their respective national governments. Other kinds of funding sources include support from industry, grants from agencies that support research, cost recovery through fees-for-service, development of databases, and other tools that complement the core role of BRCs, for example, even funding from charitable sources, especially those associated with public health or sustainable development. Furthermore, BRCs should be encouraged to coordinate their pricing policies and other activities to best serve their essential functions in response to the needs of sectors that depend on their biological resources.

The different approaches do not only rely on the expertise and function of the different collection hosts; but additionally, the needs and capacities of individual countries vary. Specifically, the needs of developing countries must be understood and accommodated. The OECD BRC Task Force advocated that national governments should identify collections and centers already capable of being designated as BRCs or forming a network and build upon and improve these rather than starting up new BRCs, especially in developing countries where resources are limited. Similarly, partnerships must be developed among BRCs and appropriate existing agencies, identifying their capacities and interests in terms of support for BRCs. A survey carried out by Stromberg et al. (2012) of 103 WFCC affiliated member collections, comprising mainly public service collections, demonstrated quite different balances between revenues and public support. Fifteen percent receive no public funding; 11 % received 1–40 % funding; 8 % receive 41–60 %; 13 % are 61–80 % funded; 54 % receive more than 80 % public funding. These differ widely from the total statistics for the WDCM which shows that the majority of the remaining 450 plus collections are not publicly funded and overall only about half the registered collections receive governmental support.

It is evident that at the outset when a collection is being considered, and before it is established, the financial plan and its sustainability must be designed. The WFCC guidelines (Anon 2010) state that the long-term support needed to enable collections to provide professional services must be considered, including appropriate operational facilities, the staffing levels to allow operation at a high standard and the training level of staff with research expertise related to the aims of the collection. The WFCC guidance presents funding as a key consideration. Administration and funding arrangements for collections require a long-term commitment from the parent organization. Support solely in the form of short-term contracts or without any allocation of core funding is inappropriate for service collections, aiming to provide long-term storage and supply services. Even the establishment of small in-house collections requires an ongoing source of direct, or indirect, financial support from a parent body. It is important to consider the level of funding, both now, and what it is likely to be in the future. This must be adequate to provide the range of services being planned and at a standard that users would expect. If secure resources are limited, in general, it is preferable to restrict the primary objectives of the collection to those which it has a strong probability of maintaining in the long-term. The financial models provided by existing culture collections of various types are well recognized and include

- The "General Collection" – often a national/regional facility.
  - "Popular" items for distribution can guarantee income.
  - Archive function requires subsidy.
- The "Specialist Collection" – usually more localized.
- The "Institutional Collection" can provide internal institutional service or wider external community/network service.
- The "Research Collection" provides a service relevant to one or more research interest.

These models vary considerably in the proportion of income derived from the various sources defined below. It must be emphasized, however, that the larger the archiving function carried out for strategic reasons rather than supply, the greater is the need for public and private subsidy.

## Financial Models for Biological Resource Centers

The diversification of activities in the transition from the "Culture Collection" to a BRC anticipates additional sources of revenue, both from existing activities and projects related to new technology-based partnerships. Two types of income streams are recognized. "Existing" income streams are those that support existing models of culture collections. "Anticipated" income streams which represent activities in which BRCs will or may participate in and that may generate recoverable income from stakeholders.

*Existing Income Streams for BRCs*

- Government support
- Private industrial support for participation in the functioning of BRCs
- Private industrial support for internal restricted BRC activities
- Public and private foundation support
- Public fundraising
- Fees for supply of biological resources and technical materials
- Provision of specialist services and technical consulting expertise
- Research income (grants and contracts)
- Fees for repository service (safe deposits and patent strain maintenance)
- Provision of technical courses
- Exploitation of and adding value to genetic resources

The understanding of current income lines for culture collections was assisted by the survey carried out by Stromberg et al. (2012) where they report that 67 % of affiliated member collections of the WFCC charge supply fees for strains provided. They go on to report that on average, 23 % of the recipients of cultures are

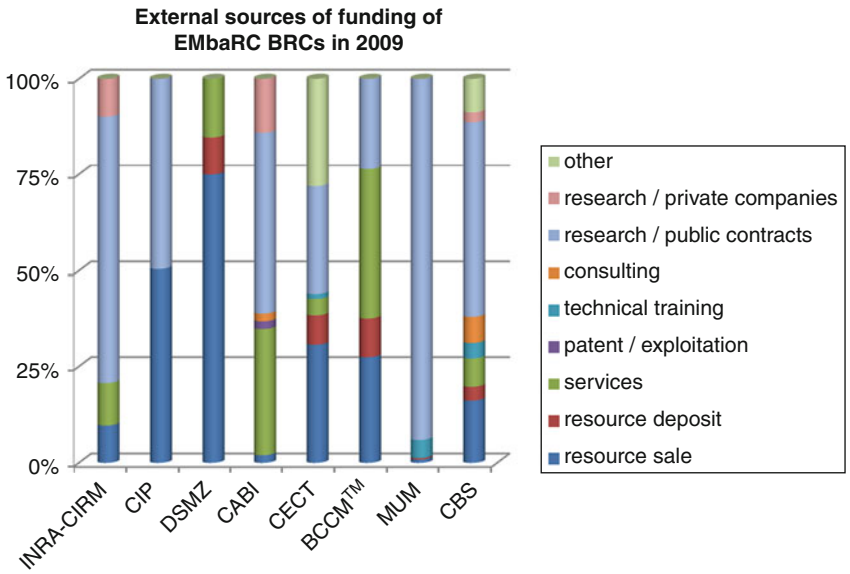


**Fig. 11.3**
**Funding sources of EMbaRC BRCs compared for 2009 (see Abbreviations)**

in industry, whereas 60 % are in academia or hospitals. They also report that there is a relatively small overlap in strains held, with between 1 % and 17 % of their holdings sourced from other collections. They report that 38 % are received with some sort of MTA, providing terms and conditions of supply. There is, however, an imbalance on where these collections are, with 82 % of them being in OECD countries and 10 % in the USA. Individually, although having overlap in the most popular reference strains, they have limited coverage of biodiversity, but together in a network, they could offer good coverage. Inevitably, there is an element of competition for the market, hence the determination of collections to store the "best sellers."

*Potential or "Anticipated" Income Streams for BRCs from, for example, the supply of services for*

- cDNA libraries, genomic libraries, filter sets, clones, plates, and PCR products
- Microarrays and reagents
- RNAi resources
- Accreditation/standardization-added value products and services
- Data storage and retrieval
- Software development/collaborations – data mining tools
- Technology development/collaborations – LIMS/robotics
- Sequence database annotation/phenotypic analysis
- Linking genomics databases to proteomics
- MLST (multilocus sequence typing) and population studies
- Product discovery, manufacture, and supply (potentially spun out to independent companies)

However, it is debatable whether all these activities have a market and offer worthwhile returns. Often such services are offered by specialist organizations, and the competition can be quite tough.

## Culture Collection Funding

There should be a balance between governmental support, commercial, and other income lines to provide support for collections. There are several collections that are supported by governments but rarely are they fully supported. The government supports 235 of the 592 culture collections registered with the WDCM, a further 56 are semi-governmental, 218 are supported by university, 17 are supported by industry, and 25 are private. It can be argued that governmental funding is essential and appropriate but even long-term stability of such funds may eventually be under threat. Culture collections perform many functions for governments not least helping them meet their obligations to the Convention on Biological Diversity and making available biological resources to underpin science, education, and the economy. Such government funding is usually balanced against the income received for the various services and products offered by the collection. This leaves very little for investment and to enable the collections to improve their coverage and incorporate new and advancing technologies. Collections need sound and innovative business plans to allow them to keep pace with the ever-increasing demands of their users.

The EMbaRC project is examining sustainability of BRCs and has compared the revenue lines of the partner collections. These collections are in the main well-established public service collections with a long history of providing products and services. They show that they have common products and services, but the balance on how important each individual line is to each collection is significantly different.

The funding sources of some major West European collections (EMbaRC) for the year 2009 are shown in ❯ *Fig. 11.3*.

The level of public funding was mainly in the range of 65–92 % but with the exception of CABI which has no specific national funding but which has overall member country contribution of around 3 % with more invested in the collection maintenance activity. Other funding sources for the EMbaRC collections in 2009 were

- Maintenance and bench fees – 0–16 %
- Resource supply – 2–75 %
- Resource deposit – 0–10 %
- Services – 0–39 %
- Technical training – 0–1 %
- Consulting – 0–7 %
- Research/public contracts – 0–94 %
- Research/private companies – 0–14 %

## Developing Income Lines

Not only do collections need to find novel ways of funding but also need to keep abreast and harness new technologies to produce information on the strains they hold, adding value with the aim to provide today's users with the information they need. It is not always possible to establish these technologies in-house, but it is possible to establish partnerships with manufacturers, other collections, or institutions with the expertise and facilities. Bioinformatics is of increasing importance to the operation of collections, and new ways of collecting, storing, analyzing, presenting, and interrogating information are required to make best use of biodiversity information. Molecular techniques are increasing in use to differentiate between strains and identification. Collections should be adopting such techniques to offer as services to users to counter the costs of utilizing these techniques for checking stability and authenticity of the strains they supply.

There are a number of ways BRCs can develop their individual business plans. However, it is crucial that BRCs do not become commercial entities; they must not compromise their public service role. Having said that they must do all they can do to reduce their public cost, a delicate balance is required. Some avenues that can be explored are outlined below.

### Commercial

- Development and ownership of spin-off biotechnology companies, generally through partnerships, sale of products, and services as well as consultancy

### Research Program Funding

- A series of projects to meet donor requirements, engaging research program funders to protect their investments by paying for deposits in collections

### Government Department Support

- Provision of services to governments to help them achieve their conservation and utilization of biodiversity commitments, their environmental policies, and their commitment to poverty alleviation

### Sponsorship

- Attracting donations to cover costs of biological resource provision, establishing a consortium of research program funders and sponsors

### Other Financial Aspects of Operating a BRC Network (as Identified by the OECD Workshop on Funding Models)

While a uniform structure of funding is not necessarily critical, many BRCs will require a significant component of government funding. Some guarantee of ongoing funding is necessary to ensure that their essential functions remain reliable for R&D and support of biotechnology. Collections will be put at risk if a BRC network operates at the expense of individual BRC funding resulting in the individual BRC's folding for lack of support. The following points were derived from the SWOT (**S**trengths, **W**eaknesses, **O**pportunities, and **T**hreats) analysis of financial models of BRCs and a proposed model for BRC development.

## Strategic Implications

- A prerequisite for any network is that it is built upon standards and accreditation. This defines the network. This may require further investment.
- A national strategy that provides core financial support for a national BRC (or BRCs) should be viewed as a prerequisite for participation in the international BRC network, to ensure that the network is sustainable.
- The international BRC network will be built upon national initiatives that in turn will evolve from existing activities (including culture collections). These activities are already based upon a range of income streams with varying levels of government support.
- Governments will be fundamental partners in the creation of national BRCs contributing to the international network, regardless of the level of financial support.
- Many existing culture collections will not wish to participate in the BRC network if this is inappropriate to their aims or goals, or if this is not justifiable given the level of investment required to raise/alter standards. Links to enable BRCs to draw resources from such centers will need to be created.
- Governments need to recognize that BRCs will take a regulated role in the supply and maintenance of

dangerous/pathogenic organisms. This important core aspect of BRCs provides a controlled framework for the availability of these sensitive resources. In turn, fulfilling this role requires a level of financial commitment.
- BRCs must use the opportunity of establishing an international network to seek sponsorship from a variety of new sources of support (national, international, public, private, and industry).

### Operational Implications

- BRCs have to take a prominent role in capacity building and ensure a link between research-based collections and the BRC and the ultimate user.
- BRCs need to function as a strategic, national repository for key academic and industrial research resources, which will in turn provide an income stream. This is unlikely to operate on the basis of full cost recovery from supply income.
- Governments and their funding agencies must ensure that products derived from publicly funded research programs are deposited in BRCs as part of the conditions attached to any award. (This could result in a small element of the grant allocated to this task as appropriate – see below.)
- BRCs need to provide greater support to research-based collections in terms of training and advice on standards, quality control and integrate more with the national activities in key-related priority research areas (e.g., model organism research consortia).
- Governments must ensure that infrastructure aspects of the support for research are funded through relevant research programs.
- BRCs must create partnerships with centers of excellence and developing new technologies and databases to ensure that linkage is possible between these leading edge aspects of research and the physical resources held in BRCs.

It is anticipated that all of these strategic and operational changes relevant to the national role of BRCs will enhance their position in providing services of benefit to the scientific community and thus in turn benefit them by maximizing the potential for financial support.

A key element for discussion, however, remains the degree to which BRCs may benefit from the direct commercial exploitation of the resources that they hold. "Ownership" as a concept has, to a large degree, been avoided in the past with the BRC acting as a "custodian" of the resource. Widespread introduction of Material Transfer Agreements and implications that IPR and reach-through are requirements for access to resources would fundamentally alter the relationship between depositor, user, and the BRC. National mechanisms for implementing the Nagoya ABS protocol (CBD 2011) could impact heavily here.

## Collections and Their Users: The Need to Know Each Other Better

Though not commonly encountered, collections are encouraged to conduct market studies and carry out regular surveys on customer satisfaction and buying behavior. Collections requiring a substantial proportion of their budget by generating revenues usually have in place a dedicated user-oriented management plan which, according to a functioning quality management, must be improved constantly. Knowledge about user demands and requests is indispensible for strengthening their market position. Therefore, collections should have access to certain basic information, such as

- Who is the user?
  For example, where are they working: in academia, bio-industry, food, or clinical sector or in public health or schools?
- What are the needs of the user? Are curators aware of them and is the management in a position to react quickly to satisfy user demands? Examples are as follows:
  Post-order communication
  Quality and type of packaging
  Modalities of shipment
  Correctness of delivered goods, such as authenticity and product information on safety aspects and handling
  Option to establish contacts
  Amicability and qualification of collections' contact person
  Goodwill policy in case of replacement shipment
  Handling of complaints
  Lead and delivery times
  Internet accessibility on information to
    Cultivation conditions
    Spectrum of services
    Databases
    New resources and products and new developments

The necessity to develop a more intense communication between collections and users is driven by the need to establish long-term and repeated use of the collection and its services. Once satisfied with some basic principles, such as high quality, short delivery time, and correct and timely information, the user will more likely as not become a loyal customer, independent of the fees for resources and services. The collection should develop a specific affiliation with the user (worldwide highly recognized brands have achieved this goal), and the collection should facilitate this relationship by documenting the advantages to be linked to justify this very resource center. Some are as follows: contact to a nationally/internationally leading collection; process reliability, such as provision of non-contaminated resources allowing reproducible results; range of a defined (either broad or specific) selection of products and services; close scientific support and consultancy, guaranteeing quality in products and processes; and long experience in taxonomy and identification, handling of recalcitrant, pathogenic and other delicate material, as well as expert knowledge in shipping packaging and import and export rules and regulations.

As compared to the collection-user relationship of 10 years ago, significant changes have already been introduced, for example, by establishment of a quality management (QM) system; one of the central features of a functioning QM system is the continuous process of improvement. This not only includes the strengthening of the collection-user relationship as mentioned above but also offers some of the advantages the customer is used to receive from well-managed online shops in other market segments. Top priority of online shopping is the option to pay by credit card. The latter issue is still a moot point in Europe as, in contrast to the situation in North America and a few other countries, most organizations do not allow payment by this means or do not provide credit cards to their employers. Another sector with room for improvement is the need for the collection to accompany the customer through the ordering and delivery process. Once an order is launched, basic information on entry date, confirmation of an order, order status, and date of dispatch should be provided. It must be the goal to have the material shipped within a few days – if not, the user should at least be informed about possible delays. Such requirements are clearly described in the OECD best practice guidelines (OECD 2007).

We are aware that online shopping for consumer goods cannot be fully compared with the provision of living organisms, which require a lengthy process of customer authorization, and administrative effort on export, import, and shipping regulations, not to mention delays of shipment of active cultures. It is, however, not overstated to indicate that most public service collections have not attempted to assess the satisfaction level of their users. Here, one can learn from commercial resource centers with which noncommercial collections compete in the same market. Collections should not hesitate to learn from the best cases of other organizations, and they should learn to react quickly to customer needs and demands. This requires the establishment of a client-led marketing policy, most efficiently executed by a professional marketing unit. Public service collections must recognize the need to make themselves more attractive through regular press releases of collection-related scientific headlines, by increased publications in international peer-reviewed journals, attractive training courses, and involvement in teaching and public lectures. These activities are already followed by larger public collections but continuously necessary to accompany measures of the core mandate and motive, that is, the provision of high-quality and non-contaminated biological references to support scientists in their goal to obtain reproducible data at the highest scientific level.

## Scientific-Technical Cooperation Among Microbial Culture Collections

Although culture collection organizations have existed for many decades, they or their modern-day versions, the BRCs, have never been fully networked. National, regional and global organizations have endeavored to help promote collections and have coordinated some efforts. They have brought together metadata on their members to central points and have helped keep members up to date with the progress of science, changing legislation, and collaborative opportunities through newsletters, conferences, and workshops. However, coordinated strategies for ensuring comprehensive coverage of species and the diversity within them are yet to be put in place. Projects and individual initiatives have made some progress, but consolidating the many initiatives that are working toward this goal is crucial to establish a systematic and networked approach. This would bring advantages to both the users and the collections themselves but importantly provide an infrastructure to underpin research and development, enabling the harnessing of microbial and cell diversity to contribute toward providing solutions to the world's big challenges.

The WFCC has been promoting the activities of culture collections for over four decades and has done a tremendous job to help establish a sound operational basis (Box 11.2). It was first to try and establish minimum standards through their guidelines (Anon 2010), common standards form the platform on which networking is based. The WFCC, as are most culture collection organizations, is a community that exchanges views and ideas. Often, this results in the uptake of common approaches, but the organization has no mandate to affect institutional changes in policies and practices. This impedes the introduction of coordinated approaches. At the regional level organizations such as the European (ECCO, Box 11.4) and Asian (ACM, Box 11.5) networks work on behalf of collections. They have been very successful in bringing project consortia together to seek project funding to solve common operational problems or address common research issues. There are over 20 national federations that do similar things at the country level. However, a lot of work still needs to be done both by collections and governments if the goal to harness the power of microbial diversity is to be realized. We need to harness the properties and products of microorganisms more efficiently if we are to tackle the big global challenges of today in poverty alleviation, food security, healthcare, climate change, and the environment.

The OECD emphasizes that biological resources, such as microorganisms and their derivatives, are the essential raw material for the advancement of biotechnology (OECD 2001). However, they go on; scientific progress and the resulting growth of the knowledge-based bio-economy will depend on the facilitated and safe access to ex-situ held living biological material and its availability in an adequate and comparable quality worldwide. It is understood that this, in turn, requires putting in place coordinated policy actions by all stakeholders involved. To meet the increasing demands of the scientific community for comprehensive, up-to-date, and easy to access living biological material available from microbiological culture collections and related information, a series of coordinated activities were initiated in different regions worldwide, leading to network activities to foster communication and research among collections for the benefits of users and science.

## Box 11.2 World Federation for Culture Collections

The World Federation for Culture Collections (WFCC) is a key global organization originated from an IAMS "section on Culture Collections" formed in 1963, which was reorganized as the World Federation for Culture Collections in 1970. From 1973, it was recognized as a multidisciplinary commission of the International Union of Biological Sciences (IUBS) and since the separation of the International Union of Microbiological Societies (IUMS) from IUBS in 1979, it has operated as an inter-union commission. It seeks to promote activities that support the interests of culture collections and their users. Member collections of the WFCC register with the World Data Center for Micro-organisms (WDCM, *Box 11.3*). A congress is held every 3 years to discuss advances in technology and common policies with regard to biodiversity and the role of culture collections. The WFCC keeps its members informed on matters relevant to collections in its Newsletter and has working programmes addressing patent depositions, biosafety and biosecurity, safeguard of endangered collections, capacity building, and quality standards. Since 1986, the WFCC has overseen the activities of the WDCM which is now the data center for the WFCC and Microbial Resource Centers (MIRCENs) Network.

The WFCC is the largest independent global organisation that represents professional individuals and culture collections, which preserve biodiversity and enable their proper use. They target living microorganisms, cell lines, viruses and parts and derivatives of them. Key values are authenticity and genetic integrity of the material and validity of the information provided. The WFCC supports the professionals, organizations and individuals with interests in culture collection activities through networking, providing information and expertise, and facilitating communication; facilitating access to the collection resources; providing training and promoting partnerships; encouraging the development and implementation of quality and security procedures and the use of common standards and regulations; representing member interests in international organizations and fora; and promoting the establishment of culture collections and their perpetuation.

There are over 120 culture collections affiliated to the WFCC who have agreed to implement the WFCC guidelines (*Guidelines for the Establishment and Operation of Culture Collections*- Anon 2010) and who contribute to the delivery of its objectives. In the growing bio-economy, WFCC's members face increasing global demands for worldwide and controlled access to biological resources, public security, industrial quality of their holdings and associated data and long-term genetic stability of the material. Key to the use of microorganisms from culture collections is the retention of their properties as research and development must be based on authentic and well-preserved biological material. The WFCC have been helping collections in this respect for over 4 decades. It is a goal that strains of organisms be supplied from member collections with traceability, conforming to national and international regulatory requirements, and that are preserved in such a way as to retain their full potential.

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## Box 11.3 The World Data Center for Microorganisms

In 1982, the World Data Center on Microorganisms hosted by the University of Queensland in Australia issued the World Directory of Collections of Cultures of Microorganisms (*Fig. 11.1*). The editors of the directory, Vicki F. McGowan and V. B. D. Skerman, articulated the roles of culture collections and the data center, "Culture collections occupy a central position in microbiology because effective research demands adequate and reliable sources of properly preserve cultures. As a result of their function as repositories of living organisms, culture collections promote microbiological research. Increased demands for historical information and strain data have created a need for easily accessible and up-to-date files of important information on the location and characteristics of cultures. Such needs can be met by the development of an adaptable system for storing, retrieving and exchanging information which can be used by all microbiologists."

The WDCM relocated in 1986 to RIKEN, Saitama, Japan, and then again in 1999 to the National Institute of Genetics, Japan, and introduced an online database and website to capture and diffuse information on culture collections and their holding. In the meantime, the number of culture collections registered in WDCM has increased year by year. However, it is to be noted that WDCM has issued 983 IDs to culture collections, that is, the community has lost about 300 culture collections since the first was registered. The WDCM collections hold in excess of 1.7 million strains: 44% are fungi, 43% bacteria, 2% viruses, 1% live cells, and 10% others (including plasmids, plant, animal cells, and algae).

In 1999, WDCM organized a symposium with the title of "Microbial Resources Centers in 21st Century – New Paradigm" back to back with the 1st OECD Meeting on Culture Collections. This was the moment when the concept of Biological Resource Centers was born. The participants of the two meetings recognized the impacts of biodiversity, genomics, and informatics on culture collections and agreed that culture collections had to evolve to become BRCs to meet their needs and those of users.

The online database of the world directory named CCINFO includes information on 592 culture collections in 68 countries as of March 2011; 235 of them are supported by the government, 56 of them are semi-governmental, 218 of them are supported by university, 17 of them are supported by industry, and 25 of them are private; 226 collections produce catalogues of holdings and there are 3,051 people working in them. These culture collections preserve 1,751,439 microbes. WDCM functions as an information hub of culture collections and their customers. The WDCM is now hosted by the Chinese Academy of Science Institute of Microbiology since April 2011 and it is expected that the functions of WDCM will be expanded to cover aspects of biodiversity, genomics, and advanced information and communication technologies (ICT). The URL addresses of the websites of WFCC and WDCM stay as they are, namely, http://www.wfcc.info/ and http://www.wdcm.org/, after the relocation of WDCM.

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Such activities are not new for regional networks (*Boxes 11.4* and *11.5*) or national networks, for example, the Brazilian initiative (*Box 11.6*).

**Box 11.4 European Culture Collections' Organization and its Networking Activities**

The European culture collections have collaborated since 1982 when the European Culture Collection Curators' Organisation was established to bring together the managers of the major public service collections in Europe to discuss common policy, exchange technologies, and seek collaborative projects. The organization opened itself to staff and users of microorganisms and is now named the European Culture Collections' Organisation (ECCO). There are currently >65 members, including 57 collections holding approximately 350,000 strains. The members have been involved in producing practical approaches to international rules and regulations. An initiative led by the Belgian Coordinated Collections of Microorganisms (BCCM$^{TM}$) produced a code of practice for collections to operate within the Budapest Treaty and the EU project Microorganisms, sustainable access and use, International Code of Conduct (MOSAICC) provided model guidelines for the operation within the spirit of the Convention on Biological Diversity. Several collaborative projects originated through discussions between ECCO members that have placed the European Collections at the cutting edge of culture collection activities and research. The most recent initiative is the EMbaRC project. They have resulted in technical guidelines and focused information documents covering requirements with which modern-day microbial collections are challenged. Substantial input was given by ECCO to the BRC initiative and the recent demonstration project for a Global Biological Resource Center Network (GBRCN). On a global level, the latter project aims to build a structured long-lasting network which will pave the way for collections to meet user needs. It addresses technical, legal, and administrative challenges presented in this globalized, fast-developing world.

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**Box 11.5 The Asian Consortium for Conservation and Sustainable Utilization of Microbial Resources (ACM)**

The Asian Consortium for the Conservation and Sustainable Use of Microbial Resources was established by the consensus of participants of 12 Asian countries during the 10th International Congress of Culture Collection (ICCC-10) held at Tsukuba in 2004. Heads of culture collections and government officers were involved in the meeting as well as research microbiologists. The objective of the consortium is to promote collaboration among Government or public organizations in Asian countries for the purposes of enhancing conservation and suitable use of microbial resources in Asia. Currently the members are from Cambodia, China, Indonesia, Japan, Korea, Laos, Malaysia, Mongolia, Myanmar, Philippines, Thailand, and Vietnam.

The ongoing activities of the consortium are:

1. Exchange of views and information of the current policy of the Asian countries for science, technology, and the related matters
2. Establishment and management of the network of culture collections including a common database
3. Enhancement of public awareness on the consortium's activities for the conservation and sustainable use of microbial resources in consideration of the Convention of Biological Diversity
4. Development of human resources for handling of microbial resources technically and legally
5. Promotion of research and development on microbial resources and their application in industrial and other uses
6. Establishment of a common scheme for international transfer of microbial resources
7. Scientific meetings (seminars, workshops, training courses, etc.) and other related activities

The General Assembly Meeting of the ACM has been held annually since 2004 in different countries. Task Forces for Bioresource Information Management, for Human Resource Development and for Management of Material Transfer are set up. These activities will be of value for the standardization and authorization of international transfer of microbial resources. Many Asian microbiologists are eager to study the microbiological diversity in the nature of various natural environments. ACM is also expected to achieve the rule of International Code of Prokaryote Nomenclature in compliance with the laws and regulations relevant to the Convention on Biological Diversity.

The 7th ACM meeting was held in Japan again in 2010, the International Year of Biodiversity, and adopted the Kazusa Statement on 15th October as follows:

- Kazusa Statement
  In the 7th ACM Meeting at Department of Biotechnology, National Institute of Technology and Evaluation (NITE) in Kazusa, Chiba Prefecture in Japan, members of the Asian Consortium for the Conservation and Sustainable Use of Microbial Resources (ACM) recognize that:
  Microorganisms such as filamentous fungi, yeasts, mushrooms, bacteria, archaea, and microalgae play important roles in the global ecosystem either directly or indirectly
  The diversity of isolated microbes only account for less than 10% of the total species, which means that many novel and yet-to be-discovered microbes inhabit the earth

The diversity of microbes is endangered by global climate changes, habitat changes, over exploitation, and ecosystem destruction

Long-term laboratory preservation of microbes is technically well attainable

Microorganisms are crucial biological resources to academia, biotechnology, and bio-industries contributing to technology, economy and social developments

They have also reached the following agreements:

1. Prompt action of each country toward ex situ conservation of microbes is imperative.
2. For effective ex situ conservation of microbes, international research cooperation is essential.
3. Active international research cooperation needs to be promoted by establishing a scheme to facilitate international transfer of microbial resources, further provision of technical cooperation, and capacity building in full compliance with the principles of the Convention on Biological Diversity.
4. For clarification of endangered microbes and conservation areas, a list of domestic microbes should be created.
5. Microbial taxonomists should take an initiative on the creation of such a list with the support of international research cooperation.
6. Demand for and importance of microbial taxonomists should therefore be well recognized in each country, so that having training programs in place for microbial taxonomists who can keep inter-generational continuity seems imperative.

To achieve the intention of this Kazusa Statement, the establishment of a Microbial Resource Center (MRC) in each country is necessary. By establishing the MRC, the training program for microbial taxonomists, legal management of microbial resources, and the creation of the list of domestic microbes through exploration, characterization, conservation, and sustainable utilization of these microbial resources can be carried out. Furthermore, the MRC can make a significant contribution to the development of the bio-industry by providing scientific and technical services to various users. The MRCs in countries must endeavor to maintain close coordination with each other and dedicate to exploration and promotion of utilization of microbes.

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**Box 11.6 The Brazilian Network**

The increasing demand for high-quality biological material and information as a consequence of the growth of the Brazilian bio-based economy is requesting the implementation of strategies and funding mechanisms to enhance and consolidate an integrated infrastructure of distributed biological resource centers. The goal is not only to underpin the actual needs for biological material from industry and academia, but to foster innovation in biotechnology, addressing issues related to emerging legal, technical, and sanitary barriers associated with the access of biological material and genetic resources to the global market.

## History of the Brazilian Resource Centers Network

The need for consolidating a network of microbial collections in Brazil was discussed for the first time at the Second International Congress of Culture Collections held in São Paulo in 1973. The recommendations from this congress organized by the Brazilian Society of Microbiology (SBM) in collaboration with the WFCC influenced the 1980s and 1990s strategies for development of biotechnology in Brazil. A key enabling instrument for improving microbial resource centers in Brazil was the implementation of the Program for Human Resources in Strategic Areas (*Programa de Recursos Humanos em Áreas Estratégicas -RHAE*) funded by the Ministry of Science and Technology (MCT). The RHAE program developed in collaboration with WFCC promoted the training of a number of experts in international collections associated with the organization of nearly 50 training courses and seminars focused on issues related to collections management, preservation techniques, and microbial taxonomy.

In the late 1990s the effort carried out by the Organization for Economic Cooperation and Development (OECD) to discuss the critical role of microbial collections as infrastructure to underpin biotechnological innovation was crucial to renew the discussion on the need to improve the bio-collections infrastructure in Brazil. The OECD report on "Biological Resource Centers: underpinning the future of life sciences and biotechnology" (http://www.oecd.org/dataoecd/55/48/2487422.pdf) published in 2001 was the catalyst factor for the establishment of a Task Force to discuss the conformity assessment of biological material. The result of the work carried out by the Task Force is summarized in the document *Sistema de Avaliação da Conformidade de Material Biológico* (MCT 2002, System for the Conformity Assessment of Biological Material) (http://www.ctnbio.gov.br/upd_blob/0000/10.pdf). This timely report summarizes the state of the art of Brazilian collections within the framework of the international scenario and discusses the challenges and opportunities associated with the implementation of a conformity assessment system for biological material in Brazil. The assessment of the local legal framework compared to international norms and guidelines, associated with a proposal for capacity building was key to guide the participation of Brazilian experts at the OECD Biological Resource Center Task Force that resulted in the publication of the "OECD Best Practice Guidelines for Biological Resource Centers" (http://www.gbrcn.org/fileadmin/gbrcn/media/OECD_guidelines_for_brc.pdf) and the "OECD Best Practice Guidelines for on Biosecurity for BRCs" (http://www.oecd.org/dataoecd/6/27/38778261.pdf) in 2007. The Brazilian document on conformity assessment and the OECD guidelines were incorporated as appropriate in the

MCT capacity building strategy and discussed at biannual events organized by the Brazilian Society of Microbiology (SBM). The importance of SBM support to Brazilian microbial collections in this decade was reviewed by Canhos et al. (2007).

Based on the recommendations of the ''System for the Conformity Assessment of Biological Material,'' the MCT launched a capacity building program to improve quality management in selected service collections. The institutional arrangements and the effort to reorganize the institutional systems of collections in Brazil were reviewed by Canhos et al. (2009).

The need to develop strategies focused on the reorganization and consolidation of the infrastructure to support biotechnological innovation in Brazil was addressed by the Presidential Decree 604 (http://www.planalto.gov.br/ccivil_03/_ato2007-2010/2007/decreto/d6041.htm) signed on 8 February 2007. The decree establishes the National Policy for the Development of Biotechnology and makes specific recommendations for the modernization of microbial collections as a key step in the implementation of the Brazilian Network of Biological Resource Centers (Br-BRCN).

## Networking Model and Institutional Arrangements

As opposed to establishing a large national center to provide a wide range of biological materials and specialized services, the Brazilian strategy is focused on the consolidation of a distributed network of specialized resource centers to meet the growing demands of the user community. The reorganization and quality management enhancement of networked collections in organizations such as Fiocruz (*Fundação Oswaldo Cruz*) (http://www.fiocruz.br//cgi/cgilua.exe/sys/start.htm?infoid=5574&sid=17) and Embrapa (Empresa Brasileira de Agropecuária) (http://www.embrapa.br), and specialized collections like CBMAI, the Brazilian Collection of Environmental and Industrial Microorganisms (http://webdrm.cpqba.unicamp.br/cbmai/english/index.php) represent important starters in the strategy for the implementation of the Br-BRCN.

Fiocruz coordinates one of the best-structured networks of epidemiological control and public health in the world and hosts several microbial collections with holdings ranging from archaea, bacteria, and fungi to protozoa. For the last 5 years, Fiocruz has been working on the harmonization of procedures and protocols, focusing on quality management based on ISO/IEC 17025/05 and OECD Best Practice Guidelines for Biological Resource Centers. This program is supported by the installation of the information management software, which is at the moment integrating data from 11 collections at Fiocruz with the System for Collections of Biotechnological Interest (SICol*Net*) (http://sicol.splink.org.br/). The Fiocruz *Leishmania* Collection (CLIOC) (http://clioc.fiocruz.br/index?) a Reference Collection of the World Health Organization (WHO) which is being prepared to be the *core* collection of the Fiocruz BRC. Its experience will be replicated to the other culture collections at the institution. CLIOC has a specialized holding with more than 1,000 *Leishmania* strains, mainly from the New World. CLIOC's mission is dedicated to preservation, storage, distribution, taxonomic characterization, and identification of *Leishmania* and associated information. CLIOC services meet the needs of public research and educational institutions, industry in general, offering assistance and technical and scientific consultancy, training and development of specific research projects.

The MCT's capacity building program focused on quality management in selected microbial collections as candidates to acquire the status of Biological Resource Center (BRC). It allowed the participation of CBMAI and CLIOC in the Demonstration project for a Global Biological Resource Center Network (GBRCN) (http://www.gbrcn.org/). This project is supported by the German Federal Ministry of Research and Education (BMBF) following work in the OECD to improve access to high-quality biological resources and information to support research and biotechnology as a platform for a knowledge-based bioeconomy.

MCT's program aiming at the establishment of the BR-BRCN is being implemented in close coordination with the activities sponsored by Brazilian Ministry of Development; Industry and Foreign Trade (MDIC) focused on the establishment of a Depository Authority for patent purposes at the National Institute of Metrology, Standardization, and Industrial Quality (INMETRO) in association with the National Institute for Industrial Property (INPI); and the implementation of the INMETRO program for certification and/or accreditation of Biological Resource Centers in Brazil.

## Information System Architecture

To support the consolidation of the Brazilian network of resource centers the MCT is funding the development of the μSICol software and implementation of SICol*Net*.

The μSICol is a collection management software to support digital documentation and traceability of all processes associated with day-to-day management of microbial collections, including methods and procedures for strain authentication, preservation techniques, stock control, quality management procedures, and distribution of strains and biological reagents. The software is a multiplatform system, designed to be compatible with different data management systems. It has multi-user and multi-language capability and supports the installation of multiple collections and sub-collections. It is designed to document specific fields of importance to microbial collections based on the WFCC Guidelines for Operation and Management of Collections of Cultures of Microorganisms (Second Edition, 1999) (http://www.wfcc.nig.ac.jp/GuideFinal.html), the Common Access on Biological Resource and Information (CABRI) Guidelines (http://www.cabri.org/guidelines.html), and the OECD Guidelines for Quality Management and is fully compatible with the DarwinCore extension for microbial strains (http://rs.tdwg.org/dwc/). The database provides a specific view that allows the exchange of data using TDWG Access Protocol for information Retrieval (Tapir) (http://www.tdwg.org/dav/subgroups/tapir/1.0/docs/tdwg_tapir_specification_2010-05-05.htm) in a simple and immediate way. The system is being

continuously developed to accommodate new features and requirements, including reports of daily activities and indicators of the collection holdings including taxonomic profile, geographic distribution of deposits, clients, and services provided.

SICol*Net* allows the dynamic integration of strain data available in Brazilian collections with relevant information sources ranging from molecular to ecosystems databases. Alignment with emerging technologies and adoption of internationally agreed standards and protocols to secure systems interoperability are key features of SICol*Net* architecture. The Virtual Catalogue of Strains based on the architecture developed for *species*Link (http://splink.cria.org.br) allows the dynamic integration of strain data with information on host organisms (botanical and zoological information). Using a simple system of mapping and filtering of sensitive data, the system allows data providers to have full control over the data served to the network, with an appropriate crediting system. Each collection determines what data is restricted and what is public. Through a web interface, users may search and retrieve nonsensitive data in different formats, may rapidly and efficiently visualize species occurrence data on maps, and also have access to a number of indicators. The system also provides reports on each collection's profile, based on metadata and on the analysis of online data and reports on data quality.

## Future Developments

It is expected that, in 2012, the Depository Authority for patent purposes at the National Institute of Metrology, Standardization, and Industrial Quality (INMETRO) will be in operation and that the regulatory framework for the accreditation of resource centers candidates to acquire the BRC status will be in place in Brazil. Fiocruz is working on the implementation of a large-scale/long-term effort to establish a Biological Resource Center for Health (BRC - Health) – unique in the world –focused on the study, preservation and distribution of microorganisms and biological materials relevant to neglected diseases; innovation in epidemiology surveillance; as well as the development and production of bio-compounds directed to diagnosis, vaccines, and drugs.

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Similarly, individual collections cooperated in other global initiatives or in those of other communities. All of these initiatives have common goals: that the enormous amount and range of both the living biological material itself and the data pertaining to this material are placed at the rapid disposal of researchers in academia and industry under full legal compliance and curated in a pure and authentic form.

## Examples for Joint Activities and Global Cooperation

### EU Projects Tackling Quality Issues of Data and the Biological Material

The Microbial Information Europe (MINE) project was an early ambitious, taxonomy-based start with a clear user-driven data selection. The main goals were the harmonization and digitization of data on over 150,000 catalogue strains, standardization of formats, and contents of fields of databases, as well as common thesauri (Gams et al. 1988; Stalpers et al. 1990).

On this important base, the Common Access to Biological Material and Information (CABRI) project was built to offer online access to data across various collection databases in Europe with search options through individual and combined catalogues of collections. The great merit of this project was to develop guidelines for two of the service aspects of culture collections: (1) for the handling of the biological material and (2) for the handling of the related data. The laboratory side of the guidelines covered aspects of accession, authentication, maintenance, storage, and supply for such kinds of biological material as bacteria, archaea, fungi, yeasts, animal, human, and plant cells, and genomic material. A compilation of model in-house procedures was added. On the data side, minimal data sets (MDS) and recommended data sets (RDS) were agreed outlining the minimum amount of data that should accompany a particular strain or culture when it is put into a publicly accessible catalogue. Both types of guidelines aimed at raising scientific and technical quality of holdings and data to better support modern research and application. CABRI was later incorporated as a core activity into the EBRCN project, and CABRI guidelines are still available today.

A project with minor microbial participation was the ENBI a regional complementation of GBIF (see below). It formed an intermediate level between national GBIF activities and the global GBIF level. One interesting outcome of this work was a monograph on digital imaging of biological specimens from the zoological, botanical, and microbiological areas. This work aimed at setting and publishing standards for improved quality photography. For the microbial side, among other items, the microscopical mounting method of "agar slides" was presented (Fritze 2005).

Providers and users of biological material worked together in the MOSAICC project on the development of a system for appropriate management of access to and transfer of microbiological resources (see report on http://bccm.belspo.be/projects/). The goal was in particular to help implement the provisions of the CBD concerning Prior Informed Consent (PIC) and mutually agreed terms (MAT) in the context of monitoring transborder movements of biological material.

Within the European Biological Resource Centers' Network project (EBRCN, 2001–2004), emphasis was laid on the development of information documents concerning the various regulatory issues around collection work, such as classification of microorganisms on the basis of risk, Convention on Biological Diversity (CBD), intellectual property rights, regulations

governing packaging and shipping (2010 update available) and control of distribution of possibly dangerous microorganisms (http://www.ebrcn.eu/).

### Recent EU Activities

The ongoing European Consortium of Microbial Resources Centers (EMbaRC; 2009–2012) project includes work on improving protocols for the authentication and preservation of cultures and combines the additional aspects of training and research activities. Within the area of compliance with regulatory requirements, emphasis is laid on the development of a Biosecurity Code of conduct. It aims to improve, coordinate, and validate microbial resource center delivery to European and international researchers from both public and private sectors. The EMbaRC project is a mixture of networking, access, training, and research (http://www.embarc.eu).

### MIRRI: A New Initiative Within the EU Strategy Level of ESFRI

A new initiative to strengthen the European innovation capacities was developed by the European Strategy Forum for Research Infrastructures. The microbiology collection community led by the GBRCN secretariat (see below) and supported by ECCO and EMbaRC worked with ESFRI state representatives to place the Microbial Resources Research Infrastructure (MIRRI) on the ESFRI road map and as such is a priority for research funding. The resultant high-quality global platform will be designed to accommodate the future needs of biotechnology and biomedicine.

MIRRI will bring together European microbial resource collections with stakeholders (their users, policy makers, potential funders and the plethora of microbial research efforts) aiming at improving access to enhanced quality microbial resources in an appropriate legal framework, thus underpinning and driving life sciences research. Emphasis will be laid on the conservation of biodiversity, on services for research and application, as well as CBD-ABS, biosafety, biosecurity, and bio-risk matters. The overall aim is to support research, development, and bio-economy by improving access to and use of the microbial material. On this platform, strong interaction of all kinds of stakeholder working with microbiological material will be enabled, ranging from scientific and industrial providers and users to collections and policy makers, as well as to regulatory bodies and others. Non-European participation is strongly encouraged. MIRRI will integrate services and resources, bridging the gap between the organism and provision of innovative solutions. MIRRI will as well provide coherence in the application of quality standards, homogeneity in data storage and management, and workload sharing to help release the hidden potential of microorganisms. All 57 microbial resource center members of ECCO in the 26 European countries are invited to join the initial consortium of collaborators in this initiative.

## Toward a Global Network

To deliver their services, BRCs preserve their holdings using long-term storage techniques such as cryopreservation and lyophilization depending upon organism type. Quite often, these techniques require optimization to enable not only survival but also retention of properties. Seldom can a single collection invest in preservation research, and often the improvement and testing of new techniques is done through projects. Networks can support each other to carry out research. Not every collection has the ability to handle every strain they are offered, and networks can share the burden with organisms being deposited in BRCs which have the expertise and facilities to handle them. There is extensive legislation that impacts upon access to, the safe handling, distribution, and use of biological resources (Fritze and Weihs 2000; Smith and Rohde 2007, 2008). A number of culture collection organizations exist to help collections keep up to date in a constantly changing legal framework notably biosecurity, shipping regulations and ethical access and use, common information resources can be established and common procedures implemented across the network to ensure compliance. Therefore, networks can increase single BRC capacity.

The work of the culture collection organizations has been invaluable and has only been limited by their voluntary nature, relying on input of dedicated people as and when they can contribute. Collections need to increase the availability of biological material for the verification of experimental data and the authenticity of reference material used in research. Deplorably, the scientific literature is full of data which cannot be verified because the material is either no longer available and/or the material once used to generate the data has changed or deteriorated. This challenge needs to be met with a coordinated approach requiring an infrastructure to support it. Such strategies cannot be achieved by projects with a defined lifespan. At a global level, the GBRCN aims at bringing together regional efforts such as those of ECCO help disseminate the outputs of projects such as EMbaRC as well as the Asian initiatives (*Box 11.5*) and national activities such as those in Brazil (*Box 11.6*) will play a key role.

The GBRCN demonstration project emanates from an OECD Working Party on biotechnology initiative. For boosting the activities, a small central secretariat is presently supported by the German Ministry of Research and Education (BMBF) to coordinate activities to deliver improved support to the life sciences. High-quality research in the life sciences and innovative solutions to global problems requires access to high-quality biological materials and associated information. No one single entity can provide the necessary coverage of organisms and data; therefore, the enormous task of maintaining biodiversity must be shared. Although the goal of the GBRCN would be to bring together BRCs from all four domains, animal, plants, human-derived material and microorganisms, the project focus was on microorganisms.

The GBRCN demonstration project secretariat coordinates some activities of candidate microbial domain BRCs in 15 countries in order to deliver:

- The establishment of a network differentiated from existing organizations
- The implementation of OECD best practice in BRCs (OECD 2007) assessed by independent third parties
- A strategy for the full GBRCN defining its infrastructure and governance mechanisms, its secretariat's structure, and function with a program of activities

A GBRCN will also help the science community address the maintenance of biodiversity range and magnitude, meeting biosecurity requirements, bridging gaps in our knowledge and protecting investments in research. A GBRCN will support the BRCs keeping abreast of modern scientific developments, meeting quality needs for research, supplying authentic cultures, supplying standardized biological material for testing and quality control, developing comparable methodology and harmonizing procedures and reconciling research and development demands with compliance with regulations.

Exploitation of biological materials must be in compliance with conventions, treaties, and law, for example, the CBD. The CBD requires that Prior Informed Consent (PIC) be obtained in the country where organisms are to be collected. Terms, on which any benefits will be shared, must be agreed. The benefits may be monetary but could be nonmonetary such as information, technology transfer or training. If the organism is passed on to a third party, it must be under terms agreed by the country of origin. This will entail the use of material transfer agreements between supplier and recipient to ensure benefit sharing with, at least, the country of origin. Access and benefit sharing rules must be followed by those countries having signed the Nagoya ABS Protocol (CBD 2011). The national implementation of the protocol may well impede access and exchange of materials and information. In this context, the collection community will have to work toward a mutually beneficial multilateral operational framework to facilitate science and the discovery process.

Biosecurity (Box 11.7) impacts heavily on the operations of public service microbial domain Biological Resource Centers, hence the activities of the WFCC and GBRCN. The GBRCN and the European EMbaRC project promote the implementation of OECD BRC best practice which includes the biosecurity guidance as well as aspects of biosafety, particularly in regard to implementation of national legislation. Concerns exist on financial constraints of BRCs/culture collections to implement best practice regarding biosecurity, particularly with the requirement of risk assessment. Another key concern is the lack of easy access to regulations and other information regarding national rules and regulations governing the movement of materials. It is evident that culture collections adopt compliant procedures firstly governed by national laws but specifically compliant with the Biological and Toxin Weapons Convention (BTWC). They must endeavor to reduce the potential for misuse of biological agents, toxins, or associated information or technologies. To this end, the GBRCN and EMbaRC projects have designed a Biosecurity Code of conduct for BRCs which, when finalized, will be (morally) binding for GBRCN members. The Biosecurity Code of conduct for BRCs sets out an undertaking by microbial BRCs to tackle their responsibilities and provides a baseline for their operation.

**Box 11.7 Biosecurity**

Research on biological material and the resulting knowledge have benefitted mankind in many respects, ranging from basic science to applied agriculture to medicine and biotechnology. However, as so often, scientific results can also be used for malicious purposes – the dual use potential. This possibility includes not only information, but also access to the biological material itself. At first the political accent was on biological warfare. Bioweapons are attractive because they are relatively cheap, leave the infrastructure intact, are self-perpetuating but may allow immunization, and have a delayed onset. Hence political activities concentrated on arms control, resulting in the Biological and Toxic Weapons Convention (1972, BTWC) with the aim to prohibit the development, possession, and use of biological weapons, and in the Australia group, which intends to prevent the supply of harmful organisms to malafide third parties.

When Ivins in 2001 sent a series of letters with contents contaminated with *Bacillus anthrax* spores, the risk of misuse of microorganisms suddenly became apparent; he changed the microbial world. Although the number of victims was limited (22 infected, 7 deceased), the consequences were severe as the public was shocked. The horror scenario of a mad scientist threatening society had suddenly become reality. The trust in a world containing only scientific institutions with sufficient instruments of self-control had been shattered. Biosecurity issues became a major concern for politicians, who immediately reacted by increasing the budget for biological warfare research and regionally by radiating imported parcels, thus jeopardizing the sharing of all biological material. The latter was remedied quickly, but the need for well-executed and transparent biosecurity regulations and the raising of public awareness remained. The task to restore the trust was taken up by both international organizations and the scientific community, and two major contributions were made to provide clear and reliable guidance: the OECD Best Practice guidelines for BRCs (2007), including the OECD Best Practice guidelines on Biosecurity for BRCs, and the Laboratory Bio-risk Management Standard, CWA 15793 (2008).

In analogy with biosafety, biosecurity also recognizes four risk categories. They are labeled negligible, low, moderate, and high risk, and by necessity the definitions for these categories do not allow unambiguous classification. Moreover, they are based on the biosafety classification, and hence focused on threats against humans, not crops.

The international standardization of lists dealing with the organism content of biosecurity risk groups, or at least with those directly affecting humans, would be advantageous, but

the political impediments are considerable and have not been solved yet. With regards to plant pathogens, the situation is even worse, because national legislation only concentrates on national interests, e.g., in the absence of a host in the respective country, a pathogen for that host is not considered to represent a risk. However, potential abusers may obtain such material from research groups or BRCs in those countries where such an organism is not on the quarantine list. This must be prevented by all means.

In order to decide on the necessary biosecurity measurements for a specific organism, a risk assessment has to be performed. As the potential targets for dual use are not only humans but also crops, life stock, or the human environment in general; these elements have also to be considered, and the biosecurity classification cannot be a simple translation or adaptation of an existing biosafety classification. Although for the human aspects there may be a considerable similarity, there will also be significant differences. For example, the highest biosafety category contains only viruses, while the highest biosecurity category contains also *Bacillus anthracis*, *Francisella tularensis*, and *Yersinia pestis*, next to the toxin producer *Clostridium botulinum*. Especially where risks for crops are concerned, the role of fungi (which with a single possible exception does not qualify for the highest biosecurity level with regard to humans) becomes important.

The requirements for a risk assessment in compliance with the OECD Best Practice guidelines are high. When sources of potential harm have been identified, the following elements have to be considered to assess the potential for misuse:

- Availability of the organism in nature
- Requirements (necessary skills and knowledge) for isolation and reproduction
- Environmental viability (survival chances)
- Conditions for dispersal (air or contact)

In case of virulence, knowledge on the infective dose, pathogenicity, lethality, incubation time, and transmissibility is required, as is information on the presence of effective countermeasures.

It will be clear that due to the high demand, many organizations and institutions cannot fully comply with these requirements, and ways have to be found to remedy this. In practice, bio-risk assessment is performed by comparison, which includes the biological material, its interaction with the substrate, dispersal system, knowledge of properties of taxonomic relatives, even tests on the organism itself. Data on virulence are usually absent, or scattered in the literature, and a tedious search for such properties need to be performed. In practice it has worked well as far as the author is aware, but in order to comply with the guidelines, collaboration with other facilities and access to specialist knowledge has to be established. In this scenario, the outreach of international societies could play an essential role.

Within an organization having to deal with biosecurity issues, any respective measures need to be implemented as part of the quality management system and regulated and supported by the senior management. It is their task to integrate bio-risk management throughout the organization, provide adequate resources and identify opportunities for improvement and prevention. They are responsible for the appointment of qualified staff and for subsequent training to maintain the desired quality. They have to convince the funding bodies of the necessity for good biosafety and biosecurity management and to provide the personnel with a supportive environment, involving working space and equipment. They also are responsible for compliance with legal requirements, communication to staff and relevant third parties, and for a reliable and appropriate risk assessment. Finally they are responsible for screening the outgoing information on potential dual use. In practice they should appoint a Biosecurity Officer to ensure internal compliance with the adopted regulations.

In order to obtain maximal collaboration of staff, it is essential that the awareness level be high. It is necessary to devote specific and sufficient attention to the education and additional training of all staff to the risks of misuse of biological material, information and life sciences research and the requirements of regulations in this context. This requires not only training in, but also auditing of, knowledge and practices with regards to biosecurity. Moreover it is also the responsibility of a biological resource collection (BRC) or research group to inform involved third parties on their responsibilities, for example, when high-risk biological material is shipped to authorized users.

Within an organization, accountability is an essential element. Both management and staff should be aware of the presence, location, and risk of the organism they are using. BRCs should maintain and update inventories of the biological material in their custody. Any finding or suspicion of misuse of biological material, information, or technology has to be reported immediately and directly to competent persons or commissions within the organization. To maintain trust in these institutions, persons reporting on misuse have to be protected. They must not suffer any adverse effects from their actions.

Restriction to the accessibility of potential dual use biological material is a vital element in biosecurity management. Depending on the appropriate biosecurity risk level resulting from the biosecurity risk assessment, physical security has to be selected. For a low level, a generally secure area is sufficient, but for a moderate or high-risk level, respectively, a restricted area or a high-security area, with different degrees of containment, are necessary. Staff and visitors have to be screened before access is allowed to areas in which potential dual-use biological materials are stored or used.

Scientists and BRCs should undertake an information risk assessment to determine what information presents a potential biosecurity risk and steps need to be taken to protect such information that could be used to locate the material and facilitate theft. During assessment or application procedures and during the execution of research projects on potential dual-use aspects, emerging threads have to be considered. Any risk that publication of results on potential dual-use organisms will contribute to misuse of that knowledge has to be minimized.

Access of unauthorized persons to internal and external e-mails, post, telephone calls, and data storage concerning

information about potential dual-use research or potential dual-use materials has to be prevented and communication of sensitive information has to be regulated.

Transport of biological material classified as moderate or high requires special conditions, both inside and outside the organization. Inside the organization, transport containers are required and high-risk material may never be unattended outside the high-security area. In case of transport to third parties, both these parties and the transporters have to be screened for both their capacity to deal with the material and their intentions to prevent dual use, in consultation with the relevant authorities and parties. Export control has to be performed in accordance with applicable regulations.

For packaging, the WHO guidelines on International Regulations for the Packaging and Transport of Infectious Substances and the International Air Transport Association (IATA) Dangerous Goods Regulations (DGR), or other applicable regulations, for example, for road transport regulations in various countries, should be utilized.

International projects and organizations are now working on a Code of Conduct for BRCs, combined with an inventory of problems occurring in practice when implementing the guidelines. They are also involved in the setting up of a database, which should allow fast and reliable information on legislation and regulations per country, for example, import and export regulations for microorganisms, transport regulations, quarantine organisms, biosafety and biosecurity regulations, classification lists for human pathogens, animal pathogens and plant pathogens. It should also contain a list of experts that could advise on biosecurity items.

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## Capacity Building

The paucity of national BRCs and the challenges outlined above necessitate an efficient coordinated program for capacity building. Again, a task that is better organized and delivered by a network. The major regions or countries of the world are serviced by collections providing whole organisms, but there are gaps particularly in countries rich in diversity but poor in resources. There are about 60 major collections supplying cultures in Europe while there are very few in Africa. It is necessary to establish expertise and facilities to access conserve and utilize microbial diversity. Capacity building is one of GBRCN's pillars for the sustainable development of Biological Resource Centers. Through the transfer of knowledge as well as skills and abilities, GBRCN supports BRC candidates as well as acknowledged BRCs and accompanies them through the development process. The focus of the capacity building is

- To help countries to establish BRCs
- To enable BRC candidates to become BRCs and GBRCN members
- To assist approved BRCs to gain higher levels of compliance to the OECD best practice guidelines (BPG)
- To support political and socioeconomic decision-making processes in the fields of BRC related bio-economy

## Authenticity Check of Microorganisms

One of the most crucial tasks of public collections is the delivery of authentic material that matches most closely material originally accepted by the collection. Changes can occur through human error during routine maintenance steps of subcultivation or intrinsically due to one of the laws of biology, namely, populations of organisms change genetically and irreversibly through time (http://hunstem.uhd.edu/ABOUT/). Especially in microorganisms, detection of such changes is challenging as short generation times and high mutation rates unavoidably lead to the formation of heterogeneous cultures. Diversification occurs at the genetic and epigenetic level during growth and repeated cultivation as has been observed for decades and experimentally proven more recently (Lenski and Travisano 1994; Rainey and Travisano 1998). As pointed out by Arber (2003) using *Escherichia coli* as an example, spontaneous genetic mutations occur in single cells at a rate of 0.1–1 % of cells per generation during exponential growth and also in the stationary phase. If cells were singled out, the genetic variation would be subjected to natural selection and consequently to reproductive and geographic isolation. The observation of ecotypes reported for strain-rich species, differing in physiology, genome content, and ecology (Cohan and Perry 2007), already starts at the strain level.

Collection curators are aware of the risk of increased microevolution by repeated sub-cultivation, most obvious by the loss of plasmids and changes in colony morphology, and have reacted by keeping the numbers of culture transfers to a minimum. As minute changes may occur during growth and sudden environmental changes such as refrigeration and freezing, most public service collections in their terms of delivery include a clause in the paragraph concerning the quality of products phrases such as the one used by the DSMZ: "Customer is aware of the products and services being biological material and therefore subject to changes of quality beyond the control of DSMZ. DSMZ's high quality standard supports availability of pure, viable and authentic biological material. DSMZ shall not be deemed to have guaranteed certain properties of the products and services except if it has expressly confirmed such guarantee in writing."

In order to ensure as far as possible the originality of the cultures, strains deposited are preserved with a minimum number of transfers. The type of preservation method depends on the organism, but freezing a few straws or alternatively glass capillaries with material from the original culture received in liquid nitrogen is good practice (see http://www.cabri.org/guidelines/micro-organisms/M204.html). Depending on the estimated number of requests by users, this master stock should contain between ~6 and ~50 straws/capillaries, one of which is used for

the production of lyophilized cultures. The number of replicates of this stock, too, depends upon the estimated user demand.

Authentication differs from the characterization of a strain in goals and effort. Characterization attempts to circumscribe a novel organism by as many properties of taxonomic value as possible to allow subsequent unambiguous identification. The range of properties to be determined is usually set by the properties defined to be useful for members of taxa (usually species and genera) described previously. Authentication may only use a subset of such properties or, especially today, molecular traits that had not been included in the original description. In both cases, characterization and authentication, purity checks of the culture under investigation, is mandatory. In the pre-molecular era, authenticity checks relied upon microscopy, culture characteristics (e.g., pigmentation or colony characteristics) and basic physiological tests. As the latter requires time for growth, confirmation of identity took at least a few days. The introduction of semiautomated commercial substrate utilization and enzymatic reaction panels shortened the time for authentication. These tests work well for those taxa which were among the hundreds of strains used for the construction of associated databases, such as those used in the food and pharmaceutical industry and on species found routinely in the clinical environment. As databases are not available for strains of new species, the use of such identification systems often delivers disappointing identification results and misidentifications. Also, as nowadays descriptions of newly named species embrace a single strain only, the type strain, the spectrum of reactions is usually too narrow to unambiguously identify additional strains of such species which may deviate in their physiological reaction from the type strain. Nevertheless, species descriptions published over the past 20 years rely on commercial kits for circumscribing substrate utilization patterns, acid production and the activity of enzymes and only rarely manually prepared media for checking such properties (e.g., Smibert and Krieg 1994). Consequently, in order to obtain more reliable test results an ad hoc committee for the reevaluation of the species definition in bacteriology (Stackebrandt et al. 2002), and more recently Tindall et al. (2010) strongly encourage authors of new descriptions to refrain from commercial kits for studying metabolic reactions.

For culture collections, the molecular era started with the introduction of one-dimensional protein patterns (Kersters and De Ley 1975), restriction enzyme and PCR-based techniques and 16S rRNA gene sequence analyses (Fischer et al. 1983). Although the latter method has the advantage of being highly reproducible and allows the generation of a cumulative database, the highly conservative primary structure does not allow discrimination at the strain level. Two other molecular techniques, DNA-DNA hybridization (DDH) (Johnson and Ordal 1968) and DNA-rRNA hybridization (DeLey and De Smedt 1975), have been used for classification at the family and genus level, respectively. However, they have failed to authenticate at the strain level. Even the DDH method lacks reproducibility to affiliate isolates to strains. Several molecular typing methods (PFGE, AFLP, RAPD, RFLP, and more) are more reliable for strain authentication though the lack of a portable database somewhat restricts the applicability of these techniques to the level of individual laboratories. Molecular methods can differ widely in their ability to differentiate strains, and the user should be aware of the strength and restrictions of the individual methods. An excellent summary of early techniques available in the 1990s, their intra- and interlaboratory reproducibility, equipment needed, costs, and duration, has been given by Olive and Bean (1999).

Among the typing methods, the ribotyping (Grimont and Grimont 1986) approach has been successfully established in several public and industrial collections, executed either manually or by the automated Riboprinter microbial characterization system (E. I. du Pont de Nemours & Co., Inc.). It has especially facilitated strain identification over the past 10 years through the ease and reproducibility of the robot functionality. The advantage of ribotyping over the amplified rDNA restriction analysis (ARDRA) (Pukall et al. 1998) approach lies in the use of the entire rrn operons rather than of individual 16S rRNA genes. In that restriction sites also occur within structural genes, the spacer regions including tRNAs and the flanking DNA regions, the diversity of fragments hybridizing to a fluorescently labeled rrn probe is much higher than those of ARDRA.

The recent introduction of the MALDI-TOF approach is a major step forward in a fast and cheap, though highly reliable, authentication at the strain level. While riboprinting works on the gene level, the highly amplified ribosomal proteins are the main targets for the MALDI-TOF spectrometric method. The resolution power of this method is significantly higher than that of comparative 16S rRNA gene sequence, often being suitable for affiliation of strains to the subspecies level. However, as the species as defined today does not necessarily reflect genomic coherency, the range of intraspecies dissimilarities determined for strains may vary between different taxa. A linear TOF mass spectrometer operates on the principle that when a group of ions of differing mass/charge (m/z) ratios move through a region of constant electric field, they will traverse this region in a time which depends upon their m/z ratios (for more details and the principle of a reflection TOF-MS, see http://www.abrf.org/abrfnews/1997/june1997/jun97lennon.html). As not all proteins occurring in a cell will be charged, only a small fraction of protein masses are detected, usually in the mass range between 2,000 and 12,000 m/z. The advantages of working with this approach, especially for the identification of those species for which a high number of strains (mainly of clinical origin), have been analyzed, and spectra deposited in databases have been outlined in Chapter X of Konstantinidis and Stackebrandt (2011). Public service collections usually cover a broad range of diverse microorganisms for which commercial MALDI-TOF databases are less well suited. In this case, the authentication laboratory has to generate tailor-made in-house databases for specific taxa. The method has been used to verify the authenticity of many type strains of different taxa maintained in different EMbaRC collections (Schumann, Bizet, Arahal, and de Vos, unpublished). The majority of these strains showed highly similar MALDI-TOF protein profiles, indicating that microevolution does not affect the masses of housekeeping proteins and ribosomal proteins; the few strains identified as being

misclassified or mislabeled have been discarded and exchanged with authentic ones. MALDI-TOF appears to be the method of choice for routine identity checks of strains in long-term storage and for authenticity checks before shipment.

## Access to Microbial Biodiversity Data

A wealth of invaluable information for academic and industrial users as well as regulatory bodies is available in resource collections, and research data are scattered in hundreds of thousands of publications since the beginning of microbiology. To put this treasure at hand, individual approaches will not suffice any more to meet the global needs. Modern information technology developments will offer increasingly easier access to data, while working toward interoperability of databases will make searching through countless databases all over the world practicable. In order to archive a comprehensive overview on published data and to offer these data to the user, standardization of data production, data recording, and data presentation should be harmonized, at least to allow text mining software to efficiently extract data. The unstructured recording of phenotypic data in current species descriptions of prokaryotes is a simple example of the present-day failure to cope with strain-associated data. It is, however, promising to see recent attempts to standardize the electronic exchange of meta-information about microorganisms which led to the definition of the Microbiological Common Language (MCL) (Verslyppe et al. 2010) within the framework of the Bacterial Commons (Dawyndt et al. 2006).

### CODATA, MINE, CABRI, and GBIF: Examples of Past and Ongoing Activities

A number of initiatives aiming at the coordination and standardization of activities to give access to information available at culture collections have been set up. Among them, WDCM (see *Box 11.3*), CODATA, MINE, and CABRI have set baselines and provided model roles.

CODATA, the Committee on Data for Science and Technology, is an interdisciplinary Scientific Committee of the International Council for Science (ICSU) founded over 30 years ago. Its main objective is to foster and advance science and technology through developing and sharing knowledge about data and the activities that work with data (www.codata.org).

MINE (see for details further above) was designed to harmonize and computerize data on >150,000 strains of microorganisms in European culture collections and to develop a common database on microorganisms held in culture collections. Requirements for efficient data recording and computerization were established by the 12 participating European countries together with details of the data structure used, the hard- and software configurations, data entry procedures and online access. The main merit of MINE has been the development of an internationally agreed format: 135 fields for fungi and yeasts and 145 fields for bacteria which cover the most important aspects of microbial taxonomy, ecology, physiology, and biochemistry, also including data pertaining to the practical applications of microorganisms. The disadvantage is that entries need to be added manually, thus prone to errors and omissions.

The Common Access to Biological Resources and Information (CABRI, see further details above) has its roots directly in the previous MINE project from which it inherited the dedicated taxonomic data structure. Its main goals are to increase awareness of the scientific user community of the quality and variety of European culture collections and to ease access to information and material. In order to reach this objective, the project has implemented a unified access to culture collection catalogues of participating collections, by also guaranteeing a common level of quality of material and related information. The final achievement of the project has been the development of an online "one-stop-shop" for biological resources (www.cabri.org) which allows the user to check on the availability of a particular item, interrogating one or more catalogues at the same time, and to pre-order the required biological resources, once located. CABRI membership is open to any recognized European BRC, willing and able to work at the CABRI quality levels.

Participating resource centers agree to find consensus for data fields and content type, harmonize procedures and agreeing on equivalent methods and procedures and producing guidelines for each type of biological material. CABRI can be seen as a pioneer model for an integrated, while distributed database which is searchable through a common gate. CABRI is currently available through the main website (see http://www.cabri.org/) and four web mirrors (see http://www.it.cabri.org/, http://www.be.cabri.org/, http://www.fr.cabri.org/, http://www.cn.cabri.org/).

Three distinct data sets were defined for each biological material. The minimum data set (MDS) consists of mandatory information needed to identify a unique strain or cell line: strains for which this information is not available cannot be inserted into the catalogue since they lack essential data. The recommended data set (RDS) includes useful information for an improved description of the material. These data should always be included in the catalogue, when available. The Full Data Set (FDS) provides all remaining information that is available at the collection for a strain or cell line. Since the individual CABRI catalogues are independently built, each collection can have its own FDS, although information which is available in the FDS undergoes a harmonization effort (http://www.cabri.org/guidelines/catalogue/CPexport.html).

At an international level, major attention has recently been devoted to bioinformatics for biodiversity. Discussions initiated through the OECD Working Group on Biological Informatics 1996–1999 have led to the Global Biodiversity Information Facility (GBIF). GBIF aims to provide a worldwide network of interlinked biodiversity databases so that the worlds' scientific biodiversity data can be made freely available. It is based on an implementing agreement signed by governments and interested organizations. The initial focus of GBIF is on species- and specimen-level data. It works in close contact with existing and ongoing activities with similar or complementary goals on

a national and international level. Implementation of GBIF is nationally and/or regionally driven (see http://www.gbif.org/). However, microbial data are still today represented only on a smaller scale. The extended Darwin Core (as developed by the Brazilian group) reflects better the needs of microbiology than the ABCD Schema and will be the basis for the envisioned future information resource within GBRCN.

## The OECD Best Practice Guidelines

In the OECD BRC best practice guidelines, detailed requirements are formulated for the processing of biodiversity data. Clear reference is taken to previous works such as CABRI, GBIF and WDCM. The following is an excerpt from OECD best practice guidelines – general (DSTI/STP/BIO(2007)9/FINAL), heading Data and Informatics which defines the responsibilities of resource centers and depositors concerning quality assurance:

**Box 11.8 Data and informatics**

39. The BRC should manage and store data and produce electronic catalogues based on authenticated and validated information.

### Data Management

40. Depositors are responsible for assuring the quality of data associated with the biological material. The BRC may require evidence to assure the validity of the data.

41. The authentication of data may differ from center to center, but a BRC should:

- Provide traceability of data through a history of modifications (dates and signatures of inputs, validations, modifications and deletions).
- Give signature for data entry, validation, modification or deletion.

42. The BRC should use a standard terminology and formats for data management and exchange and standard protocols for data transmission to networks (domain, regional or global networks):

1. Select data format, data representation and data transportation taking into consideration existing standards for data processing, e.g. DarwinCore/DiGIR and ABCD schema/BioCASE for strain data, CCINFO for the organizational information of BRCs.
2. Check vocabulary against standard reference lists or thesauri.
3. Keep consistency among BRCs for searching and retrieving of information from catalogues and databases:
   - Each biological material record should contain a Minimum Data Set, a Recommended Data Set, and/or a Full Data Set in accordance with domain specific criteria.
   - Spell checking for every field should be a basic requirement.
   - International English should be chosen as a preferred language of data (in addition to local language if different).
   - A standardised approach should be adopted to certain scientific symbols (to avoid any errors due to incorrect reading of a character set, standard ASCII alternatives to symbols should be used (examples follow):

43. BRCs should adopt procedures to detect errors in data to improve their quality and consistency. This is an essential part of information management and should be both applied to the input of new data as well as to preexisting information in current databases:

- For existing data, a series of checks should be carried out to ascertain their validity and completeness. As more BRCs become associated, more searches should be made for common classes of error to allow more efficient error correction.
- For new data, wherever possible, inputting should be checked against authorized lists of not only scientific names but also thesaurus/ontology to prevent errors such as mistyping.
- BRCs should present evidence that they have applied a recognised protocol appropriate for each data element (A comprehensive treatment of Data Cleaning can be found in Chapman, A.D., *Principles and Methods of Data Cleaning – Primary Species and Species-Occurrence Data*, Version 1.0, Publisher - Global Biodiversity Information Facility (GBIF), 2005.

### Data Processing

44. The informatics system employed by BRCs should provide appropriate facilities for information management, linkage and exchange.

45. The databases should contain either information relating to strains held by a BRC (which at least, should be retained as long as a strain remains viable), or other relevant data items or composite data needed by the BRC (e.g. users records). On the loss of a strain the database record should be either printed and stored on file or copied to a digital archive before the entry is removed from the working database, placed in reserve or annotated to indicate that it is no longer available as living material.

46. The BRC should preferably choose standard data schema and protocols to make the databases distributed and interoperable. Confidential data should be clearly identified in relation with user authentication capability, encryption techniques and other related information security tools.

47. The informatics system should ensure regular data back-up. Off-site storage of data is desirable. Data archives should be maintained in accordance with the maintenance of the biological resource storage policy. The support of these archives should be regularly updated according to its physical characteristics (obsolescence) and to software compatibility.

48. BRCs should introduce appropriate measures (protocols, tools and standards) in their own informatics systems to assure reasonable security of information. There are existing systems, e.g. authentication by user ID and password, encryption, encryption of messages and restriction of IP addresses that may provide the basis for such measures. Backup-files should be stored in secure cabinets.

From: OECD Best Practice Guidelines –General (DSTI/STP/BIO (2007)9/FINAL)

### Minimum and Recommended Data Sets

Based on the previous works in MINE and CABRI, the concept of minimum and recommended data sets was adopted by the OECD guidelines and slightly updated, in particular with a view to the necessary information of "country of origin" for implementing the requirements of the Convention on Biological Diversity. It should be noted, however, that these attempts defined data fields but have failed to set up a standardized, open infrastructure that allows electronic processing. *Table 11.3* shows as example the data sets for bacteria. Similar data sets are also available for filamentous fungi, yeasts, protozoa, cyanobacteria, archaea, virus, plasmids, phages, and cDNA/cDNA libraries.

**Table 11.3**
**Minimum (MDS) and recommended data set (RDS) bacteria**

| Minimum data set | Recommended data set (in addition to MDS) |
|---|---|
| Accession number | Serovar |
| Other collection numbers | Other names |
| Name | Isolated from |
| Infrasubspecific names | Mutant |
| Organism type | Genotype |
| Restrictions on distribution | Literature |
| Status | |
| History of deposit | |
| Geographic origin | |
| Conditions for growth | |
| Form of supply | |

## Long-Term Storage of Microorganisms: At the Very Heart of Collection Activities and Responsibilities

### Why Do We Need Long-Term Preservation?

Proper preservation methods suspend metabolic activities instantly while retaining viability and genetic and physiological stability of the specimen. This is the basis for the safe and long-term maintenance of strains of microorganisms of scientific or industrial interest, which is an inevitable prerequisite for continuous and efficient research and production. Availability of strains maintained in a genetic and physiological unchanged state must be guaranteed over years. For example, important production strains, reference strains for research and testing and other strains with valuable properties should be available for comparative examinations even after decades. Or, to give another example, sometimes years after a certain bacterial species has been described, it becomes clear that strains of this species have important, useful properties. It is then most helpful to have the reference strain available in the most original state and ready to be shipped. If such a strain is lost, time, information, and research funds are lost as well, and the re-isolation of strains with exactly the same properties as that of the type strain is highly unlikely.

Despite the fundamental significance of reliable availability of pure and stable cultures, quite often, only little attention is paid to the maintenance of these cultures in research laboratories. It is obvious that in terms of strain maintenance, these facilities have different requirements than those required in a public culture collection. As usually neither equipment nor staff experience is available in the research laboratories, some basic procedures for proper curation of strains and information should be implemented. Still today, bacterial cultures are often maintained over years through periodical transfer onto fresh media. However, this practice is not only considerably time and material consuming but presents risks of contamination, selection of mutants, loss, and mislabeling, and therefore the method should be avoided. Scientists should identify as early as possible those strains worth maintaining from their research. As has been outlined above (*Box 11.1*), scientists have the obligation to share with peers those strains included in scientific publications, meaning that subcultures of such strains need to be available and prepared in a manner that optimally preserves the properties of the original culture. It is our recommendation to contact public service collections to guarantee availability and dispatch according to national rules and regulations.

A number of reliable methods for short, medium, and long-term maintenance of microorganisms have been developed (Kirsop and Doyle 1991; Day and Stacey 2007). Some of the short-term methods are simple and may be performed in any laboratory but may not be successful for a broad range of organisms. For long-term methods, more sophisticated equipment is required; these, in turn, seem to be effective for most microorganisms. However, it must be stated that there is no universal preservation method for all microorganisms. Different taxonomical groups, even strains within a species, may react differently to the various preservation conditions. *Table 11.4* gives an overview of different long-term preservation methods.

**Table 11.4**
**Comparison of some methods used for long-term preservation**

| Method | Costs for material and equipment | Working time consumed initially | Working time consumed over storage time | Working time consumed on supply | Survival of organisms | Genetic stability | Space needed | Suitability for shipping | Danger of contamination | Dependence from power supply |
|---|---|---|---|---|---|---|---|---|---|---|
| Periodic transfer | Low | Low | High | High | 2–6 months[a] | Low | High | Active cultures acceptable | High | Cool room required |
| Drying over silica gel | Low | Medium | Medium | High | 1/2–7 years[a] | High | Low | Difficult for individual replica; needs reactivation | High | Can be independent |
| Deep freezing −80 °C | Medium | Medium | Medium | High | 1/2–7 years[a] | Medium | Medium | Needs reactivation or shipping on dry ice | Depending on method; high | Dependent |
| Freeze drying | High | High | Low | Low | > 40 years[b] | High[d] | Medium | Very good | Low | Can be independent |
| Cryo-storage in $LN_2$ | High | Low | Low | High | > 40 years "indefinite"[b] | High | Low | Needs reactivation | Low | Can be independent |

[a]Depending upon taxon and strain
[b]In service collections, experience indicates that organisms that survive the first ~5 years will survive "indefinitely" in all probability
[c]Experience shows that if organisms survive the initial freezing and, upon recovery, the thawing process, they will survive indefinitely
[d]If drying is performed too extensively, bound $H_2O$ may be removed; this may result in DNA damage

## Choice of Preservation Technique

Each of the different methods has its advantages and disadvantages which we need to know to ensure the right one is applied to meet the special conditions or needs in each circumstance. The selection should be decided upon after comparison of the conditioning factors of a given method, the available equipment and the needs of the user.

Some general aspects should be considered with any choice of methodology:

- Viability should be maintained as high as possible.
- Genetic changes should be avoided as far as possible (this is also most likely supported by a high survival rate).
- The risk of contaminating the preserved culture should be as low as possible.

Some technical aspects refer to effort/efficiency balance which is especially important when larger numbers of organisms need to be processed:

- The number of ampoules or replicas to be prepared should be considered according to the procedure chosen (expense of work, material or space for storage, authentication procedure, demand).
- If cultures are to be supplied to third parties, they must be present in an appropriate form; public collections almost always assume that strains will be requested.
- The present or envisaged market need for a given culture.
- The availability of space in the facility, its financial situation, and personnel expertise and availability.

1. Drying methods have the advantage that, once the culture has been successfully preserved, material can be stored independently from power supply. This can be done in the dark at ambient temperature, though storage at lower temperatures between ∼+10 °C and ∼−20 °C extends shelf life considerably. Dried specimen ("ampoules") can be used perfectly for shipping of cultures, and the method bears relative low costs for material.
2. Storage at ultralow temperatures (below −139 °C), for example, the procedure of preservation in liquid nitrogen ($LN_2$) at −196 °C ("cryo-storage") is much faster and more reliable than any other methods. As a drawback, costs for material are higher, and preserved cultures need to be revitalized and incubated before shipping.
3. Temperatures of −70° C to −90 °C as generated by electrical deep freezers or by solid $CO_2$ (below −78 °C) have been shown to give useful results for the preservation of some types of microorganisms. Freezing at −80°C is often applied especially in research groups. This can be an acceptable method for medium-term storage of cultures maintained for own, in-house use within a research group. However, these temperatures range within the margin where water migration into cells is possible, and therefore, cells have to be carefully protected by appropriate additives to obtain reasonable periods of storage.

As a conclusion, the safest storage for microorganisms to be preserved long-term is provided by liquid nitrogen. Nitrogen is also much safer than other liquefied gases as it does not burn, is not toxic, and is cheaper than other gases. Nevertheless, care must be taken to avoid suffocation due to displacement of oxygen. However, when considering continuous supply of cultures, drying, and storage of microorganisms under vacuum is the method of choice for preservation.

## Factors Influencing the Survival Rate of Microorganisms During Freezing and Drying

Successful preservation of microorganisms not only depends on the application of an appropriate cooling, drying, thawing or rehydration regime. Other factors (*Table 11.5*) determined by the organism itself have been shown to be important, such as type and strain of the organism, growth conditions, nutritional status, and growth phase. Additionally, the diluting medium and growth medium used for reactivation and determination of viability will influence their recovery.

Nevertheless, for most procedures, basic factors determining survival of the preserved organisms are similar.

### Protective Suspension Media for Freezing or (Freeze-) Drying

For the protection of cells against damage during drying, freezing, and freeze-drying as well as during storage, microorganisms have to be suspended in a protective suspension medium. The composition of these media may depend upon the type of organism to be preserved. Some examples of protective suspension media are given below. However, as far as possible, widely applicable routine methods should be established. Especially with larger collections, the laborious and time-consuming individual treatment of each culture cannot be afforded.

Protecting effects of compounds have been assigned to maintaining macromolecular structures (replacing $H_2O$ molecules) (Suggett 1975), protecting against $O_2$ or oxygen radicals (Lion and Bergmann 1961), avoiding damage to membranes (Morichi 1970) or maintaining a certain level of residual moisture (Nei 1974; Danilova et al. 1980).

For drying processes, complex organic substances, for example, skimmed milk, serum, and peptones and also pure substances, for example, sugars, amino acids, and mixtures thereof have been proven supportive for keeping high viability.

Substances protecting living cells against freeze-thaw injury (cryoprotectants) can, on the one hand, be compounds with defined low molecular weight, such as glycerol, dimethylsulfoxide (DMSO), methanol, or sugars. On the other hand, these can be compounds with defined high molecular weight, such as starch, hydroxyethyl starch (HES), or polyvinylpyrrolidone (PVP) and undefined substances, such as proteins, malt extracts, or blood (Farrant 1969; Fry 1966; Fuller 2004; Heckly 1978).

**Table 11.5**
**Factors influencing preservation**

| Factors | | Comments |
|---|---|---|
| General | Kind of organism | Size, taxon, strain, suspendability |
| | Culture conditions | Nutritional state |
| | Age | Mid to late log phase |
| | Concentration of cells | The higher the better[a] |
| | Media for preparing the suspension | Protective media, stabilizing structure, replacing $H_2O$, protection against $O_2$, retention of residual moisture |
| Freezing | Freezing and storage temperature | Storage at as low as possible |
| | Freezing velocity | |
| | Cryoprotectant | Depending on the permeability of the membranes |
| Drying | Drying temperature | |
| | Drying velocity | |
| | Residual moisture | Influenced by length of the drying process, temperature of cold trap and final vacuum (ideally $10^{-1}$ to $10^{-2}$ mbar) |
| | Storage conditions like: Gas atmosphere Temperature | Best under vacuum (without $O_2$) 4–10 °C recommended |
| Method of reactivation | Medium | |
| | Temperature | |
| | Rehydration time | With freeze-drying: allow the material to rehydrate for 10 min |
| | Thawing velocity | With $LN_2$: plunge into 37 °C warm water |

[a]Concerning the cell concentration mentioned above, it has been demonstrated that the survival rate is positively influenced by an initially higher concentration of the cell suspension. While for various organisms with an initial cell density between $10^7$ and $10^8$, a drop by two log levels was observed; no drop was observed when the initial cell density ranged between $10^{10}$ and $10^{11}$

The effects of penetrating cryoprotectants are manifold, as they

- Partially replace intracellular water
- Thus prevent a too high increase of salt concentration
- May also replace water molecules for the stabilization of proteins and membranes
- Influence ice crystal formation
- Like DMSO (NOTE: toxic), increases the permeability of membranes

Non-penetrating cryoprotectants work differently as they

- Cause an osmotic dehydration of cells
- Reduce extracellular salt concentration
- Influence extracellular ice formation
- May stabilize membrane structures

An extensive compilation of protective suspension media suitable for the freezing and freeze-drying of microbial strains can be found on www.cabri.org (> guidelines > microorganisms > Part 3: Guidelines for maintaining deposits – Appendixes – M/1998/3.00 Appendix 3).

## Simple Methods for In-House Purposes

Cultures may need to be maintained by simple methods, for example, as cultures on slants which are over-layered with sterile paraffin oil, in distilled water or by simple drying methods. In any case, periodical transfer onto fresh media should be avoided due to the fairly high danger of contamination and physiological and genetic changes (see above).

Cultures may be dried in earth, sand, pumice-stone, above silica gel, or on porcelain or glass beads; however, some facts should be kept in mind:

- A protective medium like skimmed milk or skimmed milk with myo-inositol, serum, or nutrient broth should be used.
- The amount to be dried should be as small as possible.
- The drying process should not take too long.
- The dried cultures should be stored in the cold, if possible under vacuum and dark.

It is recommended to use such preparations rather for in-house purposes than to use them for supply to third parties. The problem usually accompanied with these methods is that the storage receptacle needs to be opened many times to remove the required sample of the dried organism. This immediately presents the danger of contamination and negative impact on survival rates.

When drying over silica gel on glass or porcelain beads is chosen, the bacterial suspension is surface-dried but without direct contact with the drying agent. Silica gel develops considerable heat when taking up water, which may be harmful for the organisms attached. Silica gel with blue indicator (toxic $CoCl_2$) may be used, though today it is recommended to avoid $CoCl_2$ and silica gel with other indicators are available. The amount of drying agent should be sufficiently large so that only the smaller part of the silica gel will change in color during the drying process.

The method of drying over silica gel in gelatine disks, originally described by Stamp (1947), uses a protective medium containing peptone, meat extract, gelatin, and sodium ascorbate. The complex organic compounds are used for stabilizing macromolecular structures and/or to serve as physical barriers to maintain a certain residual water content. Na ascorbate is added as an oxygen radical trap, as it is suspected that cell damage occurs through oxygen radicals. During hardening and

drying of the gelatine drops, small disks are formed which may be stored in presterilized screw cap tubes containing dry silica gel.

## Preferred Methodologies for Long-Term Storage: Freeze-Drying and Cryo-storage in Liquid Nitrogen

### General Aspects of the Freeze-Drying Process

Preservation of microorganisms by drying under vacuum from the frozen state (through sublimation of ice) has been used for more than 60 years. Methods and equipment have been developed over the years and nowadays present a reliable and effective preservation method for most bacteria, fungi, and yeasts. During the freeze-drying process, wet material is frozen and the ice directly transferred into the gas phase. The ice sublimes without melting. The porous cake resulting from this has, in principle, the same size and shape as the original frozen mass. Through adding of water or culture medium, the original state is reconstituted. In general, freeze-dried material is highly soluble. However, it should be noted that freeze-dried organisms are extremely susceptible to oxygen. To exclude this negative effect, the cultures should be stored in glass ampoules sealed under vacuum.

### Practical Aspects of the Freeze-Drying Process

#### Media for Cultivation

Microorganisms should be cultivated on media which allow good growth and from which they can be harvested easily. Incubation on agar slopes is preferred. In the case of liquid cultures, these must be centrifuged before suspending in the protective medium.

#### Age of Cultures

Fast-growing organisms are harvested generally after about 24 h of incubation. This is around the mid to late logarithmic phase. Slow growing organisms must be incubated adequately. Spore-forming bacteria and fungi are incubated until optimal spore formation.

#### Ampoules for Freeze-Drying

Within a small margin, the dimensions of the ampoules are of minor importance. However, with the "single-vial-method," vials may be constricted by hand if the inner diameter is around 6 mm. When preparing the "double-vial ampoule" (see CABRI guideline for more details), outer tubes with an inner diameter of about 14 mm are recommended. With these, using an ampoule constrictor machine is recommended.

#### Protective Medium

For many microorganisms, skimmed milk has been proven an effective protective agent. To avoid caramelization, skimmed milk should be autoclaved in small amounts at 115 °C for only 13 min. Thorough sterility testing is therefore necessary, particularly for the presence of heat-resistant spores of thermophilic organisms. Thus, testing should be performed at 30 °C as well as 55 °C.

#### Sterility

During preparation of freeze-dried cultures, both, the cultures as well as the personnel, must be protected from contamination or hazard. In parallel to the common safety precautions for microbiological work, it must be observed that during the drying process cell material may escape the ampoule in the form of fine particles and contaminate the vacuum chamber or the whole freeze-drying apparatus. To avoid this, the ampoules must be provided with a filtering closure, which, simultaneously, will avoid contamination of the culture when air is allowed to enter the vacuum chamber after the first or primary drying (when true freeze-drying is applied).

#### End Vacuum

The evacuation process must be monitored. Optimally, a final vacuum between $10^{-1}$ and $10^{-2}$ mbar should be reached to guarantee good survival rates over longer periods.

*Note*: The use of silica gel as moisture indicator as used with the double-vial ampoule is meant as an "optical help" only to indicate loss of vacuum during storage. The change in color of the indicator early on in the drying process does not mean that a sufficiently deep vacuum has already been reached.

#### Sealing Off of Ampoules

Sealing off ampoules is done to maintain a vacuum. Care should be taken that the tips are perfectly sealed and rounded so that cracks or breakage during storage can be avoided. In practice, freeze-drying is performed in various ways adapted to specific needs.

### True Freeze-Drying Process

With this method, the bacterial suspension is mixed with protective medium, then frozen and transferred to the vacuum chamber in the frozen state. Vacuum is applied before the suspension starts melting, and water is removed by sublimation.

A full description of the procedure can be found in www.cabri.org (Guidelines > "Click here to read the guidelines" > Microorganisms > Part 3: Maintaining deposits > Appendixes; edited and amended > M/1998/3.00 Appendix 5.08 'Preservation of Bacteria by Freeze – Drying (True Freeze-drying)). In M/1998/

3.00 Appendix 5.08.1, a flow chart of the freeze-drying procedure is shown. For recording each step of the preservation procedure and results of viability checks, protocol form M/1998/3.00 Appendix 5.08.2 is suggested.

## Centrifugal Freezing

To shorten the exposure time to air/oxygen, the decreasing temperature in the freeze-drying chamber due to evaporation can be an alternative method for freezing, as the removal of water under vacuum results in a quick loss of about 10 % water in a relative short time. As this is an energy-consuming process, the residual suspension will freeze. To avoid the strong frothing, which would normally occur and which would expel some of the contents from the ampoules due to the release of gas, the samples are centrifuged during the evaporation process until the material is frozen.

A full description of the procedure can be found in www.cabri.org (Guidelines > "Click here to read the guidelines" > Microorganisms > Part 3: Maintaining deposits > Appendixes; edited and amended. Flow Diagram M/1998/3.00 Appendix 5.10.1 "Centrifugal Freeze-Drying").

## The Double-Vial, Liquid-Drying Method, as Applied in the DSMZ for Bacteria and Fungi

This modified method, applied for example by the DSMZ for a wide spectrum of prokaryotes and fungi, includes a drying step from the liquid state. The advantage is less stressful for the cells and less water vapor developed. Due to the much smaller amount of water vapor, the drying process may be even run without a freezing chamber.

The principle includes the transfer of a small drop of a heavy suspension of organisms in fresh medium onto the porous cake of a pre-dried skim milk pellet. The proportion of the drop of suspension to dried skim milk is such that the amount of liquid is absorbed at once and totally. This method can then be combined with the "double-vial method," where the small, cotton plug stoppered vial, containing the dried skim milk and the drop of suspension, is inserted into a bigger tube. This tube is then constricted and connected to the manifold of the freeze-drying machine.

*Note*: As cells are under extreme stress, the time lapse between transfer of drops onto the milk cake and connection of the constricted vials to the manifold of the freeze-dryer should be as short as possible.

A full description of the method can be found under www.cabri.org (> guidelines > microorganisms. Part 3: Guidelines for maintaining deposits – Appendixes M/1998/3.00 Appendix 5.11).

## Opening of Ampoules

When opening ampoules that had been sealed under vacuum, especially the one vial preparations, care should be taken to avoid the following hazards:

- Contamination of the culture through air entering the ampoule when opening
- Release of fine particles of the dried bacterial mass into the air (the sudden inrush of air when cracking an ampoule may result in a back surge of particle-loaded air), thus contaminating the air of the laboratory

*Note*: If cultures belong to hazard group 1, (freeze-) dried cultures in ampoules sealed under vacuum can be opened in an ordinary transfer cabinet. In other cases, ampoules should be opened in a biohazard safety cabinet of the appropriate level.

A full description of the methods can be found under www.cabri.org (> guidelines > microorganisms. Part 3: Guidelines for maintaining deposits – Appendixes M/1998/3.00 M/1998/3.00 Appendix 5.14).

## Cryopreservation In or Above Liquid Nitrogen

### General Aspects

Freezing of living cells or parts of them to very low temperatures and storage at these temperatures stops metabolic activities and retains viability and genetic stability of the specimens. Even molecular motions are significantly reduced at sufficiently low temperatures and cease below −139 °C. These characteristics make cryogenic storage very attractive for the long-term preservation of living cells.

Studies, as early as around 1900, have shown that microorganisms can withstand freezing down to ultralow temperatures (liquid air, liquid hydrogen). The discoveries of Polge et al. (1949) and of Lovelock and Bishop (1959), that glycerol and dimethylsulfoxide (DMSO) protect living cells against freezing damage, greatly influenced the further development of the technology in this field. Considerable progress has been made over subsequent decades with regard to the control of the freezing and thawing process to obtain optimal results. A broad range of living cells – from the small-sized prokaryotic to the larger sized eukaryotic cells (such as fungi; protozoa; algae; and plant, animal, and human cells and even tissues) – can be retained viable for long periods by low temperature storage (Reed 2008; Day and Stacey 2007).

The safest cryo-storage for both, the organisms to be preserved *and* the personnel, is that provided through liquid nitrogen: −196 °C. Compared with other liquefied gases, nitrogen is safer – it does not burn, is not toxic – and is cheaper than other more rare gases. Excellent storage containers and additional equipment is supplied by several manufacturers in many countries.

Living cells consist mainly of water, and in the protective or growth media, they are surrounded by water containing different amounts of electrolytes. Ice crystal formation occurring during freezing inside or outside the cells removes liquid water. This may impact negatively on cells which normally depend on a balanced ionic environment and hydration state of their macromolecules. Shrinkage of cells and ice crystals may be responsible for damage to the cytoplasmic membrane (Morris 1981). However, it should be borne in mind that ice crystal

formation not only occurs during freezing but also when cells are thawed slowly to subzero temperatures; therefore, rapid thawing is recommended.

To safeguard cells from freezing injuries, cryoprotectants are added to the freezing suspension. For this purpose, certain defined low molecular weight or high molecular weight (see further above) compounds or undefined complex substances are applied. A common characteristic of such compounds is that they are nonionic polar molecules with a pronounced ability to H-bonding. Those compounds penetrating the cell membrane should be applied in molar concentrations (0.5–1.5 M), while non-penetrating agents are used at much lower (around 0.01 M) concentrations.

A full description of the method can be found under www.cabri.org (> guidelines > microorganisms. Part 3: Guidelines for maintaining deposits – Appendixes M/1998/3.00 M/1998/3.00 Appendix 5.04).

### Miniaturized Method for the Cryopreservation of Bacterial Cultures: The Glass Capillary Method

The limited capacity of storage containers, together with increasing costs for equipment and nitrogen, creates problems when larger numbers of different organisms, each in multiple replicas, have to be preserved. Miniaturized methods which have been developed by several investigators may help to overcome this problem (Hippe 1991). The basic idea of these methods is to reduce the volume of cell suspension to be preserved and of the unit holding it. Use of small plastic straws or glass capillary tubes replacing the common vials or ampoules is now standard.

A full description of the glass capillary method is given in www.cabri.org (> guidelines > "click here to read the guidelines" > microorganisms > part 3: maintaining deposits: appendixes; edited and amended > m/1998/3.00 appendix 5.01 Preservation of bacteria by freezing and low temperature storage in glass capillary tubes).

Often, filamentous bacteria and fungi cannot be suspended fine enough to apply the above capillary method. Especially the fastidious, non-sporulating strains need special attention. For such organisms, a smart procedure has been developed in which young mycelium together with the agar on which the organisms is growing is punched out using short pieces of PVC straws. These can be the larger common drinking straws or the thinner straws used for artificial insemination in cattle breeding. Before punching out growth and agar, the growth is covered with a 10 % glycerol solution (take care for thorough wetting of the growth), which is poured off after about 2 h. Several of such straws can then be assembled in a cryo-vial which is stored in the vapor phase of liquid nitrogen.

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## References

Anon (2010) The WFCC Guidelines for the establishment and operation of culture collections. http://www.wfcc.info/guideline.html. Accessed 03 July 2011

Arber W (2003) Elements for a theory of molecular evolution. Gene 317:3–11

CABRI (2002) Common Access to Biological Resources and Information http//:www.cabri.org

Canhos VP, Sette LD, Cupolillo E, Tigano MS, Vazoller RF (2007) O papel da Sociedade Brasileira de Microbiologia no suporte à consolidação da Rede Brasileira de Coleções de Culturas de Microrganismos. Microbiologia in foco 2:40–48

Canhos VP, Ferraz de Souza RD, Ávila JPC (2009) Avanços na implementação da Rede Brasileira de Centros de Recursos Biológicos. Microbiologia in foco 9:46–47

CBD Nagoya Protocol (2011) https://www.cbd.int/abs/

Cohan FM, Perry EB (2007) A systematics for discovering the fundamental. Units of bacterial diversity. Curr Biol 17:R373–R386

Danilova MV, Nadirova JM, Emtseva TV (1980) The viability's dependence of lyophilized and longly stored bacteria on the quantity of residual humidity. Izv Akad Nauk SSSR, Ser Biol 3:449–451

Dawyndt P, Dedeurwaerdere T, Swings J (2006) Explorating and exploiting microbiological commons: contributions of bioinformatics and intellectual property rights in sharing biological information. Int Soc Sci J 188:249–258

Day JD, Stacey G (eds) (2007) Cryopreservation and freeze-drying protocols. In: Series: methods in molecular biology, 368, 2nd edn. Humana Press, ISBN 1-58829-377-7

DeLey J, De Smedt TJ (1975) Improvements of the membrane filter method for DNA: rRNA hybridization. Ant van Leeuwenh 41:287–307

Emerson D, Wilson W (2009) Giving microbial diversity a home. Nat Rev Microbiol 7:758

Farrant J (1969) Is there a common mechanism of protection of living cells by polyvinylpyrrolidone and glycerol during freezing? Nature 222:1175, London

Fischer A, Kroppenstedt RM, Stackebrandt E (1983) Molecular-genetic and chemotaxonomic studies on Actinomadura and Nocardiopsis. J Gen Microbiol 129:3433–3446

Fritze D (2005) Digital imaging of prokaryotes for taxonomic purposes. In: Häuser CL, Steiner A, Holstein J, Scoble MJ (eds) Digital imaging of biological type specimens. A manual of best practice. Results from a study of the european network for biodiversity information. Staatliches Museum für Naturkunde, Stuttgart, viii + 304 pp, ISBN 3-00-017240-8, pp 153–171

Fritze D (2010) A common basis for facilitated legitimate exchange of biological materials proposed by the European Culture Collections' Organisation. Int J Commons 4:507–527

Fritze D, Weihs V (2000) Systematics and legislation. In: Priest F, Goodfellow M (eds) Applied microbial systematics. Chapman and Hall, London, pp 447–469

Fry RM (1966) Freezing and drying of bacteria. In: Meryman HT (ed) Cryobiology. Academic, London/New York, pp 665–696

Fuller BJ (2004) Cryoprotectants: the essential antifreezes to protect life in the frozen state. CryoLetters 25:375–388

Gams W, Hennebert GL, Stalpers JA, Jansens D, Schipper MAA, Smith J, Yarrow D, Hawksworth DL (1988) Structuring strain data for the storage and retrieval of information on fungi and yeasts in MINE, Microbial Information Network Europe. J Gen Microbiol 134:1667–1689

Grimont F, Grimont PAD (1986) Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Ann Inst Pasteur/Microbiol 137B:165–175

Hawksworth DL, Schipper MAA (1989) Criteria for consideration in the accreditation of culture collections participating in MINE, the Microbial Information Network Europe. MIRCEN J 5:277–281

Heckly RJ (1978) Preservation of microorganisms. Adv Appl Microbiol 24:1–53

Hippe H (1991) Maintenance of methanogenic bacteria. In: Kirsop BE, Doyle A (eds) Maintenance of microorganisms and cultures cells, 2nd edn. Academic, London, pp 101–113

Johnson JL, Ordal EJ (1968) Deoxyribonucleic acid homology in bacterial taxonomy: effect of incubation temperature on reaction specificity. J Bacteriol 95:893–900

Kersters K, De Ley J (1975) Identification and grouping of bacteria by numerical analysis of their electrophoretic patterns. J Gen Microbiol 87:333–342

Kirsop BE, Doyle A (eds) (1991) Maintenance of microorganisms and cultured cells: a manual of laboratory methods. Academic, London, 308 pp

Konstantinidis K, Stackebrandt E (2011) Defining taxonomic ranks. In: Rosenberg E, DeLong EF, Thompson F, Lory S, Stackebrandt E (eds) The prokaryotes: a handbook on the biology of bacteria, 4th edn. Springer, New York

Lenski RE, Travisano M (1994) Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. Proc Natl Acad Sci USA 91:6808–6814

Lion MB, Bergmann ED (1961) The effect of oxygen on freeze-dried *Escherichia coli*. J Gen Microbiol 24:191–200

Lovelock JE, Bishop MWH (1959) Prevention of freezing damage to living cells by dimethyl sulfoxide. Nature 183:1394–1395

Morichi T (1970) Nature and action of protective solutes in freeze-drying of bacteria. In: Iizuka H, Hasegawa T (eds) Culture collections of microorganisms. University Park Press, Baltimore, pp 351–361

Morris GJ (1981) Cryopreservation: an introduction to cryopreservation in culture collections. Culture Centre of Algae and Protozoa, Cumbria

Nei T (1974) Some aspects of freezing and drying of microorganisms on the basis of cellular water. Cryobiology 10:403–408

OECD (2001) Biological resource centres – underpinning the future of life sciences and biotechnology. (http://oecdpublications.gfi-nb.com/cgi-bin/oecdbookshop.storefront)

OECD (2007) Best practice guidelines for biological resource centres (Online), http://www.oecd.org/dataoecd/7/13/GGTSPU-styx2.bba.de-7664-3281383-DAT/38777417.pdf Accessed 20 Jan 2011

OECD (2011) The bio-economy to 2030: designing a policy agenda. The Organization for Economic Cooperation and Development (OECD), Paris

Olive DM, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol 37:1661–1669

Polge C, Smith AU, Parkes S (1949) Revival of spermatozoa after dehydration at low temperatures. Nature 164:666

Pukall R, Brambilla E, Stackebrandt E (1998) Automated fragment length analysis of fluorescently-labeled 16S rDNA after digestion with 4-base cutting restriction enzymes. J Microbiol Meth 32:55–64

Rainey PB, Travisano M (1998) Adaptive radiation in a heterogeneous environment. Nature 394:69–72

Reed BM (2008) Plant cryopreservation – a practical guide. Springer, Heidelberg. ISBN 978-0-387-72275-7

Smibert RM, Krieg NR (1994) Phenotypic characterization. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC, pp 607–655

Smith D, Rohde C (2007) Biological resource centres compliance with law. Society for Microbiology, UK. http://www.sgm.ac.uk/pubs/micro_today/pdf/0299brc.pdf

Smith D, Rohde C (2008) Safety in microbiology. Laboratory Manager Issue, vol 125. Croner, UK, pp 4–6

Smith D, Ryan MJ, Day JG (2001) The UKNCC biological resource: properties, maintenance and management. UKNCC Secretariat, Egham. ISBN 0954028503

Smith D, Ryan MJ, Stackebrandt E (2008) The *ex situ* conservation of microorganisms: aiming at a certified quality management. In: Doelle HW, DaSilva EJ (eds) Biotechnology. Encyclopedia of Life Support Systems (EOLSS). Developed under the Auspices of the UNESCO, Eolss, Oxford, UK. http://wwweolss.net

Stackebrandt E (2010) Diversification and focusing: strategies of microbial culture collections. Trends Microbiol 18:283–287

Stackebrandt E (2011) Letter to the editor. Int J Syst Evol Microbiol 61:479–481

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PAD, Kämpfer P, Maiden MCJ, Nesme X, Rossello-Mora R, Swings J, Trüper HG, Vauterin L, Ward AC, Whitman WB (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

Stalpers JA, Kracht M, Jansens D, De Ley J, Van der Toorn J, Smith J, Claus D, Hippe H (1990) Structuring strain data for storage and retrieval of information on bacteria in MINE, Microbial Information Network Europe. Syst Appl Microbiol 13:92–103

Stamp TC (1947) The preservation of bacteria by drying. J Gen Microbiol 1:251–265

Stromberg PM, Dedeurwaerdere T, Pascual U (2012) The contribution of public networks to knowledge accumulation: ex situ collections in microbial research (in press)

Suggett A (1975) Water-carbohydrate interactions. In: Duckworth RB (ed) Water relations of food. Food Science and Technology, a series of monographs. Academic Press, London, pp 573–586

Tindall P, Kämpfer P, Euzéby J, Oren A (2006) Valid publication of names of prokaryotes according to the rules of nomenclature: past history and current practice. Int J Syst Evol Microbiol 56:2715–2720

Tindall BJ, Rosselló-Móra R, Busse HJ, Ludwig W, Kämpfer P (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. Int J Syst Evol Microbiol 60:249–266

Verslyppe B, Kottmann R, De Smet W, De Baets B, De Vos P, Dawyndt P (2010) Microbiological common language (MCL): a standard for electronic information exchange in the microbial commons. Res Microbiol 161:439–445

# 12 Repositories for Patented and Safeguarded Material

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## Introduction

Microorganisms have been used in a broad range of biotechnological processes for a very long time, especially for fermentation of food and drink, but their industrial use has intensified in recent decades as a consequence of advances in handling and characterization techniques for microorganisms and their products.

The intellectual property rights of many microbial-based technologies are protected by patents. The use of the patent system requires disclosure of novel aspects of the technology to be protected in order to guarantee a flux of information between inventors, and thus stimulate the development of new technologies or the improvement of existing ones.

In the case of patent applications that involve the use of biological material, a patent office can require the deposit of a viable sample in an official culture collection, so that the technology can be made available as a living organism to other parties.

This chapter discusses international arrangements for ensuring that there are culture collections able to support international patent obligations by accepting deposits of biological material. In addition, it discusses the main features of patent and safe deposit of biological material, as well as its importance for biotechnology development.

### The Role of the Intellectual Property System in Technological Development

Technological innovation has been long considered a determining factor in economical development and social changes. At the beginning of the twentieth century, Schumpeter, in his *Theory of Economic Development*, defined development as the process of discontinuous change and disequilibrium brought about by innovation. Schumpeter described innovation as the introduction of a new good or a new method of production, the opening of a new market, the conquest of a new source of supply of raw materials or half-manufactured goods, and the carrying out of the new organization of any industry (O'Hara 1994).

Intellectual property refers to a collection of specific rights, which attach to the results of intellectual activity such as in the industrial, commercial, scientific, literary, and artistic fields. An intellectual property right is a legal right based on national law encompassing that particular type of intellectual property. Industrial property is a branch of intellectual property system that covers inventions, industrial designs, trademarks, service marks, commercial names and designations, including indications of source and appellations of origin, and protection against unfair competition (Kalanje 2005; WIPO 2004). Intellectual property is considered to play an important role in creating an innovative environment since the effective use of intellectual property tools reduces risks for innovators and enhances the competitiveness of technology-based enterprises, regardless of whether they are commercializing new or improved products, or providing service on the basis of a new or improved technology (Kalanje 2005).

The territoriality principle of intellectual property determines that protection for a result of intellectual activity, such as a patent or an industrial design, is restricted to the territory

E. Rosenberg et al. (eds.), *The Prokaryotes – Prokaryotic Biology and Symbiotic Associations*, DOI 10.1007/978-3-642-30194-0_116,

where it was granted and that this granting has to be made in accordance within the legal rules and scope defined by that territory. Common ground rules have been adopted internationally over the past 150 years through multilateral treaties, which resulted in a basic harmonization of the intellectual property system at international level.

Patents play a major role in the protection of technological creations. International rules for granting patents have been the object of important treaties, such as the Paris Convention for the Protection of Industrial Property, dating from 1883 (Paris Convention 1883), and the Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS 1994). Under the Paris Convention, a patent is a temporary and exclusive right granted, upon request, by a state to an inventor or a legally entitled person, for the commercial use of a new technology. Under the TRIPS Agreement, the member states should grant patents to eligible inventions, in any field of technology, and the term of protection available should be for a minimum of 20 years.

The basic requirements for the granting of a patent are novelty, the involvement of an inventive step, and the industrial application of the subject to be protected. Full disclosure of the invention is also a common requirement for granting a patent. All patent applications are compulsorily published and therefore, the information disclosed can be easily accessed, aiming to guarantee the flux of information among developers, thus stimulating the development of new technologies, or the improvement of existing ones, by competitors. In most cases, the disclosure, which has to be sufficiently detailed as to enable others "skilled in the art" to reproduce the process or product themselves, can be presented as a written description of the invention. The patenting of inventions in which living material plays an essential role can be difficult to describe due to the inherent unpredictability and complexity of biological systems (Fritze and Weihs 2001). The problem of disclosure in the patenting of biotechnologies involving live biological material was solved by enabling patent offices to enforce the requirement that viable (living) samples be deposited in recognized culture collections; the technology can then be made available as a living organism to other inventors.

Although the patentability of microorganisms is permitted under Article 27 of the TRIPS Agreement, some signatory states do not grant patents to natural microorganisms, arguing that they by themselves are not considered inventions. However, even in these countries, besides the deposit of genetically modified microorganisms, the deposit of natural biological material can also be required when its description is essential for the reproducibility of a patentable process in which they are involved.

## Culture Collections and the Preservation of Microorganisms

Over the last decades, the international community has paid increasing attention to the adoption of measures to promote human development in a sustainable basis. The sustainable use of biological diversity is one of the three objectives set out in Article 1 of the Convention on Biological Diversity (CBD 1992), the international legal instrument signed by 150 government leaders at the 1992 Rio Earth Summit with the aim of promoting global sustainable development. Sustainable economic development includes both the discovery of new products to promote human well-being and the preservation of endangered specimens or ecosystems. The preservation of biological material used in biotechnological inventions by depositing a sample with a trusted repository institution can contribute to these major objectives of the convention.

Biological materials such as microorganisms have been used in a broad range of biotechnology-based processes for thousands of years, but their use has intensified in recent decades due to advances in our understanding of the genetic code, of its organization, and of gene expression. Microbial genetics has played a pivotal role in our understanding of metabolic pathways and enabled the production of both natural and genetically engineered products, such as antibiotics.

Microorganisms have been widely used for fermented food and beverage production for several thousand years by societies all over the world. These uses were expanded to the manufacture of different products at an industrial level in the first half of the twentieth century. This new era began during World War I, when processes to produce chemicals for munition manufacturing were developed in England and Germany. Many fermentation, bioconversion, and enzymatic processes were soon developed, yielding useful products with large markets, including amino acids, nucleotides, vitamins, organic acids, solvents, vaccines, polysaccharides, and antibiotics (Demain 2000).

A new change came in the mid-1970s with the discovery and harnessing of basic subcellular processes, such as replication, recombination, DNA repair, and gene organization and expression (gene transcription, translation, and regulation), that have revolutionized approaches to the study of life sciences. It is now possible to undertake detailed studies of many difficult and more complex organisms that were previously refractory to genetic analysis. Traditional industrial microbiology merged with recombinant DNA technology, launching the modern biotechnology era in which we have harnessed many cellular functions for the production of primary and secondary metabolites, bioconversions, and enzymes (Chandler 2008).

The advent of the modern industrial biotechnology made a major impact in the business world through a novel, high-value products such as biopharmaceuticals (recombinant protein drugs, vaccines, and monoclonal antibodies), produced a revolution in agriculture, and markedly increased the market for microbial enzymes (Demain 2000). According to O'Hara (1994), developments in biotechnology since the early 1970s have introduced a new regime of accumulation in the global economy. In this context, the patented microorganisms that underpin industrial biotechnology and the information about them have both become valuable economic resources in their own right.

A vast number of different biological materials are used in industrial biotechnology. The process of identifying an industrially useful microbial strain can require great scientific effort for its isolation, selection, investigation, and genetic

modification into different strains with diverse purposes (Donev 2001). Patent protection is often sought for useful microorganisms, especially where investments in the genetic engineering of special characteristics have been made.

The preservation and maintenance of these useful biological materials in culture collections is important for guaranteeing the continuity of the innovation process. Viable and accurately characterized biological organisms must be available to allow the development of reproducible bioprocesses and products. Controls for the purity and identity of strains are necessary to achieve optimal yields and well-defined products. These are not trivial undertakings, and in many cases, scientists do not possess the resources or interest needed for optimal long-term preservation of their material and management of associated data. This is one reason for the advent of specialized culture collections that act as long-term repositories for large numbers of organisms, stored under state-of-the-art conditions that ensure strain stability and continuity of access (Daniel and Prasad 2010).

The preservation of microbial viability, specificity, activity, and immunogenicity is necessary for standardization and industrial preparation of high-quality products and also provides a basis for future development (Donev 2001). The wide temporal gaps that may exist between the isolation and the characterization of a strain's useful properties, and its eventual industrial application, render long-term depositories essential (Daniel and Prasad 2010). The discovery of a heat-stable DNA polymerase enzyme in the thermophilic bacterium *Thermus aquaticus* illustrates the importance of the temporal gap. This novel enzyme is now used to catalyze the polymerase chain reaction (PCR) process widely used in molecular biology for amplifying DNA sequences. The PCR process revolutionized biotechnology research and won the 1993 Nobel Prize for its discoverer, Dr. Kary Mullis. While the bacterial species was identified and reported in 1969, the use of its polymerase enzyme in PCR was patented only in 1990 (Cetus Corporation 1990). Long-term storage of *T. aquaticus* strains was essential for the successful outcome of this technology during its long development period.

There are many culture collections all over the world for the long-term conservation and preservation of microbial diversity. These collections are the custodians of genetic resources of vital importance to science and society (Cameotra 2007). Culture collections are also needed to store material that can no longer be maintained by research institutions or to duplicate important reference strains so as to provide simplified user access and for safety reasons. Culture collections also establish exchange networks among themselves and with researchers (Daniel and Prasad 2010).

## The Budapest Treaty

An important characteristic of the intellectual property system is that patent laws worldwide require details of an invention to be fully disclosed in order for others skilled in the relevant field to be able to replicate it. The detailed and accurate description of the invention is a condition for receiving and maintaining a patent, and ensuring that the invention's benefits are made available to the public. Disclosure encourages innovation and dissemination of knowledge and is intended to balance the interests of the inventor with the needs of society.

The disclosure of an invention is normally achieved by means of a written description, supplemented when necessary by drawings. In the case of inventions involving the use of biological material, these patent disclosure requirements may be difficult to fulfill. Industrial property offices in many countries overcome this difficulty by recommending that the written description of an invention involving the use of a new organism should be supplemented by its deposition in a recognized culture collection, in a viable form.

The territoriality principle of intellectual property rights means that the granting of a patent is ruled by national law in each territory. Inventors seeking a patent in various countries would face a complex and costly task if they had to deposit samples in each country since the recognition of a depository institution is also independent for each patent office. This has been the driver for the rationalization of deposition procedures at an international level.

The Budapest Treaty was created to implement such recommendations. It was first proposed by the United Kingdom to the Executive Committee of the Paris Union that WIPO would study the possibilities of creating an international Treaty for the deposit of microorganisms and related material. A committee of experts was established and after three sessions (1974, 1975, and 1976) prepared a draft of the Treaty and its regulations, which was submitted to a diplomatic conference, held in Budapest in 1977.

The Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (commonly known as Budapest Treaty) was signed by 18 states on the 28 April 1977 and came into force in August 1980. The Treaty permits the deposit of microorganisms at International Depositary Authorities (IDAs), recognized for the purposes of patent procedure (Budapest Treaty 1977). As of May 2011, 75 countries are party to the Budapest Treaty. The Treaty is open to States which are party to the Paris Convention and is administered by the World Intellectual Property Organization (WIPO 2011).

Briefly, the main characteristics of the Treaty are that (1) all Contracting States recognize the deposit of a microorganism with any IDA and (2) any deposit of a microorganism with an IDA shall be accepted for the purposes of patent procedure by the patent offices of the Contracting States and by any regional office who filed a declaration of acceptance.

The main feature of the Budapest Treaty is that a Contracting State must recognize the deposit of a sample with any IDA, irrespective of whether the IDA is on or outside the territory of the said State. This allows patent applicants to make one deposit with an IDA anywhere in the world, rather than having to arrange deposits in each country where patent protection is sought. This procedure benefits patent applicants by reducing costs and setting up a mechanism to facilitate appropriate third party access to deposited biological materials. In addition, the

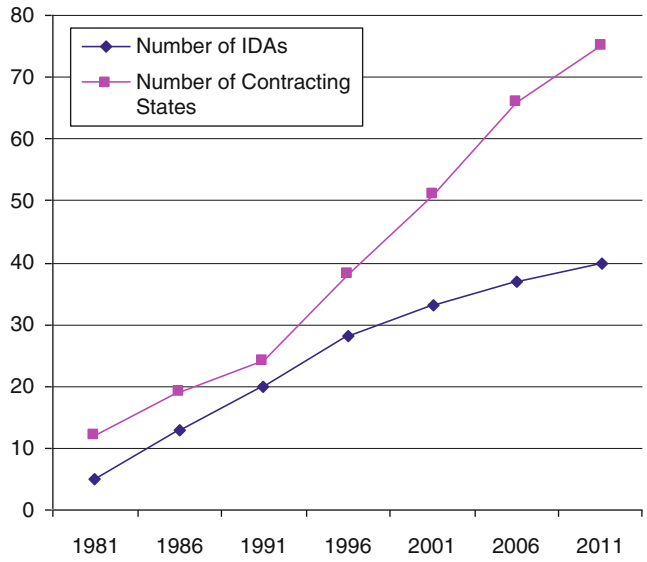


**Fig. 12.1**
**Numbers of the recognized International Depositary Authority (IDAs) and numbers of Contracting States of the Budapest Treaty since it was in force (WIPO 2011)**

Treaty increases the security of the depositor because it establishes a uniform system of deposit, recognition, and furnishing of samples of microbial samples.

The number of recognized IDAs and Contracting States of the Budapest Treaty are demonstrated in *Fig. 12.1*. Currently, 75 countries have signed the Budapest Treaty and 40 culture collections are recognized as IDAs. The latest institutions to have acquired the IDA status were CBA (Australia), recognized in February 2010; VTTCC (Finland) in August 2010; and MCC (India) in April 2011. Two countries have recently become parties to the Budapest Treaty: Morocco and Chile, since July and August 2011, respectively (WIPO 2011).

In addition to the Contracting States, other intergovernmental industrial property organizations, such as the European Patent Office (EPO), the Eurasian Patent Organization (EAPO), and the African Regional Intellectual Property Organization (ARIPO), have made declarations of acceptance of the effects of the Treaty.

As shown in *Fig. 12.1*, the numbers of Contracting States and the number of institutions that have obtained IDA status have increased in recent years. It is important to notice that among Budapest Treaty signatories, only two nations are located in South America: Peru and Chile (parties to the Treaty in 2009 and 2011 respectively), and three in Africa: South Africa, Tunisia, and Morocco (in 1997, 2004 and 2011 respectively).

## International Depositary Authorities

An IDA can be defined as a scientific institution located on the territory of a Contracting State, which accepts deposits of microorganisms, and is able to store and furnish all samples in storage. IDA status is acquired after acceptance of a communication from the Contracting State to the Director General of WIPO (Budapest Treaty, 1977—art. 7), a United Nations agency based in Switzerland that is responsible for promoting international intellectual property protection.

For a depository institution to qualify for IDA status, WIPO has set some standard guidelines, including that the IDA should be located within the territory of a Contracting State and it should have a continuous existence with the necessary staff and facilities to perform its scientific and administrative tasks under the Treaty. The IDA must be impartial and objective, which means that it should make its services available on the same terms to any depositor. The IDA can accept any or specific types of microorganism; *Fig. 12.2* describes the kinds of microorganisms received by different IDA. The Treaty does not define the term "microorganism" thus allowing a broad interpretation of the term, which includes unicellular and multicellular organisms, DNA sequences, enzymes, seeds, etc (see *Fig. 12.2* and following section for more detail).

It should test the viability of biological material promptly after receipt and issue viability statements to the depositor. In addition, the IDA could also perform training, identification, consultation, and other services, and publish catalogues on its holdings. The IDA should comply with the requirements of secrecy and, at the same time, supply samples of deposited biological material only to persons entitled to receive them according to the regulations of the Budapest Treaty (Budapest Treaty 1977; Fritze and Weihs 2001; Sekar and Kandavel 2004).

### Geographical Distribution of IDAs

Under the Budapest Treaty, Contracting States are not obliged to establish an IDA. Contracting States that decide to establish an IDA on their territory must provide assurances that the IDA fulfills Treaty requirements, and they must guarantee its permanent existence, a supply of necessary equipment, staff, and knowledge. Of the 75 Contracting States, 22 have at least one IDA on their territory. Some countries have more than one institution with IDA status: the United States of America, India, Poland, Australia, Japan, Italy; China and Spain, have two IDAs each; the Republic of Korea and Russian Federation have three IDAs each; and the United Kingdom has seven IDAs on its territory (for updates, see www.wipo.int/budapest). The majority of these institutions are concentrated in Europe, with 26 recognized IDAs; there are 9 IDAs in Asia, only 3 in North America, and 2 in Oceania. However, there are no institutions with IDA status in South and Central America or on the African continent.

### Definition of Depositable Materials

As noted above, the term "microorganism" is not defined in the Treaty, which has created considerable confusion. In general, whether a particular deposit is a microorganism or not matters

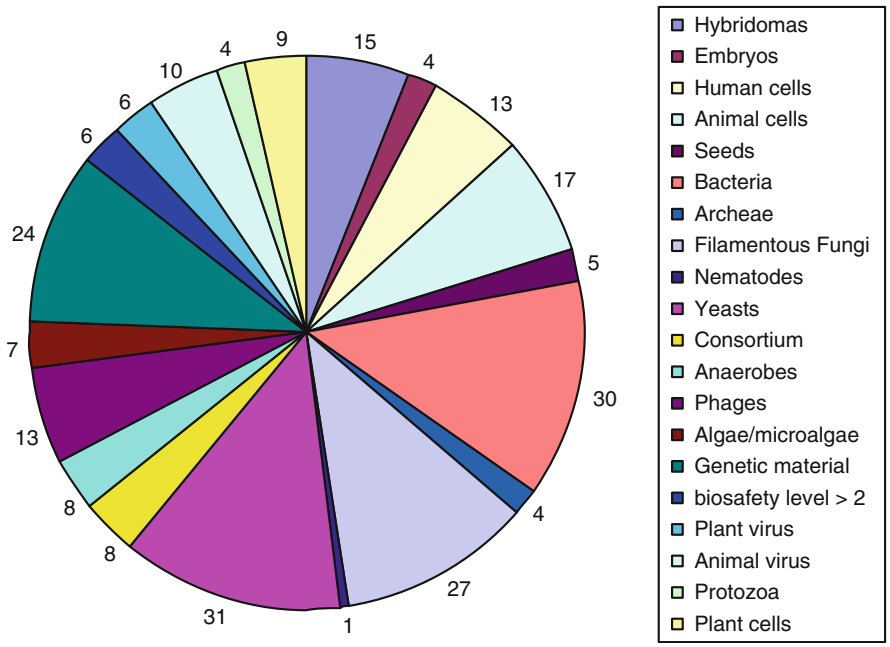


**Fig. 12.2**
**Kinds of biological material accepted for patent deposits by the 40 recognized IDAs. The numbers on graph indicate the amount of IDAs that accept the kind of microorganism listed**

less than whether deposition is necessary for the purposes of patent disclosure. Any biological material that plays an essential role in a given invention, and without which the invention would not be reproducible, needs to be deposited. For the purposes of patent protection, the term "microorganism" often applies to diverse biological material including, for example, viruses, bacteria, actinomycetes, yeasts, filamentous fungi, mushrooms, protozoa, unicellular algae, cell lines of plants or animals, fused cells, transformants and vectors used in genetic engineering, variants, plant cells, DNA, and RNA (*Fig. 12.2*).

The European Union has decided to discontinue the use of the term microorganism; instead, it has decided to use the term "biological material," encompassing any material that contains genetic information and is capable of replicating itself or of being reproduced in a biological system (Sekar and Kandavel 2004).

IDAs vary in the nature of the biological material accepted for deposition (*Fig. 12.2*). An analysis of the type of microorganism accepted by the recognized IDAs highlights wide variations in their ability to handle diverse life forms. In general, nonpathogenic bacteria and fungi and yeasts and genetic material (usually plasmids in hosts) are accepted by most IDAs (around 60–75% of IDAs depending on the material), whereas cell cultures and hybridomas are accepted by 32–37% of IDAs as demonstrated on *Fig. 12.2*.

About 17–25% of IDAs receive biological material such as algae, anaerobes (strict anaerobes are accepted by even fewer IDAs), plant cells, and animal or plant viruses. Some kinds of biological material, such as embryos, archeae, protozoa, plant seeds, pathogenic organisms (of biosafety level 2 or higher), or nematodes, are accepted only by some IDAs (personal communication from IDAs). *Figure 12.2* represents data collected by accessing all 40 IDAs in order to identify the kinds of biological material they will accept for patent purposes. The numbers of IDAs that are able to accept each kind of biological material is also shown in *Fig. 12.2*.

The technical challenge faced by IDAs and culture collections is to preserve viable life forms for a long period of time (at least 30 years), without causing genetic changes. An important aspect of an IDA is to ensure that material in storage is viable and can be cultured and propagated identically. Not every biological material meets the requirements for deposition, as some microorganisms are not amenable to preservation methods for long-term storage (Fritze and Weihs 2001).

Sekar and Kandavel (2004) have identified some future necessities for IDAs. In the near future, IDAs should prepare to deal with the emerging need to store different kinds of biological material, such as microbial consortia (natural or artificial assemblages of interacting microorganisms), or viable but not yet culturable microorganisms. At present, only eight IDAs are able to receive and store microbial consortia. However, mixtures of microbial cultures of more than two components are usually not accepted, while mixtures of two components are only accepted if these can easily be distinguished, if the composition of the mixture is defined, and its components are identifiable and can be preserved in the desired ratio (personal communication from IDAs). At present, no IDA has mentioned the possibility of preserving viable, but not yet culturable microorganisms for patent purposes.

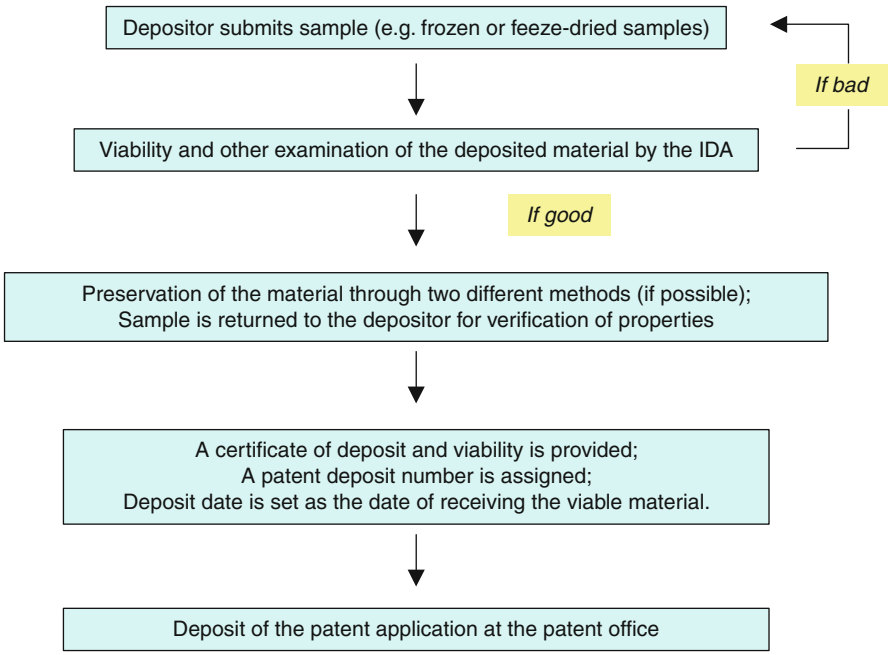


**Fig. 12.3**
**Outline of technical and administrative procedure for the deposition of biological material at an IDA (Adapted from Sekar and Kandavel (2004))**

## Patent Deposit and Safe Deposit of Biological Material

### The Deposit of Biological Material for Patent Purposes

#### Depositing a Sample

Before sending the biological samples to the chosen institution, the depositor must come into contact with it to be sure about the form and amount of material to be sent. Each IDA can determine how it should receive the material to be tested and then preserved.

The technical and administrative procedure for sample deposition is as follows: (1) A microbial culture arrives at the depositary authority. (2) Its documentation is checked. (3) The viability and purity of the organism is checked. (4) An accession number is assigned to the culture after viability and purity have been proven. (5) The depositor receives official statements of receipt and viability which should be used for filing the patent application. (6) In the case of bacteria, fungi, yeasts, and plant cell cultures, the biological material is usually subcultured and a stock of samples is—as applicable—preserved by storage in liquid nitrogen and/or freeze-drying. All other biological material will usually not be subcultured, but stored in liquid nitrogen or in freezers at around −80 °C in the same form as it was received from the depositor. (7) Viability of the stored cultures is inspected regularly during the minimum storage period of at least 30 years (Fritze and Weihs 2001; Sekar and Kandavel 2004, *Fig. 12.3*).

The cost for making a deposit under the Budapest Treaty is levied as a single fee at the beginning of the deposit process and varies depending on the depositary institution, the kind of biological material deposited, and the details of services rendered. The fee is valid for the full 30 years of storage.

#### Main Tests Performed by the IDA

After receipt of the biological material, the IDA submits the sample to its main tests for viability and purity. The depositor may be requested to indicate the most suitable methods of cultivation for conducting these tests. If the material received is not approved, a new deposit shall be made and new tests of viability and purity are conducted (*Fig. 12.3*).

After the approval of the tests, a receipt confirming reception and acceptance of material can be drafted by the institution and sent to the depositor, who should then include these data on the body of the patent document. This receipt must contain information about the IDA, the applicant, the biological material identification references supplied by the applicant, and the filing date and serial number assigned to the deposit by the IDA (Budapest Treaty 1977—Rule 7). If the scientific description and/or taxonomic designation of the material have not been listed on the deposit, the depositor may indicate them later by means of written communication (Budapest Treaty 1977—Rule 8).

IDAs are obliged to promptly test the viability of each biological material deposited with it and also at reasonable intervals, depending on the kind of material and its storage

**Table 12.1**
**Examples of biological material preservation methods (Weihs 2010)**

| Method | Successfully preserved organisms | Shelf life | Genetic stability |
|---|---|---|---|
| Storage under paraffin oil | Yeasts, fungi, some bacteria | Fungi: 5–20 years | Low |
| | | Bacteria: 2–5 years | |
| Storage in distilled water | Yeasts, filamentous fungi, actinomycetes, not enterobacteria | 1–5 years | low |
| Drying in gelatin discs | Enterobacteria, staphylococci, pseudomonas, spore-forming fungi | 1/2–7 years | Medium |
| Storage in sterile soil, sand etc. | Spore-forming bacteria and fungi | 10–15 years | Good |
| | Non-spore formers | 1–5 years | |
| L-drying | Bacteria, fungi, yeasts, animal viruses, protozoa | 2–5 years | Good |
| Drying on glass beads or porcelain rings | Fungi, bacteria, mycoplasms | 5–10 years | Good |
| | | Sporeformers: 10–15 years | |
| *Freeze-drying* | *Bacteria, some yeasts* | *>40 years* | *Good* |
| Storage at domestic refrigerator temperature | Bacteria | Several weeks/ months | Low |
| Storage at −20 °C in glycerol | Bacteria | Several months–2 years | Medium |
| Storage at −60°C to −80 °C in glycerol | Bacteria | 5 year | Good |
| *Storage in liquid nitrogen at −196°C* | *Bacteria, fungi, yeasts, plant cell cultures, animal cell cultures* | *>30 years* | *Good* |
| Maintenance on glass beads at −60°C to −80 °C | Bacteria | >10 years | Good |

conditions, or at any time, if necessary for technical reasons and at any time, upon the request of the depositor (Budapest Treaty 1977—Rule 10).

## Methods of Preservation for Biological Materials

Preservation methods can vary between different institutions, since each of these has the autonomy to decide which procedure it will follow in its daily work routine. Moreover, the intrinsic characteristics of each type of biological material to be preserved will also influence the choice of the most appropriate conservation method.

Regardless of the preservation method adopted, institutions should maintain the biological material genetically stable and without contamination. For the reduction of cellular metabolism and concomitant maintenance of material viability, the preservation methods most commonly employed are cryogenic storage, for example, through storage in liquid nitrogen at −196 °C, and deprivation of water in the cell through the process of lyophilization. These methods combine long storage times with good genetic stability.

To ensure the safety of biological material in case of unforeseen problems with a preservation method, most institutions conserve materials with at least two different preservation methods. *Table 12.1* shows some of the methods used to preserve a range of biological materials and the resulting shelf life and genetic stability.

## When Can an IDA Refuse a Sample?

In some cases an IDA can refuse to receive a depositor's biological material. This can take place (1) when the biological material is not part of the IDA's scope of work, and it cannot guarantee correct storage and handling; (2) when the properties of the biological material are so exceptional that the IDA is technically not in a position to perform tasks in relation to it; and (3) when the deposit is received in a condition which clearly indicates that the material is missing, or which precludes its acceptance for scientific reasons (Budapest Treaty 1977—Rule 6). The deposit is not considered valid in such cases. In the first two situations, a new deposit shall be held at another institution that can provide guarantees on the material deposited. In the latter situation, and at the discretion of the depositor, a new shipment of the material can be sent to the same institution (receiving a new filing date, see *Fig. 12.3*) or to another institution that can also provide guarantees on the material deposited.

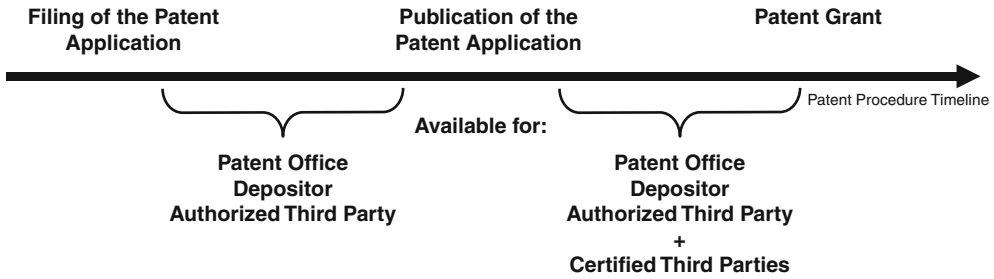


**Fig. 12.4**
**Provision of biological material samples under Budapest Treaty rules (Adapted from Weihs (2010))**

## Dispatch of the Sample

The rules for sending samples of biological material should comply with the rules established by each country. Rule 11 of the Budapest Treaty restricts the transmission of biological material samples to signatories to the Treaty or to countries that adopt Treaty rules despite not being signatories.

According to the Budapest Treaty, samples of material can be sent to:

- Interested Industrial Property Offices at any time on request to the IDA (Rule 11.1)
- The Depositor, or with the authorization of the depositor, to authorized third parties at any time on request to the IDA (Rule 11.2)
- Parties legally entitled by confirmation of the request by the responsible patent office (certified third parties) after the publication of the patent deposit (Rule 11.3)

In respect of patents granted and published by an industrial property office, that office may from time to time communicate to any IDA a list of the deposit accession numbers it has assigned to biological material referred to in specific patents it has issued. The IDA shall, under request, furnish a sample of any biological material with a listed accession number. For deposited material whose accession numbers have been communicated, the industrial property office shall not be required to provide certifications (Rule 11.3b). *Figure 12.4* shows a scheme to facilitate an understanding of how biological samples are provided under the Budapest Treaty.

Under the Treaty rules, sending samples to the patent offices is free of charge, while sending samples to the depositor, an authorized third party, or certified third parties is accomplished through the payment of fees prescribed by each IDA.

Once an IDA has furnished a sample to any interested party, other than the depositor, it shall promptly notify the depositor in writing of that fact, as well as of the date on which the said sample was furnished, and of the name and address of the industrial property office, of the authorized party, of the certified party, or of the requesting party, to whom or to which the sample was furnished.

The line graph in *Fig. 12.5* represents the total number of deposits of biological material for patent purposes on all IDAs from 2001 to 2009. The deposits dropped around 2003 and then started to increase again. In 2009 about 2,500 deposits of biological material were performed under the Budapest Treaty. *Figure 12.5* also shows the numbers of cultures furnished under the Rule 11 of the Budapest Treaty (bar graphs). Remarkably, independently of the year, very few samples of biological material were dispatched to patent offices (Rule 11.1) while about 300–500 cultures per year were furnished to depositors, authorized third parties (Rule 11.2) or legally entitled parties (Rule 11.3).

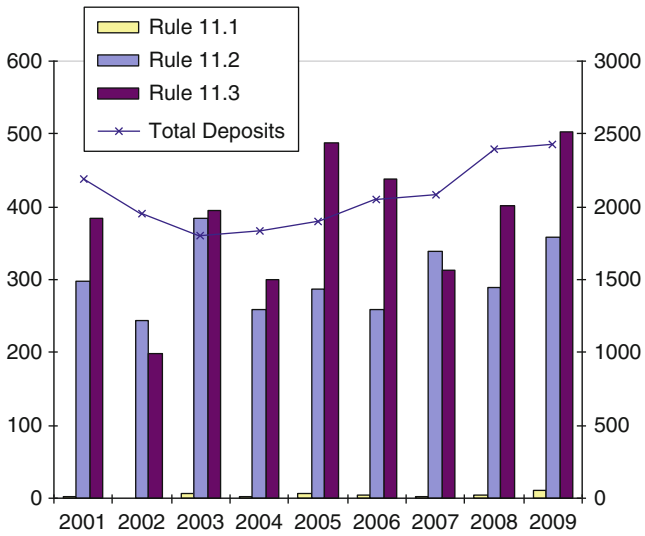


**Fig. 12.5**
**Samples dispatched by IDAs according to Rule 11 of the Budapest Treaty (*left axis*—bar graph) and the total number of deposits received by IDAs under the Budapest Treaty (*right axis*—line graph) (WIPO 2011)**

*Table 12.2* represents the ten major IDAs, the number of deposits accepted, and the number of samples furnished under Rule 11 of the Treaty Regulations from 2001 to 2009.

## Length of the Deposit

According to Rule 9 of the Treaty, any biological material deposited with an IDA shall be stored by that authority, with all the

**Table 12.2**
**Numbers of deposits of biological material for patent purposes and samples furnished under Rule 11 of the regulations of the Budapest Treaty by the major International Depositary Authorities (2001–2009) (WIPO 2011)**

| International depositary authority (*country*) | Deposited samples | Furnished samples |
|---|---|---|
| ATCC (*United States of America*) | 7,302 | 6,721 |
| CGMCC (*China*) | 3,009 | 96 |
| IPOD (*Japan*) | 2,439 | 664 |
| DSMZ (*Deutschland*) | 2,211 | 720 |
| CCTCC (*China*) | 1,793 | 52 |
| KCTC (*Republic of Korea*) | 1,727 | 152 |
| CNCM (*France*) | 1,701 | 462 |
| NRRL (*United States of America*) | 932 | 3,075 |
| KCCM (*Republic of Korea*) | 822 | 71 |
| NCIMB (*United Kingdom*) | 602 | 115 |

care necessary to keep it viable and uncontaminated, for a period of at least 5 years after the most recent request for the furnishing of a sample of the deposited material and, in any case, for a period of at least 30 years after the date of the initial deposit.

Regarding secrecy aspects, no IDA shall give information to anyone about whether a biological material has been deposited with it under the Budapest Treaty. Furthermore, it shall not disclose to anyone information concerning material deposited with it under the Treaty except to an authority or a party legally entitled to obtain a sample of a material according to Rule 11 of the Treaty.

## Safe Deposit of Biological Material

Cell cultures, microorganisms, and other biological materials are valuable assets for organizations, and the loss of these materials can be costly. Several culture collections around the world provide safe deposit services for corporations, government laboratories, academic institutions, and others.

A culture collection that offers safe deposit services must ensure security and confidentiality of the material deposited.

Preservation methods are defined for each collection and can vary depending on the type of material to be preserved. In safe deposits, the depositor can determine how long the material will remain preserved in the collection, unlike for the deposit of biological material for patent purposes.

In safe deposits all rights to cultures remain with the depositor and all information concerning deposited material is retained in confidence. Also, culture material is available only to the depositor or an individual designated in writing by the depositor. At some culture collections, vials of each culture can be returned to the depositor for analysis after a storage period to ensure the viability of material received (ATCC 2011).

## Concluding Remarks

Biotechnology processes are increasingly used for industrial production despite great advances in chemical routes to synthesize many products of interest. This is mainly due to the ability of microorganisms to naturally metabolize a wide range of compounds which are still too complex for synthetic chemical routes. Genetic engineering has given even greater control on our ability to harness the use of microbes and cell lines for industrial production. Biotechnology therefore remains a very promising field for technological and economic development, and this can be seen from the increasing number of patent applications that refer to biochemistry and genetic engineering discoveries. The preservation of biological material in internationally recognized culture collections plays a key role supporting biotechnology-related patents. The main advantages of the Budapest Treaty include the simplification and cost reduction of patent procedures, the prevention of certain risks in the field of biotechnology, enhanced research, and development through access to deposited biological material and promotion of cooperation and exchange between IDAs.

Even so, the necessity for physical deposits of biological materials for patent purposes is being increasingly questioned. This is due to the idea that DNA sequences, submitted in the form of computer data only, might already be comprehensive enough to fulfill the needs for full disclosure of an invention and be able to substitute for the deposition of complete and viable biological material. At present most applicants still prefer to deposit as comprehensively as possible to be sure that the invention is completely disclosed and rendered reworkable, so that it will not be challengeable by competitors (Fritze and Weihs 2001).

The deposit of biological material for patent purpose requires optimal conditions to guarantee long-term preservation. Due to its needs of specific expertise and resources, a culture collection is not always able to maintain a wide range of samples, and for this reason, sometimes it can be beneficial for them to operate in a coordinated network to achieve a broader preservation activity.

Several initiatives have been proposed by developing countries to achieve the necessary infrastructure to set up an IDA, which could intensify international coordination and enhance the preservation of a country or region's natural gene pool. Although a depositor can deposit a culture in any IDA belonging to any country, the presence of an IDA in the country of patent origin may result in a more affordable deposition process, particularly in developing countries, and build up important local expertise (Sekar and Kandavel 2004).

Culture collections, including suitably resourced IDAs, play a role in promoting and disseminating the use of biological materials with important characteristics, by providing controlled storage conditions and the supervision of specialized professionals. With the rapid loss of habitat, it may become important for IDAs established in developing or emerging economies to specialize not just in the storage of patented organisms but in the preservation of their regional biodiversity.

The establishment of well-structured culture collections to preserve the biodiversity of megadiverse countries would appear to be a crucial question; especially where a vast amount of material, with great potential for biologically based products and processes to benefit human welfare, has not yet been isolated and identified.

## References


ATCC (American Type Culture Collection) (2011) Available at http://www.atcc.org/DepositServices/SafeDepository/tabid/238/Default.aspx. Accessed 10 May 2011

Budapest treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure: done at 28 Apr 1977 and amended on 26 Sept 1980, and regulations (as in force since 31 Jan 1981). (Budapest): WIPO. Available at http://www.wipo.int/export/sites/www/treaties/en/registration/budapest/pdf/trtdocs_wo002.pdf. Accessed 10 May 2011

Cameotra SS (2007) Preservation of microorganisms as deposits for patent applications. Biochem Biophys Res Commun 353:849–850

Cetus Corporation, Mullis KB, Erlich HA, Gelfand DH, Horn G, Saiki RK (1990) Process for amplifying, detecting, and/or cloning nucleic acid sequences using a thermostable enzyme. US4965188. 29 Oct 1990

Chandler M (2008) Microbiology – what now? Res Microbiol 159:51–58

Convention on biological diversity. Done at 1992 (Rio de Janeiro). United Nations. Available at http://www.cbd.int/convention/text/. Accessed 20 May 2011

Daniel H-M, Prasad GS (2010) The role of culture collections as an interface between providers and users: the example of yeasts. Res Microbiol 161:488–496

Demain AL (2000) Small bugs, big business: the economic power of the microbe. Biotechnol Adv 18:499–514

Donev T (2001) Methods for conservation of industrial microorganisms. Part 1: Methods for conservation of industrial microorganisms. National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria. Available at http://www.nbimcc.org/bg/Conservation%20methods.pdf. Accessed 29 May 2011

Fritze D, Weihs V (2001) Deposition of biological material for patent protection in biotechnology. Appl Microbiol Biotechnol 57:443–450

Kalanje CM (2005) Role of intellectual property in innovation and new product development. Available at http://wipo.int/sme/en/documents/pdf/ip_innovation_development.pdf. Accessed 29 May 2011

O'Hara PA (1994) An institutionalist review of long wave theories: Schumpeterian innovation, modes of regulation, and social structures of accumulation. J Econ Issues 2:489–500

Paris convention for the protection of industrial property. Done at 20 March 1883 and amended on 28 Sept 1979. (Paris). WIPO. Available at http://www.wipo.int/treaties/en/ip/paris/trtdocs_wo020.html. Accessed 29 May 2011

Sekar S, Kandavel D (2004) The future of patent deposition of microorganisms? Trends Biotechnol 22:213–218

TRIPS – Agreement on trade-related aspects of intellectual property rights. Done at 15 Apr 1994 (Morocco). WTO. Available at http://www.wto.org/english/tratop_e/trips_e/t_agm0_e.htm. Accessed 29 May 2011

Weihs V (2010) IDAs -30 years of experiences world-wide. 12th international conference on culture collection, Florianópolis, SC, Brazil. Available at http://www.iccc12.info/presentations/vweihs.pdf. Accessed 10 May 2011

WIPO (2004) Intellectual property handbook: policy, law and use. WIPO publication no. 489. Available at http://www.wipo.int/about-ip/en/iprm/. Accessed 10 May 2011

WIPO (World Intellectual Property Organization) (2011) Available at http://www.wipo.int/treaties/en/registration/budapest/. Accessed 10 May 2011

# 13 Biotechnology and Applied Microbiology

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## Introduction

There is no agreed upon definition of biotechnology. Definitions vary from the most noble—the uses of biology for the benefit of man—to the most prosaic—the use of biology to make money. A balanced definition might be the use of living organisms (or materials from living organisms) to perform defined processes for industrial, agricultural, and health applications. Applied microbiology is a large part of biotechnology because microorganisms have a broadly diverse synthetic and degradative potential, which is easily manipulated genetically, and they can readily grow under controlled conditions in large fermenters. The so-called "new biotechnology" generally refers to the use of recombinant DNA technology to construct useful new strains. However, a considerable amount of "new biotechnology" does not use recombinant DNA technology but rather structural biology as a basis for rational drug design, monoclonal antibodies as biosensors, and enzymes from extremophiles to facilitate industrial production. In addition, conventional industrial microbiological processes apply many of the new biotechnologies.

This edition of the Prokaryotes contains an entire section dealing with the following specific biotechnology and applied microbiology topics: bioremediation, microbial biofilms, biodeterioration, bacterial enzymes, bacteria in food and beverage production, organic acid and solvent production, amino acid and vitamin production, recombinant DNA protein production in bacteria, bacterial pharmaceutical products, biofuels, biosurfactants, and repositories for patented materials. In addition, each chapter dealing with a particular genus (or a group of genera) has a section on applications. In this introductory chapter, I attempt to put applied microbiology and biotechnology into a historical perspective and give an overview of some of the general principles related to the biotechnology of prokaryotes. It should be emphasized at the outset that both applied and basic microbiology have been and always will be intertwined. Applied microbiology without basic microbiology is lame and limited, and a major driving force of basic microbiology is the solution of applied problems.

## The Fermentation Controversy

For thousands of years, microbiological processes (such as those for producing wine, beer, vinegar, bread, pickles, sauerkraut, and various milk products such as yogurts, cheeses, and butter) were developed by trial and error, without any understanding of the underlying principles. During the last half of the nineteenth century, the controversy regarding the causative

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agent of fermentation stimulated experimentation that serves as the scientific basis of today's applied microbiology and biochemistry.

For centuries fermentation had a significance that was almost equivalent to what we would now call a chemical reaction, an error that probably arose from the vigorous bubbling seen during the process. The conviction that fermentation was strictly a chemical event gained further support during the early part of the nineteenth century. French chemists led by Lavoisier and Gay-Lussac determined that alcoholic fermentation could be described as the chemical conversion of one molecule of glucose ($C_6H_{12}O_6$) into two molecules of ethanol ($C_2H_5OH$) and two molecules of $CO_2$. It was of course known that yeast must be added to the glucose solution to ensure a reproducible and rapid fermentation. The function of the yeast, according to the chemists, was merely to act as a chemical catalyst. Then, in 1837, Theodor Schwann and Charles Cagniard-Latour independently published studies that indicated yeast was a living microorganism. Prior to their publication, yeast was considered merely as a proteinaceous chemical substance. One reason it was difficult to ascertain whether yeasts were alive was that, like most other fungi, they are not motile. The cellular nature of yeast was discovered only when improved microscopes became available, and it is most likely that improved microscopes enabled the two workers to come up with the same observation at approximately the same time. Schwann and Cagniard-Latour also observed that alcoholic fermentation always began with the first appearance of yeast, progressed only with their multiplication, and ceased as soon as their growth stopped, so both scientists concluded that alcohol is a by-product of yeast growth.

The biological theory of fermentation advanced by Cagniard-Latour and Schwann was immediately attacked by the leading chemists of the time. The eminent Swedish physical chemist, Jons Jakob Berzelius, reviewed the papers of Cagniard-Latour and Schwann in his *Jahresbericht* for 1839 and dismissed microscopic evidence as of no value in what was obviously a purely chemical problem. According to Berzelius, nothing was living in yeast. "It was only a chemical substance which precipitated during the fermentation of beer and which had the usual shape of a non-crystalline precipitate." In addition, Liebig published a paper containing several important arguments against the biological theory of fermentation. Liebig's two major points can be summarized as follows:

1. Certain types of fermentation such as the lactic acid (souring of milk) and acetic acid (formation of vinegar) can occur in the complete absence of yeast.
2. It is not necessary to conclude that, even if yeast were a living organism, fermentation is a biological process. Yeast is a remarkably unstable substance, which, as a consequence of its own death and decomposition, catalyzes the splitting of sugar. Thus, fermentation is essentially a chemical change catalyzed by breakdown products of the yeast.

Liebig's views were widely accepted, partly because of his powerful influence in the scientific world and partly because of a desire among chemists to avoid conceding an important chemical change to the domain of biology.

In 1857, Pasteur published his first paper in the field of fermentation. The publication dealt with lactic acid fermentation, not alcoholic fermentation. Utilizing the finest microscopes of the time, Pasteur discovered that souring of milk was correlated with the growth of a microorganism, but one considerably smaller than the beer yeast. During the next few years Pasteur extended these studies to other fermentative processes, such as the formation of butyric acid and lactic acid. In each case he was able to demonstrate the involvement of a specific and characteristic microorganism; alcoholic fermentation was always accompanied by yeasts, lactic acid fermentation by nonmotile bacteria, and the butyric acid fermentation by motile, rod-shaped bacteria. Thus, Pasteur not only disposed of one of the opposition's strongest arguments, but also provided strong circumstantial evidence for the biological theory of fermentation. Pasteur then attacked the crucial problem, alcoholic fermentation. Liebig had argued that the proteinaceous material, released from yeast during its decomposition, catalyzed the splitting of sugar. Pasteur countered this argument by developing a protein-free medium for the growth of yeast. He found that yeast could grow in a medium composed of glucose, ammonium salts, and some incinerated yeast. If this medium was kept sterile, neither growth nor fermentation took place. As soon as the medium was inoculated with even a trace of yeast, growth commenced, fermentation ensued, and the quantity of alcohol produced paralleled the multiplication of the yeast. In this protein-free medium, Pasteur was able to show that fermentation took place without the decomposition of yeast. In fact, the yeast synthesized protein at the expense of the sugar and ammonium salts. Thus, Pasteur concluded in 1860 that "Fermentation is a biological process, it is the subvisible organisms which cause the changes in the fermentation process. What's more, there are different kinds of microbes for each kind of fermentation. I am of the opinion that alcoholic fermentation never occurs without simultaneous organization, development and multiplication of cells, or continued life of the cells already formed. The results expressed in this memoir seem to me to be completely opposed to the opinion of Liebig and Berzeliu." (Dubos 1950).

Pasteur argued effectively and, more importantly, all the data were on his side. Thus, the so-called vitalistic theory of fermentation predominated until 1897, when an accidental discovery by Eduard Buchner finally resolved the controversy and threw open the door to modern catalytic biochemistry based on enzymes.

Buchner was attempting to obtain from yeast an extract that might have medicinal value. After several unsuccessful trials, he discovered that mixing yeast cells with fine sand and grinding the mixture in a mortar and pestle disrupted the cells. After filtering the mash to remove the sand and any unbroken cells, a clear yeast juice was obtained. The juice, however, soon became contaminated with bacterial growth. Since the extract was for

human consumption, Buchner could not utilize ordinary antiseptics to prevent the spoilage. Therefore, he attempted to preserve the yeast extract by adding large quantities of sugar. The yeast extract began to bubble soon after the sugar was added. Careful analysis revealed that the sugar was decomposing to carbon dioxide and ethyl alcohol. Fermentation had proceeded *in the absence of living cells*. Buchner's achievement inaugurated a new era in the study of alcoholic fermentation and other metabolic processes. Reactions that normally took place only in vivo could now be studied in vitro. The agents which are present in cell extracts and which catalyze these reactions were called enzymes, from the Greek "en zyme," meaning "in yeast." In 1907, Eduard Buchner received the Nobel Prize in Chemistry "for his biochemical researches and his discovery of cell-less fermentation."

## Classical Applied Microbiology (1900–1940)

After it was demonstrated that living yeast was the causative agent for the alcohol fermentation, rapid progress was made in isolating and identifying the microorganisms responsible for other fermentations. As a result, considerable practical knowledge was gained, commercial media developed, producer strains improved, and the fermentation industry grew and diversified (*Table 13.1*). As an example, commercial bioproduction of citric acid was begun in New York in 1923, and by 1940 over 10 million kilograms were produced per annum. Much of this early work on industrial fermentations has been reviewed by Prescott and Dunn (1940).

With the advent of World War I, it became necessary to develop a fermentation method for making acetone, which is used for the manufacture of explosives. The US government purchased two large distilleries in Indiana and established the Weizmann process, which made acetone from maize by *Clostridium acetobutylicum* fermentation. After the war, the Weizmann process was used mainly to produce butyl alcohol needed in the manufacture of automobile lacquers. During the development of the Weizmann and other industrial fermentation processes, several important and general aspects of applied microbiology became established.

## Isolation of Cultures

Isolation of a pure culture of the desired microorganism is often the first step in an applied microbiology project. Selective media and growth conditions developed for the isolation and cultivation of specific groups of bacteria and archaea are described throughout this handbook. In addition, microorganisms that carry out a specific selectable function can be isolated by enrichment culture procedures (Kreig 1981; Veldamp 1970; Jones and Krieg 1984). For example, if the applied goal is to obtain a xylanase that is active at pH 9.0 and 70°C, then an enrichment culture that contains xylan as the sole carbon and energy source would be adjusted to pH 9.0 and incubated at 70°C. Also, the preferred inoculum would be a soil sample taken from a high temperature and pH site that contains decaying plant material. After several transfers in the same medium, the mixed culture is streaked onto an agar medium containing the same nutrients. The resulting colonies of microorganisms must contain an extracellular xylanase that is active at 70°C and pH 9.0. Enrichment cultures of this type have been successfully employed to obtain a wide variety of useful strains. The power of the enrichment culture technique depends on the enormous metabolic diversity of microorganisms and on the ingenuity of the investigator to establish proper restrictive growth conditions.

## Screening Procedures

The enrichment culture technique is valuable only when growth conditions select for the desired strain. The selection is usually based on the degradation of a particular substrate or the resistance to a particular environmental parameter, for example, high and low temperature, salt and pH values, antibiotics and toxic metals. However, it is generally not possible to select directly for microorganisms that produce or overproduce useful chemicals, such as fermentation products, antibiotics, and other drugs. In such cases, screening procedures have to be employed. The success of the Weizmann process for producing acetone and butanol depended on screening a large number of microorganisms for a strain (*C. acetobutylicum*, Weizmann) that produced high levels of these solvents. In recent years, many new screening procedures have been developed and automated for the discovery of new products (White et al. 1986). Screening procedures are based on either chemical or functional assays, using intact cells or subcellular preparations. An example of a functional assay that uses intact cells is antibacterial screening by the agar plate diffusion assay (Gerhardt 1981). The lambda prophage induction assay for screening anticancer drugs is an example of an assay that uses subcellular preparations (Price et al. 1964). In recent years, high-throughput screening (HTS) using robotics, data processing and control software, liquid handling devices, and sensitive detectors has been applied for drug discovery (Zhang 2011).

## Strain Improvement

Microorganisms, freshly isolated from nature, have highly regulated metabolic systems designed to prevent the overproduction of biochemicals. Genetically altering the strain so that it overproduces a desired product is an important step in industrial microbiology. Three such general procedures have been used successfully: mutation, genetic recombination, and gene cloning. Each technique has its advantages and disadvantages. Often two or three of these techniques can be used in tandem to obtain a stable improved strain. Success in bringing a fermentation product to market and consequently competing in that market depends on continuous strain improvement

**Table 13.1**
**Production of industrial chemicals by fermentation**[a]

| Fermentation | Microorganism | Commercial use |
|---|---|---|
| Ethyl alcohol | *Saccharomyces cerevisiae* | Industrial alcohol |
| Acetone-butanol | *Clostridium acetobutylicum* | Synthetic rubber, explosives, lacquers, and solvents |
| Acetone-ethanol | *Bacillus acetolyticus* | Solvents |
| Acetic acid | *Acetobacter* | Vinegar |
| Lactic acid | *Lactobacilli, Streptococci* | Textile and leather industries |
| Propionic acid | Propionic acid bacteria | Solvent |
| Citric acid | *Aspergillus niger* | Medicines, food and beverages |
| Gluconic acid | *Penicillium chrysogenum* | Pharmaceutical industry, cleaning |
| Gallic acid | *Penicillium glacum* | Dye industry |
| Fumaric acid | *Rhizopus* | Food and beverages |
| Mannitol | White *Asperg* | Resin production, food and beverages |
| Dihydroxy acetone | *Acetobacter* | Artificial tanning and chemical industry |

[a]See Chap. 1, "Organic Acid and Solvent Production" in Vol. 4

**Table 13.2**
**Strain improvement in penicillin production**

| Year | Penicillin strain | Origin | Yield (g/l) |
|---|---|---|---|
| 1929 | Penicillium (Fleming) | Chance contamination | 0.01 |
| 1941 | NRRL-832 | Isolated in Belgium | 0.04 |
| 1943 | NRRL-1951 | Isolated from a melon | 0.15 |
| 1944 | X-1612 | X-ray mutant of NRRL 1951 | 0.30 |
| 1945 | Q-176 | UV mutant of X-1612 | 0.55 |
| 1949 | 49–133 | Spontaneous mutants of Q-176[a] | 1.2 |
| 1990 | Commercial strains | Nitrogen mustard mutants of 49–133[a] | >7.0 |

[a]Several steps of mutation and selection were used to obtain this overproducing strain

programs. Screening and selection methods for strain improvement have been reviewed by Elander (1966), Aharonowitz and Cohen (1981), Queener and Lively (1986), Silverman et al. (1998), and Demain and Adrio (2008).

Historically, mutant screening was the first systematic method to improve industrial strains. The lineage of strain improvement for penicillin production is shown in *Table 13.2*. After an exhaustive screening of natural *Penicillium* strains, one isolate (NRRL-1951) was obtained that produced ten times more penicillin than the initial isolate of Fleming. Starting in 1944, strain NRRL-1951 was used as the parent strain for a program of mutation and selection by several groups of investigators. The results were remarkable: strain X-1612, an x-ray-induced mutant of NRRL-1951, produced 0.3 g of penicillin per liter; strain Q-176, an ultraviolet light-induced mutant of X-1612, yielded 0.55 g per liter; and descendants of strain Q-176 (e.g., strain 49–133) in turn produced 1.2 g; and currently used commercial strains yield more than 7 g per liter. The significance of this strain improvement program is that today it is possible to produce enough penicillin at a low cost to treat anyone who needs the antibiotic, whereas before only a few serious cases could be treated at a high cost.

Genetic recombination of advantageous mutations from several mutant strains is a useful procedure for strain improvement. It allows one to combine advantageous mutations from different sources. Also, genetic recombination makes possible the removal of deleterious secondary mutations. For example, when a culture is mutagenized and then a bacterium is selected, which overproduces the desired product, the bacterium may also contain mutations that interfere with growth. By backcrossing the mutant strain into the wild-type, it is possible to screen for strains that contain the useful mutation without the deleterious one. This procedure of "cleaning up" the strains is particularly important when multiple mutation steps are employed. The concept of genetic recombination for strain improvement has also been applied to natural strains (Zhang et al. 2002).

Gene manipulation is the third and most recent technique of strain improvement. This method requires a good understanding of the molecular genetics and biochemical pathway that is involved in the biosynthesis of the desired product. Gene manipulation can be used to overcome rate-limiting reactions by increasing the production of specific enzymes. This can be obtained by cloning the gene and increasing its copy number, by altering promoter strength and ribosome-binding sites, and eliminating undesirable properties such as product inhibition. In addition, gene manipulation can be used to generate new products by combining genes from different microorganisms. This latter technique—combinatorial genetics—has been used successfully to produce new macrolide antibiotics (Hutchinson and Fujii 1995).

## Biochemical Engineering

To commercialize the production of fermentation products by microorganisms, it was necessary to scale up productions from flasks to large fermentation tanks. This requires a combination of microbiological and engineering skills. The first problem that had to be overcome was the need for sterilization and prevention of contamination by undesirable microorganisms. The fundamental principles of sterilization, developed by Pasteur and others, had to be modified considerably when going from flasks and Petri dishes to very large stainless steel tanks. Not only did engineering techniques have to be applied to sterilize the medium, but techniques had to be developed for the sterile introduction of the inoculum, maintenance of pH and temperature, and the introduction of large quantities of oxygen (air) under sterile conditions.

Understanding and controlling a fermentation process depends on the data obtained from biosensors and instrumentation. The most important parameters to measure and control are pH, temperature, and concentrations of dissolved oxygen and substrate. If these parameters are not maintained within a narrow range, the synthesis of the desired product will decline and in the worst case, the culture will die. Each of these parameters must be measured online. In modern fermenters, the information obtained by the sensors is fed into computers that then automatically adjust the conditions of the fermentation broth to a predetermined value. Dissolved oxygen can be regulated by increasing air flow into the broth or agitation speed of the rotors, pH can be adjusted by pumping in acid or base, and temperature can be controlled by heating or cooling with circulating cold water. Additional parameters of biological significance that can be measured include culture turbidity, exit $O_2$ and $CO_2$, and product concentration. Instrumentation for measuring and regulating fermentation parameters and for computer control of microbial processes has been reviewed by Wang (1981) and Guerreiro et al. (1997).

Scale-up of fermentation processes generally proceeds from flasks to small fermenters to large fermenters (Gaden 1981). Certain physiological parameters can be determined in flask experiments, such as optimum temperature for growth and product formation, growth rates, growth yields, changes in pH values during growth, and nutrient requirements. Since the cost of producing a fermentation product is often influenced largely by the costs of the nutrient feed stocks, it is important to examine inexpensive commercial sources of nutrients, such as corn syrup and casein hydrolysates, even in the flask experiments. Small fermenters (1–50 l) are used to determine the optimum biochemical engineering parameters that are required in large-scale industrial fermentations, for example, oxygen demand. Many economic evaluations cannot be performed accurately using laboratory equipment, and therefore pilot plants must sometimes be built. Scale-up of fermentations has been reviewed by Trilli (1981) and Reisman (1993).

The series of steps used to concentrate and purify the desired product is referred to as the downstream process. Each downstream process is tailored to fit the specific properties of the desired product. The first step is usually the separation of the cells from the broth by continuous centrifugation or hollow fiber filtration. If the product is in the cells, then the cells are disrupted and the product purified by specific biochemical procedures. If the product is in the cell-free broth, then the next step is concentration of the product by removal of the water. Depending on the molecular size and charge of the product, the water can be removed by ultrafiltration, precipitation, or distillation of the product. Again, the final purification of the fermentation product depends on its specific biochemical properties. Typical procedures include liquid-liquid extractions, adsorption chromatography, ion-exchange chromatography, and gel filtration.

## The Antibiotic Era

See Chap. 9, "Bacterial Pharmaceutical Products" in Vol. 4.

### The Discovery of Penicillin and Aftermath

The discovery and development of antibiotics is one of the greatest scientific achievements of the twentieth century. Like many events of major historical significance, the contributing factors were varied and complex. Microbiologists, chemists, fermentation engineers, medical doctors, businessmen, industrialists, lawyers, and government officials played major roles. The motivating forces ranged from pure scientific curiosity and the desire to alleviate suffering to economic gain and the pressures of the Second World War.

The story, which has been told many times, begins in 1928 with a fortuitous observation by Alexander Fleming, a bacteriologist working in St. Mary's Hospital in London. Fleming was growing a disease-causing staphylococcus in Petri dishes containing nutrient agar. An airborne fungal spore fell inadvertently onto the agar and began to multiply. As the contaminating mold grew, a halo, or clear area, developed around the mold colony. Such accidents must surely have happened to hundreds of bacteriologists before Fleming, but the ruined agar

plates were simply discarded in disgust. Fleming, however, realized the significance of the clear area around the mold colony. The fungus must have secreted something into the medium that inhibited the growth of the staphylococci. He therefore isolated the fungus and repeated the experiment, this time intentionally adding the fungus to the bacterial culture. Again the fungus secreted a product that killed bacteria in the neighborhood of the mold colony. Fleming went one step further. He grew large batches of the mold and then separated the mycelial mass from the culture medium. The mold-free juice he prepared was still lethal to the bacteria. Since the mold was a species of *Penicillium*, Fleming called the antibacterial material in the juice penicillin. Shortly thereafter he attempted to concentrate and purify penicillin, failed, and abandoned the project. There followed a period of 10 years during which there were no significant developments in penicillin research. Several suggestions have been put forth to explain this 10-year lag between the discovery of penicillin and its development as a potent chemotherapeutic agent. Although Fleming wrote in 1929 that penicillin "may be an efficient antiseptic for applications to, or injection into, areas infested with penicillin-sensitive microbes," he failed to convince the scientific community. Fleming gave up because he was not a good enough chemist to purify the unstable molecule and lacked the money necessary to hire chemists to help him. With the millions of dollars being spent today on medical research, it is difficult to comprehend how an established scientist like Fleming could not then obtain the $5,000 per year that he needed. Finally, it should be mentioned that scientists are not always the best promoters of their own ideas. Fleming represents the classic example of a scientist whose original findings were subsequently rediscovered, developed, and exploited by others.

As a consequence of the rise of fascism in the 1930s, many outstanding scientists were forced to flee from Hitler's Germany. One of these refugees was the biochemist Ernst Chain. Shortly after arriving in England, Chain joined with Howard Florey, a pathologist at Oxford, in a systematic search for antibacterial substances. A careful library study led Chain to select penicillin as the target of research. A relatively short time after receiving the penicillin-producing mold from Fleming, the team of Chain and Florey succeeded in developing techniques for the purification of penicillin. The first animal experiment with the partially purified penicillin was extremely encouraging. Subsequent studies demonstrated that penicillin was not toxic to mammals, including humans. Preliminary tests with humans suffering from incurable bacterial infections showed that penicillin was a miraculous drug. By 1941, the pressing problem was how to produce enough penicillin for general clinical use. With the outbreak of World War II, the need for drugs effective against battle wounds became even more urgent. Florey and Chain went to the Ministry of Health for help. A decision was made at the highest level of government to assist the Oxford group in making more penicillin. Florey was sent to Peoria, Illinois, the headquarters of the newly established Fermentation Division of the US Department of Agriculture. The laboratory in Peoria was ideally suited for the project. The proper equipment was available and, more importantly, the research staff was experienced in mold fermentations. After a few days of intense discussions, Florey returned to England, leaving his trusted assistant, Norman Heatley, to teach the Americans what was then known about growing *Penicillium* and measuring the quantity of penicillin produced. Progress was rapid. As described above, strain improvement by mutation and selection yielded strains that produced higher and higher levels of antibiotic (*Table 13.2*). By 1943, the major bioengineering problems had been resolved by a cooperative effort of the Peoria laboratory, universities, and industry, and the first aerated stainless steel tank for penicillin production was put into operation.

One of the ironies of the penicillin saga is that England, where the drug was first discovered and shown to be effective, had to pay US companies royalties after the Second World War for the technical know-how to produce penicillin. Fleming, of course, never attempted to patent penicillin. It is unfortunate but true that even if he had, the patents would have expired before the drug was produced commercially. The Oxford group, on the other hand, could have obtained considerable financial reward for developing the penicillin extraction procedure, but instead they gave their knowledge freely to the world. The pioneering research on penicillin, however, did not go unrecognized: The 1944 Nobel Prize in medicine was shared by Fleming, Chain and Florey. For the immigrant biochemist Chain, there was the added pleasure of knowing that he was a major contributor to the development of a drug that saved the lives of thousands of young men who fought to liberate his former homeland—sweet justice.

## Limitations of Penicillin and the Development of Other Antibiotics

There are at least three serious limitations to the chemotherapeutic use of penicillin. First, it cannot be taken orally. The molecule is rapidly decomposed in the acid of the human stomach. Second, it is not effective against several bacteria, including a number of Gram-negative pathogens. Third, a significant number of people are allergic to it. At least three approaches have been used by applied microbiologists and chemists to overcome these limitations: (1) synthesis of chemical derivatives of penicillin (semisynthetic penicillins) that are more resistant to acid and penicillinase and inhibitory to a wider range of bacteria than the natural penicillin G, (2) use of natural inhibitors of penicillinase, such as clavulanic acid, that can work synergistically with β-lactam antibiotics to extend their range, and (3) development of many other antibiotics for clinical use.

The great success of penicillin encouraged microbiologists to search for other antibiotics. A key figure in this search was the soil microbiologist Selman Waksman of Rutgers University in New Jersey. Between 1939 and 1945, Waksman and his associates examined thousands of microbes for their ability to produce antibacterial substances. A major conclusion that emerged from the Rutgers study was that spore-forming bacteria in the soil,

**Table 13.3**
**Antibiotics used in chemotherapy**

| Antibiotic | Source | Used medically in treating | Mode of action |
|---|---|---|---|
| Penicillin | Fungus (Penicillium) | Gram-positive infections | Blocks bacterial cell-wall synthesis |
| Ampicillin (Penbritin) | Semisynthetic pencicillin | Gram-positive and Gram-negative infections | Blocks bacterial cell-wall synthesis |
| Cephalosporins | Fungus and *Streptomyces*[a] | Gram-positive and Gram-negative infections | Blocks bacterial cell-wall synthesis |
| Streptomycin | *Streptomyces* | Tuberculosis; Gram-negative intestinal tract infections | Inhibits protein synthesis in bacteria |
| Oxytetracycline (Terramycin) | *Streptomyces* | Most bacterial infections | Inhibits protein synthesis in bacteria |
| Chlortetracycline | *Streptomyces* | Most bacterial infections | Inhibits protein synthesis in bacteria |
| Chloramphenicol (Chloromycetin)[b] | *Streptomyces* | Typhoid fever; Rocky Mountain spotted fever | Inhibits protein synthesis in bacteria |
| Polymyxin (Aerosporin) | *Bacillus* | Gram-negative wound infections | Destroys bacterial cytoplasmic membrane |
| Bacitracin | *Bacillus* | Topical infections of eye and skin; burn infections | Blocks bacterial cell-wall synthesis |
| Novobiocin | *Streptomyces* | As penicillin; also penicillin-resistant staphylococci | Blocks bacterial nucleic acid synthesis |
| Erythromycin | *Streptomyces* | As penicillin; also penicillin-resistant staphylococci | Inhibits protein synthesis in bacteria |
| Griseofulvin | Fungus and *Streptomyces*[a] | Fungal infections (taken orally) | Destroys fungal cytoplasmic membrane |
| Polyenes (Nystatin) | *Streptomyces* | Systemic fungal infections | Destroys fungal cytoplasmic membrane |

[a]Initially discovered as a fungus, now known also to be produced by *Streptomyces* species
[b]Initially discovered in a *Streptomyces*, now made chemically

especially the *Streptomyces*, were a rich source of antibiotics. From the thousands of antibacterial substances tested, Waksman isolated, characterized and patented several important antibiotics, including streptomycin, neomycin, and actinomycin. Streptomycin, first reported in 1944, quickly emerged as a valuable treatment for tuberculosis and other infections caused by penicillin-resistant Gram-negative bacteria. With Waksman pointing his finger to the soil, the "gold rush" was on for new and better antibiotics. Thousands of active compounds have been found, identified chemically, and screened for potential clinical use. Although most of the active compounds were shown to be too toxic for general use, about 50 of them have proven therapeutic value and are currently produced commercially for medical and veterinary use. A partial list of important antibiotics is shown in *Table 13.3*.

What are the common characteristics of antibiotics? (1) From the chemical point of view, they are an extremely diverse group of organic molecules. The only chemical feature antibiotics seem to share is their relatively small size. Molecules have fewer than 40 carbon atoms. (2) Ecologically, antibiotics are produced almost exclusively by spore-forming soil microorganisms. Furthermore, antibiotics are synthesized at precisely the time the cells are sporulating. Because of these correlations, some microbiologists have hypothesized that the natural role of antibiotics is to regulate the formation of spores in microbes. However, many mutants do not produce antibiotics yet sporulate normally. It is, therefore, reasonable to conclude that antibiotics have no single function, playing different natural roles in different microorganisms. (3) Medically, the most important trait of an antibiotic is high therapeutic index, an indicator of selective killing and low toxicity. Each antibiotic interferes with an essential and specific microbial function.

## The Role of Antibiotic Research in the Development of Microbial Biochemistry, Molecular Genetics and Biotechnology

Much of what we now know about the bacterial cell wall and biopolymer synthesis has come from studies using specific antibiotics to inhibit synthesis. Furthermore, antibiotics were key tools in the development of microbial genetics. For example, most auxotrophic mutants were obtained using the penicillin selection method (Davis 1948). Antibiotic resistance continues

to be the most important selection marker for genetic recombination. The discovery of plasmids was initially connected to research on multidrug resistance or R factors. Even today, most of DNA recombinant technology relies heavily on the use of antibiotics and antibiotic-resistance genes to construct useful strains. Antibiotic research is an excellent example of how applied and basic research overlap, interact, and mutually benefit each other.

## The New Biotechnology: Applications of Genetic Engineering.

Cloning genes in bacteria is already a valuable technology in both basic biological research and practical applications (Glazer and Nikaido 1995). In basic research, genetic engineering in bacteria has been used to examine the underlying mechanisms of DNA replication, gene recombination, and gene expression of viral, bacterial, and eukaryotic genes. The number of commercial applications of gene engineering continues to grow (*Table 13.4*). Molecular biology of bacteria led the way for the development of the biotechnology industry and today molecular biology is the major driving force in pharmaceutical research (Ferrer-Miralles et al. 2009). Recombinant DNA technology can be used to more efficiently carry out an existing process or develop entirely new products. One of the first examples was the production of human insulin in *Escherichia coli*. After the insulin gene was chemically synthesized and inserted downstream from a suitable *E. coli* promoter, the cell was able to synthesize a fusion protein, which was then split into the separate insulin peptides. These peptides, once connected by disulfide bonds, were the final product that is identical to human pancreatic insulin and now commercially available. Microbially produced human insulin is not immunogenic and is purer and less expensive to produce than porcine or bovine insulin, which was previously used. Several other human protein pharmaceuticals are now produced in microorganisms including anticancer agents, such as α- and β- interferon; human growth hormone for the treatment of dwarfism; nerve growth factor; lysozyme for the treatment of inflammations; and several blood proteins, such as hemoglobins used as blood substitutes (Kumar 1995), erythroprotein to treat anemia, urokinase to assist in blood clotting, and tissue plasminogen activator to dissolve clots. The number of new pharmaceutical products by recombinant technology in 2005 comprised 134 approved products, with an approximate sales value of 35 billion dollars (Lawrence 2006).

Safe and highly effective vaccines can be produced by recombinant DNA technology. Instead of using killed or attenuated pathogenic bacteria or viruses, subunit vaccines are produced by cloning part of the protein that is on the outer surface of a bacterium or virus. The subunit protein vaccine is then produced in a microorganism, usually *E. coli*. The subunit vaccines produce a rapid and high level of protective immunity with no possibility of transmitting the infection. Furthermore, vaccines can be produced against microbes that are difficult to grow in vitro. The first recombinant subunit vaccine approved for use in humans was against hepatitis B virus. Recombinant vaccines are also available for animal diseases, such as Newcastle's and fowl pox diseases in poultry. Several trials are currently being carried out with polyvalent (subunits from more than one protein) vaccines against acquired immunodeficiency syndrome (AIDS). A new approach is to use specific, cloned DNA itself as the vaccine. The DNA is taken up by the animal cell and then transcribed and translated into an active immunogenic protein. If successful, these DNA vaccines would be safe and inexpensive.

**Table 13.4**
**Some of the commercial applications of recombinant DNA technology**

| Applications | Examples |
|---|---|
| Production of mammalian proteins in bacteria | Insulin, blood proteins, interferons, growth hormones, and tumor necrosis factor |
| Vaccines | Hepatitis B, rabies, measles, polyvalent vaccines, and DNA vaccines |
| Fermentation products | Increased yields of antibiotics, restriction enzymes, proteases, xylanases, amino acids, and vitamins |
| Diagnostics | Diagnosis of pathogenic microbes and genetic diseases |
| New antibiotics | Hybrid polyketides |
| Transgenic plants and animals | Herbicide, insect and microbial disease resistant plants; cows that produce pharmaceutical products in their milk |
| Gene therapy | Introduction of cloned adenosine deaminase (ADA) into immune deficient patients lacking ADA |
| Environmental biotechnology | Cloning and overexpression of genes involved in the degradation of pollutants |

The fermentation industry has benefited greatly from recombinant DNA technology. The technology is used in strain improvement programs to obtain not only more efficient processes but also new products. Most of the DNA restriction enzymes used in biotechnology are produced in *E. coli* from cloned DNA that has been put into high expression vectors. Similarly, many of the commercial enzymes (such as proteinases used in the detergent and food industries, xylanases and amylases used in the textile and paper industries, and glucose isomerase used to make high fructose corn syrup) are produced in bacteria that are easy to grow in fermenters, for example, *E. coli* and *Bacillus* species (Godfrey and West 1996). An interesting approach to the production of new antibiotics is to place selected genes from two different polyketide antibiotic-producing bacteria into a recipient bacterium that can then synthesize a new polyketide antibiotic (McDaniel et al. 1993). Another important application of recombinant DNA technology is the construction of transgenic plants and animals.

Such organisms have great potential for enhancing agricultural productivity and improving the nutritional and storage properties of meats and vegetables. Also in development are crop plants, used for the production of recombinant vaccines, including edible vaccines against cholera and enteric diarrhea. In many of the plant engineering constructs, *Agrobacterium tumefaciens* is used to introduce desired genes into the plant. Monsanto Company (St. Louis, MO) is presently marketing a transgenic soybean plant that has an *Agrobacterium-derived* gene that confers resistance to Roundup, a glyphosate herbicide. Genetic engineering also has been used to protect plants from viral diseases. Calgene Corporation (Davis, CA) produces a transgenic tomato that spoils more slowly than normal tomatoes because it contains an inhibitor of pectinase transcription. Transgenic animals are used extensively in research as well as for the commercial production of proteins of pharmaceutical value, such as α-1-antitrysin in sheep and tissue plasminogen activator in goats. Biotechnology is also involved with human genes and, in the long run, recombinant DNA technology will probably have its most dramatic effect on the human genome. As Robert Sinsheimer stated 30 years ago:

> For eon after eon, creature has given rise to creature upon this earth—blindly, each generation usually like the former, occasionally—by accident—a little different. Of all the creatures that have lived upon this earth we are the first to understand this process. The ultimate significance of this understanding of the very basis of heredity is incalculable. It will change all the eons to come.
>
> (Rosenberg 1991)

Already, PCR technology is widely used in forensic medicine to determine parentage, to analyze evolutionary relationships between modern man, early man, and pre-man, and so forth. In one of the largest biological research programs ever conducted, the human genome project, the entire nucleotide sequence of the human genome will soon be determined. By comparing the DNA sequence of healthy and diseased individuals, the precise position and aberration in the "bad" gene may be found. Gene therapy, through genetic engineering, offers the hope of replacing disease-causing genes with healthy ones, thereby providing preventive medicine in the most profound sense.

## Applied Environmental Microbiology: Bioremediation

See Chaps. 10, "Biosurfactants" and Chap. 11, "Bioremediation" in Vol. 4.

Albert Einstein defined the environment as "anything which is not me." This definition is interesting for two reasons: it has enormous scope, and it points the finger away from each of us. Individuals, industries, and societies have assumed incorrectly that if the environment is not "them," they do not have to be responsible for it. The result is worldwide pollution, which degrades the quality of our lives and threatens our very existence.

When considering pollution, one must focus on the turnover of matter, which is fundamentally a series of microbial processes. It is primarily by microorganisms that waste products, including most pollutants, are broken down. Microbes have enormous biodegradation potential. They occupy all possible ecological niches, from deep in the earth to high in the atmosphere, and collectively are able to grow over a wide range of temperatures, pH values, salt concentrations, and oxygen tensions. There is not a single known natural organic compound that cannot be degraded by one or more microbial species.

If microbes have such enormous degradative power, why do we have pollution? There are two fundamental reasons: (1) man-made conditions can become unfavorable for rapid degradation and (2) certain man-made synthetic compounds are resistant to biodegradation. The former is dictated by the "law of the minimum," that is, growth is limited by one essential component. For example, if a large amount of petroleum is dumped into the sea, biodegradation may not be at an appreciable rate because utilizable forms of nitrogen and phosphorus are not available—even if many hydrocarbon-degrading bacteria are at the site. Nitrogen and phosphorus are, of course, essential components for all living cells. Another condition, which is unfavorable for biodegradation, is high concentration of organic material. Two good examples of this are whiskey and jam in which ethyl alcohol and sugar are, respectively, at unnatural levels that resist microbial breakdown. One of the reasons that organic matter is degraded so slowly in garbage dumps is that the consolidation and concentration of materials make biodegradation difficult. In the above examples, natural bacteria can completely degrade (mineralize) these materials, whether they are hydrocarbons in crude oil or cellulose in newspaper, if the conditions are favorable. However, several man-made unnatural compounds, such as certain halogenated aromatics and plastics, are not biodegradable under any circumstances. They simply have not been on the planet long enough for bacteria to have evolved degradative mechanisms.

Bioremediation is the use of living organisms, usually bacteria, to prevent or clean up pollution. Bioremediation is a large and growing area of applied microbiology. Hundreds of companies around the world are involved in treating water and soil that is polluted with industrial wastes, such as petroleum products, herbicides, pesticides, toxic metals, dioxenes, and polychlorinated biphenyls (PCBs), and explosives like TNT. There are two general approaches to bioremediation. The first and most successful method is to optimize conditions so bacteria native to the polluted site can more rapidly degrade the pollutants. The second method, which has had limited success to date, is to introduce more efficient degraders to the polluted site, or in the case of nonbiodegradable synthetic pollutants, to construct genetically engineered microorganisms (GEMs) that can degrade the pollutant. The following examples will illustrate these two approaches.

### Petroleum Pollution and Bioremediation

During the twentieth century, the demand for petroleum as a source of energy and as a primary raw material for the chemical

**Table 13.5**
**Requirements for biodegradation of petroleum**

| |
|---|
| A. Microorganisms with: |
| 1. Hydrocarbon-oxidizing enzymes |
| 2. Ability to adhere to hydrocarbons |
| 3. Mechanism for desorption from hydrocarbons |
| 4. Emulsifier-producing potential |
| B. Water |
| C. Oxygen |
| D. Relatively large amounts of utilizable nitrogen and phosphorus |
| E. Iron, magnesium, and other essential elements |

industry has increased world production to about 3,500 million metric tons per year. Approximately 1 % or 35 million tons finds its way to soils, lakes, and seas. The toxicity of petroleum to microorganisms, plants, animals, and man is well established. The polycyclic aromatic hydrocarbon (PAH) fraction of petroleum is particularly toxic and is on the United States Environmental Protection Agency priority pollutant list (Cernilia 1992).

It has been known for over 80 years that certain bacteria are able to degrade petroleum hydrocarbons and use them as a sole source of carbon and energy. Effective bioremediation of petroleum pollution relies heavily on a fundamental understanding of the requirements for petroleum biodegradation (*Table 13.5*). Generally, the presence of hydrocarbon-degrading microorganisms is not the limiting factor in petroleum bioremediation. Hydrocarbon-oxidizing bacteria have been isolated from a wide variety of natural aquatic and terrestrial environments (Rosenberg 1991), including hydrothermal vent sites (Baylinski et al. 1989). Furthermore, the addition of oil causes a rapid shift to increased numbers of hydrocarbon degraders. For example, Song and Bartha (1990) demonstrated that the number of hydrocarbon-degrading microorganisms increased from $4 \times 10^4$ to $3 \times 10^{10}$ per g of soil 4 weeks after addition of jet fuel and inorganic nutrients. The practical conclusion from this and other studies is that seeding an oil-polluted area with microorganisms is generally not necessary, and GEMs are not required for petroleum bioremediation.

In addition to having the biochemical potential for degrading one or more classes of hydrocarbons, an efficient hydrocarbon degrader must be able to adhere to and desorb from water-insoluble hydrocarbons. The role of cell surface hydrophobicity, adhesins, and bioemulsifiers in the growth process has been reviewed (Rosenberg and Ron 1996; Ron and Rosenberg 2009).

The most important factor determining the success of a petroleum pollution bioremediation project is the ability to provide the optimum conditions for the hydrocarbon-degrading bacteria. The major requirements are adequate sources of oxygen, nitrogen, and phosphorus. Oxygen can be added to soils by

**Table 13.6**
**Bioremediation of hydrocarbon-contaminated sand at an Israeli beach**

| Day | Hydrocarbon degradation (%) | |
|---|---|---|
| | Control (untreated) | Experimental ($N_3P$ added)[b] |
| 10 | 10 | 20 |
| 20 | 20 | 41 |
| 30 | 22 | 50 |
| 40 | 18 | 60 |
| 50 | 20 | 65 |
| 70 | 10 | 70 |
| 90 | 0 | 75 |
| 120 | 0 | 88 |

[a]Data from Rosenberg et al. (1996)
[b]Approximately 30,000 $m^2$ of heavy oil contaminated beach were treated with 15 g urea-formaldehyde polymers (containing utilizable N and P) per kilogram sand. The area was plowed twice weekly

frequent plowing and to underground reservoirs by pumping aerated water through them. Soils can be amended with utilizable nitrogen and phosphorus salts. However, no satisfactory method exists that provides the nitrogen and phosphorus necessary for bioremediation of oil spills in open systems, such as the sea. The ideal nitrogen and phosphorus source should adhere to the oil, rather than be diluted with water. Recently, it has been shown that water-insoluble fertilizers, such as uric acid and urea-formaldehyde polymers, can be effective in stimulating biodegradation of hydrocarbons in the marine environment (Koren et al. 2003; Knezevich et al. 2006). An example of bioremediation of beach sand by addition of urea-formaldehyde polymers is shown in *Table 13.6*. Overall, the bioremediation treatment resulted in the degradation of 88 % of the oil after 4 months. During the same period, there was virtually no change in the oil concentration of the untreated control plot.

## The Use of GEMs in Bioremediation

The first GEM to be patented in the USA was a Pseudomonas that was designed for degrading different hydrocarbons and constructed from genes derived from different bacteria (Chakrabarty and Gunsalus 1971). The great potential of GEMs for bioremediation was publicized by the news media, which referred to them as "superbugs." Unfortunately, the potential of GEMs in bioremediation has not been realized as yet for several reasons. (1) Safety regulations have restricted the release of GEMs into the environment; in fact, most of the research carried out on GEMs and bioremediation has been diverted to studying the fate of GEMs in the environment. (2) Genetically engineered microorganisms do not appear to have any significant advantage over a mixture of existing bacteria for the in situ degradation of natural pollutants, such as crude oil. (3) Also, to construct

GEMs that effectively degrade recalcitrant synthetic pollutants has proved difficult. In spite of these problems, progress is being made in constructing GEMs for degrading haloaromatic compounds (Reineke 1998; Timmis et al. 1994; Cases and Lorenzo 2005) and other pollutants (Chen et al. 1999). When enhanced biodegradative pathways are combined with the genetic potential to catabolize these pollutants in the natural environment, then mineralization rates will likely improve. One possible approach is to clone genes for biosurfactant production into GEMs that have good catabolic potential.

## Health-Related Applied Microbiology

See Chap. 9, "Bacterial Pharmaceutical Products" in Vol. 4.

### Drug Discovery

One of the most exciting areas of applied microbiology involves the discovery of new drugs for human and animal health. Secondary metabolites produced by microorganisms provide a vast array of potentially useful drugs. While the search for new drugs from *Streptomyces* and other spore-forming bacteria continues to be productive, new sources of bacteria are now being exploited, such as marine bacteria (Fenical 1993, 1997; Hill and Fenical 2010). In addition to expanding the pool of microorganisms in the search for novel pharmaceuticals, researchers are now employing new technologies for drug screening (Steele and Stowers 1991; Lloyd 1998; Schmid et al. 1999). These include robotic techniques for plating test bacteria and delivering dilutions of bacterial extracts, use of monoclonal antibodies and fluorescent techniques and combinatorial biosynthesis (Hutchinson 1998), and screens based on specific enzymatic reactions. These new techniques allow for a more rapid and comprehensive screening for new drugs.

### Diagnostic Microbiology

Detecting, classifying, and enumerating microorganisms in drinking water, foods and beverages, and human tissues and fluids is a large area of applied microbiology that is constantly undergoing improvements. During most of the twentieth century, microbiology laboratories developed many dependable approaches for detecting microorganisms, especially pathogens, using microscopy coupled to staining techniques, culturing in selective media, and immunoserology (Koneman et al. 1992). This field of diagnostic microbiology has proved invaluable to physicians treating patients, food producers controlling the quality of their products, and health officials monitoring water supplies and consumer products. However, many of these diagnostic tests are slow, expensive, and insensitive. For example, culturing bacteria from urine or blood and then testing them for antibiotic sensitivity can take several days. During this time, the patient may be at risk, or in the case of a water supply or consumer product, many people could be infected during the diagnosis period. Therefore, it would be advantageous to have more rapid and sensitive techniques.

During the last 10 years, many new biotechnology companies have emerged that have as their major focus the development of specific diagnostic tests that do not depend on culturing microorganisms. Rather, these new biotechnology methods rely on amplification of a species-specific DNA target, of the probe, or of the signal that is generated (Hammes and Higgins 1995; Whelen and Persing 1996). In addition to great sensitivity and rapidity, these methods can detect pathogens that are difficult to culture, such as *Tropheryma whippelii* (Relman et al. 1992), *Chlamydia trachomatis* (Chapin-Robertson 1993), and *Neisseria gonorrhoeae* (Hale et al. 1993). Nucleic acid probes have already replaced classical morphology and biochemical tests in clinical laboratories for the definitive identification of mycobacteria (Keller et al. 2002). Considerable effort is now being employed to develop biotechnology-based detection methods for drug resistance in clinically important organisms (Eron et al. 1992). Advances in approaches to DNA-based diagnostics have been reviewed (Whitcombe et al. 1998; Tang and Stratton 2011).

## Food Microbiology

See Chap. 8, "Bacteria in Food and Beverage Production" in Vol. 4.

Food microbiology, one of the traditional areas of applied microbiology, is changing dramatically. Microorganisms play a direct role in producing and processing the healthy foods we eat and drink. Meat and milk are available to us only because microbes, in symbiotic relationships with certain animals, are able to digest cellulose. Different microorganisms convert milk to a variety of yogurts, cheeses, and butter. Still other microbes preserve plant materials in the form of tasty products such as pickles and sauerkraut. Alcoholic fermentation by yeast is needed for bread as well as all alcoholic beverages. Hagedom and Kaphammer (1994) have reviewed the role of microbial biocatalysis in the generation of flavor and fragrance chemicals.

An interesting recent development in food microbiology is the use of "probiotic bacteria" to improve animal and human food (Bengmark 1998; Holzapfel 1998; Guarino 1997). Probiotic bacteria are generally isolated from the mouth, stomach, and upper and lower bowel of healthy humans and animals. These normal resident bacteria are beneficial, providing vitamin K, helping to process nutrients, preventing colonization of transient bacteria (nonspecific defense mechanism), developing the immune system, and stimulating phagocytic activity. The future of probiotics will depend upon the development of safe strains that are well adapted to occupy specific niches in the body and promote health. When added to food or used as starters in food fermentations, these strains can provide the beneficial effects of probiotics to man and animals.

## Biosafety and Legal Protection in Biotechnology

The safety of research microbiologists and legal protection for their discoveries are two subjects, which have become more important in recent years in applied microbiology and biotechnology. Unfortunately, most microbiologists do not get an adequate education in these topics. Many laboratory techniques create aerosols, which can lead to the inhalation of undetected infectious agents and the occupational illnesses among laboratory workers who handle infectious materials. Based upon available data, preventative measures have been developed which provide safeguards for scientific personnel and the environment. These safeguards are collectively called containment practices. Leiberman et al. (1986) have written a concise review on biosafety in biotechnology, which should be studied by all practicing microbiologists.

To derive financial benefit from an original and potentially useful discovery in biotechnology, it should be patented (see Chap. 12, "Repositories for Patented and Safeguarded Material" in Vol. 1). This is particularly important for research scientists at universities because once a patent application has been filed, it is possible to disclose the invention (scientific publications, grant applications, seminars, etc.) and still have legal protection. In the USA, it is possible to file a patent within 1 year of the publication date. The culture of science demands free exchange of ideas and data. Patenting makes this possible, while still protecting the rights of the scientist. Another reason for patenting a discovery is that it allows the owner of the patent to more safely market the invention. An applied microbiologist at a university or research institution cannot generally convert his discovery into a commercial product. It is therefore necessary for the institution to find a partner or sell the patent and know-how. The procedures for obtaining a patent have been outlined by Saliwanchik (1986).

## Outlook

The border between basic and applied microbiology, which has always been tenuous, is disappearing. The period in which a discovery in basic microbiology becomes an applied project is often very short. Some of the most basic discoveries in microbiology are now made by scientists working on applied problems in universities, research institutions, and biotechnology companies. The rapid progress taking place in applied microbiology and biotechnology, made possible by almost a century of fundamental work on microbial biochemistry, physiology, and genetics, makes it difficult to predict which areas will develop most rapidly in the next few decades. Clearly, the use of bacteria in DNA-based technologies will continue to grow and find new applications. Already, a large percentage of the corn grown in the USA contains cloned *Bacillus thuringiensis* protein as an endogenous insecticide. In addition, I predict the following three general subjects will soon emerge as highly significant in applied microbiology and biotechnology.

### Environmental Microbiology

The rapid increase in human population and technological developments is degrading our environment at an alarming rate, causing a lowering of the quality of life and serious health problems. As a recent example, the Deepwater Horizon oil spill released 140 million gallons of crude oil into the Gulf of Mexico, causing extensive damage to marine and wildlife habitats and to the Gulf's fishing and tourism industries (Biello 2010). Since microorganisms are largely responsible for the turnover of matter on this planet, applied environmental microbiology will become more important. Not only will bioremediation develop further scientifically, technologically, and commercially, but also selected microbes will be used under controlled conditions to recycle waste and to prevent pollution by replacing pollution-producing chemical processes. The so-called "green revolution" will be carried out by microorganisms supplied by a new generation of applied microbiologists.

An outcome of research in environmental microbiology will be the emergence of new principles of ecology. For the same reasons that microbes were ideal organisms to study the principles of biochemistry and genetics, they will be used to develop fundamental principles of ecology. The use of higher animals and plants for deriving general ecological principles is limited by our inability to carry out controlled experiments. Animal and plant ecologists can make accurate observations, ask interesting questions and present hypotheses, but the microbial ecologist can obtain quantitative data in controlled experiments, using molecular probes and other tools of modern microbiology. The combined effort of animal, plant, and microbial ecologists should lead to a better understanding of how organisms interact with their environment.

### Microbial Associations with Animals, Plants, and Humans

Bacterial symbionts play a key role in maintaining the health of all animals, plants, and humans. Cooperation between the normal microbiota and the host generally leads to improved fitness of the holobiont, by the host outsourcing (Zilber-Rosenberg and Rosenberg 2008) different kinds of functions to its microbiota and vice versa. Protection against infectious disease is one of the important attributes of the resident microbiota. The exact mechanism is unknown, but it has been suggested that resident bacteria occupy binding sites needed by pathogens for adhesion in addition to releasing antibacterials active against pathogens. Another important known beneficial functions of microbiota is participation in the development and normal function of the innate and adaptive immune systems in the gut (O'Hara and Shanahan 2006; Ivanov and Littman 2011) while creating a permissive, noninflammatory environment for their own presence (Hapfelmeier et al. 2010). The microbiota also play a key role in angiogenesis, (Stappenbeck et al. 2002), they synthesize and excrete vitamins in excess of their own

needs, which can be absorbed as nutrients by their host. For example, in humans, enteric bacteria secrete Vitamin K and Vitamin B12, and lactic acid bacteria produce certain B-vitamins (Mai et al. 2010). The human gut microbiota is a complex ecosystem that plays an essential role in the catabolism of dietary fibers, and fat metabolism (obesity) and related disorders (Ley et al. 2006; Turnbaugh et al. 2006; Cani and Delzenne 2009).

As we learn more about these interactions, use of selected and improved bacterial strains will grow: in agriculture, for improving yields and preventing spoilage; in animal husbandry, for improving yields and nutritional value of meat, milk, and eggs; in humans, for maintaining health and deterring diseases (Zilber-Rosenberg and Rosenberg 2011). The development of defined prebiotics and probiotics will become an important applied microbial technology.

### Microbial Enzymes

Microbial enzymes are currently being used commercially, primarily for degradative reactions, for example, proteinases, cellulases, amylases, and lipases. This area will be further developed with the introduction of more efficient enzymes and enzymes from extremophiles with increased heat tolerance and extended pH range. However, the commercial potential of microbial enzymes used to synthesize drugs and other fine and bulk chemicals is even greater. With the accumulating data on biosynthetic pathways and the ability to overproduce specific enzymes, it should be possible to mix and match enzymes both in vivo and in vitro to efficiently produce existing valuable products as well as new materials. In this regard, bacterially produced polyhydroxybutyrate and its copolymers have attracted considerable attention as environmentally degradable plastics for a wide range of agricultural, industrial, and medical applications (Kumaravel et al. 2010).

This short list is certainly not comprehensive; many other subjects of applied microbiology will expand and new areas will be created. In short, the future of applied microbiology and biotechnology is bright.

## References


Aharonowitz Y, Cohen G (1981) The microbiological production of pharmaceuticals. Sci Am 245:106–119

Baylinski DA, Wirsen CE, Jannasch HW (1989) Microbial utilization of naturally occurring hydrocarbons at the Guaymas Basin hydrothermal vent site. Appl Environ Microbiol 55:2832–2836

Bengmark S (1998) Ecological control of the gastrointestinal tract. Gut 42:2–7

Biello D (2010) Meet the microbes eating the gulf oil spill. Sci Am 303:14–17

Cani PD, Delzenne NM (2009) The role of the gut microbiota in energy metabolism and metabolic disease. Curr Pharm Des 15:1546–1558

Cases I, Lorenzo V (2005) Genetically modified organisms for the environment: stories of success and failure and what we have learned from them. Int Microbiol 8:213–222

Cernilia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351–368

Chakrabarty AM, Gunsalus IC (1971) Degradative pathways specified by extrachromosomal gene clusters. Pseudomonas Genet 68:510

Chapin-Robertson K (1993) Use of molecular diagnostics in sexually transmitted diseases: critical assessment. Diagn Microbiol Infect Dis 16:173–184

Chen W, Bruhlmann F, Richins RD, Mulchandani A (1999) Engineering of improved microbes and enzymes for bioremediation. Curr Opin Biotechnol 10:137–141

Davis BD (1948) Isolation of biochemically deficient mutants of bacteria by penicillin. J Am Chem Soc 70:4267

Demain AL, Adrio JL (2008) Strain improvement for production of pharmaceuticals and other microbial metabolites by fermentation. Prog Drug Res 6:253–289

Dubos RJ (1950) Louis Pasteur: free lance of science. Little, Brown and Company, Boston

Elander RP (1966) Two decades of strain development in antibiotic-producing microorganisms. Dev Ind Microbiol 7:61–73

Eron JJ, Gorczyc P, Kaplan JC, D'Aquila RT (1992) Susceptibility testing by polymerase chain reaction DNA quantitation: a method to measure drug resistance of human immunodeficiency virus type 1 isolated. Proc Natl Acad Sci 89:3241–3245

Fenical W (1993) Chemical studies of marine bacteria: developing a new resource. Chem Rev 93:1673–1683

Fenical W (1997) New pharmaceuticals from marine organisms. Trends Biotechnol 15:339–341

Ferrer-Miralles N, Domingo-Espin J, Corcero JL, Vasquez E, Villaverde A (2009) Microbial factories for recombinant pharmaceuticals. Microb Cell Fact 8:17–25

Gaden EL (1981) Production methods in industrial microbiology. Sci Am 245:134–145

Gerhardt P (ed) (1981) Manual of methods for general bacteriology. ASM, Washington, DC

Glazer AN, Nikaido H (1995) Microbial biotechnology: fundamentals of applied microbiology. W. H. Freeman, New York

Godfrey T, West S (1996) Industrial enzymology. In: Godfrey T, West S (eds) The application of enzymes in industry, 2nd edn. Stockton, New York

Guarino A (1997) Oral bacterial therapy reduces the duration of symptoms and of viral excretion in children with milk diarrhea. J Pediatr Gastroenterol Nutr 25:516–519

Guerreiro MA, Andrietta SR, Maugeri F (1997) Expert system for the design of an industrial fermentation plant for the production of alcohol. J Chem Technol Biot 68:163–170

Hagedom S, Kaphammer B (1994) Microbial biocatalysis in the generation of flavor and fragrance chemicals. Annu Rev Microbiol 48:773–800

Hale YM, Melton ME, Lewis JS, Willis DE (1993) Evaluation of PACE 2 Neiseria gonorrhoeae assay by three public health laboratories. J Clin Microbiol 31:451–453

Hammes BD, Higgins SJ (1995) Gene probes: a practical approach. Oxford University Press, New York, pp 1–288

Hapfelmeier S, Lawson MAE, Slack E et al (2010) Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science 328:1705–1709

Hill RT, Fenical W (2010) Pharmaceuticals from marine natural products: surge or ebb? Curr Opin Biotechnol 6:777–779

Holzapfel HH (1998) Overview of gut flora and probiotics. Int Food Microbiol 41:85–101

Hutchinson CR (1998) Combinational biosynthesis for new drug discovery. Curr Opin Microbiol 1:319–329

Hutchinson CR, Fujii I (1995) Polyketide synthase gene manipulation: a structure-function approach in engineering novel antibiotics. Annu Rev Microbiol 49:201–238

Ivanov II, Littman DR (2011) Modulation of immune homeostasis by commensal bacteria. Curr Opin Microbiol 14:106–114

Jones D, Krieg NR (1984) Seriology and chemotaxonomy. In: Krieg NR, Holt JC (eds) Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore, pp 15–18

Keller AP, Beggs ML, Amthor B, Bruns F, Meissner P, Haas WH (2002) Evidence of the presence of IS1245 and IS1311 or closely related insertion elements in

nontuberculous mycobacteria outside of the *Mycobacterium avium* complex. J Clin Microbiol 40:1869–1872

Knezevich V, Koren O, Ron EZ, Rosenberg E (2006) Petroleum bioremediation in seawater using guano as the fertilizer. Bioremed J 10:83–91

Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC Jr (1992) Diagnostic microbiology. 4th ed. Philadelphia: JB Lippincott

Koren O, Knezvic V, Ron EZ, Rosenberg E (2003) Petroleum bioremediation using water insoluble uric acid as the nitrogen source. Appl Environ Microbiol 69:6337–6339

Kreig NR (1981) Enrichment and isolation. In: Gerhardt P (ed) Manual of methods for general bacteriology. ASM, Washington, DC, pp 112–142

Kumar R (1995) Recombinant hemoglobins as blood substitutes: a biotechnology perspective. Proc Soc Exp Biol 208:150–158

Kumaravel S, Hema R, Lakshmi R (2010) Production of polyhydroxybutyrate (bioplastic) and its biodegradation by *Pseudomonas lemoignei* and *Aspergillus niger*. E-J Chem 7(S1):S536–S542

Lawrence S (2006) Biotech blockbusters consolidate markets. Nat Biotechnol 24:1466

Leiberman DG, Fink R, Schaffer F (1986) Biosafety in biotechnology. In: Demain AL, Solomon NA (eds) Industrial microbiology and biotechnology. ASM, Washington, DC, pp 402–409

Ley RE, Turnbaugh PJ, Klein S, Gordon JI (2006) Human gut microbes associated with obesity. Nature 444:1022–1023

Lloyd A (1998) High-throughput screening. Drug Discov Today 3:566–569

Mai V, Ukhanova M, Baer DJ (2010) Understanding the extent and sources of variation in gut microbiota studies; a prerequisite for establishing associations with disease. Diversity 2:1085–1096

McDaniel R, Ebert-Khosla S, Hopwood DA, Khosla C (1993) Engineered biosynthesis of novel polyketides. Science 262:1546–1550

O'Hara AM, Shanahan F (2006) The gut flora as a forgotten organ. EMBO Rep 7:688–693

Prescott SC, Dunn CG (1940) Industrial microbiology. McGraw-Hill, New York

Price KE, Buck RE, Lein J (1964) System for detecting inducers of lysogenic *Escherichia coli* W1709 (gamma) and its applicability as a screen for antineoplastic antibiotics. Appl Microbiol 12:428–435

Queener SW, Lively DH (1986) Screening and selection for strain improvement. In: Demain AL, Solomon NA (eds) Industrial microbiology and biotechnology. ASM, Washington, DC, pp 155–169

Reineke W (1998) Development of hybrid strains for the mineralization of chloroaromatics by patchwork assembly. Annu Rev Microbiol 52:287–332

Reisman HB (1993) Problems in scale-up of biotechnology production processes. Crit Rev Biotechnol 13:195–253

Relman DA, Schmidt TM, MacDermott RP, Falkow S (1992) Identification of the uncultured bacillus of Whipple's disease. N Eng J Med 327:293–301

Ron EZ, Rosenberg E (2009) Role of biosurfactants. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Heidelberg

Rosenberg E (1991) Hydrocarbon-oxidizing bacteria. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer K-H (eds) The prokaryotes, 2nd edn. Springer, New York, pp 446–459

Rosenberg E, Ron EZ (1996) Bioremediation of petroleum contamination. In: Crawford RL, Crawford DL (eds) Bioremediation: principles and applications. Cambridge University Press, New York, pp 100–124

Rosenberg E, Legmann R, Kushmaro A, Adler E, Abir H, Ron EZ (1996) Oil bioremediation using insoluble nitrogen source. J Biotechnol 51:273–278

Saliwanchik R (1986) Legal protection for biotechnology. In: Demain AL, Solomon NA (eds) Industrial microbiology and biotechnology. ASM, Washington, DC, pp 389–401

Schmid I, Sattler I, Grabley S et al (1999) Natural products in high throughput screening: automatic high-quality sample preparation. J Biomol Screen 4:15–25

Silverman L, Campbell R, Broach JR (1998) New assay technologies for high-throughput screening. Curr Opin Chem Biol 2:397–403

Song HG, Bartha R (1990) Effects of jet fuel spills on the microbial community in soil. Appl Environ Microbiol 56:646–651

Stappenbeck TS, Hooper LV, Gordon JI (2002) Developmental regulation of intestinal angiogenesis by Peneth cells. Proc Natl Acad Sci USA 99:15451–15455

Steele DB, Stowers MD (1991) Techniques for selection of industrially important microorganisms. Annu Rev Microbiol 45:89–106

Tang YW, Stratton CW (2011) Advanced techniques in diagnostic microbiology overview. Springer, New York

Timmis KW, Steffan RJ, Unterman R (1994) Designing microorganisms for the treatment of toxic wastes. Annu Rev Microbiol 48:525–557

Trilli A (1981) Scale-up of fermentation. In: Demain AL, Solomon NA (eds) Industrial microbiology and biotechnology. ASM, Washington, DC, pp 277–307

Turnbaugh PJ, Ley RE, Mahowald MA et al (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444:1027–1031

Veldamp H (1970) Enrichment cultures of prokaryotic organisms. In: Noris JR, Ribbons DW (eds) Methods in microbiology, vol 6. Academic, New York, p 305

Wang HY (1981) Bioinstrumentation and computer control of fermentation processes. In: Demain AL, Solomon NA (eds) Industrial microbiology and biotechnology. ASM, Washington, DC, pp 308–320

Whelen AC, Persing DH (1996) The role of nucleic acid amplification and detection in the clinical microbiology laboratory. Annu Rev Microbiol 50:349–373

Whitcombe D, Newton CR, Little S (1998) DNA-based diagnostics. Curr Opin Biotechnol 9:602–608

White RJ, Maiese WM, Greenstein M (1986) In: Demain AL, Solomon NA (eds) Industrial microbiology and biotechnology. ASM Press, Washington, DC, pp 24–31

Zhang XHD (2011) Optimal high-throughput screening: practical experimental design and data analysis for genome-scale RNAi research. Cambridge University Press, Cambridge

Zhang YX, Perry K, Vinci VA et al (2002) Genome shuffling leads to rapid phenotype improvement in bacteria. Nature 415:644–646

Zilber-Rosenberg I, Rosenberg E (2008) Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. FEMS Microbiol Rev 32:723–735

Zilber-Rosenberg I, Rosenberg E (2011) Prebiotics and probiotics within the framework of the hologenome concept. J Microbial Biochem, doi:10.4172/1948-5948.S1-001

# 14 Genomes and Post-genome Technology

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## Introduction

Until a few years ago, microbial biotechnology was limited by the use of cultivated microorganisms. However, most of the microorganisms that can be seen through a microscope or be detected using molecular biology tools are labeled as non-cultivable (or not-yet-cultivated). Nowadays, this previously unreachable genomic information can be accessed without the need of having the microorganism in pure culture. Microbiology is at one of those inflection points in history, and it is expected that this newly accessible wealth of microorganisms will bring significant contributions to mankind over the next decades. This will be achieved by the development of new biotechnological products for plant and animal disease, diagnostics, management, and control. Furthermore, there will be significant contributions to improve the quality of crops and food production yields for an ever-growing human population, development of new biocatalysts, new processes, and applications for bioremediation and energy production.

Although not absolutely necessary for biotechnological application, one of the first steps to study a microorganism is its classification. DNA-DNA hybridization, 16S rRNA gene sequencing, multilocus house-keeping genes sequencing, and super-trees are the standards for bacterial classification. The first complete bacterial genome sequenced was that of *Haemophilus influenza*. This genome was published in the early 1990s (Fleischmann et al. 1995) and was undoubtedly a seminal achievement for the microbiology field. Now, with our access to whole genome information, even from "uncultivable" microbe representatives, we have a better understanding of what a microbe is capable of doing, its role in the microbial community, and its role in the environment. Access to individual genomes allowed comparison among genomes from different species, among genomes from different isolates from the same species (pan-genome), and finally metagenomes (the collective community genome) (Medini et al. 2005, 2008; Ansorge 2009; MacLean et al. 2009).

Some of the immediate benefits we have already reaped from genomic studies are the understanding of the general organization of genomes, the constituents and regulators of metabolic pathways, virulence factors, and other genes involved in host-pathogen interactions. However, as with any major scientific advancement, this knowledge paved the road for uncountable other inquiries that the structural sequence of a given genome is not capable of providing clear answers to (e.g., a universal bacteria species concept). It is clear, as billions of bases pour into databases every day, that as we move from genomes, single cell genome, pan-genomes to metagenomes and other "omes," we need to think about how to make the most of this information, not only from a basic biology standpoint but from a biotechnology perspective.

The availability of genome sequences has allowed a better understanding of genome evolution, lateral gene transfer, and genomic plasticity. Furthermore, we should be able to explore this genomic plasticity and ability to adapt to ever-changing environmental conditions to bio-prospect in a systematic fashion the universe of biochemical reactions and compounds produced and develop products – if we are clever enough to design bioassays. In addition, the tools to manipulate the genome are being developed and it is now possible to reduce genome size to a minimum to sustain life in free form. It is expected that this development will allow the custom design of a microorganism to

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perform particular reactions with high efficiency and yield such as hydrogen production (Gibson et al. 2008b; Glass et al. 2009; Lartigue et al. 2009). We are now able to not only change small parts of a genome but also to create an entire new entity with the sole purpose of providing us with a very specific function – the basis of biotechnology (Lartigue et al. 2007; Gibson et al. 2008a, 2010; Benders et al. 2010).

One important achievement obtained over the last two decades is the development of "culture-independent" techniques. Initially fingerprinting techniques such as DGGE, RISA, T-RFLP, DNA-microarrays, among others, targeting gene markers as the 16S rRNA gene (one of the most frequently used genes) allowed the detection of shifts in the microbial communities according to changes in the environment, time, and perturbations such as pollution. Soon after, metagenomics (the study of a pool of environmental genomes) became more popular, allowing the metabolic potential of the microbial community to be probed and increased the understanding of the functional aspects of these communities (Rondon et al. 2000; Lopez-Garcia and Moreira 2008; Chistoserdova 2010; Chistoserdovai 2010; Fernandez-Arrojo et al. 2010; Simon and Daniel 2011).

Growing a microorganism in the laboratory has its advantages. For example, if an organism is already naturally capable of producing a certain compound, it is expected that this organism can cope with its production. For instance, a microorganism producing an antibiotic compound, invariably, will not succumb to its expression, whereas heterologous expression will certainly need several adaptations to avoid killing the host. However, it is well known that several microorganisms that produce compounds of biotechnological interest are not yet cultivated. Now, with the development of single cell genomics, we have the ability to locate a specific cell in a complex microbial background, to isolate it, and sequence its genome (Walker and Parkhill 2008; Siegl et al. 2010; de Jager and Siezen 2011). With its genome information in hand, we can now ask why this microorganism is not able to grow in standard laboratory culture medium and adapt the growth conditions accordingly to culture it.

Regardless of one´s scientific interest, one major concern today is the incredible amount of information pouring from hundreds and hundreds of next generation sequencing (NGS) machines every single day worldwide (Gomez-Alvarez et al. 2009; Kunin et al. 2009; Morgan et al. 2010; Schwartz et al. 2011; Suzuki et al. 2011). This amount of information overwhelms the most prepared bioinformaticians, not to mention the poor microbiologist trying to understand his single favorite bug in the laboratory. Trained personnel, infrastructure for processing, storing and analyzing data have become some of the major bottlenecks in the field of genomics and post-genomics (Several Authors 2008; Horner et al. 2009; Schadt et al. 2010).

This chapter will cover all the aspects mentioned above of how the information acquired by genome sequencing has impacted microbiology and what are the emerging post-genomic technologies. Initially, we will discuss prokaryotic intraspecies genome diversity. Next, we will cover the diversity at the community level and how this can be exploited, then we will discuss the sequencing of the genome from an individual cell, and we end describing the advancements in the field of bioinformatics. Whenever possible, the implications and advancements of these post-genome technologies to biotechnology will be discussed. A schematic form of how these themes are connected can be found in ❯ *Fig. 14.1*.

## Application of Prokaryotic Intraspecies Genomic Diversity

Since the landmark of 1,000 bacterial genomes in 2010, and 100 archaeal genomes in 2011, several strains belonging to the same species were sequenced revealing that the intraspecies genetic diversity of prokaryotes was higher than previously thought (Lagesen et al. 2010; Brochier-Armanet et al. 2011). The genomic analysis of multiple strains revealed that the number of genes belonging to a single bacterial species was larger than the number of genes present in one strain, leading to the concept of a pan-genome for bacteria (Medini et al. 2005; Tettelin et al. 2008; Mira et al. 2010).

The pan-genome is the sum of all genes present in members of one taxonomic group; initially, it was used to describe the repertoire of genes shared by one species but later was applied to other taxonomic groups, such as genus, and even for all bacteria (Lefébure and Stanhope 2007; Tettelin et al. 2008; Lapierre and Gogarten 2009; Ussery et al. 2009; Lukjancenko et al. 2010; Mira et al. 2010). This concept was first used in 2005 in a study on *Streptococcus agalactiae* strains, 10 years after the first bacterial genome was published. Eight genomes of *S. agalactiae* were compared in order to determine the set of genes shared by all strains for further development of a universal vaccine against Group B *Streptococcus* (GBS) (Tettelin et al. 2005). The average number of identified genes was 2,200 genes per genome; 1,800 of these genes were present in all strains studied. In other words, approximately 80 % of the genes were part of the *S. agalactiae* common core genome. However, the estimations on the number of genomes required to fully describe *S. agalactiae* pan-genome would be over hundreds, since it was estimated that for every extra genome added to the analysis, an average of 33 new genes would be revealed (Maione et al. 2005; Tettelin et al. 2005). The analysis of this pan-genome indicated a series of putative antigens that could be used to generate a vaccine against GBS. Among them only four were considered good antigens, and although they were effective against a wide range of strains, none alone could be considered a universal vaccine (Maione et al. 2005; Muzzi et al. 2007).

Similar to the *S. agalactiae* pan-genome study, clinical applications were among the objectives of a study comparing the genomes of 13 pathogenic and nonpathogenic strains of *Haemophilus influenzae*. In this case, the goal was the development of a hybridization chip to identify clinical pathogenic isolates at low cost. The *H. influenzae* pan-genome was estimated to be between 4,425 and 6,052 genes, which is at least three times larger than the number of genes that a single strain may contain. The core genome was estimated to contain only 51 % of the genes (Hogg et al. 2007).

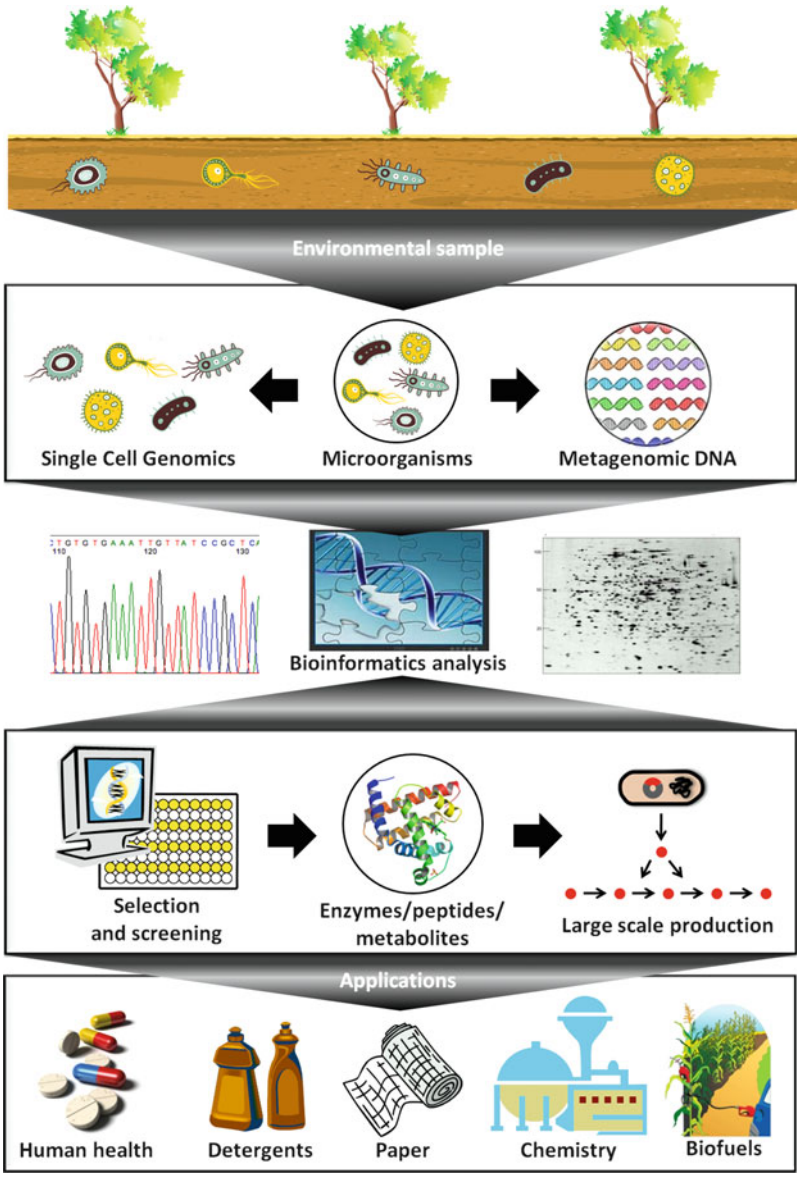


**Fig. 14.1**

**Schematic of the main topics covered in this chapter. The genomes of microorganisms present in the environment can be easily sequenced, regardless if we are able to cultivate them in the laboratory, if they are present as part of a community, or if we can only separate them as single cells. Bioinformatics is crucial for the organization and analyzes of genome sequencing data. The metabolic wealth present in these microbial genomes is being used for the development of products such as enzymes with applications in various industries**

It is only natural to think about the *Escherichia coli* pan-genome; after all, this is the bacterial species with the largest number of strains with genome sequenced. A study on the pan-genome of *E. coli* using 51 strains showed that, considering an average size of 5,000 genes for a genome, approximately 20 % of the genes are shared among all strains. A previous work using 21 strains had suggested that the core genome, defined by the presence of a gene in all strains, contained roughly 50 % of these genes. The *E. coli* pan-genome is currently estimated to contain 13,926 gene families, which correspond to approximately three times the average number of gene families described for *Escherichia coli* 0157:H7 str. EC4196i (Rasko et al. 2008; Lukjancenko et al. 2010).

Species such as *S. agalactiae, H. influenza*, and *E. coli* are considered to have "open pan-genomes" which means that every time a genome is added to the analysis, new genes, or genes not previously found in any strain, will be identified. Some species present a "closed pan-genome" indicating that the number of new genes to be identified by the addition of a genome is zero. That is the case of *Bacillus anthracis*, *Staphylococcus aureus*, and,

*Ureaplasma urealyticum* (Tettelin et al. 2008). *B. anthracis* was the first example of a closed pan-genome since the analysis of eight genomes showed that the number of new genes added to the pan-genome is zero as the fourth genome was added. In fact, *B. anthracis* pan-genome was considered an extreme case of a closed pan-genome and it was previously described that these strains are a clonal population derived from *Bacillus cereus* that carries the anthrax toxin plasmid (Tettelin et al. 2005).

It is important to understand that the models used for each pan-genome analysis were distinct depending on the study, and in some cases, the interpretation whether a pan-genome should be considered open or closed may differ among authors; similarly, the choice of methods for analysis on comparative genomics is far from being standardized (Hogg et al. 2007; Tettelin et al. 2008; Klenk and Göker 2010). Whether or not these terms will be largely used to define the intraspecies diversity in bacterial genomes is yet to be determined.

The core genome usually comprises house-keeping genes involved in cell envelope synthesis, regulation of gene expression, and protein transport; however, several genes are "hypothetical proteins" or "proteins of unknown function" (Tettelin et al. 2005; Mira et al. 2010). Genes that are present in some strains, but not all, are known by different designations: "expandable genome," "distributed genome," or "accessory genome"; and usually contain a large number of transposable elements. Some authors prefer a more relaxed concept for a core genome, for example, a gene should be present in at least 99 % or 95 % of the strains, which maybe more useful for taxonomic purposes (Konstantinidis et al. 2006; Lapierre and Gogarten 2009; Kislyuk et al. 2011).

On the other hand, the study of the accessory genome recognized the extent of the importance of horizontal gene transfer (HGT) to bacterial evolution. It was first believed that the major source of evolutionary variation in bacteria would come from spontaneous mutations, the present number of genomes available demonstrated that HGT is one of the major contributors to the genetic variability found in prokaryotic genomes. The majority of sequences present in the accessory genomes are involved in GHT processes, they are genomic islands, phage-related sequences, and transposon-related sequences (Tettelin et al. 2005; Doolittle and Papke 2006; Ehrlich et al. 2010).

Initially, it was proposed that a core set of genes could be used to define a bacterial species (Lan and Reeves 2001); however, for the reasons explained above, defining a core genome is neither a simple task nor a standardized procedure. Studies have compared genomic analysis of multiple strains with other methods for typing such as "multilocus sequence typing" or to the "gold standard" method to define bacterial species: DNA-DNA hybridization (DDH) or the "average nucleotide identity" (ANI) (Konstantinidis and Tiedje 2005; Konstantinidis et al. 2006; Deloger et al. 2009; Hall et al. 2010). It was previously demonstrated that ANI can be used to determine the level of relatedness between two bacteria and that this index correlates nicely with DDH, in general two strains that present higher than 95 % ANI will present higher than 70 % DDH; therefore, they would belong to the same species (Konstantinidis et al. 2006; Goris et al. 2007). It should be mentioned that the relationship between ANI and 16S rRNA gene phylogeny seems to be strong as well; in general, if two species show higher than 97 % similarity in the 16S rRNA gene nucleotide sequence, they will present higher than 95 % ANI (Konstantinidis and Tiedje 2005). Although it may seem that there are many available sequences of bacterial genomes, they represent a small fraction of the bacterial diversity and to use genome data for defining bacterial species seems premature.

Another important observation of intraspecies variation is that, in general, bacteria with larger genomes tend to show higher intraspecific variations, and it has been proposed that free-living species will present a larger pan-genome (Konstantinidis et al. 2006). The pan-genome of *Burkholderia pseudomallei* and other *Burkholderia* was estimated and, as observed for *E. coli*, the *B. pseudomallei* pan-genome was estimated to be three times larger than a typical genome, based on a strain average of 5,000 genes. However, in this case, the core genome corresponds to 80–85 % of one strain genome. For *B. mallei*, the core genome was estimated to comprise 58 % of the genes present in all strains, although the genome is smaller probably because it is a pathogen whereas some *B. pseudomallei* strains are free-living bacteria (Konstantinidis et al. 2006; Ussery et al. 2009).

The majority of multi-strain genomic analysis focused on pathogenic bacteria, not only for the clinical applications but because these are the ones with the highest number of cultivated representatives. Some studies on intraspecies variation included pathogenic and nonpathogenic bacteria, but there are few including only free-living microorganisms. Archaeal pan-genomes are rare and often these studies overlap with metagenomic analysis. For example, comparative genomic analysis of *Haloquadratum walsbyi*, a haloarchaeon common in saltern crystallizer ponds, showed that this species presents low genetic variability among strains isolated from geographically distant ponds as far as Spain and Australia. In fact, *H. walsbyi* seems to be the only species in this genus and strains vary in their 16S rRNA gene sequences by less than 2 %. In one study comprising sequences from three distant ponds located in Australia, almost all sequences present in the genome of *H. walsbyi* type species were retrieved from the collective genome of the microbes inhabiting a saltern pond. It was estimated that the core genome would contain 84 % of the genes found in the type species. This low level of variation is not a common feature identified in halophilic archaea; other genera inhabiting saltern ponds show a higher number of species, but their pan-genomes are yet to be studied (Bolhuis et al. 2006; Legault et al. 2006; Cuadros-Orellana et al. 2007; Oh et al. 2010; Dyall-Smith et al. 2011).

The pan-genome concept was created to improve the identification of clinically relevant bacteria and to develop new strategies for pathogen control, but the striking discovery of the intraspecies genetic variation has contributed to bacterial ecology and evolution in at least in two ways: (1) it has led to an in-depth debate on a genome-based definition for prokaryotic

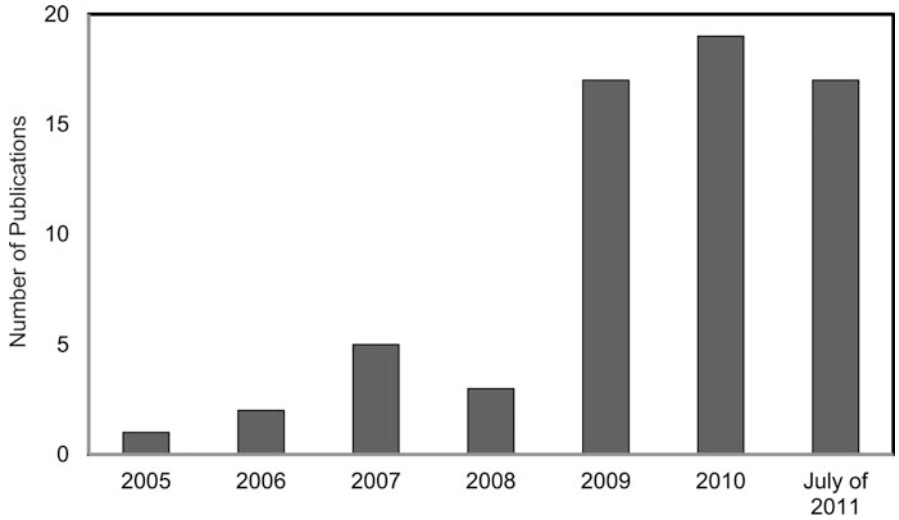


**Fig. 14.2**
**Number of publications in PubMed that presented the word "pan-genome" in the title or abstract from 2005 to the present (July 2011)**

species, and (2) it has shown the extent and importance of horizontal gene transfer to prokaryotic evolution. Other applications of this knowledge may come in the future. In fact, the number of publications that include the word pan-genome has increased every year (*Fig. 14.2*), demonstrating that microbiologists find this concept useful enough to continue applying it.

## Culture-Independent Exploitation of Microbial Genomes: Metagenomics

A major challenge in post-genome biology is to uncover gene function and assemble together gene sets into useful metabolic pathways for biotechnological purposes. Bioinformatics has been instrumental for the organization of the avalanche of genome sequence information so that gene families and specific domains are identified. However, there are still single genes, gene families, and entire gene clusters for which there are no leads regarding their biological function, despite bioinformatics analyses. Therefore, there is no substitute for lab bench work in this post-genome era. There are many studies published in which the function of a specific gene has been uncovered by mutational analysis (e.g., targeted mutagenesis, transposon mutagenesis) and comparison with the wild type strain under a number of growth conditions.

A different approach focuses on the biotechnological application rather than on any specific gene and is common in the metagenomics field. As previously mentioned, the term "metagenome" here is used in its original context referring to the collection of the bacterial genomes from a microbial community (Rondon et al. 2000). In the metagenomics approach, total DNA of the microbial community of choice is directly extracted from the environment and can be used to construct DNA libraries that are then screened by various means to reveal clones with a biological activity of interest.

The first step to construct a metagenomic DNA library is to obtain total microbial community DNA. Direct DNA extraction allows both the cultivated and uncultivated components of a microbial community to be represented in DNA libraries, and these can be exploited for biotechnological purposes. While some studies describe the selection of their samples for DNA extraction and library construction based on the overall microbial diversity, others have attempted to exploit the natural enrichment of a specific microbial community – sometimes over long evolutionary time – to a particular environment. For example, one can look for enzymes that can withstand high temperatures in hot spring microbial communities (Rhee et al. 2005), for esterases that work in cold conditions in the arctic soil (Yu et al. 2011), for cellulases and other enzymes involved in deconstructing plant cell walls in the microbial communities of the gut, rumen, and feces of various vertebrate and invertebrate animals (e.g., cow (Zhao et al. 2010), wallaby (Pope et al. 2010), termites (Liu et al. 2011), earthworm (Beloqui et al. 2010)) and for bioremediation enzymes in the microbial communities of herbicide contaminated soil (Lu et al. 2011). However, knowledge about bacterial physiology is warranted when choosing a specific microbial community for a particular project. For instance, many acidophiles have a circumneutral internal pH due to a variety of homeostasis mechanisms (reviewed in (Baker-Austin and Dopson 2007)), and therefore a microbial community from a low pH environment would not necessarily be an appropriate source of enzymes tolerant to low pH.

Although there are many interesting environments to be explored, there are technical difficulties to obtain microbial DNA for library construction. Each environmental sample will bring different challenges. Some microorganisms may be hard to lyse. When extracting DNA to construct an expression library

from the cow's rumen, one needs to ensure that DNA from the plant fraction is not overrepresented in the library. When the sample of choice is water, the scarcity of microbial cells in the sample may require concentration by filtration prior to processing. When dealing with low biomass samples, it may also be necessary to amplify the small amount of extracted DNA before library construction by multiple displacement amplification (MDA). Sampling and obtaining replicates may also require special attention in projects dealing with aquatic microbial communities as the species composition of these environments may vary significantly according to the depth, season, currents, and temperature in which the water samples were collected. For other environments, contaminants that may degrade DNA and that are incompatible with downstream molecular biology applications are the main challenge. Humic acids that are co-extracted with DNA inhibit enzymatic reaction and are a common problem when dealing with soil samples. Clay particles can absorb organic matter including DNA and interfere with DNA extraction (Purdy 2005). High iron content in soils can lead to DNA breakage by Fenton reaction in which hydroxyl radicals are produced from the reaction of iron with hydrogen peroxides (Harrison and Arosio 1996). Therefore, there is no universal protocol to obtain microbial DNA from the environment; new protocols need to be developed or old ones from similar environments optimized for each new environment explored (de Castro et al. 2011).

Once metagenomic DNA is obtained in high enough quantity and quality (e.g., not degraded and free of inhibiting substances), three main approaches can be taken to study gene function. Firstly, traditional technologies or NGS can be used to sequence metagenomic DNA that can be assembled into near-complete or partial individual genomes when the complexity of the community is low. This has been accomplished for different microbial communities including that of an acidophilic biofilm, the nutrient-limited open ocean in the Sargasso sea, and anaerobic ammonium oxidation bioreactor (Tyson et al. 2004; Venter et al. 2004; Strous et al. 2006). Once assembled, the genome is analyzed in various ways and annotated. The genes identified are categorized according to their putative functions such as signal transduction, transcription, energy metabolism, and unknown function. New genes such as the 782 rhodopsin-like photoreceptors found in the Sargasso Sea work can be identified (Venter et al. 2004). Information about which biochemical pathways are present is obtained and sometimes new ones can be deduced. An example of the latter is a new metabolic pathway for anaerobic ammonium oxidation, which is a sink to fixed nitrogen in the oceans (Strous et al. 2006). These metabolic genomic analyses can also be useful to understand how microorganisms adapt to specific environments and to understand the role a particular microorganism plays in a microbial community. Microbial diversity as well as the species richness present in the original sample can also be estimated.

More commonly, sequencing of metagenomic DNA will allow the analysis of gene function without the individual genomes present being assembled. An interesting example of this approach was the study of the microbiota of fungus gardens kept by leaf-cutter ants from Panama (Suen et al. 2010). These ants harvest fresh leaves to cultivate a fungus – which can be considered a form of farming – that is its primary food source. Pyrosequencing of the community metagenome followed by a carbohydrate-active enzyme characterization was performed and 28 families of glycosyl hydrolases, carbohydrate esterases, and polysaccharide lyases were identified. The presence of these enzymes able to degrade cellulose and hemicellulose was most similar to the enzyme profile of the bovine rumen, which can be used as evidence that the fungus garden can be considered an external digestive system (Suen et al. 2010). Similar work has been performed to study the cow rumen and the human feces microbiota (Brulc et al. 2009; Arumugam et al. 2011).

Another approach to perform a functional study of a microbial community is to sequence the metatranscriptome (i.e., sequencing the environmental cDNA derived from mRNA). Although the mRNA complement of a microbial community reflects more accurately its metabolic functions, there are fewer studies using this approach than the metagenomic approach due to the technical difficulties associated with mRNA extraction from the environment. Interestingly, comparison between the total microbial community (metagenome) and the most active community (metatranscriptome) may reveal differences such as those found in the coniferous forest soil where low abundance fungal species seem to be highly active in plant decomposition (Baldrian et al. 2011). Furthermore, metatranscriptome data has been used to design a growth medium for the cultivation of a *Rikenella*-like bacterium that inhabits the medicinal leech gut, a previously uncultivated and the most abundant endosymbiont of this microbiome (Bomar et al. 2011).

Although functional analysis of genes identified in the microbial community of interest through sequencing and comparison with databases can be quite useful, as previously discussed, these sequence-based approaches will not identify genes that are not similar to previously known genes. A third sequence-independent approach to study gene function within a metagenome starts with the construction of metagenomic DNA libraries. These can be used to screen for the activity of interest. Indeed, this approach has allowed the identification of enzymes with low similarity to known enzymes and even a new family of enzymes (Beloqui et al. 2006; Kim et al. 2009; Pang et al. 2009; Park et al. 2011).

For metagenomic DNA library construction, different vectors including plasmids, phages, fosmids, cosmids have been successfully used. Most works have used *E. coli* as an expression host, although there are reports of libraries constructed in α-proteobacteria (e.g., *Agrobacterium tumefaciens*, *Caulobacter vibrioides*, *Rhizobium leguminosarum*), β-proteobacteria (e.g., *Burkholderia graminis*, *Ralstonia metallidurans*), and γ-proteobacteria (e.g., *Pseudomonas fluorescens*, *P. putida*, *Xanthomonas campestris*) (reviewed in Taupp et al. 2011). The choice of insert size for construction of expression metagenomic libraries will affect how intense the library will need to be screened, as the same number of clones from a large insert library will represent more metagenomic DNA if a large insert

library is being screened than a small insert library. However, once a clone showing the activity of interest is identified, it is harder to identify the gene responsible for the activity in large insert clones.

A metagenomic DNA library can be submitted to essentially three different types of functional screens to identify clones with the biological activity of interest: (1) direct activity screens, (2) promoter activity screens, and (3) selection of clones by complementation. Direct activity screens are still the most common. They are well suited for the identification of activities with biotechnological application such as antibiotic, amylase, amidase, nitrilase, oxidoreductase, esterase/lipase, cellulase, xylanase, pectinase, β-glucosidase, protease, agarase, chitinase, and phytase (reviewed in Simon et al. 2009). Some of these screens rely on the hydrolysis of the substrate molecule present in the solid growth medium (e.g., starch for amylase, milk protein for protease) and thus formation of a clear halo around positive clones. Other screens are based on the hydrolysis of a synthetic substrate added to the growth medium that mimics the natural substrate releasing a molecule that can be detected by a change in fluorescence when excited by the appropriate wavelength (e.g., 4-methylumbelliferyl-β-D-glucoside, methylumbelliferyl-β-D-cellobioside). Some screens such as those for cellulase activity where halos around positive colonies are sought for in soluble cellulose (CMC) containing-plates followed by Congo red staining are popular because they are inexpensive. Although they are labor intensive as they are destructive, requiring that the clones are stored prior to the assay or that a replica of each plate is made, the local accumulation of an enzyme at a high concentration may facilitate detection of certain enzyme activities. Liquid-based screens are also used and have the advantage of being quantitative.

Screens using solid as well as liquid growth medium can be automated for high-throughput screens of metagenomic libraries. Colony picker robots, liquid handlers, and integrated spectrophotometers are commercially available. These can be used on 96- or 384-well format microplates. Robotization of screening procedures allows high-throughput experiments. There are a few examples of the success of this strategy from the company Verenium (U.S.A.). One such example is the alpha-amylase Fuelzyme®, used commercially for the production of ethanol by corn wet milling process. Fuelzyme® is the chimeric product obtained by direct evolution of three archaeal enzymes, each with different properties, obtained by screening metagenomic DNA from the depths of the ocean floor (Richardson et al. 2002). Another example of a metagenomic DNA library derived-product is a phospholipase C commercialized under the name Purifine® PLC. This enzyme is used in the edible oil industry for the removal of the phosphorus from phospholipids. High phosphorus oils are present in different species such as soybean and canola. The removal of phosphorus is part of the oil refining process (www.verenium.com). Yet another example of a product from metagenomic origin is a xylanase used in the paper industry commercialized under the name Luminase® PB-100. This xylanase hydrolyses hemicellulose making the pulp bleaching process (i.e., process used to make paper whiter) easier and thus requiring less harsh chemicals to be used. In this case, the original xylanase obtained from soil environmental samples was mutagenized to improve its thermostability (Dumon et al. 2008). One mutant with changes at multiple sites had a Tm 25 °C higher than the parent enzyme, while retaining similar catalytic properties (Dumon et al. 2008).

As with any method, function-based screening of metagenomic DNA libraries has limitations. The first hurdle that needs to be overcome is to get the proteins expressed. As previously mentioned, different hosts with different codon bias and cellular machinery for protein expression may be useful. There are patents for broad-host range vectors that will function in different hosts. Furthermore, it is important that the host strain is devoid of proteases that may hydrolyze the protein one is interested in expressing. Another problem is the low level of expression of clones which may lead to false negatives. This problem can be partly addressed by optimizing assay protocols such as by extending incubation times to increase sensitivity. The opposite problem where a clone will initially give a positive result that cannot latter be reproduced (i.e., false positives) is also common. Furthermore, when dealing with the functional diversity and metabolic complexity of metagenomes in a screen, one may discover that the method used to target a particular activity is not as specific as previously thought. For example, in our lab (Quirino et al., unpublished data), we have found that halos around clones plated in starch-containing medium and staining with $I_2$ vapor may lead not only to clones with amylase activity but may also reveal clones with amydase activity, an entirely different enzyme class.

Promoter activity–based screens have been designed to identify novel catabolic genes based on the induction of operons by a substrate of choice. This technique is called substrate-induced gene expression screening or SIGEX (Uchiyama et al. 2005) and is based on the fact that in many cases, catabolic gene expression is generally trigged by a substrate and that catabolic genes are often part of operons. Therefore, a system was developed where operons from metagenomic origin are inserted upstream of the reporter gene GFP. Clones in liquid culture from such libraries are screened in the presence of the desired inducer molecule using fluorescence-activated cell sorting (FACS). This procedure allows the selection of positive clones in high throughput.

Although promising, there have been few examples of the biotechnological exploitation of metagenomes for the discovery of new regulatory elements per se rather than enzymes. For example, constitutive promoters can be identified by cloning metagenomic DNA fragments upstream of promoter-less reporter genes such as GFP or DsRed and screening for the reporter gene activity (Han et al. 2008). One of the possible problems in metagenomic screens may be the insufficient recognition of promoters, and one application of this technology is to identify new promoters from metagenomic DNA to construct new expression vectors.

A variation of function-based screens is targeted screens for a particular activity by complementation of a cell mutant. In this approach, borrowed from traditional microbial genetics, the metagenomic library clones are used to complement the phenotype of a mutant of interest. When the phenotype is no growth

under specific conditions and this condition can be restored by metagenomic clones, a selection rather than a screen can be performed. Phenotypic selection is a powerful method for finding an activity of interest as it allows much greater numbers of clones to be analyzed than traditional screens. This approach has been now used to find a number of gene activities from metagenomic libraries (Majernik et al. 2001; Riesenfeld et al. 2004), many of which with biotechnological applications. Such approach was used to obtain a xylose isomerase from a garden compost metagenomic library (Parachin and Gorwa-Grauslund 2011). The plasmid library was used to complement an *E. coli* strain that had a nonfunctional *xylA* gene to restore growth in xylose-containing medium. Xylose is present in hemicellulose, and for complete fermentation of plant biomass to make cellulosic ethanol by yeast, it is necessary that the yeast strain be able to ferment xylose.

DNA polymerases have a number of applications in molecular biology such as DNA sequencing, probe labeling, and mutagenic PCR. Nine new genes encoding DNA polymerase I proteins or domains were identified by complementation of the *E. coli polA* mutant with metagenomic library clones derived from glacial ice (Simon et al. 2009).

Novel polyhydroxyalkanoate (PHA) synthase genes that may be used industrially to produce biodegradable plastics were identified from a soil metagenomic library by complementation of *Sinorhizobium meliloti* PHA synthesis mutants (Schallmey et al. 2011). The metagenomic library was transferred to *S. meliloti* by triparental conjugation and the screens for PHA synthase were based on the restoration of mucoid phenotype or Nile red staining on YM agar.

Although function-based screens are the method of choice when one is interested in finding new classes of proteins, one can also use the library to screen for specific activities in sequence-based screens. Depending on how the sequence-based screen is designed, entirely novel proteins can also be identified using this approach. For instance, a new blue-light sensitive protein was uncovered in a microarray-based screen of 2,500 clones of a soil metagenome cosmid library in which the microarray contained oligonucleotides derived from LOV (light, oxygen, and voltage) domain consensus sequences of blue-light photoreceptor genes (Pathak et al. 2009).

Once positive clones are obtained in functional screens, the gene responsible for the phenotype needs to be identified. This can be accomplished by sequencing the insert by traditional (Sanger) or high-throughput sequencing methods and subcloning of open reading frames for expression followed by testing for the activity of interest. This can be quite laborious if one is dealing with large fragments. However, transposon mutagenesis can be used to narrow in to the gene (Wang et al. 2006).

Screens are constantly evolving and allowing new proteins to be identified and studied. As more high-throughput and miniaturized screens are developed, more products of metagenome origin will certainly emerge. However, for more businesses based on the genetic wealth of metagenomes to become lucrative, not only technical problems need to be solved, but it will necessary to be in tune with the market.

## Single Cell Genomics

The metagenomic approach has revolutionized microbiology as it has widened its boundaries revealing a whole new world of microorganisms. However, access to the rare components of the community is still a challenge. It is not known what the importance of these low abundance species in the community is but it is believed that if environmental conditions change, these species may become dominant in the community (Sogin et al. 2006). Therefore, these species can be thought of as genetic reservoirs of biological functions. While next generation sequencing of 16S rRNA gene will reveal the presence of these rare species, single cell genomics – the ability to obtain a full (or partial) genome derived from a single cell – provides a strategy to access the gene content of a cell in a targeted fashion.

From a practical standpoint, the first step in obtaining whole genome information from a single cell is isolating it. There are various methods published in the literature that describe isolation of single microbial cells. Traditionally, single cells have been isolated by dilution to extinction (Button et al. 1993). Although this method has a great advantage for certain cultivation procedures (Connon and Giovannoni 2002), it is seldom applied to amplify the genome of a single cell since it routinely operates with too large of a volume which is incompatible with downstream amplification procedures. More suitable methods to obtain a single cell for subsequent genome amplification include optical tweezers and microfluidics, laser-capture microscopy, as well as fluorescence-activated cell sorting (FACS) (Porter et al. 1993; Frohlich and Konig 1999; Ferrari et al. 2004; Ishoy et al. 2006; Marcy et al. 2007; Taylor et al. 2009). Methods that employ a microscope, such as microfluidics and laser-capture microscopy, have the advantage that the cell can be visualized before amplification where obvious contamination will easily be spotted. Furthermore, effects of environmental perturbations can be observed in real time. Based on its high-throughput nature, FACS has an advantage when underrepresented microbes within a community are being targeted. A combination of FACS with fluorescence in situ hybridization (FISH) allows for the targeting of organisms that represent only a small fraction of the total community (Podar et al. 2007).

Following successful single cell isolation, nucleic acids can be amplified. Amplification can target a set of genes or the whole genome. A set of genes can be amplified (e.g., by digital PCR) to screen for rare variables within a population, to link a particular organism to a particular function, or for example to study viral infection patterns within a population (Ottesen et al. 2006; Zeng et al. 2010; Tadmor et al. 2011). Whole genomes from single cells can be amplified using multiple displacement amplification (MDA) (Dean et al. 2001). Due to the nature of MDA, the coverage for microorganisms is typically less than 100 % (Woyke et al. 2010) and can vary substantially depending on the organism (Raghunathan et al. 2005; Zhang et al. 2006; Marcy et al. 2007; Mussmann et al. 2007; Podar et al. 2007; Stepanauskas and Sieracki 2007; Rodrigue et al. 2009; Woyke et al. 2009; Blainey et al. 2011). Similar to accomplishments for *Archaea* and *Bacteria*, whole genome amplification has

successfully been applied to gain insights into the genomes and lifestyles of eukaryotic organisms, such as marine protists (Heywood et al. 2011; Yoon et al. 2011).

Subsequent sequencing of amplified genomic DNA can be performed with various sequencing and next generation sequencing platforms. Depending on sequence quality and length, the genome assembly relies on computational tools to stitch the genome together (Nagarajan et al. 2010; Chitsaz et al. 2011). This genetic information is providing insight into the diversity that exists on the genomic level within a natural population. We learned from pure culture studies that cell individuality can be much larger than originally expected. Studying the evolution of bacterial genomes in the laboratory confirmed that microbial genomes exhibit an enormous plasticity (Portnoy et al. 2011). Single cell genomics now provides a suitable tool to evaluate this phenomenon in the natural environment. However, cell individuality is often controlled on the transcriptional and translational level (Zengler 2009). Deciphering cell individuality on the transcriptional level for single cells thus far requires conversion of mRNA to cDNA and subsequent amplification. Although progress has been made studying the transcriptome of a single cell for eukaryotes (Tang et al. 2009; Taniguchi et al. 2009), mRNA-seq for single bacterial cells is still in its infancy and has proven more difficult because of the reduced amount of mRNA that can be obtained from bacterial cells (Taniguchi et al. 2010). However, recent developments have made it possible to amplify mRNA from a single bacterial cell, thus enabling single cell transcriptomic studies for bacteria (Kang et al. 2011).

Where metagenomic and other top-down approaches cast a wide net to obtain as much information from the environment as possible, single cell genome sequencing can be considered a bottom-up approach in which detailed information for single organisms is stitched together to generate the bigger picture (Zengler 2009).

## Using Genome and Post-genome Information to Make a Product

Genomes and post-genomic tools are at the heart of any genetic or metabolic engineering effort. The genes involved in the synthesis of molecules of biotechnological interest can be identified in the genomes of cultivated or uncultivated microorganisms or from metagenomes. After the desired trait is linked to a gene or a pathway, the next step is to find a strategy to obtain the product in commercially compatible quantities.

Several examples from the natural products field show that a desired chemical entity found in nature often cannot be retrieved in large enough quantities to make it a viable commercial product (Newman and Cragg 2005). Natural products are often constituted of complex chemical structures which are difficult to synthesize organically in a cost-effective manner (Wender and Miller 2009). Identifying the organism that produces it, its chemical structure, and the genes encoding for its synthesis can be a long-term project. This is true especially in symbiotic or other host-associated microorganisms, where transcription and translation of a biomolecule can be tightly correlated with the host physiology, making it even harder to decipher by traditional methods. For example, the isolation and structure determination of bryostatin (i.e., a compound which demonstrated potential in treating certain types of cancer and Alzheimer's disease), the link of its biosynthesis to a bacterial endosymbiont, and the identification of genes involved in the synthesis took almost two decades (Sharp et al. 2007).

After a biologically active compound is identified, it is necessary to decide between two major options in order to produce it. The first includes a robust microorganism (mainly *Escherichia, Bacillus, Pseudomonas*, or *Saccharomyces*) that is genetically engineered to produce a desired product. The second option takes advantage of an organism that possesses a desired biosynthetic pathway which can subsequently be isolated and propagated in the laboratory. Although less common for most industrial applications, the isolation of new organisms can become necessary, especially when synthesis of the biomolecule is encoded in complex pathways and spread throughout the organisms' genome, as is often the case in natural products. Having established production strains with a long history in industrial settings, however, is greatly advantageous over newly isolated microorganisms when it comes to producing biomolecules for applied purposes, since critical factors, such as scale-up processes and phage resistance, have already been evaluated.

As stated before, a metagenomic approach can be used to obtain sequence information or, in case of expression libraries, to screen for a desired trait. Using recombinantly generated expression libraries has been shown to be successful for individual enzymes (Robertson et al. 2004), but it is more challenging for whole biosynthetic pathways because various enzymes involved in a pathway can be located on different parts of a genome and therefore will not be represented in a single large insert clone. In principle, it is possible to express any particular enzyme or whole pathways recombinantly in a production host. Regarding the latter, the field of metabolic engineering has made great advances in understanding metabolic processes and using these for obtaining this product. This can be achieved by modifying a microorganism by recombinant DNA methods in various ways such as by removing competing networks, adding new ones to feed or to speed up the major one, and adding new enzymes to create new products (*Fig. 14.3*). In this approach, it is important to monitor the yields of the desired product and the other components of the metabolic network and make the necessary fine adjustments to improve it even further. Usually, it is not possible to predict the overall behavior of the complete network. Therefore, it is important to obtain information about the final product yield, as well as about other systems' components to identify bottlenecks. Modifications in the engineered metabolic pathway can then be proposed and tested, starting another cycle of improvements. As expected, this procedure can be very time and resource consuming. It may be necessary, for example, to reverse enzymatic reactions,

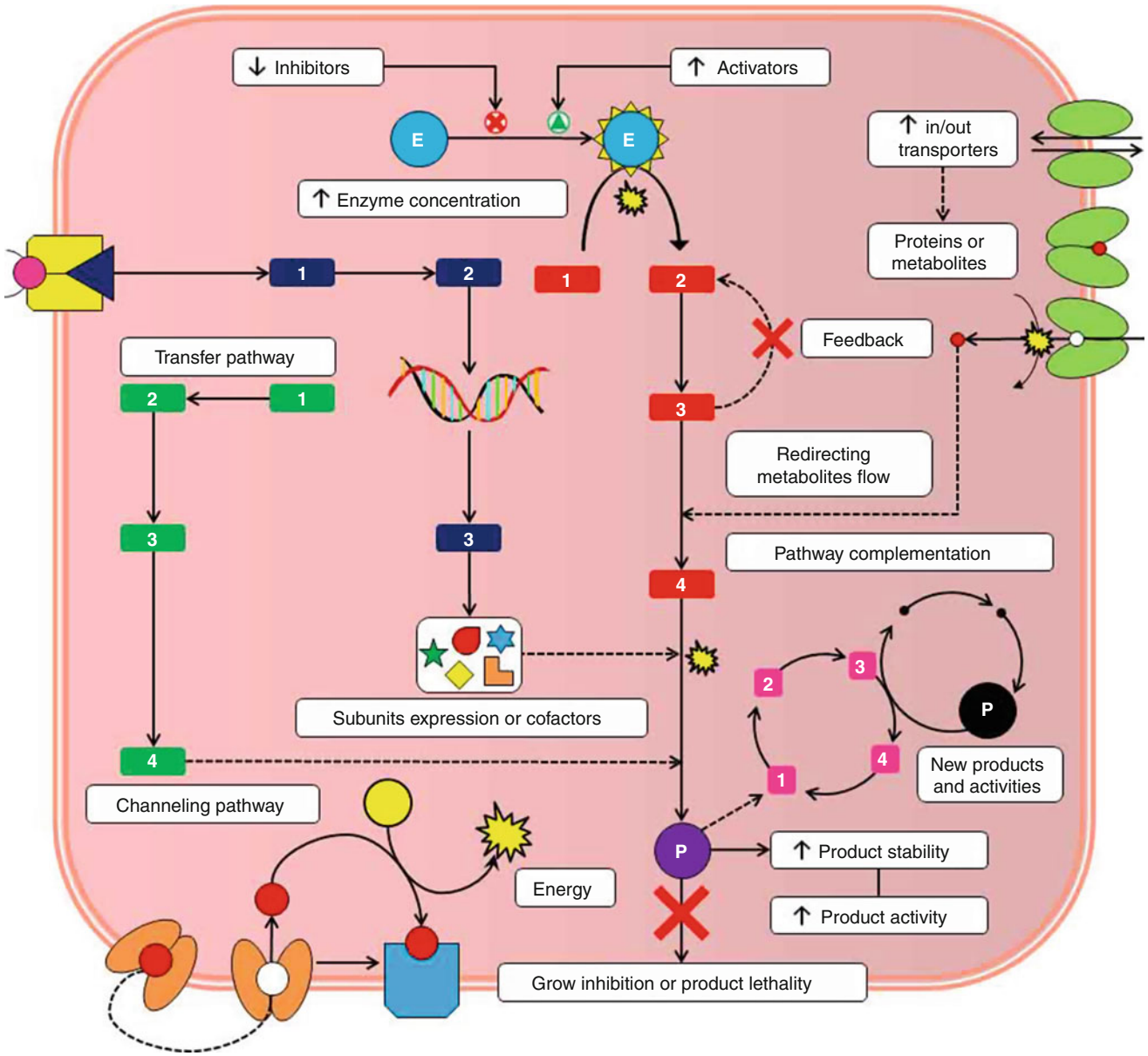


**Fig. 14.3**
**Schematic of some network modifications accomplished by metabolic engineering. *Red rectangles* and the *purple circle* ("P") represent the synthetic pathway and the product of interest. The *blue circle* ("E") represents the first enzyme of this pathway. The *magenta rectangles* represent regulators/activators necessary for the production of new enzyme subunits or cofactors responsible for changing or enhancing the product P activity. The *green rectangles* represent an entirely new pathway transplanted to the host to cross-feed directly or indirectly into the main synthetic pathway. *Pink squares* represent a new added pathway to modify the final product further as a consequence producing a new one (*black circle* "P"). *Red* and *yellow circles* represent pathways added or enhanced to increase the energy (*yellow stars*) availability**

synchronize transcription and translation levels, change a particular enzyme for another with more suitable kinetic properties, or avoid the undesirable formation of inclusion bodies. An example of a successful metabolically engineered product is the production of amorpha-4,11-diene, the precursor of artemisinin which is used in the treatment of malaria by metabolic engineering (Tsuruta et al. 2009).

## DNA Sequencing and Bioinformatics

### Working with Large Amounts of Data

The advent of next generation sequencing technologies (NGS) resulted in an unprecedented gain of resolution in genomic and metagenomic experiments as a result of orders of magnitude

**Table 14.1**

**Storage requirements at two stages of data analysis, for the different sequencing technologies**

| Data units: Gigabyte (GB)[a] | Roche/454 | | | Illumina/Solexa | | | ABI/SOLiD | | |
|---|---|---|---|---|---|---|---|---|---|
| | GS20 | FLX | Ti | I | II | IIx | 1 | 2 | 3 |
| *Stage 1* images | 10 | 10 | 30 | 500 | 1,100 | 2,800 | 1,800 | 2,500 | 1,900 |
| *Stage 2* sequence reads | 0.5 | 1 | 4 | 30 | 50 | [b] | 100 | 140 | 600 |

[a]The first row of numbers at each stage refers to fragment data, while the second row is for paired-end library data

[b]Data are not available (Source: Genome Center at Washington University, Dooling 2010, http://www.politigenomics.com/next-generation-sequencing-informatics)

sampling capacity. One generally overlooked aspect is that the amount of data generated by NGS surpasses processing capabilities of conventional desktop computers and that a sizeable computational infrastructure is necessary to manipulate gigabytes of raw data. Three data generation and transformation stages are present during genome sequencing: The first involves collection of the light intensity values captured by the image sensor of the instrument and emitted by the fluorescent nucleotides added during each sequencing cycle; the second stage data results from quantification and quality check of the intensities and their conversion to corresponding nucleotides and sequence reads using specialized algorithms; and the third where scientific value is produced from the sequence data, by applying assembly algorithms to the sequence reads in order to reconstruct the complete genome. Having acquired the complete genome sequence, it is then processed further using bioinformatic pipelines for gene calling, functional annotation, and discovery of other interesting biological features on the genome.

As sequencing data go from first to third stage, their scientific value is increased, while their size is reduced. For example, the light intensity values are saved by the sequence instrument in large image files which have no value for scientific analysis after they are quantified and converted to sequence reads, other than used as quality reference for the reads. On the other hand, sequence reads are small, plain text files which are used to reconstruct the complete genome sequence with assembly algorithms. Assembled genomes have the most value, as they can be used in discovery of new genes, comparative genomics, and evolutionary studies. Examples of data size generated from specific sequencing instruments are available on the market presented in *Table 14.1*, and it is apparent that data transformations from stage one to stage three result in data reduction from 22-fold (Illumina II) to 3-fold (SOLiD 3).

Since the introduction of the ABI SOLiD instrument in 2007, sequencing technologies continue to move in a direction where throughput is increasing while the cost per sequenced DNA base is decreasing, dropping in half about every 5 months. Furthermore, the introduction of benchtop, low-cost genome sequencers such as MiSeq by Illumina (http://www.illumina.com/systems/miseq.ilmn) and GS Junior by 454 (http://www.gsjunior.com) has made complete sequencing of bacterial genomes affordable to small laboratories. Nonetheless, acquiring the sequence is only the first step, and must be followed by large-scale computational analysis to process the data, test hypotheses, and draw scientific insights. Therefore, investment in a sequencing instrument must be accompanied by substantial investment in computer hardware, skilled informatics support, and bioinformaticians competent in configuring and using specific software to analyze the data.

For laboratories acquiring a sequencing instrument, web-based bioinformatic tools are not an option for data analysis since they do not offer enough computational and storage capacity. For example, bioinformatics tools available through the website of the National Center for Biotechnology Information (NCBI, http://ncbi.nlm.nih.gov) allow input of only few MegaBytes in size, while most sequence datasets are in the order of gigabytes or even terabytes (*Table 14.1*). Few sequencing runs can overwhelm disk systems available to most laboratories, and costs for data disks must be considered in addition to the expense for acquiring the sequencing instrument. Hard disk prices per GB have been following a downward trend similar to the price per sequenced DNA base, but this has been happening at a reduced rate, halving roughly every 14 months. Therefore, the required investment for data storage systems and overall costs for building an informatics infrastructure can surpass the cost of the sequencer by many orders of magnitude.

Given the significant costs that can result from data storage and computation systems, data management plans should be drawn on the early stages of designing sequencing experiments and submitting proposals involving generation of large amounts of genomic data. An alternative option to investing in informatics infrastructure is obtaining storage capacity from a cloud computing service. Cloud services provide researchers with the ability to store data and perform bioinformatic analysis on a practically unlimited pool of virtual machine (VM) servers, without owning or maintaining any computer hardware. The charge model used by cloud service providers is similar to utilities such as electricity, and customers are billed based on the amount of computational resources consumed. This can work better for smaller research laboratories acquiring a low-cost, benchtop sequencing instrument, instead of investing in computer hardware and data center infrastructure for which the cost cannot be justified for only a few experiments.

An example of a bioinformatics platform using cloud computing technologies is J. Craig Venter Institute's (JCVI) Cloud Biolinux (http://www.cloudbiolinux.org), which consists of a VM designed to run on cloud computing platforms. The system offers a suite of bioinformatics software including more than 100 preinstalled and configured sequence analysis tools, coupled with an intuitive graphical interface. The software included with JCVI Cloud Biolinux runs on a practically unlimited pool of high capacity VM servers that can be rented on demand from cloud computing vendors such as Amazon EC2. This provides researchers with a large-scale, virtualized informatics infrastructure, without the financial or time burden of owning and maintaining hardware. Research on cloud and virtualized computing solutions for bioinformatics can democratize access to computational resources for smaller laboratories, which use next generation sequencing or other high-throughput genomics technologies for biological experiments.

### Analytical Pipelines

Once the raw sequencing data is generated, it should be stressed that a series of steps are necessary before any biological insights could be drawn, since all sequencing technologies have inherent technical biases. As such, the exploration of metagenomic datasets, both at quantitative and qualitative levels, requires elaborate analytical protocols to face this complex setting.

General guidelines for both functional and community metagenomics processing have been comprehensively described previously (Wooley et al. 2010). A detailed protocol for functional (shotgun) metagenomics data processing was described recently (Tanenbaum et al. 2010) and is illustrative of how several programs should be chained together to accomplish a reliable functional annotation. This process involves the prediction of coding and noncoding genes followed by application of several similarity search algorithms to assign biologically relevant attributes like gene ontology, Enzyme Commission (EC) numbers, and protein domains.

### Metagenomics Pipelines

As seen previously, the analytical process of metagenomic datasets involves the coordinated execution of several specialized programs, each receiving and emitting data files in different formats, in a concerted flow of events. There is an intrinsic modularity and, for each task, a marked heterogeneity in terms of software options, parameter settings, and execution times. As more data and analytical tools are continuously generated, it is important to stress the complexity of the whole process, which is increasingly turning out to be more demanding than data generation itself.

Automated web-based workflows provide a user-friendly alternative to execute initial metagenomic analyses, effectively abstracting the end user from software installation and maintenance. Galaxy (Kosakovsky Pond et al. 2009) is a generic web workflow management system that offers highly customizable pipelines for metagenomic data processing. The CAMERA portal (Sun et al. 2011) not only provides metagenomic dataset querying and download capabilities, but it also makes available a series of pre-built workflows acting on common metagenomic data analyses, such as raw data preprocessing, BLAST-based similarity searches, sequence clustering and assembly, functional annotation, and community diversity measures. More importantly, it offers a collaborative environment for data sharing and integration.

MG-RAST is a comprehensive analysis server providing annotation and comparative analysis of metagenomic data, as well as quality control and multivariate sample comparison tools (Meyer et al. 2008). The latest version (3.0) is cloud computing ready, capable of handling short reads (at least 75 bp), and supports metadata to describe samples. WebMGA (Wu et al. 2011) is a web server providing a wide range of tools for metagenomic analysis, such as gene prediction, taxonomic analysis, and functional annotation.

Nevertheless, web-based workflows face some limitations, such as the general lack of flexibility to adapt the workflows and data security. Also some constraints can be set by the remote servers to reduce storage and processing loads. Stand-alone software packages like SmashCommunity (Arumugam et al. 2010) provide an all-encompassing list of features to perform functional annotation of metagenomes. Another option is to completely avoid pre-built pipelines and manually invoke a series of task-specific programs to perform the analysis. There are advantages in this scenario, such as greater control of program-specific parameters, maintenance of intermediate files, and freedom to create streamlined processing protocols. This comes at the expense of requiring bioinformatics experts to cope with installation, maintenance, and programming in the Linux operating system, the "de facto" bioinformatics computational environment. In this respect, CloVR (Angiuoli et al. 2011) was recently released, providing a Linux virtual machine with preinstalled software to perform automated pipelines for single-genome, shotgun, or 16S rRNA gene metagenomic data.

## Concluding Remarks

A number of post-genomic tools have been developed to study genomes, regardless if they come from a cultivated or uncultivated microorganism, if they come from a microbial community or single cell. The challenge now is to make this information useful through the development of products. Whenever possible, scientists should be even more proactive in seeking new application opportunities for their research keeping a strong link with the market. If there is no understanding of the market needs from the early stages of the process, the result may be an interesting academic work with no market value. With the scarcity of public funding and an ever greater interest of taxpayers in how their money is spent, scientists should push creative solutions to convert the knowledge about genomes into everyday technologies that can benefit the average citizen.

This is a great opportunity to show the value of science and how basic and applied science are interconnected, as the knowledge about genomes is transformed into post-genome technologies and commercial products.

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## References

Angiuoli SV, Matalka M, Gussman A, Galens K, Vangala M, Riley DR, Arze C, White JR, White O, Fricke WF (2011) CloVR: a virtual machine for automated and portable sequence analysis from the desktop using cloud computing. BMC Bioinformatics 12:356

Ansorge WJ (2009) Next-generation DNA sequencing techniques. N Biotechnol 25:195–203

Arumugam M, Harrington ED, Foerstner KU, Raes J, Bork P (2010) SmashCommunity: a metagenomic annotation and analysis tool. Bioinformatics 26:2977–2978

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D, Ugarte E, Zoetendal EG, Wang J, Guarner F, Pedersen O, de Vos WM, Brunak S, Dore J, Antolin M, Artiguenave F, Blottiere HM, Almeida M, Brechot C, Cara C, Chervaux C, Cultrone A, Delorme C, Denariaz G, Dervyn R, Foerstner KU, Friss C, van de Guchte M, Guedon E, Haimet F, Huber W, van Hylckama-Vlieg J, Jamet A, Juste C, Kaci G, Knol J, Lakhdari O, Layec S, Le Roux K, Maguin E, Merieux A, Melo Minardi R, M'Rini C, Muller J, Oozeer R, Parkhill J, Renault P, Rescigno M, Sanchez N, Sunagawa S, Torrejon A, Turner K, Vandemeulebrouck G, Varela E, Winogradsky Y, Zeller G, Weissenbach J, Ehrlich SD, Bork P (2011) Enterotypes of the human gut microbiome. Nature 473:174–180

Baker-Austin C, Dopson M (2007) Life in acid: pH homeostasis in acidophiles. Trends Microbiol 15:165–171

Baldrian P, Kolarik M, Stursova M, Kopecky J, Valaskova V, Vetrovsky T, Zifcakova L, Snajdr J, Ridl J, Vlcek C, Voriskova J (2011) Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. ISME J 6:248–258

Beloqui A, Pita M, Polaina J, Martinez-Arias A, Golyshina OV, Zumarraga M, Yakimov MM, Garcia-Arellano H, Alcalde M, Fernandez VM, Elborough K, Andreu JM, Ballesteros A, Plou FJ, Timmis KN, Ferrer M, Golyshin PN (2006) Novel polyphenol oxidase mined from a metagenome expression library of bovine rumen: biochemical properties, structural analysis, and phylogenetic relationships. J Biol Chem 281:22933–22942

Beloqui A, Nechitaylo TY, Lopez-Cortes N, Ghazi A, Guazzaroni ME, Polaina J, Strittmatter AW, Reva O, Waliczek A, Yakimov MM, Golyshina OV, Ferrer M, Golyshin PN (2010) Diversity of glycosyl hydrolases from cellulose-depleting communities enriched from casts of two earthworm species. Appl Environ Microbiol 76:5934–5946

Benders GA, Noskov VN, Denisova EA, Lartigue C, Gibson DG, Assad-Garcia N, Chuang RY, Carrera W, Moodie M, Algire MA, Phan Q, Alperovich N, Vashee S, Merryman C, Venter JC, Smith HO, Glass JI, Hutchison CA 3rd (2010) Cloning whole bacterial genomes in yeast. Nucleic Acids Res 38:2558–2569

Several Authors (2008) Big data. Nature 455:1. Nature special issue

Blainey PC, Mosier AC, Potanina A, Francis CA, Quake SR (2011) Genome of a low-salinity ammonia-oxidizing archaeon determined by single-cell and metagenomic analysis. PLoS One 6:e16626

Bolhuis H, Palm P, Wende A, Falb M, Rampp M, Rodriguez-Valera F, Pfeiffer F, Oesterhelt D (2006) The genome of the square archaeon *Haloquadratum walsbyi*: life at the limits of water activity. BMC Genomics 7:169

Bomar L, Maltz M, Colston S, Graf J (2011) Directed culturing of microorganisms using metatranscriptomics. MBio 2(2):e00012-11

Brochier-Armanet C, Forterre P, Gribaldo S (2011) Phylogeny and evolution of the Archaea: one hundred genomes later. Curr Opin Microbiol 14:274

Brulc JM, Antonopoulos DA, Miller ME, Wilson MK, Yannarell AC, Dinsdale EA, Edwards RE, Frank ED, Emerson JB, Wacklin P, Coutinho PM, Henrissat B, Nelson KE, White BA (2009) Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. Proc Natl Acad Sci USA 106:1948–1953

Button DK, Schut F, Quang P, Martin R, Robertson BR (1993) Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. Appl Environ Microbiol 59:881–891

Chistoserdova L (2010) Recent progress and new challenges in metagenomics for biotechnology. Biotechnol Lett 32:1351–1359

Chistoserdovai L (2010) Functional metagenomics: recent advances and future challenges. Biotechnol Genet Eng Rev 26:335–352

Chitsaz H, Yee-Greenbaum JL, Tesler G, Lombardo M-J, Dupont CL, Badger JH, Novotny M, Rusch DB, Fraser LJ, Gormley NA, Schulz-Trieglaff O, Smith GP, Evers DJ, Pevzner PA, Lasken RS (2011) De Novo assembly of bacterial genomes from single cells. Nat Biotechnol 29:915

Connon SA, Giovannoni SJ (2002) High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl Environ Microbiol 68:3878–3885

Cuadros-Orellana S, Martin-Cuadrado AB, Legault B, D'Auria G, Zhaxybayeva O, Papke RT, Rodriguez-Valera F (2007) Genomic plasticity in prokaryotes: the case of the square haloarchaeon. ISME J 1:235–245

de Castro AP, Quirino BF, Allen H, Williamson LL, Handelsman J, Kruger RH (2011) Construction and validation of two metagenomic DNA libraries from Cerrado soil with high clay content. Biotechnol Lett 33:2169

de Jager V, Siezen RJ (2011) Single-cell genomics: unravelling the genomes of unculturable microorganisms. Microb Biotechnol 4:431–437

Dean FB, Nelson JR, Giesler TL, Lasken RS (2001) Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. Genome Res 11:1095–1099

Deloger M, El Karoui M, Petit MA (2009) A genomic distance based on MUM indicates discontinuity between most bacterial species and genera. J Bacteriol 191:91–99

Doolittle WF, Papke RT (2006) Genomics and the bacterial species problem. Genome Biol 7:116

Dumon C, Varvak A, Wall MA, Flint JE, Lewis RJ, Lakey JH, Morland C, Luginbuhl P, Healey S, Todaro T, DeSantis G, Sun M, Parra-Gessert L, Tan X, Weiner DP, Gilbert HJ (2008) Engineering hyperthermostability into a GH11 xylanase is mediated by subtle changes to protein structure. J Biol Chem 283:22557–22564

Dyall-Smith ML, Pfeiffer F, Klee K, Palm P, Gross K, Schuster SC, Rampp M, Oesterhelt D (2011) *Haloquadratum walsbyi*: limited diversity in a global pond. PLoS One 6:e20968

Ehrlich GD, Ahmed A, Earl J, Hiller NL, Costerton JW, Stoodley P, Post JC, DeMeo P, Hu FZ (2010) The distributed genome hypothesis as a rubric for understanding evolution in situ during chronic bacterial biofilm infectious processes. FEMS Immunol Med Microbiol 59:269–279

Fernandez-Arrojo L, Guazzaroni ME, Lopez-Cortes N, Beloqui A, Ferrer M (2010) Metagenomic era for biocatalyst identification. Curr Opin Biotechnol 21:725–733

Ferrari BC, Oregaard G, Sorensen SJ (2004) Recovery of GFP-labeled bacteria for culturing and molecular analysis after cell sorting using a benchtop flow cytometer. Microb Ecol 48:239–245

Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM et al (1995) Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. Science 269:496–512

Frohlich J, Konig H (1999) Rapid isolation of single microbial cells from mixed natural and laboratory populations with the aid of a micromanipulator. Syst Appl Microbiol 22:249–257

Gibson DG, Benders GA, Andrews-Pfannkoch C, Denisova EA, Baden-Tillson H, Zaveri J, Stockwell TB, Brownley A, Thomas DW, Algire MA, Merryman C, Young L, Noskov VN, Glass JI, Venter JC, Hutchison CA 3rd, Smith HO (2008a) Complete chemical synthesis, assembly, and cloning of a Mycoplasma genitalium genome. Science 319:1215–1220

Gibson DG, Benders GA, Axelrod KC, Zaveri J, Algire MA, Moodie M, Montague MG, Venter JC, Smith HO, Hutchison CA 3rd (2008b) One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic *Mycoplasma genitalium* genome. Proc Natl Acad Sci USA 105:20404–20409

Gibson DG, Glass JI, Lartigue C, Noskov VN, Chuang RY, Algire MA, Benders GA, Montague MG, Ma L, Moodie MM, Merryman C, Vashee S, Krishnakumar R, Assad-Garcia N, Andrews-Pfannkoch C, Denisova EA, Young L, Qi ZQ, Segall-Shapiro TH, Calvey CH, Parmar PP, Hutchison CA 3rd, Smith HO, Venter JC (2010) Creation of a bacterial cell controlled by a chemically synthesized genome. Science 329:52–56

Glass JI, Hutchison CA 3rd, Smith HO, Venter JC (2009) A systems biology tour de force for a near-minimal bacterium. Mol Syst Biol 5:330

Gomez-Alvarez V, Teal TK, Schmidt TM (2009) Systematic artifacts in metagenomes from complex microbial communities. ISME J 3:1314–1317

Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM (2007) DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57:81–91

Hall BG, Ehrlich GD, Hu FZ (2010) Pan-genome analysis provides much higher strain typing resolution than multi-locus sequence typing. Microbiology 156:1060–1068

Han S, Lee J, Kim W, Shin HJ, Kim G (2008) Screening of promoters from metagenomic DNA and their use for the construction of expression vectors. J Microbiol Biotechnol 18:1634–1640

Harrison PM, Arosio P (1996) The ferritins: molecular properties, iron storage function and cellular regulation. Biochim Biophys Acta 1275:161–203

Heywood JL, Sieracki ME, Bellows W, Poulton NJ, Stepanauskas R (2011) Capturing diversity of marine heterotrophic protists: one cell at a time. ISME J 5:674–684

Hogg JS, Hu FZ, Janto B, Boissy R, Hayes J, Keefe R, Post JC, Ehrlich GD (2007) Characterization and modeling of the *Haemophilus influenzae* core and supragenomes based on the complete genomic sequences of Rd and 12 clinical nontypeable strains. Genome Biol 8:R103

Horner DS, Pavesi G, Castrignano T, De Meo PD, Liuni S, Sammeth M, Picardi E, Pesole G (2009) Bioinformatics approaches for genomics and post genomics applications of next-generation sequencing. Brief Bioinform 11:181–197

Ishoy T, Kvist T, Westermann P, Ahring BK (2006) An improved method for single cell isolation of prokaryotes from meso-, thermo- and hyperthermophilic environments using micromanipulation. Appl Microbiol Biotechnol 69:510–514

Kang Y, Norris MH, Zarzycki-Siek J, Nierman WC, Donachie SP, Hoang TT (2011) Transcript amplification from single bacterium for transcriptome analysis. Genome Res 21:925–935

Kim EY, Oh KH, Lee MH, Kang CH, Oh TK, Yoon JH (2009) Novel cold-adapted alkaline lipase from an intertidal flat metagenome and proposal for a new family of bacterial lipases. Appl Environ Microbiol 75:257–260

Kislyuk AO, Haegeman B, Bergman NH, Weitz JS (2011) Genomic fluidity: an integrative view of gene diversity within microbial populations. BMC Genomics 12:32

Klenk HP, Göker M (2010) En route to a genome-based classification of *Archaea* and *Bacteria*? Syst Appl Microbiol 33:175–182

Konstantinidis KT, Tiedje JM (2005) Genomic insights that advance the species definition for prokaryotes. Proc Natl Acad Sci USA 102:2567–2572

Konstantinidis KT, Ramette A, Tiedje JM (2006) The bacterial species definition in the genomic era. Philos Trans R Soc Lond B Biol Sci 361:1929–1940

Kosakovsky Pond S, Wadhawan S, Chiaromonte F, Ananda G, Chung WY, Taylor J, Nekrutenko A (2009) Windshield splatter analysis with the Galaxy metagenomic pipeline. Genome Res 19:2144–2153

Kunin V, Engelbrektson A, Ochman H, Hugenholtz P (2009) Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. Environ Microbiol 12:118–123

Lagesen K, Ussery DW, Wassenaar TM (2010) Genome update: the 1000th genome–a cautionary tale. Microbiology 156:603–608

Lan R, Reeves PR (2001) When does a clone deserve a name? A perspective on bacterial species based on population genetics. Trends Microbiol 9:419–424

Lapierre P, Gogarten JP (2009) Estimating the size of the bacterial pan-genome. Trends Genet 25:107–110

Lartigue C, Glass JI, Alperovich N, Pieper R, Parmar PP, Hutchison CA 3rd, Smith HO, Venter JC (2007) Genome transplantation in bacteria: changing one species to another. Science 317:632–638

Lartigue C, Vashee S, Algire MA, Chuang RY, Benders GA, Ma L, Noskov VN, Denisova EA, Gibson DG, Assad-Garcia N, Alperovich N, Thomas DW, Merryman C, Hutchison CA 3rd, Smith HO, Venter JC, Glass JI (2009) Creating bacterial strains from genomes that have been cloned and engineered in yeast. Science 325:1693–1696

Lefébure T, Stanhope MJ (2007) Evolution of the core and pan-genome of *Streptococcus*: positive selection, recombination, and genome composition. Genome Biol 8:R71

Legault BA, Lopez-Lopez A, Alba-Casado JC, Doolittle WF, Bolhuis H, Rodriguez-Valera F, Papke RT (2006) Environmental genomics of "*Haloquadratum walsbyi*" in a saltern crystallizer indicates a large pool of accessory genes in an otherwise coherent species. BMC Genomics 7:171

Liu N, Yan X, Zhang M, Xie L, Wang Q, Huang Y, Zhou X, Wang S, Zhou Z (2011) Microbiome of fungus-growing termites: a new reservoir for lignocellulase genes. Appl Environ Microbiol 77:48–56

Lopez-Garcia P, Moreira D (2008) Tracking microbial biodiversity through molecular and genomic ecology. Res Microbiol 159:67–73

Lu Y, Yu Y, Zhou R, Sun W, Dai C, Wan P, Zhang L, Hao D, Ren H (2011) Cloning and characterisation of a novel 2,4-dichlorophenol hydroxylase from a metagenomic library derived from polychlorinated biphenyl-contaminated soil. Biotechnol Lett 33:1159–1167

Lukjancenko O, Wassenaar TM, Ussery DW (2010) Comparison of 61 Sequenced *Escherichia coli* Genomes. Microb Ecol 60:708–720

MacLean D, Jones JD, Studholme DJ (2009) Application of 'next-generation' sequencing technologies to microbial genetics. Nat Rev Microbiol 7:287–296

Maione D, Margarit I, Rinaudo CD, Masignani V, Mora M, Scarselli M, Tettelin H, Brettoni C, Iacobini ET, Rosini R, D'Agostino N, Miorin L, Buccato S, Mariani M, Galli G, Nogarotto R, Nardi Dei V, Vegni F, Fraser C, Mancuso G, Teti G, Madoff LC, Paoletti LC, Rappuoli R, Kasper DL, Telford JL, Grandi G (2005) Identification of a universal Group B *Streptococcus* vaccine by multiple genome screen. Science 309:148–150

Majernik A, Gottschalk G, Daniel R (2001) Screening of environmental DNA libraries for the presence of genes conferring Na(+)(Li(+))/H(+) antiporter activity on *Escherichia coli*: characterization of the recovered genes and the corresponding gene products. J Bacteriol 183:6645–6653

Marcy Y, Ouverney C, Bik EM, Losekann T, Ivanova N, Martin HG, Szeto E, Platt D, Hugenholtz P, Relman DA, Quake SR (2007) Dissecting biological "dark matter" with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. Proc Natl Acad Sci USA 104:11889–11894

Medini D, Donati C, Tettelin H, Masignani V, Rappuoli R (2005) The microbial pan-genome. Curr Opin Genet Dev 15:589–594

Medini D, Serruto D, Parkhill J, Relman DA, Donati C, Moxon R, Falkow S, Rappuoli R (2008) Microbiology in the post-genomic era. Nat Rev Microbiol 6:419–430

Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A, Wilkening J, Edwards RA (2008) The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 9:386

Mira A, Martin-Cuadrado AB, D'Auria G, Rodriguez-Valera F (2010) The bacterial pan-genome:a new paradigm in microbiology. Int Microbiol 13:45–57

Morgan JL, Darling AE, Eisen JA (2010) Metagenomic sequencing of an in vitro-simulated microbial community. PLoS One 5:e10209

Mussmann M, Hu FZ, Richter M, de Beer D, Preisler A, Jorgensen BB, Huntemann M, Glockner FO, Amann R, Koopman WJ, Lasken RS, Janto

B, Hogg J, Stoodley P, Boissy R, Ehrlich GD (2007) Insights into the genome of large sulfur bacteria revealed by analysis of single filaments. PLoS Biol 5:e230

Muzzi A, Masignani V, Rappuoli R (2007) The pan-genome: towards a knowledge-based discovery of novel targets for vaccines and antibacterials. Drug Discov Today 12:429–439

Nagarajan H, Butler JE, Klimes A, Qiu Y, Zengler K, Ward J, Young ND, Methe BA, Palsson BO, Lovley DR, Barrett CL (2010) De Novo assembly of the complete genome of an enhanced electricity-producing variant of Geobacter sulfurreducens using only short reads. PLoS One 5:e10922

Newman DJ, Cragg GM (2005) The discovery of anticancer drugs from natural sources. In: Zhang L, Demain AL (eds) Natural products: drug discovery and therapeutic medicine. Humana Press, Totowan, pp 128–168

Oh D, Porter K, Russ B, Burns D, Dyall-Smith M (2010) Diversity of *Haloquadratum* and other haloarchaea in three, geographically distant, Australian saltern crystallizer ponds. Extremophiles 14:161–169

Ottesen EA, Hong JW, Quake SR, Leadbetter JR (2006) Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. Science 314:1464–1467

Pang H, Zhang P, Duan CJ, Mo XC, Tang JL, Feng JX (2009) Identification of cellulase genes from the metagenomes of compost soils and functional characterization of one novel endoglucanase. Curr Microbiol 58:404–408

Parachin NS, Gorwa-Grauslund MF (2011) Isolation of xylose isomerases by sequence- and function-based screening from a soil metagenome library. Biotechnol Biofuels 4:9

Park SY, Shin HJ, Kim GJ (2011) Screening and identification of a novel esterase EstPE from a metagenomic DNA library. J Microbiol 49:7–14

Pathak GP, Ehrenreich A, Losi A, Streit WR, Gartner W (2009) Novel blue light-sensitive proteins from a metagenomic approach. Environ Microbiol 11:2388–2399

Podar M, Abulencia CB, Walcher M, Hutchison D, Zengler K, Garcia JA, Holland T, Cotton D, Hauser L, Keller M (2007) Targeted access to the genomes of low-abundance organisms in complex microbial communities. Appl Environ Microbiol 73:3205–3214

Pope PB, Denman SE, Jones M, Tringe SG, Barry K, Malfatti SA, McHardy AC, Cheng JF, Hugenholtz P, McSweeney CS, Morrison M (2010) Adaptation to herbivory by the Tammar wallaby includes bacterial and glycoside hydrolase profiles different from other herbivores. Proc Natl Acad Sci USA 107:14793–14798

Porter J, Edwards C, Morgan JA, Pickup RW (1993) Rapid, automated separation of specific bacteria from lake water and sewage by flow cytometry and cell sorting. Appl Environ Microbiol 59:3327–3333

Portnoy VA, Bezdan D, Zengler K (2011) Adaptive laboratory evolution-harnessing the power of biology for metabolic engineering. Curr Opin Biotechnol 22:590–594

Purdy KJ (2005) Nucleic acid recovery from complex environmental samples. Methods Enzymol 397:271–292

Raghunathan A, Ferguson HR Jr, Bornarth CJ, Song W, Driscoll M, Lasken RS (2005) Genomic DNA amplification from a single bacterium. Appl Environ Microbiol 71:3342–3347

Rasko DA, Rosovitz MJ, Myers GSA, Mongodin EF, Fricke WF, Gajer P, Crabtree J, Sebaihia M, Thomson NR, Chaudhuri R, Henderson IR, Sperandio V, Ravel J (2008) The pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal and pathogenic isolates. J Bacteriol 190:6881–6893

Rhee JK, Ahn DG, Kim YG, Oh JW (2005) New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. Appl Environ Microbiol 71:817–825

Richardson TH, Tan X, Frey G, Callen W, Cabell M, Lam D, Macomber J, Short JM, Robertson DE, Miller C (2002) A novel, high performance enzyme for starch liquefaction. Discovery and optimization of a low pH, thermostable alpha-amylase. J Biol Chem 277:26501–26507

Riesenfeld CS, Goodman RM, Handelsman J (2004) Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ Microbiol 6:981–989

Robertson DE, Chaplin JA, DeSantis G, Podar M, Madden M, Chi E, Richardson T, Milan A, Miller M, Weiner DP, Wong K, McQuaid J, Farwell B, Preston LA, Tan X, Snead MA, Keller M, Mathur E, Kretz PL, Burk MJ, Short JM (2004) Exploring nitrilase sequence space for enantioselective catalysis. Appl Environ Microbiol 70:2429–2436

Rodrigue S, Malmstrom RR, Berlin AM, Birren BW, Henn MR, Chisholm SW (2009) Whole genome amplification and de novo assembly of single bacterial cells. PLoS One 4:e6864

Rondon MR, August PR, Bettermann AD, Brady SF, Grossman TH, Liles MR, Loiacono KA, Lynch BA, MacNeil IA, Minor C, Tiong CL, Gilman M, Osburne MS, Clardy J, Handelsman J, Goodman RM (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl Environ Microbiol 66:2541–2547

Schadt EE, Linderman MD, Sorenson J, Lee L, Nolan GP (2010) Computational solutions to large-scale data management and analysis. Nat Rev Genet 11:647–657

Schallmey M, Ly A, Wang C, Meglei G, Voget S, Streit WR, Driscoll BT, Charles TC (2011) Harvesting of novel polyhydroxyalkanaote (PHA) synthase encoding genes from a soil metagenome library using phenotypic screening. FEMS Microbiol Lett 321:150–156

Schwartz S, Oren R, Ast G (2011) Detection and removal of biases in the analysis of next-generation sequencing reads. PLoS One 6:e16685

Sharp KH, Davidson SK, Haygood MG (2007) Localization of 'Candidatus Endobugula sertula' and the bryostatins throughout the life cycle of the bryozoan *Bugula neritina*. ISME J 1:693–702

Siegl A, Kamke J, Hochmuth T, Piel J, Richter M, Liang C, Dandekar T, Hentschel U (2010) Single-cell genomics reveals the lifestyle of Poribacteria, a candidate phylum symbiotically associated with marine sponges. ISME J 5:61–70

Simon C, Daniel R (2011) Metagenomic analyses: past and future trends. Appl Environ Microbiol 77:1153–1161

Simon C, Herath J, Rockstroh S, Daniel R (2009) Rapid identification of genes encoding DNA polymerases by function-based screening of metagenomic libraries derived from glacial ice. Appl Environ Microbiol 75:2964–2968

Sogin ML, Morrison HG, Huber JA, Mark Welch D, Huse SM, Neal PR, Arrieta JM, Herndl GJ (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc Natl Acad Sci USA 103:12115–12120

Stepanauskas R, Sieracki ME (2007) Matching phylogeny and metabolism in the uncultured marine bacteria, one cell at a time. Proc Natl Acad Sci USA 104:9052–9057

Strous M, Pelletier E, Mangenot S, Rattei T, Lehner A, Taylor MW, Horn M, Daims H, Bartol-Mavel D, Wincker P, Barbe V, Fonknechten N, Vallenet D, Segurens B, Schenowitz-Truong C, Medigue C, Collingro A, Snel B, Dutilh BE, Op den Camp HJ, van der Drift C, Cirpus I, van de Pas-Schoonen KT, Harhangi HR, van Niftrik L, Schmid M, Keltjens J, van de Vossenberg J, Kartal B, Meier H, Frishman D, Huynen MA, Mewes HW, Weissenbach J, Jetten MS, Wagner M, Le Paslier D (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. Nature 440:790–794

Suen G, Scott JJ, Aylward FO, Adams SM, Tringe SG, Pinto-Tomas AA, Foster CE, Pauly M, Weimer PJ, Barry KW, Goodwin LA, Bouffard P, Li L, Osterberger J, Harkins TT, Slater SC, Donohue TJ, Currie CR (2010) An insect herbivore microbiome with high plant biomass-degrading capacity. PLoS Genet 6:e1001129

Sun S, Chen J, Li W, Altintas I, Lin A, Peltier S, Stocks K, Allen EE, Ellisman M, Grethe J, Wooley J (2011) Community cyberinfrastructure for advanced microbial ecology research and analysis: the CAMERA resource. Nucleic Acids Res 39:D546–D551

Suzuki S, Ono N, Furusawa C, Ying BW, Yomo T (2011) Comparison of sequence reads obtained from three next-generation sequencing platforms. PLoS One 6:e19534

Tadmor AD, Ottesen EA, Leadbetter JR, Phillips R (2011) Probing individual environmental bacteria for viruses by using microfluidic digital PCR. Science 333:58–62

Tanenbaum DM, Goll J, Murphy S, Kumar P, Zafar N, Thiagarajan M, Madupu R, Davidsen T, Kagan L, Kravitz S, Rusch DB, Yooseph S (2010) The JCVI standard operating procedure for annotating prokaryotic metagenomic shotgun sequencing data. Stand Genomic Sci 2:229–237

Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, Wang X, Bodeau J, Tuch BB, Siddiqui A, Lao K, Surani MA (2009) mRNA-Seq whole-transcriptome analysis of a single cell. Nat Methods 6:377–382

Taniguchi K, Kajiyama T, Kambara H (2009) Quantitative analysis of gene expression in a single cell by qPCR. Nat Methods 6:503–506

Taniguchi Y, Choi PJ, Li GW, Chen H, Babu M, Hearn J, Emili A, Xie XS (2010) Quantifying E. coli proteome and transcriptome with single-molecule sensitivity in single cells. Science 329:533–538

Taupp M, Mewis K, Hallam SJ (2011) The art and design of functional metagenomic screens. Curr Opin Biotechnol 22:465–472

Taylor RJ, Falconnet D, Niemisto A, Ramsey SA, Prinz S, Shmulevich I, Galitski T, Hansen CL (2009) Dynamic analysis of MAPK signaling using a high-throughput microfluidic single-cell imaging platform. Proc Natl Acad Sci USA 106:3758–3763

Tettelin H, Masignani V, Cieslewicz MJ, Donati C, Medini D, Ward NL, Angiuoli SV, Crabtree J, Jones AL, Durkin AS, Deboy RT, Davidsen TM, Mora M, Scarselli M, Margarity Ros I, Peterson JD, Hauser CR, Sundaram JP, Nelson WC, Madupu R, Brinkac LM, Dodson RJ, Rosovitz MJ, Sullivan SA, Daugherty SC, Haft DH, Selengut J, Gwinn ML, Zhou L, Zafar N, Khouri H, Radune D, Dimitrov G, Watkins K, O'Connor KJ, Smith S, Utterback TR, White O, Rubens CE, Grandi G, Madoff LC, Kasper DL, Telford JL, Wessels MR, Rappuoli R, Fraser CM (2005) Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome". Proc Natl Acad Sci USA 102:13950–13955

Tettelin H, Riley D, Cattuto C, Medini D (2008) Comparative genomics: the bacterial pan-genome. Curr Opin Microbiol 11:472–477

Tsuruta H, Paddon CJ, Eng D, Lenihan JR, Horning T, Anthony LC, Regentin R, Keasling JD, Renninger NS, Newman JD (2009) High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in *Escherichia coli*. PLoS One 4:e4489

Tyson GW, Chapman J, Hugenholtz P, Allen EE, Ram RJ, Richardson PM, Solovyev VV, Rubin EM, Rokhsar DS, Banfield JF (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 428:37–43

Uchiyama T, Abe T, Ikemura T, Watanabe K (2005) Substrate-induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. Nat Biotechnol 23:88–93

Ussery DW, Kiil K, Lagesen K, Sicheritz-Ponten T, Bohlin J, Wassenaar TM (2009) The genus *Burkholderia*: analysis of 56 genomic sequences. Genome Dyn 6:140–157

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH, Smith HO (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304:66–74

Walker A, Parkhill J (2008) Single-cell genomics. Nat Rev Microbiol 6:176–177

Wang C, Meek DJ, Panchal P, Boruvka N, Archibald FS, Driscoll BT, Charles TC (2006) Isolation of poly-3-hydroxybutyrate metabolism genes from complex microbial communities by phenotypic complementation of bacterial mutants. Appl Environ Microbiol 72:384–391

Wender PA, Miller BL (2009) Synthesis at the molecular frontier. Nature 460: 197–201

Wooley JC, Godzik A, Friedberg I (2010) A primer on metagenomics. PLoS Comput Biol 6:e1000667

Woyke T, Xie G, Copeland A, Gonzalez JM, Han C, Kiss H, Saw JH, Senin P, Yang C, Chatterji S, Cheng JF, Eisen JA, Sieracki ME, Stepanauskas R (2009) Assembling the marine metagenome, one cell at a time. PLoS One 4:e5299

Woyke T, Tighe D, Mavromatis K, Clum A, Copeland A, Schackwitz W, Lapidus A, Wu D, McCutcheon JP, McDonald BR, Moran NA, Bristow J, Cheng JF (2010) One bacterial cell, one complete genome. PLoS One 5:e10314

Wu S, Zhu Z, Fu L, Niu B, Li W (2011) WebMGA: a customizable web server for fast metagenomic sequence analysis. BMC Genomics 12:444

Yoon HS, Price DC, Stepanauskas R, Rajah VD, Sieracki ME, Wilson WH, Yang EC, Duffy S, Bhattacharya D (2011) Single-cell genomics reveals organismal interactions in uncultivated marine protists. Science 332:714–717

Yu EY, Kwon MA, Lee M, Oh JY, Choi JE, Lee JY, Song BK, Hahm DH, Song JK (2011) Isolation and characterization of cold-active family VIII esterases from an arctic soil metagenome. Appl Microbiol Biotechnol 90:573–581

Zeng Y, Novak R, Shuga J, Smith MT, Mathies RA (2010) High-performance single cell genetic analysis using microfluidic emulsion generator arrays. Anal Chem 82:3183–3190

Zengler K (2009) Central role of the cell in microbial ecology. Microbiol Mol Biol Rev 73:712–729

Zhang K, Martiny AC, Reppas NB, Barry KW, Malek J, Chisholm SW, Church GM (2006) Sequencing genomes from single cells by polymerase cloning. Nat Biotechnol 24:680–686

Zhao S, Wang J, Bu D, Liu K, Zhu Y, Dong Z, Yu Z (2010) Novel glycoside hydrolases identified by screening a Chinese Holstein dairy cow rumen-derived metagenome library. Appl Environ Microbiol 76:6701–6705

# Symbiotic Associations

# 15 Role of Microorganisms in Adaptation, Development, and Evolution of Animals and Plants: The Hologenome Concept

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## Introduction

It is useful to begin by providing some common definitions. The term *symbiosis* was first coined by Anton de Bary in the mid nineteenth century as "the living together of different species." This broad definition is still generally accepted. The symbiotic system is usually constructed from a large partner termed the *host* and smaller partners called *symbionts*. This arbitrary division by dimension between host and symbiont may not fit all systems because size can be measured by cell number or by genome size, and in the case of many systems, the microbiota outnumbers its host. In spite of these limitations, the definition of host and symbiont based on size continues to be widely used. *Endosymbionts* and *exosymbionts* refer to microbiota living inside or outside host cells, respectively. Symbioses take many forms, mostly different levels of *mutualism*, where both the host and the symbiont benefit from the interaction, and to a much smaller degree—*parasitism*, where the symbiont benefits and the host suffers damage. *Commensalism* is defined as the close association of two or more dissimilar organisms where the association is advantageous to one and does not affect the other(s). These types of symbioses may change under different circumstances. For example, *Wolbachia* is generally detrimental (parasitic) to its insect host; however, it can also be beneficial (mutualistic): *Wolbachia* is essential for the production of mature oocytes in a parasitic wasp (Dedeine et al. 2001). Another example is the bacterium *Vibrio shiloi*, which is only parasitic when the temperature is raised and virulence genes are expressed (Rosenberg and Falkovitz 2004). The *holobiont* refers to the host and its symbiotic microbiota (Margulis 1993; Rohwer et al. 2002), and the *hologenome* is defined as the sum of the genetic information of the host and its microbiota (Rosenberg et al. 2007; Zilber-Rosenberg and Rosenberg 2008). The term *microbiome* was defined by J. Lederberg as the sum of the genetic information of the microbiota (Hooper and Gordon 2001).

Until recently, studies on symbioses have concentrated on a single primary symbiont and its host. However, with the advent of molecular (culture-independent) techniques in microbiology during the last 20 years, it is now clear that all animals and plants live in close association with hundreds or thousands of different microbial species. In many cases, the number of symbiotic microorganisms and their combined genetic information far exceed that of their host. In the last few years, it has been demonstrated that these diverse microbiota with their large microbiomes play a remarkable role in the lives of animals and plants.

Evolutionary developmental biology is based on the principle that evolution arises from hereditable changes in development (Gilbert et al. 2010). In the past, the focus of these changes has been on the host genome (genetic and epigenetic) and

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occasionally on the genome of a specific primary symbiont (co-evolution). In this chapter, we shall summarize the hologenome concept and then discuss the contribution of diverse microbial symbionts to fitness (adaptation, survival, development, growth, and reproduction) and evolution of representative animals and plants, thereby demonstrating the idea that holobionts have developed, lived, survived, and evolved together. The other chapters in this section deal with specific host: bacteria symbioses.

## Microbes and the Origin of Eukaryotic Cells and Multicellular Organisms: The First Hologenomes

Prokaryotic life first appeared on Earth about 3.5 billion years ago (Schopf 1993), whereas animals first appear in the fossil record approximately 500 million years ago (Hickman 2005). Thus, microbes were the only form of life on Earth for most of its history. During the initial 3 billion years of prokaryotic life, there was time for metabolic diversification and specialization. According to the endosymbiotic theory, eukaryotic cells arose when free-living bacteria were taken inside another bacterial cell as an endosymbiont. Mitochondria developed from proteobacteria, in particular, *Rickettsia prowazekii* or close relatives (Andersson et al. 1998) and chloroplasts from cyanobacteria (Falcon et al. 2010). The origin of the nucleus is not clear, but one hypothesis is that it arose when ancient archaea, similar to modern methanogenic archaea, invaded and lived within bacteria similar to modern myxobacteria, eventually forming the early nucleus (Pennisi 2004). This theory is analogous to the accepted theory for the origin of eukaryotic mitochondria and chloroplasts. According to Margulis and Sagan (2001), "Life did not take over the globe by combat, but by networking" (i.e., by cooperation or formation of hologenomes).

The concept that mitochondria are bacteria goes back more than 100 years. Portier (1918) wrote "Each living cell contains in its protoplasm formations which histologists designate by the name mitochondria. These organelles are, for me, nothing other than symbiotic bacteria, which I call symbiotes." However, it was not until the 1970s that biochemical evidence demonstrated the bacterial origin of mitochondria, chloroplasts, and probably centrioles. Recently, Davidov and Jurkevitch (2009) suggested that eukaryotes arose from predatory bacteria, like Bdellovibrio, that penetrated into larger bacteria and became endosymbionts.

With regard to multicellularity, it is reasonable to assume that early prokaryotes developed means of interacting among themselves and with other groups in a manner that was advantageous, i.e., allowed them to survive and multiply more efficiently. Cell-to-cell adhesion and signaling are two mechanisms that are widespread in the bacterial world. Myxobacteria, for example, when starved of nutrients, produce signals and aggregate by gliding chemotaxis in order to construct species-specific fruiting bodies consisting of thousands of cells (Reichenbach 1984). The interactions of different groups of bacteria in biofilms and microbial mats with the exchange of metabolites can be considered symbioses. The origin of the first multicellular eukaryotic organism is purely speculative. It is reasonable to assume that at least some of the early eukaryotic cells, formed by the fusion of two or more prokaryotes, had the genetic information that would allow for cell-to-cell interactions and the formation of multicellular eukaryotic structures as described above. The earliest animal that still exists is the sponge. What can it tell us about the early evolution of animals? Costerton and colleagues (1995) have compared modern sponges to biofilms because both lack tissues and organs, but are composed of a three-dimensional matrix that allows for flow of water, nutrients, metabolites, and oxygen. Modern sponges are well known for containing large complex microbial symbiotic communities—more than half of the biomass of a sponge is bacteria (Reitner and Schumann-Kindel 1997). The fossil record of sponges demonstrates their ancient association with bacteria, further indicating that prokaryotic symbionts were essential components of animals from their very beginning (Hickman 2005).

In light of the available information, it is likely that animal and plant cells arose from prokaryotic organisms by fusion, aggregated into multicellular complexes, initially using prokaryotic genetic information, and differentiated into animals and plants, always in close association with microorganisms. During evolution, animals and plants acquired additional structures and functions either by changing their DNA or by acquiring additional symbionts. Good examples of the latter are ruminants (Dehority 2003) and termites (Minkley et al. 2006), which evolved the ability to utilize cellulose as a nutrient by incorporating cellulose-decomposing microorganisms, thereby avoiding the very slow process of evolving novel efficient enzymes by themselves. This is an example of outsourcing functions from eukaryotes to prokaryotes that continues to the present.

## The Hologenome Concept

The hologenome theory of evolution considers the holobiont with its hologenome, acting in consortium, as a unit of selection in evolution (Rosenberg et al. 2007; Zilber-Rosenberg and Rosenberg 2008; Sharon et al. 2010). The hologenome theory posits that (1) all animals and plants harbor abundant and diverse microorganisms acquiring from their host a sheltered and nutrient-rich environment, (2) these microbial symbionts affect the fitness of the holobiont and in turn are affected by it, (3) variation in the hologenome can be brought about by changes in either the host genome or the microbial population genomes (microbiome), and (4) these variations, including those of the microbiome, can be transmitted from one generation to the next with fidelity and thus may also influence evolution of the holobiont.

## All Animals and Plants Establish Symbiotic Relationships with Microorganisms

As discussed above, eukaryotes presumably arose from prokaryotes and have remained in close relationship with them ever

since. It is therefore not surprising that the surfaces of animals and plants contain a great abundance and variety of microorganisms. In addition, some microorganisms are able to grow inside animal or plant cells, i.e., endosymbionts. The number of microbial cells and their combined genetic information often far exceed that of their hosts. For example, using the powerful technique of metagenomics, it has recently been reported that the number of bacterial genes present in the human gut exceeds 3.3 million (Qin et al. 2010). By comparison, the human host genome contains about 20,500 genes (She et al. 2004). This large number of microbial genes considerably increases the potential for adaptation by the holobiont. In addition, wherever microbes exist, bacteriophages also occur, and they probably play an important role in microbiota dynamics, which will be discussed later.

Because the vast majority of microorganisms which have been observed on or in animal and plant tissues cannot be cultured, current research on the diversity of microorganisms associated with a particular species relies primarily on culture-free DNA-based technology. Although censuses of microorganisms associated with different animal and plant species are only in an early stage, certain interesting generalizations have emerged: (1) The diversity of microbial species associated with a particular animal or plant species is high (*Table 15.1*). (2) The host associated microbial community is very different from the community in the surrounding environment (Chelius and Triplett 2001; Rohwer et al. 2002; Sharp et al. 2007). (3) In some cases, it has been shown that similar, but not identical, microbial populations are found on the same eukaryote species that are geographically separated, while different populations are found on different species at the same location (Rohwer et al. 2002; Lambais et al. 2006; Fraune and Bosch 2007). (4) Different microbial communities often dominate different tissues of the same organism (Tannock 1995; Koren and Rosenberg 2006; Dethlefsen et al. 2006; Reid et al. 2011). (5) In several cases where a large diversity of associated bacterial species exists, certain bacterial groups dominate (Koren and Rosenberg 2006; Ley et al. 2008; Redford et al. 2010).

The association of microorganisms with hosts can take many different forms. Some may be transitory and have little effect on adaptation or evolution of the holobiont. At the other extreme, there are several examples of well-studied long-lasting interactions (e.g., the rumen system) between host and microorganisms, which can lead to total dependence of one on the other. Between these two extremes lies a gradient of interactions of varying strengths, including pathogenesis. Let us consider some factors that determine the diversity of microorganisms associated with the holobiont. We shall first consider those characteristics that would result in high diversity. Many microorganisms are specialists. Given that hosts provide a variety of different niches that can change with the developmental stage of the host, the diet, and other environmental factors, a diverse microbial community is established, with different microbial strains filling the different niches. This microbial diversity, and therefore its versatility, may allow the holobiont as a whole to function more optimally and adapt more rapidly to changing conditions. The idea that microbial diversity can play a critical role under conditions of fluctuating environments has been referred to as the insurance policy hypothesis (Yachi and Loreau 1999). Another factor that contributes to bacterial diversity is bacteriophages. It has been established that high concentrations of bacteriophages are present in animal and plant tissues (Breibart et al. 2003). If any microorganism becomes too abundant, it may be lysed by bacteriophages. This concept, referred to as the "kill the winners" hypothesis (Thingstad and Lignell 1997), is supported by mathematical models of the bacteria: bacteriophage dynamics (Weitz et al. 2005).

On the other hand, there exist opposing forces that limit the number of strains that can survive and become established in the holobiont, notably the innate and adaptive immune systems. The innate or nonspecific immune system is the first line of defense and includes physical barriers, antimicrobial molecules,

**Table 15.1**
**Number of microbial species associated with representative animals and plants**[a]

| Host | Minimum number of microbial species | References |
|---|---|---|
| ***Invertebrates*** | | |
| *Drosophila melanogaster* | 74 | Mateos et al. (2006) |
| Marine sponge | 1,694 | Webster et al. (2001) |
| Coral *Oculina patagonica* | 400 | Koren and Rosenberg (2006) |
| Tunicate | 40 | Martínez-García et al. (2007) |
| Termite gut | 367 | Hongoh et al. (2005) |
| ***Vertebrates*** | | |
| Human skin | 113 | Grice et al. (2009) |
| Human gut | 1,050 | Qin et al. (2010) |
| Great ape gut | 8,914 | Ochman et al. (2010) |
| Human subgingival plaque | 756 | Dewhirst et al. (2010) |
| Bovine rumen | 341 | Edwards et al. (2004) |
| Reindeer rumen | 700 | Sundset et al. (2007) |
| Pig gut | 375 | Leser et al. (2002) |
| ***Plants*** | | |
| Leaf of the plant *Trichilia catigua* | 617 | Lambais et al. (2006) |
| Plant seed | 46 | Cankar et al. (2005) |
| Roots of *Zea mays* | 74 | Chelius and Triplett (2001) |

[a]These are minimum numbers because no one has sequenced enough to detect rare species

enzymes, specific binding proteins for microbial attachment (e.g., the peptidoglycan-binding protein and lectin complement system), production of reactive oxygen species and phagocytes (Iwanaga and Lee 2005). Interestingly, resident symbiotic bacteria are also part of the innate immune system—by occupying potent adhesion sites and by producing antibiotics (Ritchie 2006). The adaptive or the specific immune system in vertebrates includes specific recognition of "foreign" microorganisms, generation of responses to eliminate these microorganisms, and development of immunological memory to hasten the response to subsequent infections with the same microbe. In essence, the immune system of the host is responsible for both limiting the types of microorganisms that can survive within the host and recognizing and accommodating the normal microbiota, thereby regulating the kinds of microorganisms that can reside in the holobiont. Also, it is important to note that plants have evolved myriad phytochemicals, whose purpose is to prevent infection by harmful microorganisms (Wallace 2004) and enable coexistence with beneficial ones (Smith et al. 1999; Stougaard 2000; Wilkinson 2001). The human gut is an example in which, although one finds a substantial diversity of bacterial strains, they belong by and large to only 30–40 species, which themselves belong to two main bacterial divisions (out of 70 divisions identified), the Firmicutes and the Bacteroidetes, while the Archaea are represented mainly by only one strain, *Methanobrevibacter smithii* (Ley et al. 2006a).

## Cooperation Between the Host and the Microbiota Contributes to the Fitness of the Holobiont

Considering the hologenome concept, we argue that the cooperation between the normal microbiota and the host generally leads to improved fitness of the holobiont, by the host outsourcing (Gilbert et al. 2010) different kinds of functions to its microbiota and vice versa. ❯ *Table 15.2* displays various representative symbiotic systems, indicating some of the ways in which the microorganisms contribute to the fitness of the holobiont. The first cases listed in the table are the mitochondria and chloroplasts, which can be considered (extreme) symbionts which are responsible for respiration and photosynthesis, respectively. Animal–microbe symbioses take many forms: Few are endocellular and termed "primary" symbionts (P-symbionts) and the vast majority are extracellular symbionts. While some of the symbioses show absolute dependency, most of the symbiosis systems, as indicated in ❯ *Table 15.2*, are not based on life or death interactions, but rather the microbial partners contribute in different degrees to the holobiont's well-being. The hologenome concept emphasizes the importance not only of the intracellular symbionts but also of the diverse and dynamic extracellular microbial symbionts that are present in all animals. The enormous genetic richness of these symbionts can play a major role in adaptation and evolution of holobionts during times of environmental change. Some examples of well-described symbioses are summarized below.

## Invertebrates

### Insect Holobionts

Insects are the most diverse animal group on earth, embracing several million species (Wilson 1992). Insect–microbe symbioses take many forms: Some are endocellular and many more are extracellular. In some insects, obligate mutualistic bacteria are harbored in specialized host cells. For example, *Buchnera aphidicola* is harbored intracellularly within bacteriocytes in the abdominal body cavity of almost all aphids and provides essential amino acids that are lacking in the phloem sap diet of the insects (Douglas 1998), and *Wigglesworthia glossinidia* is localized in a midgut-associated bacteriome of tsetse flies and plays pivotal roles in biosynthesis of B vitamins that are deficient in the blood diet of the insects (Akman et al. 2002). These obligate P-symbionts usually share long evolutionary histories with their hosts and, in most cases, the host cannot survive without the endosymbiont, or the elimination of the endosymbiont has a deleterious effect on the fitness of the host (Baumann et al. 2006).

*Wolbachia*, a Gram-negative bacterium of the alpha-proteobacteria group, is a common obligate intracellular parasite of insects and other invertebrates. It is probably the most ubiquitous endosymbiont on the planet (Dedeine et al. 2001) and is maternally transmitted through the cytoplasm of eggs. First recognized as the cause of some incompatible crosses in insects (Yen and Barr 1971), *Wolbachia* has since been identified as a cause of parthenogenesis, feminization of male hosts, and male killing in different arthropod taxa (Veneti et al. 2005).

Recent studies using molecular techniques have brought new insights into the mechanisms by which microbial symbionts digest cellulose in the small intestine of insects (Watanabe and Tokuda 2010). If the available diet of an insect changes from simple sugars to complex polysaccharides, those symbionts, which contain the appropriate polysaccharidases will amplify in number, depolymerize the polysaccharides, and allow the insect to grow efficiently. Termites, for example, have a multitude of different microorganisms in their hindgut (Warnecke et al. 2007) that are largely responsible for the breakdown of lignocelluloses (Breznak and Brune 1994) and nitrogen fixation (Golichenkov et al. 2002). It has been shown that different bacterial phylogenetic groups are present in the different gut compartments (Schmitt-Wagner et al. 2003).

It has also been suggested that microbes have been powerful selective agents in the development of social behavior in insects, such as ants, bees, wasps, and termites (Stow and Beattie 2008): On one hand, close contact between community members ensures that beneficial microorganisms are transmitted from one generation to the next; on the other hand, it provides ideal conditions for transfer of contagious diseases. To help solve this problem, many social insects contain symbiotic bacteria, which produce antibiotics active against pathogens (Currie et al. 2006).

Diet-induced mating preference in *Drosophila* was reported many years ago (Dodd 1989); however, the mechanism was unknown until a recent demonstration that changing the diet caused an amplification of a particular bacterial symbiont,

**Table 15.2**

**Modes of transmission of symbionts and their contribution to the fitness of the holobiont**

| Holobiont: microbiota | Mode of transmission of microorganisms | Microbial contribution | References |
|---|---|---|---|
| **General** | | | |
| All eukaryotes: Mitochondria | Cytoplasmic inheritance | Respiration | Andersson et al. (1998) |
| Plants: Chloroplasts | Cytoplasmic inheritance | Photosynthesis | Falcon et al. (2010) |
| **Invertebrates** | | | |
| Aphids: *Buchnera* sp. (primary-endosymbiont) | Via intracellular bacteria in bacteriocytes; present in ova | Provision of specific required amino acids lacking in the plant sap diet | Baumann et al. (2006), Douglas (1998) |
| Aphids: Secondary endosymbionts | Via intracellular bacteria in addition to environment | Growth at high temperature; resistance to parasites | Sandström et al. (2001), Russell et al. (2003) |
| Termite: Microbiota in hind gut | Feces of adult termites fed to newly hatched juveniles | Utilizable energy and carbon; nitrogen metabolism, recognition signal from odor of bacterial metabolites | Abe et al. (2000), Minkley et al. (2006), Watanabe and Tokuda (2010) |
| Anthropods/nematodes: *Wolbachia* spp. | Intracellular transmission via egg cytoplasm | Fertility and sex determination | Veneti et al. (2005) |
| Drosophila: *Vibrio plantarum* | Feces of mother placed on egg mass | Mating preference | Sharon et al. (2010) |
| Stinkbug midgut: *Burkholderia* | Specific transmission via environment | More efficient food utilization | Kikuchi et al. (2007) |
| Squid nidamental gland: Microbiota | Via cover of eggs originating from the gland | Protection of eggs and embryos against pathogens | Kaufman et al. (1998), Barbieri et al. (2001) |
| Squid light organ: *Vibrio fischeri* | Environmental from surrounding water | Camouflage against predators | McFall-Ngai (1999) |
| Corals: Microbiota | From the environment and by vegetative reproduction | Photosynthesis (intracellular algae); nitrogen fixation; protection against pathogens | Rohwer et al. (2002), Buddemeier et al. (2004), Rosenberg et al. (2007) |
| Sponges: Microbiota | Environmental in addition to possible transmission from parent | Breakdown of complex polymers; nitrogen cycling; protection against pathogens | Webster et al. (2001), Hickman (2005), Taylor et al. (2007) |
| **Vertebrates** | | | |
| Cow rumen: Microbiota | Physical contact with parents and via grass contaminated with feces and sputum | Provision of all nutritional needs from cellulose | Dehority (2003), Russell and Rychlik (2001) |
| Whale forestomach: Microbiota | Physical contact with mother | Provision of nutrition from chitin and other complex organics | Herwig et al. (1984), Olsen et al. (2000) |
| Human gut and mouse model: Microbiota | Via passage through the birth canal, physical contact and from environment | Protection against pathogens; stimulation of immune system; angiogenesis; vitamin synthesis; fiber breakdown; fat metabolism, obesity and related disorders | Silva et al. (2004), Stecher and Hardt (2008), Stappenbeck et al. (2002), O'Hara and Shanahan (2006), Mai et al. (2010), Dethlefsen et al. (2006), Ley et al. (2006a), Cani and Delzenne (2009) |
| **Plants** | | | |
| Legume plants: Rhizobium | Environmental from surrounding | Nitrogen fixation | Stougaard (2000), Jones et al. (2007), Ott et al. (2009) |
| Plant: Growth promoting rhizobacteria | Environmental from surrounding soil | Protection against pathogens; nitrogen metabolism; acceleration of mineralization; carbon cycling; salt tolerance | Singh et al. (2004), Lugtenberg and Kamilova (2009), Lindow and Brandle (2003), Whipps et al. (2008) |
| Rice plants: *Azoarcus* sp. | From surrounding soil | Associative nitrogen fixation | Hurek and Reinhold-Hurek (2003) |

*Lactobacillus plantarum*, and that this bacterium was responsible for the mating preference (Sharon et al. 2010). The combination of partial geographic separation and bacterial-induced mating preference could reduce interbreeding of the populations. Slower changes in the host genome would further enhance the mating preference. The stronger the mating preference, the greater the chance that two populations will become sexually isolated, and biologists (Coyne 1992; Schluter 2009) have argued that the emergence of sexual isolation is the central event in the evolution of species. These data strongly support the hologenome theory of evolution which posits that microorganisms can play a key role in the evolution of animals and plants (Zilber-Rosenberg and Rosenberg 2008).

### Squid Light Organ–*Vibrio* Symbiosis

The symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri* is one of best studied systems that demonstrate how a bacterial symbiont can play a role in the development of an animal organ (Ruby 1996; Nyholm and McFall-Ngai 2004). Following fertilization of the eggs within the female, the embryos develop an immature light organ that is free of bacteria but has three pores leading to separate epithelial-lined crypts. The female host lays clutches of hundreds of eggs, which hatch almost synchronously at dusk. Within hours after hatching, the juvenile squid becomes colonized by *V. fischeri*, which triggers morphogenesis of the light organ (Montgomery and McFall-Ngai 1994).

### Coral: Bacteria Symbiosis

The hundreds of different bacterial species that are associated with the coral mucus, tissues, and skeleton are essential for coral health. A substantial part of the coral nitrogen requirement is provided by nitrogen-fixing coral bacteria (Shashar et al. 1994). Some of the bacterial symbionts degrade complex polysaccharides, such as chitin (Ducklow and Mitchel 1979), thereby providing nutrients to the coral holobiont; others are able to protect the coral against pathogens by producing antibiotics (Ritchie 2006; Nissimov et al. 2009; Shnit-Orland and Kushmaro 2009).

Microbial symbionts of corals also play a major role in adaptation to changing environmental conditions. When seawater temperature exceeds the normal maximum by a few degrees, corals lose their symbiotic zooxanthellae, a process referred to as bleaching. If the process is not reversed in a reasonable time, the coral will die. The adaptive hypothesis of coral bleaching (Buddemeier et al. 2004) puts forth the concept that expulsion of the algae allows more temperature-resistant zooxanthellae to infect the coral and establish a more favorable symbiosis. Another adaptive process of bleached corals is the amplification of cyanobacteria in the coral skeleton. The photosynthetic products of these bacteria are transferred to the host and have been suggested to help them survive the bleaching episodes (Fine and Loya 2002).

## Vertebrates

Symbioses between diverse microbiota and vertebrates have been studied in a variety of animals, including ruminants (Dehority 2003), chickens (Abbas Hilmi et al. 2007), whales (Olsen et al. 1994), gorillas (Frey et al. 2006), and rats (Brooks et al. 2003). We would like to present here briefly what is probably the best studied metabolic system in vertebrates, namely, the human/mouse gut symbiosis. This system has provided a wealth of detailed information on how diverse extracellular symbionts contribute to the health of animal holobionts. The number of bacteria in and on a typical healthy human is $10^{14}$ to $10^{15}$. Most of these microbes are not merely in transit but rather inhabit defined niches. Although about 90% of the human microbiota are found in the gastrointestinal tract, there is a high abundance and diversity of microbes on all surfaces of the human body, including skin, oral cavity, nasal cavity, pharynx, esophagus, and urogenital tract.

Protection against infectious disease is one of the important attributes of the resident microbiota. Most bacterial pathogens infect their human hosts predominantly via mucosal surfaces of the respiratory, urogenital, or gastrointestinal tracts. In addition to mechanical and immunological barriers, mucosal surfaces are protected against pathogen infection by the high concentration of microbiota colonizing the mucosa. The exact mechanism is unknown, but it has been suggested that resident bacteria occupy binding sites needed by pathogens for adhesion in addition to releasing antibacterials active against pathogens (Belden and Harris 2007). As an experimental example of bacterial protection against infection, mice were treated with *Bifidobacterium longum*, part of the normal microbiota, and then infected with the pathogen *Salmonella typhimurium*. The mice that received *B. longum* survived, whereas the control group (Salmonella alone) all died within a few days (Silva et al. 2004). Interestingly, it has been demonstrated that immune response to integral microbiota via IgM differs from its reaction to pathogenic microorganisms (Hapfelmeier et al. 2010).

Another important known beneficial function of microbiota is participation in the development and normal function of the innate and adaptive immune systems in the gut (O'Hara and Shanahan 2006; Ivanov and Littman 2011) while creating a permissive, noninflammatory environment for their own presence (Hapfelmeier et al. 2010). The gut, in spite of providing a diversity of niches for microbial colonization, imposes strict requirements for their survival, the important ones being adaptation to digestive enzymes, evading the potent innate and adaptive immune systems, escaping washout from the gut, and the ability to live anaerobically. These strict requirements will force a narrowing of the variability of microorganisms, leaving only those that are able to create a viable and well-adapted holobiont with their host. Bacteria are critical in promoting the early development of the gut's mucosal immune system both in terms of its physical components and its function and continue to play a role later in life in its operation. The microbiota also plays a key role in angiogenesis, the structural buildup of blood vessels (Stappenbeck et al. 2002). Bacteria

found in the gut synthesize and excrete vitamins in excess of their own needs, which can be absorbed as nutrients by their host. For example, in humans, enteric bacteria secrete Vitamin K and Vitamin B12, and lactic acid bacteria produce certain B vitamins (Mai et al. 2010).

Moreover, germ-free animals are deficient in Vitamin K to the extent that it is necessary to supplement their diets (Komai et al. 1988). The human gut microbiota is a complex ecosystem that plays an essential role in the catabolism of dietary fibers, the part of plant material in our diet that is not metabolized in the upper digestive tract, because the human genome does not encode adequate enzymes (Dethlefsen et al. 2006).

Germ-free animals, born and grown under sterile conditions, are a useful tool for studying the relationship between host and its microbiota. Studies on germ-free mice exhibit significant differences in gut development, function, and regulation when compared with mice grown conventionally, i.e., possessing normal gut microbiota. The germ-free mice demonstrate enlarged ceca (Wostmann 1981), a slow digested food transit time (Abrams and Bishop 1967), altered kinetics of epithelia turnover in the small intestine (Savage et al. 1981), an increased caloric intake (Wostmann et al. 1983), and a greater susceptibility to infection (Silva et al. 2004). The influence of microbiota on energy metabolism in germ-free conventionalized mice was observed within 2 weeks of the introduction of microbiota (Bäckhed et al. 2007). It included microbial fermentation of polysaccharides not digested by the host, absorption of the microbially produced short-chain fatty acids, more efficient absorption of the monosaccharides from the intestine, conversion of breakdown products in the liver to more complex lipids, and microbial regulation of host genes that promote fat deposition in adipocytes. These events were accompanied by lower food intake and a higher metabolic rate. It has been shown in mice (Ley et al. 2005; Turnbaugh et al. 2006) and humans (Ley et al. 2006b) that obesity is correlated with different bacterial communities and that a gradual transition occurs in humans from the obese microbiota to the lean microbiota during a course of a restrictive energy intake (Ley et al. 2006b). Moreover, obese microbiota have been implicated in obesity-related metabolic disorders such as type 2 diabetes, inflammation, disordered lipid metabolism, and atherosclerosis, in addition to fatty liver, primarily via bacterial Gram-negative LPS (lipopolysaccharide) metabolic effects (Cani and Delzenne 2009; Caesar et al. 2010; Abu-Shanab et al. 2010).

## Plant: Microbe Symbioses

Plant abundance, diversity, and activities are essential for life on our planet, and microorganisms play a central role in all three phenomena. Microorganisms supply plants with nutrients, play a role in establishment of plants and the development of root systems and in protection against pathogens and other environmental stress conditions. Moreover, it is estimated that about 20,000 species of plants are obligatorily dependant on microbial cooperation for development, growth, and survival (van der Heijden et al. 2008).

Studies on the microbiology of plants have been performed with microorganisms found in three main locations: around the roots (rhizosphere), on the leaves, stems, flowers, and fruit (phyllosphere), and inside plant cells (endophytes). The great majority of microorganisms have different degrees of beneficial relationships with plants, whereas only a small minority is parasitic. The close cooperation between plants and microorganisms necessitates overcoming the plant's immune response and often using some of its components together with other elements of the plant and some functions of the microbiota for enabling this interaction to occur (Bucher et al. 2009; Bednarek et al. 2010).

In addition to fungi, many bacterial species interact with plant roots. The multitude of bacterial species contribute to carbon transfer to soil, nitrogen fixation, nitrate reduction, mineralization of organic materials, maintenance of soil structure and water cycling, protection against pathogens and other stress conditions, all of which promote plant growth directly or indirectly (Singh et al. 2004; Lugtenberg and Kamilova 2009). Rhizosphere microbial communities differ between plant species (Innes et al. 2004; Berg and Smalla 2009), between ecotypes within species (Micallef et al. 2009), between different developmental stages of a given plant (Weisskopf et al. 2006), and from those present in bulk soil (Broz et al. 2007). Microorganisms in the rhizosphere are selected for their functional abilities no less than for their taxonomy (Singh et al. 2004). Moreover, it has been shown that plant's specific exudates are major contributors to the plant specificity of rhizosphere microbiota (Somers et al. 2004; Singh et al. 2004; Berg and Smalla 2009).

Large populations of microorganisms live also in the phyllosphere. Archaea, filamentous fungi, and yeast are present in the phyllosphere, but bacteria are considered to be the dominant microbial inhabitants present on the plant surface and within the plant tissue (Whipps et al. 2008). Stressful conditions on the leaves, such as extreme temperatures and dryness, irradiation, and oxidative stress in addition to poor nutrient availability, determine the kinds of bacteria, their mode of growth, and their activities (Lindow and Brandl 2003). Most information on the microbial communities in the phyllosphere has been established using culture-dependant methods, and much of it is on pathogenic bacteria and fungi. The global surface area of the phyllosphere, estimated to be $4 \times 10^8$ km$^2$, harbors a bacterial population in the region of $10^{26}$ cells including $2–3 \times 10^6$ species (Whipps et al. 2008). Interestingly, culture-independent techniques have revealed that similarly to the human gut, these species fall within a relatively small number of dominant phyla, the proteobacteria being the most abundant on leaves (Delmotte et al. 2009; Redford et al. 2010). This phenomenon is in accordance with the special conditions known to occur in the phyllosphere, demanding specific adaptations and activities (Delmotte et al. 2009). Given the high mass of phyllosphere microbiota, it is likely that they play an important role in global transformation of matter, including recycling of carbon and nitrogen. In addition, they contribute to the plant's fitness mainly through spatial protection against pathogens, promotion

of growth, and deterrence of herbivores (Lindow and Brandl 2003; Whipps et al. 2008). Microorganisms are unevenly distributed, mainly on the lower part of leaves, as single or aggregated microorganisms (Whipps et al. 2008). Culture-independent methods have shown that by and large community pattern of the phyllosphere bacteria correlates with the tree phylogeny even across continents (Yang et al. 2001; Redford et al. 2010), though not all studies are in agreement (Whipps et al. 2008). In addition, though bacterial leaf communities differ between seasons, similar ones are found on leaves sampled during the same season, and this pattern is predictable from year to year (Ercolani 1991; Redford and Fierer 2009).

The best studied plant: bacteria symbiosis involves nitrogen-fixing legume holobionts. Several specialized kinds of bacteria, including the most studied, *Rhizobium*, engage in symbiotic relationships with peas, soybeans, and other legumes to convert nitrogen gas into ammonia and further into organic nitrogen-containing compounds. *Rhizobia* are highly specific for their plant host. Their specificity arises, in part, from chemical "cross-talk" between the bacteria and plant (Hardison 1996). Thus, a two-way conversation between the bacterium and its plant host is responsible for the development of the nodule and its nitrogen-fixing capability. The Rhizobium–legume symbiosis is discussed in detail in another chapter of this section of the book.

## Transmission of Symbionts Between Holobiont Generations

The hologenome theory of evolution relies on ensuring the continuity of partnerships between holobiont generations. *Table 15.2* presents some of the diverse modes of transmission of symbionts in animals and plants. Mitochondria and chloroplasts are transmitted by the most direct mode, namely, cytoplasmic inheritance. Direct transmission from parent to offspring also occurs with other symbionts where the microorganisms are in or on the reproductive cells. For example, in the aphid–*Buchnera* symbiosis, bacteria are intracellularly situated in bacteriocytes and are transferred to and transmitted via the eggs (Baumann et al. 2006). Another slightly less direct mode of transmission is used in the termite hindgut: microbiota symbiosis where feces of adult termites (containing abundant microorganisms) are fed to newly hatched juveniles by workers in the colony (Abe et al. 2000). Similarly, in the bovine rumen–microbiota symbiosis, the offspring acquire the microbiota by feeding on grass that is contaminated with feces and sputum from their parents, as well as by passage through the birth canal (Dehority 2003). In general, inoculating newborns with the feces of their parents is widespread in the animal world.

A less direct, but precise, mode of transmission is exemplified in the squid light organ: *Vibrio fischeri* symbiosis where the high specificity of the light organ for *V. fischeri* has evolved together with the need to acquire the motile bacteria from the surrounding seawater. The adult squid releases large amounts of *V. fischeri* into the water at dawn every day, assuring that sufficient symbionts are available to colonize the hatchlings (McFall-Ngai 1999). Furthermore, the squid provides a habitat in which only *V. fischeri* that emits light is able to maintain a stable association (McFall-Ngai 1999; Visick et al. 2000). Thus, even in transfer via the environment (often referred to as horizontal transfer), the holobiont is reconstituted faithfully.

Direct contact is another slightly less direct mode of transmission demonstrated in mammals in which many of the symbionts are derived during passage through the birth canal or subsequently by close physical contact with parent or family and community members. In humans, for example, a greater similarity was observed within-family members as compared to between families (Zoetendal et al. 2001) and within the same European population as compared with between different European populations (Mueller et al. 2006).

Some animals and most plants can develop from cells other than gametes, namely, from somatic cells. The most striking example is vegetative reproduction in plants. When a fragment of a plant falls to the earth, it may root and grow into a fully developed plant. In such cases, it will clearly contain some of the symbionts of the original plant (direct transfer). In addition, it will most likely incorporate microorganisms from the soil adjacent to the parent.

Most studies demonstrating the transfer of symbionts from parent to offspring are short term. However, there is also evidence that symbionts are maintained for many generations. Fraune and Bosch (2007) demonstrated the specificity and long-term accuracy of transmission in the metazoan Hydra. Similarly to many plants, Hydra reproduce vegetatively (by budding) and sexually. The researchers showed, first, that two different species of Hydra were colonized by different communities of microorganisms and, second, in both cases, the two species of Hydra were populated with similar microorganisms both in the laboratory and in nature, even after more than 30 years of maintaining the animals in the laboratory. In addition, it has been reported recently that the gut microbiota among great ape species is phylogenetically conserved over evolutionary timescales and has diverged in a manner consistent with vertical inheritance (Ochman et al. 2010). In humans, the conservation of specific strains of *Helicobacter pylori* between generations has been used as a window into human migration (Devi et al. 2006).

## Genetic Variation in Holobionts

Genetic variation is the raw material for evolution. Genetic variation in holobionts can arise from changes in either the host or the symbiotic microbiota genomes. Variation in host genomes occurs during sexual reproduction, chromosome rearrangements, and ultimately by mutation. These same processes occur in microorganisms with the noteworthy difference that in haploid bacteria, recombination occurs, within the same species, by conjugation, transduction, and DNA transformation, and, between species, by horizontal gene transfer. In addition, changes in the hologenome of the holobiont can occur by two other processes that are specific to holobionts: microbial amplification and acquisition of novel strains from the environment.

Microbial amplification is the most rapid and easy to understand mode of variation in holobionts. It involves changes in the relative numbers of the diverse types of associated microorganisms that can occur as a result of changing temperatures (for plants and invertebrates), nutrient availability or quality, exposure to antibiotics, or other environmental factors. The holobiont is a dynamic entity with certain microorganisms multiplying and others decreasing in number as a function of local conditions. Accordingly, an increase in the number of a particular microbe is equivalent to gene amplification. Considering the large amount of genetic information encoded in the diverse microbial population of holobionts, as mentioned above, microbial amplification is a powerful mechanism for adapting to changing conditions. In fact, changes of symbiont populations as a function of external factors are well documented (Weimer et al. 1999; Russell and Rychlik 2001; de la Cruz and Davies 2005; Koren and Rosenberg 2006; Blaser and Falkow 2009). Acquiring new symbionts from the environment is another mechanism for introducing variation into holobionts. Animals and plants come in contact with billions of microorganisms during their lifetime. It is reasonable to assume that occasionally, as a random event, some of these microorganisms will find a niche and become established in the host. Unlike microbial amplification, acquiring novel symbionts can introduce entirely new genes into the holobiont. Under the appropriate conditions, the new symbionts may become more abundant and affect the phenotype of the holobiont. It is often technically difficult to distinguish between amplification of a low abundant indigenous microbe and acquisition from the environment.

## Prebiotics and Probiotics as Applications of the Hologenome Concept

Prebiotics and probiotics are two technological applications of the hologenome concept (Zilber-Rosenberg and Rosenberg 2011). Prebiotics has been defined as follows: "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Gibson et al. 2004). The implication of this definition is that prebiotics in the form of functional carbohydrates change the gut microbiota and its metabolic activity similarly to natural fiber present in the normal human diet, except that prebiotics are targeted at specific bacteria and are degraded in a specific form and therefore are more controlled. In essence, prebiotics cause variation in the holobiont by amplification of beneficial bacteria. Both fiber and prebiotics have been shown to exert health benefits, the main ones being: improving or stabilizing microbiota, improvement of intestinal function, increase of mineral absorption, modulation of gastrointestinal excretions, improving energy metabolism and satiety, modulation of intestinal immune functions, and reduction in the risk of intestinal infection.

Probiotics have been defined in different ways since 1965 when initially proposed. The following accepted definition of probiotics appears to capture our current knowledge of the subject: "Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Hoffman et al. 2008). Unlike variation of the hologenome by acquisition of microbes from the environment, probiotic technology involves the nonrandom introduction of specific bacteria to improve the health of the host. The effects can even be transferred to the next generation as was shown in a study (Luoto et al. 2010a, b) where pregnant women treated with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* had reduced frequency of gestational diabetes mellitus and diminished risk of larger birth size in affected cases. In view of the fact that large birth size is a risk factor for later obesity, the present results are of significance for public health in demonstrating that this risk is not only modifiable but also transferred from one generation to the next. Going one step further, we speculate that since probiotics can be transmitted to future generations, they could, in principle, be used to treat not only metabolic and alimentary tract diseases such as diabetes, coronary heart disease, and alimentary tract diseases, but also genetic and behavioral human diseases. In addition, probiotics could also be used more intensely in agriculture to combat diseases and improve yields and achieve specific characteristics in plants and animals.

## References


Abbas Hilmi HT, Surakka A, Apajalahti J, Saris PE (2007) Identification of the most abundant Lactobacillus species in the crop of 1- and 5-week-old broiler chickens. Appl Environ Microbiol 73:7867–7873

Abe T, Bignell DE, Higashi M (eds) (2000) Termites: evolution, sociality, symbioses, ecology. Kluwer, Dordrecht

Abrams GD, Bishop JE (1967) Effect of normal microbial flora on gastrointestinal motility. Proc Soc Exp Biol Med 126:301–304

Abu-Shanab A, Eamonn MM, Quigley EMM (2010) The role of the gut microbiota in nonalcoholic fatty liver disease. Nat Rev Gastro Hepatol 7:691–701

Akman LA, Yamashita H, Watanabe A et al (2002) Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glosssinidia*. Nat Genet 32:402–407

Andersson SGE, Zomorodipour A, Andersson JO (1998) The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. Nature 396:133–143

Barbieri E, Paster BJ, Hughes D, Zurek L, Moser DP, Teske A, Sogin ML (2001) Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules of the squid Loligo pealei (Cephalopoda: Loliginidae). Environ Microbiol 3:151–167

Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI (2007) Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci USA 104:979–984

Baumann P, Moran NA, Baumann L (2006) Bacteriocyte-associated endosymbionts of insects. In: Dworkin M, Rosenberg E, Schleifer KH, Stackebrandt E (eds) The prokaryotes, vol 1. Springer, New York, pp 403–438

Bednarek P, Kwon C, Schulze-Lefert P (2010) Not a peripheral issue: secretion in plant–microbe interactions. Curr Opin Plant Biol 13:378–387

Belden LK, Harris RN (2007) Infectious diseases in wildlife: the community ecology context. Front Ecol Environ 5(10):533–539

Berg G, Smalla K (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol Ecol 68:1–13

Blaser MJ, Falkow S (2009) What are the consequences of the disappearing human microbiota? Nature Rev Microbiol 7:887–894

Breibart M, Hewson I, Felts B et al (2003) Metagenomic analysis of an uncultured viral community from human feces. J Bacteriol 185:6220–6223

Breznak JA, Brune A (1994) Role of microorganisms in the digestion of lignocellulose by termites. Ann Rev Entomol 39:453–487

Brooks SPJ, McAllister M, Sandoz M, Kalmokoff ML (2003) Culture-independent phylogenetic analysis of the faecal flora of the rat. Can J Microbiol 49:589–601

Broz AK, Manter DK, Vivanco JM (2007) Soil fungal abundance and diversity: another victim of the invasive plant *Centaurea maculosa*. ISMEJ 1:763–765

Bucher M, Wegmüller S, Drissner D (2009) Chasing the structures of small molecules in arbuscular mycorrhizal signaling. Curr Opin Plant Biol 12:500–507

Buddemeier RW, Baker AC, Fautin DG, Jacobs JR (2004) The adaptive hypothesis of bleaching. In: Rosenberg E, Loya Y (eds) Coral health and disease. Springer, New York, pp 427–444

Caesar R, Fåk F, Bäckhed F (2010) Effects of gut microbiota on obesity and atherosclerosis via modulation of inflammation and lipid metabolism. J Intern Med 268:320–328

Cani PD, Delzenne NM (2009) The role of the gut microbiota in energy metabolism and metabolic disease. Curr Pharm Des 15:1546–1558

Cankar K, Kraigher H, Ravnikar M, Rupnik M (2005) Bacterial endophytes from seeds of Norway spruce (*Picea abies* L. Karst). FEMS Microbiol Lett 244:341–345

Chelius MK, Triplett EW (2001) The diversity of Archaea and bacteria in association with the roots of *Zea mays*. Microb Ecol 41:252–263

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995) Microbial biofilms. Annu Rev Microbiol 49:711–745

Coyne JA (1992) Genetics and speciation. Nature 355:511–515

Currie CR, Poulsen M, Mendenhall J et al (2006) Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science 311:81–83

Davidov Y, Jurkevitch E (2009) Predation between prokaryotes and the origin of eukaryotes. Bioessays 31:748–757

Dedeine F, Vavre F, Fleury F et al (2001) Removing symbiotic Wolbachia bacteria specifically inhibits oogenesis in a parasitic wasp. Proc Natl Acad Sci USA 98:6247–6252

Dehority BA (2003) Rumen microbiology. Nottingham University Press, Nottingham

de la Cruz F, Davies J (2005) Industrial revolution and microbial evolution. In: McFall-Ngai MJ, Henderson B, Ruby EG (eds) (2005) The influence of cooperative bacteria on animal host biology. Cambridge University Press, New York, pp 73–82

Delmotte N, Knief C, Chaffron S et al (2009) Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proc Natl Acad Sci USA 106:16428–16433

Dethlefsen L, Eckburg PB, Bik EM, Relman DA (2006) Assembly of the human intestinal microbiota. Trends Ecol Evol 21:517–523

Devi SM, Ahmed I, Khan AA et al (2006) Genomes of *Helicobacter pylori* from native Peruvians suggest a mixture of ancestral and modern lineages and reveal a western type cag-pathogenicity island. BMC Genomics 7:191

Dewhirst FE, Chen T, Izard J et al (2010) The human oral microbiome. J Bacteriol 192:341–345

Dodd DMB (1989) Reproductive isolation as a consequence of adaptive divergence in *Drosophila pseudoobscura*. Evolution 43:1308–1311

Douglas AE (1998) Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. Annu Rev Entomol 43:17–37

Ducklow HW, Mitchel R (1979) Bacterial populations and adaptations in the mucus layers on living corals. Limnol Oceanogr 24:715–725

Edwards JE, McEwan NR, Travis AJ, Wallace RJ (2004) 16S rDNA library-based analysis of ruminal bacterial diversity. Anton Van Leeuw 86:263–281

Ercolani GL (1991) Distribution of epiphytic bacteria on olive leaves and the influence of leaf age and sampling time. Microb Ecol 21:35–48

Falcon L, Magallon S, Castillo A (2010) Dating the cyanobacterial ancestor of the chloroplast. ISME J 4:777–783

Fine M, Loya Y (2002) Endolithic algae: an alternative source of photoassimilates during coral bleaching. Proc R Soc Lond B 269:1205–1210

Fraune S, Bosch TCH (2007) Long-term maintenance of species-specific bacterial microbiota in the basal metazoan Hydra. Proc Natl Acad Sci USA 104:13146–13151

Frey JC, Rothman JM, Pell AN et al (2006) Fecal bacterial diversity in a wild gorilla. Appl Environ Microbiol 72:3788–3792

Gibson GR, Probert HM, Van Loo J, Rastall RA, Roberfroid MB (2004) Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev 17(2):259–275

Gilbert SF, McDonald E, Boyle N et al (2010) Symbiosis as a source of selectable epigenetic variation: taking the heat for the big guy. Philos Trans R Soc Lond B 365:671–678

Golichenkov MV, Kostina NV, Ul'yanova TA et al (2002) Specific features of nitrogen fixation and denitrification in termites *Neotermes castaneus*, *Zootermopsis angusticollis*, and *Reticulitermes lucifugus*. Biol Bull 29:172–175

Grice EA, Kong HH, Contan S et al (2009) Topographical and temporal diversity of the human skin microbiome. Science 324:1190–1192

Hapfelmeier S, Lawson MAE, Slack E et al (2010) Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science 328:1705–1709

Hardison RC (1996) A brief history of hemoglobins: plant, animal, protist, and bacteria. Proc Natl Acad Sci USA 93:5675–5679

Herwig RP, Staley JT, Nerini MK, Braham HW (1984) Baleen whales: preliminary evidence for forestomach microbial fermentation. Appl Environ Microbiol 47:421–423

Hickman CS (2005) How have bacteria contributed to the evolution of multicellular animals? In: McFall-Ngai MJ, Henderson B, Ruby EG (eds) The influence of cooperative bacteria on animal host biology. Cambridge University Press, New York, pp 3–33

Hoffman FA, Heimbach JT, Sanders ME, Hibberd PL (2008) Executive Summary: Scientific and regulatory challenges of development of probiotics as food and drugs. Clinical Infect Dis 46:S53–S57

Hongoh Y, Deevong P, Inoue T et al (2005) Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. Appl Environ Microbiol 71:6590–6599

Hooper LV, Gordon JI (2001) Commensal host-bacterial relationships in the gut. Science 292:1115–1118

Hurek T, Reinhold-Hurek B (2003) *Azoarcus* sp. strain BH72 as a model for nitrogen-fixing grass endophytes. J Biotechnol 106:169–178

Innes L, Hobbs PJ, Bardgett RD (2004) The impacts of individual plant species on rhizosphere microbial communities in soils of different fertility. Biol Fertil Soils 40:7–13

Ivanov II, Littman DR (2011) Modulation of immune homeostasis by commensal bacteria. Curr Opin Microbiol 14:106–114

Iwanaga S, Lee BL (2005) Recent advances in the innate immunity of invertebrate animals. J Biochem Mol Biol 38:128–150

Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007) How rhizobial symbionts invade plants: the *Sinorhizobium medicago* model. Nat Rev Microbiol 5:619–633

Kaufman MR, Ikeda Y, Patton C, Van Dykhuizen G, Epel D (1998) Bacterial symbionts colonize the accessory nidamental gland of the squid *Loligo opalescens* via horizontal transmission. Bio Bull 194:36–43

Kikuchi Y, Hosokawa T, Fukatsu T (2007) Insect–microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. Appl Environ Microbiol 73:4308–4316

Komai M, Shirakawa H, Kimura S (1988) Newly developed model for vitamin K deficiency in germfree mice. Int J Vitam Nutr Res 58(1):55–59

Koren O, Rosenberg E (2006) Bacteria associated with mucus and tissues of the coral *Oculina patagonica* in summer and winter. Appl Environ Microbiol 72:5254–5259

Lambais MR, Crowley DE, Cury JC, Büll RC, Rodrigues RR (2006) Bacterial diversity in tree canopies of the Atlantic Forest. Science 312:1917

Leser TD, Amenuvor JZ, Jensen TK et al (2002) Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl Environ Microbiol 68:673–690

Ley RE, Bäckhed F, Turnbaugh P et al (2005) Obesity alters gut microbial ecology. Proc Natl Acad Sci USA 102:11070–11075

Ley RE, Peterson DA, Gordon JI (2006a) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124:837–848

Ley RE, Turnbaugh PJ, Klein S, Gordon JI (2006b) Human gut microbes associated with obesity. Nature 444:1022–1023

Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI (2008) Worlds within worlds: evolution of the vertebrate gut microbiota. Nature Rev Microbiol 6:776–788

Lindow SE, Brandl MT (2003) Microbiology of the phyllosphere. Appl Environ Microbiol 69:1875–1883

Lugtenberg B, Kamilova F (2009) Plant-growth-promoting Rhizobacteria. Annu Rev Microbiol 63:541–556

Luoto R, Laitinen K, Nermes M, Isolauri E (2010a) Impact of maternal probiotic-supplemented dietary counseling on pregnancy outcome and prenatal and postnatal growth: a double-blind, placebo-controlled study. Br J Nutr 103:1792–1799

Luoto R, Kalliomaki M, Laitinen K, Isolalauri E (2010b) The impact of perinatal intervention on the development of overweight and obesity: follow-up study from birth to 10 years. Int J Obes 34:1531–1537

Mai V, Ukhanova M, Baer DJ (2010) Understanding the extent and sources of variation in gut microbiota studies; a prerequisite for establishing associations with disease. Diversity 2:1085–1096

Margulis L (1993) Symbiosis in cell evolution: microbial communities in the Archean and Proterozoic eons, 2nd edn. Freeman, New York

Margulis L, Sagan D (2001) Marvellous microbes. Resurgence 206:10–12

Mart´ınez-Garc´ıa M, D´ıaz-Vald´ez M,Wanner G et al (2007) Microbial community associated with the colonial ascidian *Cyctodytes dellechiajei*. Environ Microbiol 9:521–534

Mateos M, Castrezana SJ, Nankivell BJ, Estes AM, Markow TA, Moran NA (2006) Heritable endosymbionts of Drosophila. Genetics 174:363–376

McFall-Ngai MJ (1999) Consequences of evolving with bacterial symbionts: insights from the squid–Vibrio association. Ann Rev Ecol Syst 30:235–256

Micallef SA, Shiaris MP, Colon-Carmona A (2009) Influence of *Arabidopsis thaliana* accessions on rhizobacterial communities and natural variation. J Exp Bot 60:1729–1742

Minkley N, Fujita A, Brune A, Kirchner WH (2006) Nest specificity of the bacterial community in termite guts (*Hodotermes mossambicus*). Insect Soc 53:339–344

Montgomery MK, McFall-Ngai M (1994) Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*. Development 120:1719–1729

Mueller S, Saunier K, Hanisch C et al (2006) Differences in fecal microbiota in different European study populations in relation to age, gender and country: a cross-sectional study. Appl Environ Microbiol 72:1027–1033

Nissimov J, Rosenberg E, Munn C (2009) Antimicrobial properties of resident coral mucus bacteria of *Oculina patagonica*. FEMS Microb Lett 292:210–215

Nyholm SV, McFall-Ngai M (2004) The winnowing: establishing the squid vibrio symbiosis. Nat Rev Microbiol 2:632–642

Ochman HM, Worobey CH, Kuo JB et al (2010) Evolutionary relationships of wild hominids recapitulated by gut microbial communities. PLoS Biol 8: e1000546. online

O'Hara AM, Shanahan F (2006) The gut flora as a forgotten organ. EMBO Rep 7:688–693

Olsen MA, Aagnes TH, Mathiesen SD (1994) Digestion of herring by indigenous bacteria in the minke whale forestomach. Appl Environ Microbiol 60:4445–4455

Ott T, Sullivan J, James EK et al (2009) Absence of symbiotic leghemoglobins alters bacteroid and plant cell differentiation during development of *Lotus japonicus* root nodules. Mol Plant Microbe Interact 22:800–808

Pennisi E (2004) Evolutionary biology. The birth of the nucleus. Science 305:766–768

Portier P (1918) Les symbiotes. Masson, Paris

Qin J, Li R, Raes J et al (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464:59–65

Redford AJ, Fierer N (2009) Bacterial succession on the leaf surface: a novel system for studying successional dynamics. Microb Ecol 58:189–198

Redford AJ, Bowers RM, Knight R et al (2010) The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. Environ Microbiol 12:2885–2893

Reichenbach H (1984) Myxobacteria: a most peculiar group of social prokaryotes. In: Rosenberg E (ed) Myxobacteria: development and cell interactions. Springer, New York, pp 1–50

Reid G, Younes JA, Van der Mei HC et al (2011) Microbiota restoration: natural and supplemented recovery of human microbial communities. Nat Rev Microbiol 9:27–38

Reitner J, Schumann-Kindel G (1997) Pyrite in mineralized sponge tissue-product of sulfate reducing sponge related bacteria. Facies 36:272–276

Ritchie KB (2006) Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. Mar Ecol Prog Ser 322:1–14

Rohwer F, Seguritan V, Azam F, Knowlton N (2002) Diversity and distribution of coral-associated bacteria. Mar Ecol Prog Ser 243:1–10

Rosenberg E, Falkovitz L (2004) The *Vibrio shiloi/Oculina patagonica* model system of coral bleaching. Ann Rev Microbiol 58:143–159

Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I (2007) The role of microorganisms in coral health, disease and evolution. Nat Rev Microbiol 5:355–362

Ruby EG (1996) Lessons from a cooperative bacterial-animal association: the *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. Annu Rev Microbiol 50:591–624

Russell JB, Rychlik JL (2001) Factors that alter rumen ecology. Science 292:1119–1122

Russell JA, Latorre A, Sabater-Muñoz B, Moya A, Moran NA (2003) Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. Mol Ecol 12:1061–1075

Sandström JP, Russel JA, White JP, Moran NA (2001) Independent origins and horizontal transfer of bacterial symbionts of aphids. Mol Ecol 10:217–228

Savage DC, Siegel JD, Snellen JE, Whitt DD (1981) Transit time of epithelial cells in the small intestines of germfree mice and ex-germfree mice associated with indigenous microorganisms. Appl Environ Microbiol 42:996–1001

Schluter D (2009) Evidence for ecological speciation and its alternative. Science 323:737–741

Schmitt-Wagner D, Friedrich MW, Wagner B, Brune A (2003) Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites. Appl Environ Microbiol 69:6007–6017

Schopf JW (1993) Microfossils of the early Archean Apex chert: new evidence of the antiquity of life. Science 260:640–646

Sharon G, Segal D, Ringo JM, Zilber-Rosenberg I, Rosenberg E (2010) Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. Proc Natl Acad Sci USA 107:20051–20056

Sharp KH, Eam B, Faulkner DJ, Haygood MG (2007) Vertical transmission of diverse microbes in the tropical sponge Corticium sp. Appl Environ Microbiol 73:622–629

Shashar N, Cohen Y, Loya Y, Sar N (1994) Nitrogen fixation (acetylene reduction) in stony corals: evidence for coral–bacteria interactions. Mar Ecol Prog Ser 111:259–264

She X, Jiang Z, Clark RA, Liu G et al (2004) Shotgun sequence assembly and recent segmental duplications within the human genome. Nature 431:927–930

Shnit-Orland M, Kushmaro A (2009) Coral mucus-associated bacteria: a possible first line of defense. FEMS Microbiol Ecol 67:371–380

Silva AM, Barbosa FH, Duarte R et al (2004) Effect of *Bifidobacterium longum* ingestion on experimental salmonellosis in mice. J Appl Microbiol 97:29–37

Singh BK, Millard P, Whiteley AS, Murrell JC (2004) Unravelling rhizosphere–microbial interactions: opportunities and limitations. Trends Microbiol 12:386–393

Smith KP, Handelsman J, Goodman RM (1999) Genetic basis in plants for interactions with disease-suppressive bacteria. Proc Natl Acad Sci USA 96:4786–4790

Somers E, Vanderleyden J, Srinivasan M (2004) Rhizosphere bacterial signalling: a love parade beneath our feet. Crit Rev Microbiol 30:205–240

Stappenbeck TS, Hooper LV, Gordon JI (2002) Developmental regulation of intestinal angiogenesis by Peneth cells. Proc Natl Acad Sci USA 99:15451–15455

Stecher B, Hardt WD (2008) The role of microbiota in infectious disease. Trends Microbiol 16:107–114

Stougaard J (2000) Regulators and regulation of legume root nodule development. Plant Physiol 124:531–540

Stow A, Beattie A (2008) Chemical and genetic defenses against disease in insect societies. Brain Behav Immun 22:1009–1013

Sundset MA, Praesteng KE, Cann IK, Mathiesen SD, Mackie RI (2007) Novel rumen bacterial diversity in two geographically separated sub-species of reindeer. Microb Ecol 54:424–438

Tannock G (1995) Normal microflora. Chapman & Hall, London

Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge associated microorganisms: evolution, ecology and biotechnological potentials. Microbiol Mol Biol Rev 71:295–347

Thingstad TF, Lignell R (1997) Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. Aquat Microb Ecol 13:19–27

Turnbaugh PJ, Ley RE, Mahowald MA et al (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444:1027–1031

van der Heijden MGA, Bardgett RD, van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol Lett 11:296–310

Veneti ZL, Reuter M, Montenegro H et al (2005) Interactions between inherited bacteria and their hosts: the Wolbachia paradigm. In: McFall Ngai MJ, Henderson B, Ruby EG (eds) The influence of cooperative bacteria on animal host biology. Cambridge University Press, New York, pp 119–141

Visick KL, Foster J, Doino J, McFall-Ngai MJ, Ruby EG (2000) *Vibrio fischeri* lux genes play an important role in colonization and development of the host light organ. J Bact 182:4578–4586

Wallace RJ (2004) Antimicrobial properties of plant secondary metabolites. Proc Nutr Soc 63:621–629

Warnecke F, Luginbu P, Ivanova N et al (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature 450:560–565

Watanabe H, Tokuda G (2010) Cellulolytic systems in insects. Annu Rev Entomol 55:609–632

Webster NS, Wilson KJ, Blackall LL, Hill RT (2001) Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. Appl Environ Microbiol 67:434–444

Weimer PJ, Waghorn GC, Odt CL et al (1999) Effect of diet on populations of three species of ruminal cellulolytic bacteria in lactating dairy cows. J Dairy Sci 82:122–134

Weisskopf L, Abou-Mansour E, Fromin N et al (2006) White lupin has developed a complex strategy to limit microbial degradation of secreted citrate required for phosphate acquisition. Plant Cell Environ 29:919–927

Weitz JS, Hartman H, Levin SA (2005) Coevolutionary arms races between bacteria and bacteriophage. Proc Natl Acad Sci USA 102:9535–9540

Whipps JM, Hand P, Pink D, Bending GD (2008) Phyllosphere microbiology with special reference to diversity and plant genotype. J Appl Microbiol 105:1744–1755

Wilkinson DM (2001) Mycorrhizal evolution. Trends Ecol Evol 16:64–65

Wilson EO (1992) The diversity of life. W. W. Norton, New York

Wostmann BS (1981) The germ-free animal in nutritional studies. Annu Rev Nutr 1:257–297

Wostmann BS, Larkin C, Moriarty A, Bruckner-Kardoss E (1983) Dietary intake, energy metabolism and excretory losses of adult male germfree Wistar rats. Lab Anim Sci 33:46–50

Yachi S, Loreau M (1999) Biodiversity and ecosystem productivity in a fluctuating environment: the insurance hypothesis. Proc Natl Acad Sci USA 96:1463–1468

Yang CH, Crowley DE, Borneman J, Keen NT (2001) Microbial phyllosphere populations are more complex than previously realized. Proc Natl Acad Sci USA 98:3889–3894

Yen JH, Barr AR (1971) New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipens*. Nature 232:657–658

Zilber-Rosenberg I, Rosenberg E (2008) Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. FEMS Microbiol Rev 32:723–735

Zilber-Rosenberg I, Rosenberg E (2011) Prebiotics and probiotics within the framework of the hologenome concept. J Microb Biochem Technol http://dx.doi.org/10.4172/1948-5948.S1-001

Zoetendal EG, Akkermans ADL, Akkermans-van Vliet WM et al (2001) A host genotype affects the bacterial community in the human gastrointestinal tract. Microb Ecol Health Dis 13:129–134

# 16 Cyanobacterial-Plant Symbioses

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## Introduction

Cyanobacteria are unique in the wide range of symbiotic associations they form with eukaryotic hosts including plants, fungi, sponges, and protists (for reviews, see Adams 2000; Adams and Duggan 2012; Adams et al. 2012; Rai et al. 2000; Rai et al. 2002; Bergman et al. 2003, 2008). Cyanobacteria are photoautotrophs, and in many cases facultative heterotrophs and nitrogen fixers, and can provide nonphotosynthetic hosts with both nitrogen and carbon. Even if the benefit to the host is clear, that to the cyanobacteria is less obvious. They often receive carbon from photosynthetic hosts, but they are capable of carbon fixation themselves. Perhaps, in the enclosed environment provided by the host, a more likely advantage is protection from predation and from environmental extremes, such as high light intensity and desiccation.

The cyanobacterial symbionts of plants all possess at least two essential characteristics—the ability to differentiate heterocysts, which are specialized nitrogen-fixing cells (for reviews, see Adams and Duggan 1999; Zhang et al. 2006), and hormogonia, which are short, gliding filaments that lack heterocysts and provide a means of dispersal (Adams 2000; Meeks 2003, 2009; Gusev et al. 2002; Meeks et al. 2002; Meeks and Elhai 2002; Bergman et al. 2007). The hormogonia serve as the infective agents in most plant symbioses; some plants enhance their chances of infection by producing chemical signals that stimulate hormogonia formation and also chemoattractants that direct hormogonia into the plant tissue. Cyanobacteria are not restricted to the roots of plants but can infect thalli, stems, and leaves. The major hosts are bryophytes (see the section "Cyanobacterial Symbioses with Hornworts and Liverworts" in this chapter), the angiosperm *Gunnera* (see the section "Interactions in the *Nostoc*-*Gunnera* Symbiosis" in this chapter), the aquatic fern *Azolla* (see the section "The *Azolla* Symbiosis" in this chapter), fungi (forming lichens; see the section "Cyanolichens" in this chapter), the fungus *Geosiphon* (see the section "The *Geosiphon pyriformis*: *Nostoc* Endocyanosis and its Relationship to the Arbuscular Mycorrhiza (AM)" in this chapter), and cycads (see the section on "The Cycad Symbioses" in this chapter).

## Cyanobacterial Symbioses with Hornworts and Liverworts

### Bryophyte Symbioses

The division Bryophyta consists of the Hepaticae (liverworts), the Anthocerotae (hornworts), and the Musci (mosses), all of which are small, nonvascular terrestrial plants, some of which form epiphytic or endophytic associations with cyanobacteria, primarily of the genus *Nostoc* (Adams 2002a, b; Meeks 2003; Solheim et al. 2004; Adams et al. 2006, 2012; Adams and Duggan 2008; Bergman et al. 2007, 2008). Moss-associated cyanobacteria are mostly epiphytic (Solheim and Zielke 2002; Solheim et al. 2004; Gentili et al. 2005), apart from those found in two *Sphagnum* species in which the cyanobacteria are found in water-filled, hyaline (dead) cells, where they may be protected from the acidic bog environment (Solheim and Zielke 2002). Even these associations can be considered epiphytic as the hyaline cells are connected via pores to the outside environment. A wide range of cyanobacteria, including members of the non-heterocystous, filamentous genera *Phormidium* and *Oscillatoria* and even the unicellular *Microcystis*, have been found as moss epiphytes (Solheim et al. 2004), although members of the filamentous, heterocyst-producing genera *Nostoc*, *Stigonema*, and *Calothrix* are the most common (DeLuca et al. 2002, 2007; Gentili et al. 2005; Houle et al. 2006). These epiphytic associations will not be discussed further here, but they are of ecological importance as they are commonly the major source of combined nitrogen in ecosystems where mosses are abundant, such as northern hemisphere forests (Zielke et al. 2002, 2005; Solheim and Zielke 2002; Nilsson and Wardle 2005; DeLuca et al. 2008; see also Adams et al. 2012).

In their natural habitat, the liverworts and hornworts grow as a prostrate gametophyte thallus a few centimeters in length, attached to the substratum by primitive roots known as rhizoids. Mature symbiotic colonies can be seen as dark spots 0.5–1.0 mm in diameter within the plant tissue (*Fig. 16.1*). Of the more than 340 liverwort genera, only four are known to develop

**Fig. 16.1**
**The liverwort *Blasia pusilla*, collected from the wild, showing the thick midribs of the thallus surrounded by the dark spots of *Nostoc* colonies (From Adams (2000), with permission)**

associations with cyanobacteria: two (*Marchantia* and *Porella*) forming epiphytic associations and two (*Blasia* and *Cavicularia*) forming endophytic associations (Meeks 1990). Four of the six hornwort genera (*Anthoceros*, *Phaeoceros*, *Notothylas*, and *Dendroceros*) form endophytic associations (Meeks 1990). The epiphytic associations are more common than once thought but are poorly understood (Dalton and Chatfield 1985; Brasell et al. 1986), whereas the endophytic associations have been well studied because of the ease with which they can be grown in the laboratory. The hornworts *Anthoceros* and *Phaeoceros* and the liverwort *Blasia* can all be grown conveniently in shaken liquid culture (*Fig. 16.3b*), with or without their symbiotic partners, and can be readily reinfected with their original partner or with cyanobionts from *Gunnera*, cycads, lichens, and even some free-living strains (Enderlin and Meeks 1983; Meeks 1988, 1990, 2003; Kimura and Nakano 1990; Babic 1996; West and Adams 1997; Adams 2002a, b; Duckett et al. 2004; Adams and Duggan 2008; *Fig. 16.3c*).

## The Symbionts

For a cyanobacterium to establish a successful plant symbiosis, it must possess at least two essential characteristics—the ability to differentiate both heterocysts, which are specialized nitrogen-fixing cells (for reviews, see Adams and Duggan 1999; Zhang et al. 2006), and hormogonia, which are short, gliding filaments that lack heterocysts and provide a means of dispersal (Campbell and Meeks 1989; Johansson and Bergman 1994; Bergman et al. 1996). Heterocysts fix dinitrogen for both partners, and the motile hormogonia, which are a transient phase of the life cycle, enable the otherwise immotile cyanobacterial filaments to gain entry to the plant host (see the section "Bryophyte Structures and Their Infection" in this chapter). The symbiotically competent cyanobacteria are hormogonia-forming strains of mostly the genus *Nostoc*, although *Calothrix* and *Chlorogloeopsis* strains have been shown to reconstitute the symbiosis with *Blasia* and *Phaeoceros* (West and Adams 1997). In the field, a single liverwort or hornwort thallus can become infected by many different *Nostoc* strains (West and Adams 1997; West et al. 1999; Costa et al. 2001; Rasmussen and Nilsson 2002; Adams and Duggan 2008).

Hormogonia differentiation is triggered by environmental stimuli, including the dilution of liquid cultures, or their transfer to solid medium or exposure to red light (Herdman and Rippka 1988; Tandeau de Marsac 1994). Their formation can also be triggered by exudates from plants such as *Anthoceros* (Campbell and Meeks 1989), *Blasia* (Knight and Adams 1996), *Gunnera* (Rasmussen et al. 1994), and wheat roots (Gantar et al. 1993; Knight and Adams 1996). The first 24 h of *Nostoc punctiforme* hormogonia development, induced by hormogonia-inducing factor (HIF, see section "Bryophyte Structures and Their Infection" in this chapter) or combined nitrogen starvation (Campbell et al. 2007, 2008), is characterized by many changes in gene expression, with the transcription of 944 genes upregulated and 856 downregulated (Campbell et al. 2007). The upregulated genes reflect the importance of signal sensing and chemotaxis because a majority of the encoded proteins are involved in signal transduction and transcriptional regulation, and others have putative roles in chemotaxis and pilus biogenesis (Meeks et al. 2001; Klint et al. 2006; Campbell et al. 2007).

Abundant type IV pili (Tfp) cover the surface of *Nostoc* hormogonia but are absent from vegetative cells (*Fig. 16.2*). In a wide range of bacteria, Tfp have roles in adhesion, motility, pathogenesis, and DNA uptake (Mattick 2002; Nudleman and Kaiser 2004; Burrows 2005). Both adhesion (to the plant surface) and motility (together with chemotaxis, to locate the host plant symbiotic structures) are likely to be essential factors in the successful infection of plants. Tfp are involved in motility in some unicellular cyanobacteria (Bhaya 2004) and may also have a role in the gliding of hormogonia, although this is so far unproven (Duggan et al. 2007). In *Nostoc punctiforme*, the mutation of genes such as *pilT* and *pilD*, thought to be involved in Tfp function, greatly reduces the infectivity of the mutant hormogonia in the liverwort *Blasia* (Duggan et al. 2007). However, it is not clear if this is due to loss of motility (and with it, chemotaxis) or interference with another potential function of the pili, such as recognition of, or adhesion to, the plant surface.

The ability of hormogonia to infect a particular host can be affected by subtle aspects of their behavior. For example, the infection frequency of *Nostoc punctiforme* hormogonia in the liverwort *Blasia* is influenced by mutations in *cyaC*, which encodes adenylate cyclase, the enzyme responsible for the

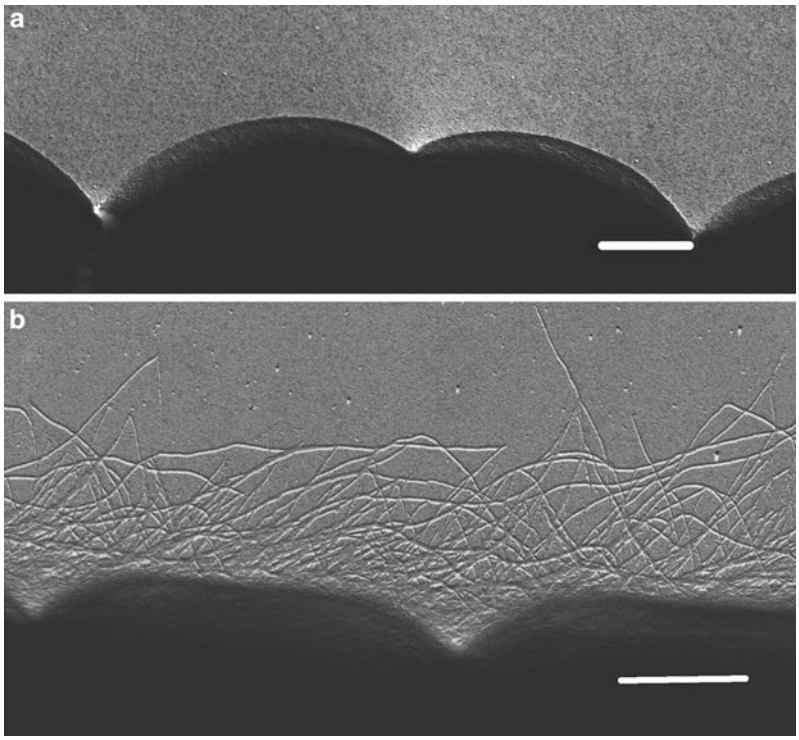



**Fig. 16.2**
**Pili on the surface of *Nostoc punctiforme* hormogonia. Pili are absent from the cell surface of vegetative filaments (a) but are abundant on the surface of hormogonia (b). Scale bars represent 1 μm. For electron microscopy, platinum was evaporated onto the surface of each sample which was then viewed using a JEOL1200EX transmission electron microscope at 80 kV (From Duggan et al. (2007) with permission)**

biosynthesis of the intracellular messenger cAMP, adenosine 3′, 5′-cyclic monophosphate (Adams and Duggan 2008; Chapman et al. 2008). However, mutation in two different domains of this multi-domain enzyme results in hormogonia with very different infection frequencies in *Blasia*, one having a three- to fourfold greater infection frequency than the wild type and the other showing a 75 % reduction in frequency compared with the wild type (Chapman et al. 2008). The explanation of these different infection phenotypes is not readily apparent, as both mutants have cellular cAMP levels 25 % of the wild type, and the mutant hormogonia, induced in the presence of *Blasia*, show no differences in their frequency, motility, or piliation.

## Bryophyte Structures and Their Infection

In the bryophyte-cyanobacteria symbioses, the symbionts infect existing plant structures. In the liverwort *Blasia*, the cyanobacteria occupy roughly spherical structures, known as auricles, on the underside of the thallus (*Fig. 16.3c, d*). These develop from a three-celled mucilage hair that undergoes extensive elaboration (Renzaglia et al. 2000). The thallus of the hornworts *Anthoceros* and *Phaeoceros* is much thicker than that of *Blasia*, and the cyanobacteria are found in slime cavities, within the thallus, that open to the ventral surface via slit-like pores or mucilage clefts (*Fig. 16.3a*). The mucilage clefts, which resemble stomata but are not thought to be related (Villarreal and Renzaglia 2006), are formed by the separation of adjacent epidermal cells, and their formation is followed by the development of a slime cavity directly beneath the cleft (Renzaglia et al. 2000). *Blasia* auricles have two slime papillae, one of which (the inner slime papilla) partly fills the auricle cavity, whereas the other (the outer slime papilla) arises from the thallus adjacent to the auricle (*Fig. 16.3d*). In the hornwort *Leiosporoceros dussii* (*Fig. 16.4a*), the slime cavities take the form of elongated mucilage-filled "canals" (*Fig. 16.4b*) that result from the separation of plant cell walls along their middle lamellae and are connected to the outside by mucilage clefts (*Figs. 16.4c, d*) through which *Nostoc* can gain entry. Branching of the canals results in an integrated network, enabling the symbiont to invade the whole thallus (Villarreal and Renzaglia 2006). The cyanobacteria enter *Blasia* auricles, and presumably hornwort slime cavities, as hormogonia (see the preceding section "The Symbionts"), whereupon they lose motility and differentiate heterocysts (Kimura and Nakano 1990; Babic 1996).

## Bryophyte-Cyanobacterium Signal Exchange

*Anthoceros punctatus* releases an unidentified, low-molecular-mass, heat-labile product that stimulates

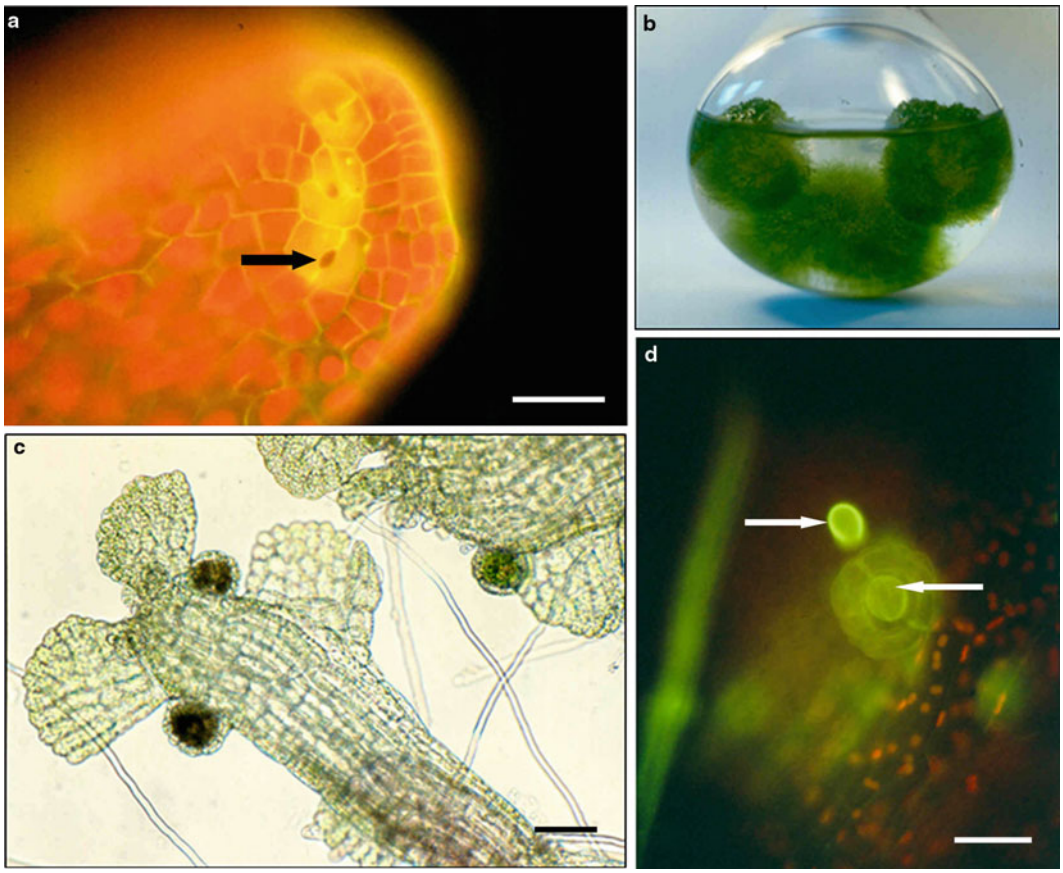



**Fig. 16.3**

**The hornwort and liverwort symbioses. (a) Fluorescence micrograph of the hornwort *Phaeoceros* sp. stained with calcofluor. Hormogonia gain entry to the slime cavities within the thallus via slit-like entrances (one of which is *arrowed*). (b) View of the liverwort *Blasia pusilla* grown free of cyanobacteria in shaken liquid medium in an Erlenmeyer flask (viewed from *below*). (c) Liquid-grown *Blasia pusilla* infected in the laboratory with two different *Nostoc* strains, one *brown* pigmented (the two auricles to the *left*) and the other blue-green. (d) Fluorescence micrograph of an uninfected *Blasia* auricle stained with calcofluor. The auricle has one inner (*lower arrow*) and one outer (*upper arrow*) slime papilla. Bars 50 μm (Photographs (a) and (d) courtesy of S. Babic. (a, d) From Adams (2000) with permission; (b) from Adams (2002a) with permission; (d) from Adams and Duggan (1999) with permission)**

hormogonia formation in *Nostoc* strains (Campbell and Meeks 1989). This hormogonia-inducing factor (HIF) seems to be produced as a result of nitrogen starvation, as it is not present when the hornwort is cultured in medium containing excess $NH_4^+$. Compounds with similar activity to HIF are found in *Gunnera* stem gland mucilage (Rasmussen et al. 1994), wheat root exudates (Gantar et al. 1993), and *Blasia* exudates (Babic 1996; Watts et al. 1999; Watts 2000). To attract hormogonia, a potential host must release a chemoattractant, such as that produced by the liverwort *Blasia* when nitrogen starved (Knight and Adams 1996; Watts 2000; Adams and Duggan 2008). However, hormogonia chemoattractants can also be produced by nonhost plants such as *Trifolium repens* (Nilsson et al. 2006) and germinating wheat seeds (Knight and Adams 1996; Watts 2000; Adams and Duggan 2008). Although the chemical identity of these chemoattractants is not known, they are thought to be sugar-based molecules (Watts 2000), and in keeping with this, simple sugars such as arabinose, glucose, and galactose are known to attract hormogonia (Nilsson et al. 2006).

As a symbiotic colony develops, filamentous protrusions grow from the host plant into the colony, possibly to enhance nutrient exchange between host and symbiont (see the section "Morphological Modifications to Bryophyte and Symbiont" in this chapter). What signal induces these changes in the host is not known; however, arabinogalactan proteins (AGPs) are released by many cyanobacteria (Bergman et al. 1996; Jackson et al. 2012), and such AGPs are thought to have important roles in plant growth and development (Pennell 1992). Liverworts also produce AGPs (Basile 1990), the inner and outer slime papillae of *Blasia* and the slime cavity of *Phaeoceros* staining with both Yariv reagent, which is specific for AGPs, and with anti-AGP monoclonal antibodies (Watts 2000; Jackson et al. 2012).

Another group of potential signaling molecules in cyanobacteria-plant symbioses is the flavonoids; these are secreted by legumes and are involved in the initial signaling in the symbiosis with *Rhizobium*, by binding to the transcriptional activator NodD (Fisher and Long 1992). Seed rinse from *Gunnera*, an angiosperm that forms symbiosis with *Nostoc*, can

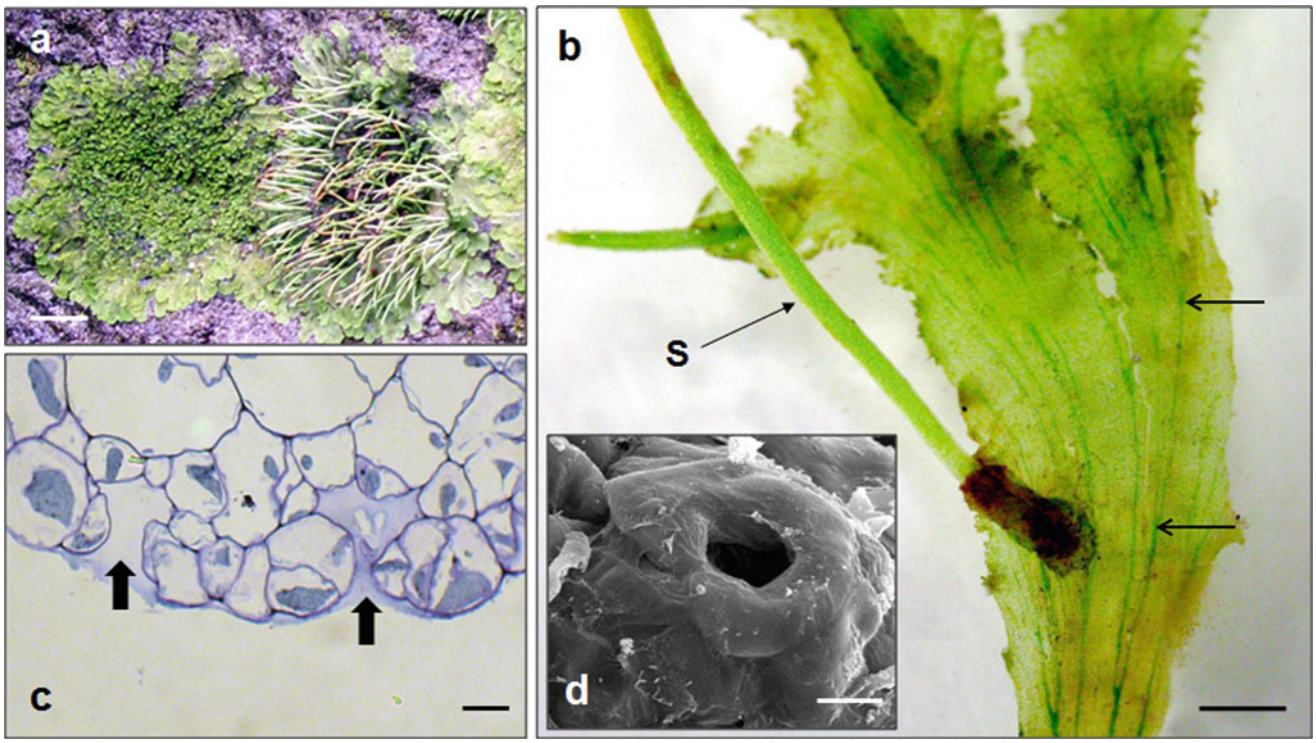



**Fig. 16.4**
**The hornwort *Leiosporoceros dussii* with symbiotic *Nostoc*. (a) The young rosette to the *left* lacks the upright sporophytes that are abundant on the surface of the older thallus to the *right*. (b) The *Nostoc* colonies can be seen as long "strands" (some of which are *arrowed*) within the thallus, parallel to the main axis. S = sporophyte. (c) Light micrograph of a nearly transverse section of the mucilage clefts (*arrows*) that serve as the point of entry for cyanobacterial infection; the filaments of *Nostoc* subsequently spread through channels that result from the separation of hornwort cells along their middle lamellae. (d) Scanning electron micrograph of a mucilage cleft. Bars 10 mm in (a), 2 mm in (b), 15 μm in (c) and 20 μm in (d) (From Villarreal and Renzaglia (2006) with permission)**

induce expression of *nod* genes in *Rhizobium* (Bergman et al. 1996; Rasmussen et al. 1996; Rai et al. 2000), and the flavonoid naringin induces expression of *hrmA* (see the sections "Cell Division Control and Hormogonia Formation" and "The *Hrm* Operon" in this chapter) in *Nostoc punctiforme* (Cohen and Yamasaki 2000). Expression of the *N. punctiforme hrmA* gene is also induced by a combination of components, including deoxyanthocyanins, found in extracts of the water fern *Azolla* which forms symbioses with *Anabaena* (Cohen et al. 2002).

The lectins are another group of signaling compounds of importance in bacterial symbioses. Although little is known about their potential involvement in cyanobacteria-plant symbioses, they are produced by the plant host in bryophyte and *Azolla* symbioses and bind to sugars on the surface of symbiotic *Nostoc* strains (Lehr et al. 2000; see also: Rai et al. 2000; Adams 2000; Rikkinen 2002; Adams et al. 2006, 2012). They have also been suggested to be involved in fungus-partner recognition in lichens (Lehr et al. 2000; Elifio et al. 2000; Rikkinen 2002; Legaz et al. 2004; Sacristan et al. 2006).

## Host-Cyanobiont Interactions Post Infection

### Cell Division Control and Hormogonia Formation

In symbiosis with *Anthoceros*, the doubling time of *Nostoc* can be 240 h, compared with 45 h in the free-living state (Meeks 1990). This slowed growth of the cyanobiont ensures that its growth rate matches that of the host plant. The mechanism of this growth control is unknown, but it seems not to be nitrogen limitation, even though the host takes most of the nitrogen fixed by its partner (see the section "Nitrogen Fixation and Transfer of Fixed Nitrogen" in this chapter).

As well as controlling the growth rate of the cyanobiont, the host must control hormogonia formation. Prior to infection, the host plant stimulates the development of hormogonia in potential partners by releasing HIF (see the section "Bryophyte-Cyanobacterium Signal Exchange" in this chapter). However, once infection has occurred, the plant must prevent hormogonia differentiation because hormogonia lack heterocysts and so cannot form a viable, nitrogen-fixing colony.

A hormogonia repressing factor (HRF), found in aqueous extracts of *Anthoceros* tissue (Cohen and Meeks 1997; Meeks 1998), inhibits HIF-induced hormogonia formation in wild-type *N. punctiforme*. The expression of two genes, *hrmA* and *hrmU*, is induced by HRF but not by HIF. These observations imply that the gene products of the *hrmUA* operon block hormogonium formation, perhaps by the production of an inhibitor or by the catabolism of an activator (Cohen and Meeks 1997; see the section "Genetic Analysis of the *Nostoc-Anthoceros* Association" in this chapter).

## Morphological Modifications to Bryophyte and Symbiont

The cells of hornwort-associated *Nostoc* are often enlarged and show irregularities of shape compared with the same strains grown free living (Meeks and Elhai 2002). In free-living cyanobacteria, heterocyst frequency is typically 4–10 % of cells, whereas in symbiosis with hornworts and liverworts, frequencies are usually considerably higher (Adams 2000; Adams et al. 2012; *Table 16.1*). Although, in at least *Anthoceros*, some heterocysts seem to be senescent or dead (Meeks 1990), the increase in heterocyst frequency is still correlated with elevated rates of nitrogen fixation. Because heterocysts are unable to fix $CO_2$, this elevated heterocyst frequency results in a loss of $CO_2$-fixing capacity, which can be compensated by the supply of carbon skeletons by the host. In *Anthoceros*, and presumably all endophytic bryophyte associations, nitrogenase gene expression and heterocyst development in the symbiotically associated *Nostoc* appear to be controlled by plant signals and are independent of the nitrogen status of the cyanobiont (Campbell and Meeks 1992; Meeks 2003, 2009).

Morphological changes are also observed in the bryophyte following infection. In both *Blasia* and *Anthoceros*, branched, multicellular filaments grow from the wall of the symbiotic cavity and invade the colony, increasing the surface area of contact between the cyanobacteria and the bryophyte (Rodgers and Stewart 1974; Rodgers and Stewart 1977; Duckett et al. 1977; Renzaglia 1982; Kimura and Nakano 1990; Gorelova et al. 1996). In *Blasia*, these filaments are derived from the inner slime papilla and possess transfer cell morphology, implying an involvement in nutrient exchange. However, such wall ingrowths are not found in other hornworts, including *Leiosporoceros* (Villarreal and Renzaglia 2006).

**Table 16.1**
**Summary of morphological and physiological changes in cyanobacteria symbiotically associated with hornworts and liverworts**

| Characteristic | Hornworts | Liverworts |
|---|---|---|
| Plant structure infected | Slime cavities | Auricles |
| Cyanobiont | *Nostoc* | *Nostoc* [a] |
| Location of cyanobiont | Intercellular | Intercellular |
| Heterocyst frequency (%)[b] | 30–50 | 30–50 |
| Nitrogenase specific activity[d] | 443 | n.d. |
| Glutamine synthetase: | | |
| Amount of protein[c] | ~86 | n.d. |
| Specific activity[c] | ~38 | n.d. |
| Form of combined N released | $NH_4^+$ | $NH_4^+$ |
| Light-dependent $CO_2$ fixation (%)[d] | 12 | n.d. |
| RuBisCo: | | |
| Amount of protein[d] | 100 | n.d. |
| Specific activity[d] | 12 | n.d. |

*Abbreviations: RuBisCo* ribulose bisphosphate carboxylase/oxygenase, *n.d.*, not determined, though likely to be similar to hornwort data
[a]The symbionts are *Nostoc* spp. in almost all cases; there have been rare reports of *Calothrix* spp. as symbionts
[b]Heterocyst frequencies are expressed as a percentage of total cells. Typical values for free-living cyanobacteria are 4–10 %
[c]Values are for the symbiont as a percentage of the same cyanobacterium in the free-living state
[d]Values are expressed as a percentage of those for the free-living cyanobacteria
From Steinberg and Meeks (1989, 1991), Meeks (1990), Rai (1990), and Bergman et al. (1992a)

## Nitrogen Fixation and Transfer of Fixed Nitrogen

The elevated rate of nitrogen ($N_2$) fixation in bryophyte-associated cyanobacteria broadly correlates with the increased heterocyst frequency in symbiosis (*Table 16.1*). The $N_2$ fixation rate of the *Anthoceros-Nostoc* association is 4- to 35-fold higher than that of free-living *Nostoc* (Steinberg and Meeks 1991). Such a high rate of $N_2$ fixation cannot be supported by the reduced photosynthetic capacity of the cyanobiont and must rely on reduced carbon derived from the plant.

Nitrogen fixed by the cyanobiont is released to the plant as ammonia (*Table 16.1*) in both *Anthoceros* (Rodgers and Stewart 1974; Stewart and Rogers 1977; Meeks et al. 1985a; Meeks et al. 1985b) and *Blasia* (Rodgers and Stewart 1974; Stewart and Rogers 1977), and initial uptake of the ammonia occurs via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway of the host (Meeks et al. 1983, 1985b; Meeks 1990; Rai 1990). In *Anthoceros*, the cyanobiont retains as little as 20 % of the nitrogen it fixes (Meeks et al. 1985a) yet shows no signs of nitrogen deprivation. Ammonia is released by the cyanobiont as a consequence of decreased activity of glutamine synthetase, the first enzyme in the GS-GOGAT pathway, which is the primary route of ammonia assimilation in cyanobacteria (Muro-Pastor et al. 2005; Flores and Herrero 2005). In *Anthoceros*, the decreased activity of GS appears to be the result of an undetermined posttranslational modification of the enzyme because the amount of GS protein differs little in filaments of free-living and symbiotically associated *Nostoc* (Joseph and Meeks 1987; Lee et al. 1988; Meeks 1990, 2003, 2009; Meeks and Elhai 2002; *Table 16.1*).

## Carbon Dioxide Assimilation and Transfer of Carbon

The Calvin cycle is the primary route of $CO_2$ fixation in free-living and symbiotically associated cyanobacteria, with ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo) as the primary carboxylating enzyme (Tabita 1994). The rate of light-dependent $CO_2$ fixation in the *Nostoc* symbiont of *Anthoceros* immediately after its separation from symbiosis is eightfold lower than that of the same cyanobacterium in the free-living state (Steinberg and Meeks 1989; Meeks 1990; *Table 16.1*). However, the level of RuBisCo protein is similar in the two cases (Rai et al. 1989; Steinberg and Meeks 1989; Meeks 1990, 2003; Meeks and Elhai 2002), implying that activity is regulated by an unidentified posttranslational modification of the enzyme (Steinberg and Meeks 1989; Meeks 1990, 2003; Meeks and Elhai 2002). The cyanobiont therefore grows photoheterotrophically, receiving fixed carbon from its photosynthetic host, probably in the form of sucrose (Stewart and Rogers 1977; Steinberg and Meeks 1991). In at least *Anthoceros*, the presence of glycogen granules in the cells of symbiotically associated *Nostoc* implies that the symbiont is not starved of carbon (Meeks 1990).

## Genetic Analysis of the *Nostoc-Anthoceros* Association

Meeks and coworkers have developed genetic techniques, including transposon mutagenesis, for the analysis of the symbiotically competent cyanobacterium *Nostoc punctiforme* strain ATCC 29133 (Cohen et al. 1994, 1998) and have used these techniques to identify a number of genes involved in the initial infection of *Anthoceros*. This has been aided by the availability of the complete genome sequence of *Nostoc punctiforme* {DOE Joint Genome Institute website} (see [{http://www.jgi.doe.gov}]).

## The *hrm* Operon

In a transposon mutant of *Nostoc* 29133, characterized by an increased rate of initial infection of *Anthoceros* (Cohen and Meeks 1997; *Table 16.2*), Meeks et al. (1999) identified two open reading frames (ORFs), *hrmU* and *hrmA*, flanking the site of transposition (*Fig. 16.5*). *hrmA* has no significant similarity to sequences in major databases, whereas *hrmU* has similarity to the sequences of mannonate oxidoreductase genes and 2-keto-3-deoxygluconate dehydrogenase genes. Expression of *hrmUA* is induced by an aqueous extract of *A. punctatus* but not by the hormogonium-inducing factor, HIF. The aqueous extract appears to contain a hormogonium-repressing factor (HRF) because it suppresses HIF-induced hormogonia formation in the wild type but not in the mutant. Whereas HIF is released into the growth medium, HRF is probably released into the symbiotic cavity, suppressing further hormogonium formation and permitting heterocyst differentiation.

At the 5′ end of *hrmUA*, three other ORFs (*hrmI*, *hrmR*, and *hrmK*) are followed by two ORFs coding for unknown proteins, followed by *hrmE*, which has similarity to an aldehyde reductase (*Fig. 16.5*). HrmI shows similarity to uronate isomerase, HrmR to the LacI/GalR family of transcriptional

**Table 16.2**

**Effect of insertion mutations on the symbiotic infectiveness (expressed in column two as the number of symbiotic colonies per unit of host tissue) and effectiveness (expressed in column three as acetylene reduction activity per g fresh weight of host tissue and in column four as acetylene reduction activity per symbiotic colony) of *Nostoc* 29133 strains in association with *Anthoceros punctatus***

| Strain (gene) | Colonies per mg dry wt per μg Chl *a* | nmol $C_2H_2$ reduced per min per: | | Gene induction factor(s) |
|---|---|---|---|---|
| | | g of fresh weight | Colony ($\times 10^{-3}$) | |
| ATCC 29133 (WT) | 0.21 | 6.3 | 12.4 | n.d. |
| UCD 328 (*hrmA*) | 1.6 | 6.1 | 8.6 | HRF |
| UCD 398 (*sigH*) | 1.2 | 8.0 | 10.1 | HIF |
| UCD 400 (*tprN*) | 0.49 | 10.4 | 6.7 | HIF and HRF |

*Abbreviations:* Chl *a*, chlorophyll *a*; HRF, aqueous extract of *A. punctatus* containing hormogonium-repressing factor identified as inducing the *hrm* operon; WT, wild type; HIF, exudate of *A. punctatus* containing hormogonium-inducing factor; and n.d., not determined (From Meeks et al. (1999). The standard deviations and number of replicates have been omitted for simplicity)

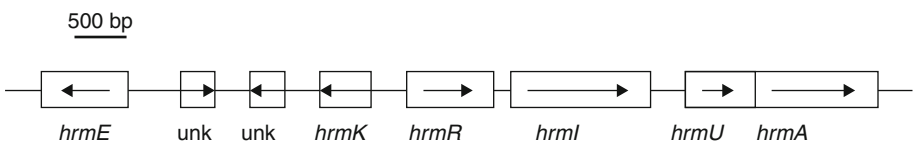



**Fig. 16.5**

**Map of the open reading frames in the *hrm* locus of *Nostoc punctiforme*. The direction of transcription is indicated by the *arrows*. Unk are unknown proteins. Sizes are approximate (Adapted from Campbell et al. (2003))**

repressors, and HrmK to gluconate kinases. HrmR is a DNA-binding protein that binds sugar ligands and represses transcription of *hrmR* and *hrmE* (Campbell et al. 2003). Galacturonate abolishes in vitro binding of HrmR to DNA, implying that the in vivo inducer may be a sugar molecule similar to or containing galacturonate. These observations led Meeks and coworkers to propose the following model for the way in which the HRF external signal is transduced into *Nostoc*. HRF enters the *Nostoc* cell and it, or a derivative similar to galacturonate, binds to HrmR, rendering it incapable of binding to the *hrmR* and *hrmE* operator regions; this derepresses transcription of these genes, leading to inhibition of hormogonia formation (Campbell et al. 2003).

### *sigH* and *ctpH*

Mutation of the *Nostoc* 29133 *sigH* gene, which encodes an alternative RNA polymerase sigma subunit, produces no obvious phenotype in filaments grown in medium with or without combined nitrogen but results in an increased infection phenotype when they are cocultured with *A. punctatus* (Campbell et al. 1998; Meeks et al. 1999; Meeks and Elhai 2002; Meeks 2003; *Table 16.2*). Transcription of *sigH* is induced by *Anthoceros* HIF, but not by HRF, and *hrmA* transcription is not altered in a *sigH* mutant. Thus, although the *hrmA* and *sigH* mutants both have an increased infection phenotype, it seems likely that increased infection has a different basis in the two strains (Meeks et al. 1999).

The gene *ctpH* lies immediately 5′ of *sigH* and encodes a protein with significant similarity to carboxy-terminal proteases of the cyanobacterium *Synechocystis* PCC 6803 (Meeks et al. 1999). In *Synechocystis* 6803, this gene is required for processing the carboxy-terminal portion of the photosystem II D1 protein in the thylakoid lumen (Anbudurai et al. 1994). However, in *Nostoc* 29133, *ctpH* seems to have a different physiological role because it is not transcribed under vegetative growth conditions, but transcription is induced by *Anthoceros* HIF. The significance of this is not understood.

### *tprN*

Lying 3′ of the gene *devR*, expression of which is essential for heterocyst maturation is the gene *tprN*, which encodes a protein with similarity to tetratricopeptide repeat proteins (Campbell et al. 1996). These proteins have been studied primarily in eukaryotes in which they are required for a variety of functions from cell cycle control to transcription repression and protein transport (Lamb et al. 1995). Inactivation of *tprN* in *Nostoc* 29133 has no apparent phenotypic effect in the free-living growth state, but the mutant infects *Anthoceros* at about twice the level of the wild type (*Table 16.2*). Transcription of *tprN* occurs during vegetative growth but increases in the presence of both HIF and HRF (Meeks et al. 1999). The significance of this in the infection process is not known.

### *ntcA*, *hetR*, and *hetF*

*Nostoc punctiforme* (*Nostoc* 29133) strains unable to develop heterocysts because of mutations in either *hetR* or *hetF* can still infect *Anthoceros* at a frequency similar to that of the wild type, despite being incapable of forming a functional nitrogen-fixing symbiosis (Wong and Meeks 2002). *hetR* is thought to be the primary activator of heterocyst development (Wolk 2000; Golden and Yoon 2003; Zhang et al. 2006), and the HetF protein seems to be a positive activator of heterocyst differentiation, enhancing transcription of *hetR* and ensuring that HetR is localized to developing heterocysts (Wong and Meeks 2001).

In cyanobacteria, NtcA functions as a nitrogen-dependent global regulator (Herrero et al. 2004) and controls the transcription of a number of genes, including *hetR* (Fiedler et al. 2001; Herrero et al. 2001). The *Nostoc punctiforme ntcA* mutant, UCD 444, forms motile hormogonia with wild-type morphology but at only 5–15 % of the wild-type frequency (Wong and Meeks 2002). However, rather than infecting *Anthoceros* at a reduced frequency, as might be expected, the *ntcA* mutant fails to infect at all. This noninfective phenotype can be complemented with copies of *ntcA*.

## Interactions in the *Nostoc-Gunnera* Symbiosis

### The *Nostoc-Gunnera* Symbiosis

Although cyanobacterial-plant symbioses are the most widespread of the nitrogen-fixing symbioses, with hosts throughout the plant kingdom, those symbioses with angiosperms (flowering plants) are presently restricted to one monogeneric family, the Gunneraceae. This contrasts with the more recently evolved rhizobia- or *Frankia*-angiosperm symbioses, which involve a considerably wider angiosperm host range. The scarcity is also unexpected as angiosperms form the ecologically most successful plant division on earth, an area discussed in recent reviews by Osborne and Bergman (2009) and by Usher et al. (2007). In addition, cyanobacteria are globally widespread with a morphological variation surpassing most other prokaryotes. In spite of this, the cyanobacterial range is narrow, with only one cyanobacterial genus, *Nostoc*, functioning as microsymbiont in *Gunnera*. However, as the Gunneraceae is one of the oldest angiosperm families and with *Gunnera* and cyanobacterial fossils dating to some 90 million years ago (Ma) and three billion years ago (Ba), respectively, this symbiosis is likely to have persisted for a long time. Prior to the establishment of the *Nostoc-Gunnera* symbiosis, however, the same or a similar cyanobacterial genus may also have given rise to chloroplasts by entering some ancestral eukaryotic cell/organism. Indeed, the chloroplast genome of *Arabidopsis* is more similar to that of *Nostoc* than to the unicellular cyanobacteria tested (Martin et al. 2002; Deusch et al. 2008). This ancient endosymbiotic event (or series of events)

was the origin of all plants and algae and therefore totally revolutionized our biosphere and atmosphere (via oxygenic photosynthesis).

## A Unique Endosymbiosis

Although the *Nostoc-Gunnera* symbiosis was first described by Reinke in 1873 (Reinke 1873), understanding of the infection mechanism in this unique angiosperm symbiosis is incomplete. In contrast to the other cyanobacterial-plant symbioses, the *Gunnera* symbiosis is exclusively intracellular. Still, being a facultative symbiosis, the cyanobiont is easily separated from the plant and may be grown independently, and the symbiosis can be reconstituted under laboratory conditions. This makes the *Nostoc-Gunnera* symbiosis an excellent model for identifying mechanisms involved in plant endosymbioses and indirectly in plastid evolution. Also, since it is the only plant symbiosis in which the cyanobacterium penetrates into the plant cells, the symbiotic development in *Gunnera* may have evolved further than that in all other plant symbioses in which the cyanobacterium remains extracellular.

## The Symbionts

The genus *Gunnera* was named by C. von Linné in honor of the Norwegian bishop Gunnérus, a person Linné admired. The approximately 30–50 *Gunnera* species are mostly subtropical to tropical perennial herbs, the exception being the smallest, *G. herteri*, which is annual (Wanntorp et al. 2001; Osborne and Sprent 2002). The *Gunnera* plants are composed of large compound spikes and are rhizomatous, or more seldom stoloniferous, and have rhubarb-like leaves. Plant sizes vary considerably; some are gigantic and may be the largest herbs on earth, such as species in South America, Hawaii, and Asia, whereas others are small and creeping, such as the stoloniferous species in New Zealand. In nature, *Gunnera* spp. seem to be invariably infected by cyanobacteria (Wanntorp et al. 2001; Osborne and Sprent 2002).

Ever since the discovery of this peculiar symbiosis (Reinke 1873), cyanobacteria of the genus *Nostoc*, which are filamentous and differentiate heterocysts, have been identified as the sole cyanobionts (see Meeks et al. 2001; Meeks and Elhai 2002; Bergman et al. 2003). The phenotypic range of the cyanobiont of *Gunnera* is wide in terms of morphology, pigmentation, and colony shape and size, which is obvious when isolates are cultivated (Bergman et al. 1992b; Rasmussen and Nilsson 2002; Svenning et al. 2005; Papaefthimiou et al. 2008). A genotypic variation has also been verified using genetic fingerprinting of 45 cultured isolates originating from 11 *Gunnera* species (Nilsson et al. 2000; Rasmussen and Svenning 2001) and natural cyanobacteria freshly collected from different *Gunnera* growing in Chile (Guevara et al. 2002). One specific *Gunnera* plant may also occasionally be infected with more than one *Nostoc* strain (Nilsson et al. 2000), while no variation within one plant was found in cyanobionts of *Gunnera* spp. sampled from natural stands in Chile (Guevara et al. 2002) using the same fingerprinting technique. 16S rRNA analyses also demonstrate that all *Gunnera* isolates examined belong to the genus *Nostoc* (Rasmussen and Svenning 2001). Svenning et al. (2005) demonstrated that some cyanobacteria isolated from various *Gunnera* spp. may form a distinct clade (based on the complete 16S rDNA gene sequence) suggesting host specificity, although a few *Gunnera* isolates did not conform to this clade. Later it was suggested that most cyanobionts are affiliated to two clusters in which they are intermixed with free-living cyanobacteria (Papaefthimiou et al. 2008).

## Specificity and Recognition

Although all *Nostoc* strains form hormogonia (the plant colonization units) per definition, still only certain strains of *Nostoc* are accepted as symbionts, which suggests the existence of other selective recognition mechanisms (see Rasmussen and Nilsson 2002). The intracellular position of the cyanobiont in *Gunnera* may also impose more severe restrictions on symbiotic partner recognition than in other intercellular, and possibly less intimate, plant symbioses. On the other hand, cyanobacterial isolates from cycads and bryophytes readily invade *Gunnera* cells and *vice versa*.

## The Site of *Gunnera* Infection: The Gland

Infection occurs via a peculiar bright red gland (*Fig. 16.6*), already clearly visible at the developing cotyledon (see Bergman 2002; Bergman and Osborne 2002; Bergman et al. 2003; Chiu et al. 2005; Khamar et al. 2010). The development of the glands is a response to the nitrogen status of the *Gunnera* plant, and they only fully develop under nitrogen-deplete conditions. The plant

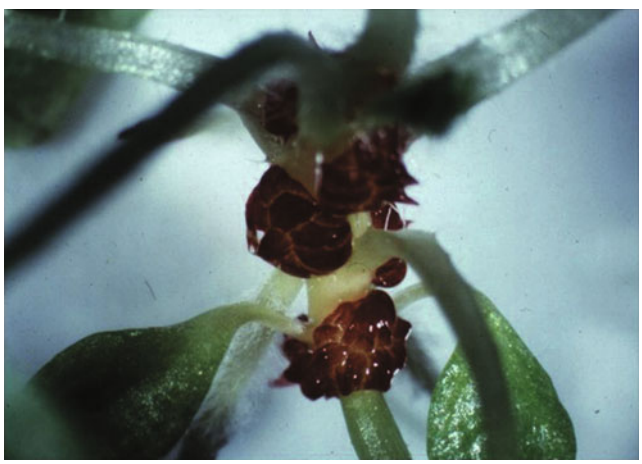


**Fig. 16.6**
***Gunnera* seedling with red stem glands out of which viscous mucilage is released**

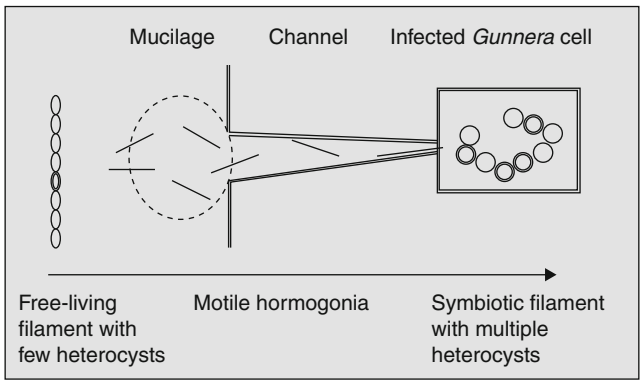



**Fig. 16.7**
**Schematic illustration of the *Nostoc* infection process in *Gunnera*. Vegetative cells of *Nostoc* with heterocysts (5–10 %) are attracted to the mucilage pouring out of the *Gunnera* stem and stolon glands. The motile hormogonial stage is induced by the mucilage, and the cyanobacterium proceeds toward the interior of the gland. At the *bottom* of the channel, the cyanobacterial filaments penetrate the plant cell walls, and intact *Nostoc* filaments enter the *Gunnera* cells. After internalization, a cyanobacterial phenotype with larger cells and supernumerous heterocysts (up to 80 %) develops. The *arrow* indicates the direction of infection**

hormone auxin is positively involved, potentially communicating the C:N status of the plant (Chiu et al. 2005; Khamar et al. 2010). Gland development is further accelerated under N-limited conditions if modest levels of sugars (0.5–1.0 % sucrose) are added (Chiu et al. 2005). This strengthens the significant role of the glands in plant nitrogen acquisition. Furthermore, high levels of carbohydrates (glucose and fructose), known to support symbiotic nitrogen fixation (Wouters et al. 2000), accumulate in the mature glands prior to the colonization by the cyanobacterium, while in *Nostoc*-colonized glands (in which nitrogen is replenished via nitrogen fixation) soluble sugar quantities are highly reduced (Khamar et al. 2010).

The glands secrete a carbohydrate-rich mucilage (*Fig. 16.7*) when non-infected, and new glands continuously develop at the base of each new leaf petiole, i.e., near the growing stem apices, which also become covered by the mucilage. Cyanobacteria-colonized glands are closed and do not release mucilage. Although root primordia were earlier suggested to be the point of entry (Schaede 1951), the present consensus is that glands are the sole cyanobacterial entry point (Silvester and McNamara 1976; Bonnett and Silvester 1981; Towata 1985; Johansson and Bergman 1992; Khamar et al. 2010). It has been proposed that these modified glands should be termed "nodules" (Silvester and McNamara 1976) and indeed a distinct and well-functioning symbiotic "organ," restricted in time and space, develops below the gland surface on colonization. Each gland, and possibly also each of the channels that penetrate into the gland (see the section "The Infection Process" in this chapter), functions as an independent colonization conduit, which would explain why several cyanobionts may be found inside one individual gland (Johansson and Bergman 1992; Nilsson et al. 2000).

The involvement of other microorganisms in the establishment of the *Gunnera* symbiosis, as proposed by Towata (1985), is not likely. This can be demonstrated by, for instance, reconstitution experiments under sterile laboratory conditions (Silvester and McNamara 1976; Johansson and Bergman 1992). In addition, some cells of the gland have heavy tannin depositions, which have been suggested to prevent the invasion of non-compatible or unwanted microorganisms (fungi and bacteria), which often reside together with cyanobacteria in the channel mucilage (Towata 1985).

## The Infection Process

The focus has so far primarily been on morphological and adaptive changes in the cyanobiont. The plasticity of *Nostoc*, in this respect, is utilized by the plant throughout the colonization process and is likely a key factor contributing to its success as a *Gunnera* symbiont. A typical feature of the *Nostoc*-*Gunnera* symbioses is the tight regulation by the plant of cyanobacterial behavior such as cell division (considerably slowed down *in planta*), cell differentiation (the development of supernumerous heterocysts), and physiological performance (high nitrogen fixation rates).

### Hormogonium Differentiation

A terrestrial cyanobacterium like *Nostoc* would (under normal free-living conditions) primarily occur as nonmotile, vegetative filaments with heterocysts at regular intervals (about 5–10 % of the total cell number; *Fig. 16.7*). On contact with *Gunnera*, the gland and the plant apex are, however, soon covered by a cyanobacterial "biofilm" composed of tightly packed hormogonia (Osborne et al. 1991; Johansson and Bergman 1992; Johansson and Bergman 1994; Chiu et al. 2005). Differentiation of these small-celled motile hormogonia is essential for the whole *Gunnera* colonization and cell penetration process; they act as a means for the cyanobacterium both to reach and to invade the *Gunnera* organ (the gland; *Fig. 16.7*). The mucilage has a pivotal role during this process (Rasmussen et al. 1994). It is composed of highly glycosylated arabinogalactan proteins (AGPs; Rasmussen et al. 1996) and stimulates not only growth but also hormogonium differentiation. A low-molecular-weight (<12 kDa), heat-labile protein, not yet characterized, which acts as hormogonium-inducing factor (HIF), has been identified in the mucilage (Rasmussen et al. 1994). In contrast, the soluble sugars of *Nostoc*-colonized glands inhibit hormogonium differentiation (Khamar et al. 2010). This is needed to stimulate heterocyst differentiation and nitrogen fixation, the "essence" of the symbiosis. Molecular mechanisms behind the induction of hormogonia and their differentiation are still largely unexplored.

Preliminary studies, using subtractive hybridization and proteomics (two-dimensional [2-D] gel electrophoresis coupled to mass spectrometry) of soluble *Nostoc* proteins treated with *Gunnera* mucilage show that the induction of hormogonium differentiation is also reflected in a differential expression of

genes and proteins, whose expression is either up- or downshifted or both. For instance, three mucilage-induced *hie* (host-induced expression) genes have been identified, including a putative precursor of a pheromone-like signaling peptide (HieA), an outer membrane or secreted glycoprotein (HieB), and a protein probably involved in adaptation to acidity (HieC; Liaimer et al. 2001). The latter may be important as the *Gunnera* mucilage has a pH of 4–5 (Rasmussen et al. 1994), a pH at the lower limit of the cyanobacterial tolerance range. Another set of proteins was also identified as being differentially expressed in hormogonia (Klint et al. 2006). These proteins, which were predominantly surface associated, may have roles in motility, recognition, adhesion, as well as in communication with host plants. The mucilage therefore appears to have important functions at earlier stages of the *Gunnera* infection process.

### Entrance and Penetration

The *Gunnera* glands are composed of a set of up to nine papillae surrounding a central papilla (Johansson and Bergman 1992; Uheda and Silvester 2001; Chiu et al. 2005). Between the papillae, and leading into the stem tissue, are deep invaginations through which the mucilage is released. The hormogonia use these narrow channels to enter the dark interior of the *Gunnera* stems (*Fig. 16.7*). As this is against the normal positive phototactic behavior of *Nostoc*, a potent attractant must be released by the plant, possibly carried by the mucilage. Motility is crucial at this stage, as the direction of infection is opposite to that of the flow of mucilage. Upon reaching the bottom of the gland channels, the cyanobacterium penetrates the thin walls of smaller meristematic and dividing cells lining the channel (Silvester and McNamara 1976; Johansson and Bergman 1992; Johansson and Bergman 1994; Uheda and Silvester 2001). A delimited tissue of *Nostoc*-infected *Gunnera* cells is formed within a few days of inoculation. The mechanism(s) involved in the actual host cell penetration is still unknown, although Towata (1985) suggested the occurrence of pectolytic or cellulolytic activities in the mucilage of *G. kaalensis*. Also lining the channel are the thick-walled secretory cells releasing the mucilage (Towata 1985).

### Signal Exchange Between the Cyanobacterium and the Host

Besides HIFs, the plant signals involved in hormogonium differentiation still await genetic identification and chemical characterization, as do the cellular response signaling cascades in *Nostoc*. In this context, a highly interesting question is whether the differentiation of hormogonia resulting from a biotic stimulus (such as *Gunnera* mucilage) triggers specific genes (such as those involved in "symbiotic competence") but not those triggered by any abiotic stimulus (such as red light). Also interesting are mechanisms involved in the initial rapid cell division and the machinery behind motility. All studies do, however, verify that the plant influences cyanobacterial morphology and behavior at all stages of the infection process and that this includes several fundamental cyanobacterial processes such as growth, cell division, cell differentiation, ammonia assimilation, and phototactic behavior. The question is whether this is triggered by plant compounds or by the environment within the plant. For instance, the symbiotic tissue is low in oxygen and light, which may have consequences for gene expression.

Another open question is to what extent the release of the auxin IAA (indole-3-acetic acid) by *Nostoc* (Sergeeva et al. 2002) acts as a signal or influences the development of the symbiotic *Gunnera* tissues. The influence of auxin has recently been stressed (Chiu et al. 2005). Indeed, cyanobacteria seem to have the potential to produce major phytohormones (Liaimer and Bergman 2003) and also to release "AGP-like" proteoglucans, which may also influence plant development (Bergman et al. 1996).

## Host-Cyanobacterial Interactions Post Infection

Internalization of the cyanobiont elicits novel, dramatic modifications of cyanobacterial morphology and function (*Fig. 16.7*). Because hormogonia lack heterocysts, they are unable to fix nitrogen. Thus, the hormogonium stage is lethal under free-living conditions (unless combined nitrogen is available), and hence is, of necessity, transient. Redifferentiation into vegetative filaments with heterocysts occurs after 1–2 days. The maintenance of a continuous vegetative stage with heterocysts is a prerequisite for the symbiosis to persist as an efficient provider of combined nitrogen. Repression of hormogonium differentiation in *Gunnera* may be achieved by homologues to the hormogonium-repressing factor(s) (HRFs) identified in the bryophyte symbiosis (see Meeks and Elhai 2002). One component of this repression machinery may be the inhibitory effects on hormogonium development by the soluble sugars present in *Nostoc*-colonized glands (Khamar et al. 2010).

When inside the *Gunnera* cells, the cyanobacterial cells enlarge, and cell division is considerably restricted (Söderbäck and Bergman 1992). In addition, the filaments remain surrounded by the host cell plasmalemma through the pinocytosis process. This membrane, like the peribacteroid membrane in *Rhizobium*-legume symbioses, acts as the interface between the symbionts through which the exchange of metabolites takes place. The *Gunnera* cells eventually become filled with cyanobacterial filaments, which soon start to differentiate an abnormally high frequency of heterocysts (*Fig. 16.7*). Once infection is complete, the host must tightly control cyanobiont growth to avoid being outgrown, and this may explain the enlargement of cyanobacterial cells typical for the endosymbiotic stage (see Rai et al. 2000; Bergman 2002).

The dramatic morphological transitions seen in *Nostoc* on entering *Gunnera* cells are also reflected in the transcription of genes (and the corresponding proteins) related to heterocyst differentiation and nitrogen fixation (see Table 1 in Bergman 2002). For instance, the expression of the *hetR* gene (the master gene for heterocyst differentiation) correlates positively with the increase in heterocyst frequency, as does the expression of the

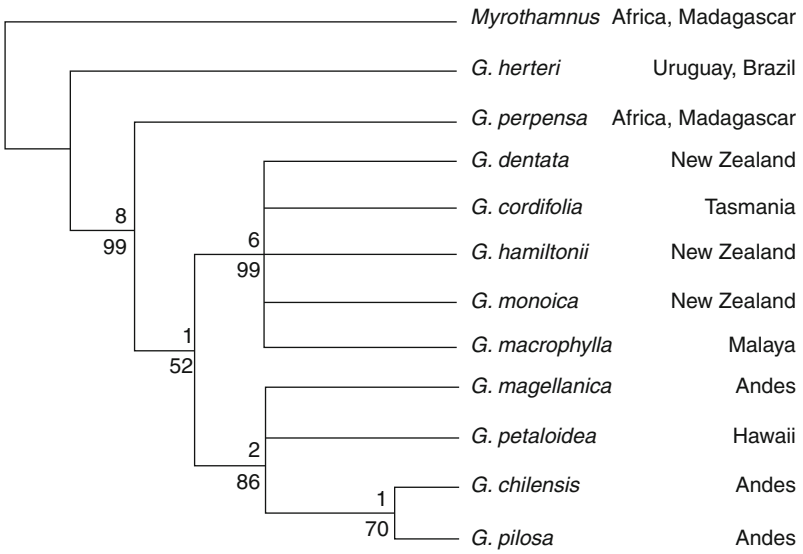



**Fig. 16.8**

**Phylogram of 11 *Gunnera* species representing varying sizes and geographical origin. *Myrothamnus* is an African shrub-like plant growing in dry areas, as opposed to *Gunnera* species that prefer wet environments characterized by high humidity and high rainfall (From Wanntorp et al. (2001))**

nitrogen-responsive transcription factor encoded by *ntcA*, whereas *nifH* expression is (as expected) already high, close to the growing apex. By contrast, the expression of the *glnB* gene, encoding the signal transduction protein $P_{II}$, decreases along the same symbiotic profile (Wang et al. 2004). The overexpression of both *hetR* and *ntcA* and the contrasting downregulation of *glnB* are features indicating important regulatory differences between the symbiotic and free-living life stages. Later, Ekman et al. (2006) identified a differential protein expression pattern in a cyanobacterium isolated from *Gunnera manicata* when using proteomic analysis. Changes were primarily related to cell envelope and membrane-associated proteins and to changes in cellular activities of C and N metabolism, including upregulation of nitrogenase and proteins of the oxidative pentose phosphate pathway and a downregulation of Calvin-Benson cycle enzymes. The significance of these findings in relation to cyanobacterial cell differentiation and the establishment and maintenance of an efficient nitrogen-fixing cyanobacterial-plant symbiosis now needs to be further explored.

Cross-sectioning of rhizomes of mature plants reveals the final outcome of the symbiosis: distinct and bright blue-green pigmented but restricted and delimited cyanobacterial colonies seen scattered in the rhizome or along the stolons of the smaller *Gunnera* plants (Osborne et al. 1991). However, the sites of infection comprise only a small proportion of the total plant biomass, particularly in the large *Gunnera* species.

## Nitrogen Fixation and the Transfer of Nitrogen

As with most other plant symbioses, the main function of the cyanobacterium in *Gunnera* is to cover the total combined nitrogen requirement of the host via nitrogen fixation (Silvester and Smith 1969; Silvester 1976; Bonnett and Silvester 1981; Osborne et al. 1992; Khamar et al. 2010). The heterocysts act as the nitrogen-producing entities, holding all the nitrogenase (Söderbäck et al. 1990; Söderbäck 1992), and are capable of supporting the entire symbiosis with combined nitrogen. In addition, the cyanobacterium attains enhanced nitrogen fixation capacities compared to its free-living relatives (Silvester 1976; Bonnett and Silvester 1981). This may be related to the high heterocyst frequency or to an enhanced nitrogen starvation signal caused by the continuous N-drainage from the cyanobiont.

Up to 90 % of the nitrogen fixed is exported from the cyanobacterium to the host (Silvester et al. 1996). This is likely due to downregulation of glutamine synthetase protein levels, specifically in heterocysts, as well as other activities in symbiosis (Söderbäck 1992). As in most nitrogen-fixing plant symbioses, the nitrogen fixed is released primarily as $NH_4^+$ (Silvester et al. 1996). The *Nostoc*-infected *Gunnera* tissues are always well invested with vascular strands that facilitate exchange of metabolites such as nitrogen and carbohydrates (see ❯ *Fig. 16.8* in Bergman et al. 1992b). Multiple vascular strands (polystele) persist in *Gunnera*, which may be reminiscent of an aquatic ancestry (Osborne et al. 1991). Stock and Silvester (1994) showed, using pulse-chase labeling with $^{15}N$, that the nitrogen fixed was efficiently transported from mature to young parts (with lower heterocyst frequencies) in *G. monoica* stolons and that N-translocation occurs via the phloem.

## Carbon Assimilation and the Transfer of Fixed Carbon

As *Nostoc* inside the *Gunnera* cells is excluded from light, the host must supply the cyanobiont with fixed carbon via its

photosynthesis. Hence, the cyanobiont must adapt to a heterotrophic, or at least a mixotrophic, mode of life to generate enough reductant and ATP to support the demanding nitrogen fixation process (Söderbäck and Bergman 1992, 1993; Black et al. 2002; Khamar et al. 2010). Nevertheless, total pigment and ribulose-1,5-bisphosphate carboxylase levels remain constant along the developmental sequence, from young to old parts, although values decrease if related to cell volume as this increases in older cells (Söderbäck and Bergman 1992). The high frequency of heterocysts also drastically diminishes the number of vegetative cells, but the use of gas chromatography with mass spectrometry (GC-MS) has shown that still only the vegetative cells are actively taking up C (Black et al. 2002). Following $^{14}C$ translocation in *Nostoc*-infected *Gunnera* stolons reveals that the *Nostoc*-infected tissues at the apex of *G. magellanica* stolons are particularly efficient sinks for newly fixed plant carbon (Söderbäck and Bergman 1993). The phloem of *Gunnera* has the unusual capacity to contemporaneously transport N outward and C inward toward the symbiotic tissue (Stock and Silvester 1994). A tight interaction of nitrogen and carbon metabolism in the *Gunnera* symbioses is also suggested (see, e.g., Chiu et al. 2005; Khamar et al. 2010). *hetR* expression in symbiotically competent *Nostoc* (PCC 9229) is negligible in the absence of a carbon source in darkness but pronounced in the presence of fructose (Wouters et al. 2000).

### Ecological Importance

Our understanding of the ecology and significance (e.g., as a nitrogen fixer) of this ancient plant and its cyanobiont is still rudimentary. For a detailed review of the ecology of *Gunnera*, the reader is referred to Osborne and Sprent (2002). The geographic range of *Gunnera* was considerably wider in the past when the climate was more favorable (Osborne et al. 1991; Osborne and Sprent 2002). Today, *Gunnera* typically grows in super-humid habitats and often at high elevations or on steep cliffs. The genus is found in all continents, except in Europe and polar regions (see Wanntorp et al. 2001; Osborne and Sprent 2002). Some large *Gunnera* species were introduced into Europe as ornamental plants at the end of the nineteenth century, and eventually some plants escaped and became invasive in, e.g., western Ireland, the Channel Islands, and the Azores (Osborne et al. 1991; Osborne and Sprent 2002).

The genetics (*rbcL* and *rps*16 introns) of *Gunnera* plant species have recently been analyzed (❯ *Fig. 16.8*). The large species in South America and Hawaii distinctly group together in one clade, the often smaller species of New Zealand and Southeast Asia group in another, while *G. perpensa* (the first *Gunnera* to be described by Linné) and *G. herteri* (with the smallest size) are sister groups, representing Africa and Brazil, respectively (Wanntorp et al. 2001).

### Conclusions

From a cyanobacterial perspective, the *Nostoc*-*Gunnera* symbiosis may on the one hand seem wasteful; the cyanobiont merely functions as an N-producing entity with highly suppressed growth and is possibly deprived of producing a new generation of cyanobionts, being enclosed in tissues in a long-lived plant. On the other hand, it may be beneficial; the cyanobiont no doubt extends its ecological niche to also include symbiotically competent cells of an angiosperm. In this way, the cyanobacterium not only gains access to plant leaves and roots and their nutrient acquisition capacities but it also finds shelter from all possible predators, being the sole organism in this "golden cage."

The data obtained so far clearly show that cyanobacterial morphology and protein and gene expression patterns are drastically affected prior to, during, and after the establishment of the *Nostoc*-*Gunnera* symbiosis, although no symbiosis-specific genes and proteins, equivalent to the *nod* genes and Nod-factors in the *Rhizobium*-legume symbioses, have yet been discovered. However, it seems logical to assume that equally advanced molecular mechanisms must persist in a cyanobacterial-angiosperm endosymbiosis to generate this potentially very long-lived, well-coordinated, and successful interaction.

## The *Azolla* Symbiosis

### Introduction

#### Taxonomy and Distribution

The *Azolla* symbiosis is a mutualistic association between the aquatic fern *Azolla*, the filamentous, heterocystous, nitrogen-fixing cyanobacterium *Nostoc* (formerly classified as *Anabaena*), and endosymbiotic bacteria. The genus *Azolla* has been reported to contain seven extant species that are divided into two sections on the basis of spore morphology. Section *Azolla* (New World species) has included *A. caroliniana*, *A. mexicana*, *A. filiculoides*, *A. microphylla*, and *A. rubra*. However, the taxonomy of the New World species of *Azolla* has been the subject of much debate. In 2004, a comprehensive review of the literature was carried out along with original observations of type specimens using optical and scanning electron microscopy (Evrard and Van Hove 2004). This study confirmed the opinion of some that *A. caroliniana* and *A. microphylla* are synonyms of the previously described *A. filiculoides*. To clarify the taxonomic classification, the authors suggested the need to rehabilitate the Mettenius concept, and then according to the priority rule, the section *Azolla* species must be named *A. cristata* and *A. filiculoides*. Section *Rhizosperma* (Old World species) includes *A. pinnata* and *A. nilotica*. Geographically, *A. pinnata* is found in Australia, New Zealand, Japan, Asia, and Africa, and *A. nilotica* is primarily found in Africa (Saunders and Fowler 1993). Species from the section *Azolla* are more widely distributed around the world and are found in Europe, Asia, Africa, Australia, and America.

However, the distribution of *A. rubra* is restricted to New Zealand and Australia (Large and Braggins 1993). The distribution of some species has been impacted by human effects (Janes 1998a).

## Morphology

The plant's shape, color, and size change significantly under different growth conditions (Janes 1998b). The rhizome is branched, bearing alternate leaves that are bilobed. The ventral lobe is transparent and serves to float the plant on the surface of the water, whereas the dorsal photosynthetic lobe contains a leaf cavity in which the symbionts are found. The roots are adventitious. The shedding of roots and branches is related to environmental and physiological factors and enables the plant to reproduce via vegetative fragmentation. Factors affecting the growth of *Azolla* include genotype, temperature, light (intensity, quality, and photoperiod), water chemistry (including pH, salinity, and nutrients), and influence of pests and diseases (see Singh and Singh 1997).

## General Characteristics

The association has been most frequently used as an alternative nitrogen fertilizer in rice fields, as well as a supplemental animal fodder. *Azolla* provides the cyanobiont with nutrients, including fixed carbon, and the cyanobiont provides the host with combined nitrogen (via nitrogen fixation). The exact role of the endosymbiotic bacteria in the association remains unclear; however, some possible functions have been suggested.

# The Symbionts

## Cyanobacterial Symbionts

### Identification

The filamentous, heterocystous, nitrogen-fixing cyanobacterial symbionts in the *Azolla* association have been extensively studied using both traditional and modern molecular techniques. In addition to characterization of the cyanobionts after they have been directly extracted from the association, there have been a number of studies in which cyanobacteria were isolated and cultured, in attempts to study the cyanobacterial symbionts in a free-living state. While numerous researchers have reported success in isolation and cultivation of the symbiotically associated cyanobacteria (Newton and Herman 1979; Tel-Or et al. 1983; Gebhardt and Nierzwicki-Bauer 1991; see Braun-Howland and Nierzwicki-Bauer 1990), molecular studies (primarily based on restriction fragment length polymorphism [RFLP] analyses) have indicated that none of the isolates represent the major cyanobacterial symbionts in the association (Gebhardt and Nierzwicki-Bauer 1991). Though not conclusively demonstrated, major as well as some minor cyanobacteria may be present in the association, with the more readily cultured cyanobacteria representing minor symbionts (Gebhardt and Nierzwicki-Bauer 1991). The other possible explanation is that the isolates presumably obtained from the association are actually epiphytes. However, recent studies of the genetic diversity of cultured cyanobionts of diverse species of *Azolla* revealed a genetic distinctness of the cultured *Azolla* cyanobionts as compared to free-living cyanobacterial strains of the genera *Anabaena* and *Nostoc* and symbiotic *Nostoc* strains from *Anthoceros*, *Cycas*, and *Gunnera* (Sood et al. 2008). These findings support the coexistence of minor species rather than epiphytes. Regardless, based on molecular studies, it has not been demonstrated that the major cyanobacterial symbiont from the association can be cultured in a free-living state. In fact, with the genome sequencing of the major cyanobiont of an *A. filiculoides* strain, there is now strong evidence (described below) that there has been ongoing selective streamlining of the cyanobiont genome which has resulted in an organism devoted to nitrogen fixation and devoid of autonomous growth (Ran et al. 2010).

Given the challenge of studying the cyanobacterial symbionts in a free-living state, direct molecular studies have been used for accurate identification. Restriction fragment length polymorphism (RFLP) analyses (Gebhardt and Nierzwicki-Bauer 1991), polymerase chain reaction (PCR) fingerprinting (Zheng et al. 1999), random amplified polymorphic DNAs (RAPDs; Van Coppenolle et al. 1995), as well as fluorescence in situ hybridizations (FISH) (Bushnell 1998) have been used to examine the identity of the symbiotic cyanobacteria. Regardless of the approach used, the cyanobiont referred to as "*Anabaena azollae*" has in most instances been described as being somewhat related to *Anabaena* or *Nostoc* (Plazinski et al. 1990b; Gebhardt and Nierzwicki-Bauer 1991). A study (Baker et al. 2003) using comparisons of sequences of the phycocyanin intergenic spacer and a fragment of the 16S rRNA gene places the *Azolla* cyanobiont in the order Nostocales but in a separate group from *Anabaena* or *Nostoc*. Additionally, near full-length (1,500 bp) 16S rRNA sequencing and phylogenetic analysis of major cyanobionts from a variety of *Azolla* species yielded similar results (Milano 2003). In 1989, Komarek and Anagnostidis placed the *Azolla* cyanobiont in a revised genus named "*Trichormus*" on the basis of morphology (Komarek and Anagnostidis 1989). This is not inconsistent with the most recent molecular-based findings.

Recently, the genome sequencing of a cyanobacterium from *Azolla filiculoides* leaf cavities has provided the most comprehensive information on its identity (Ran et al. 2010). Surprisingly, the phylogenetic analysis places the cyanobiont (*Nostoc azollae* 0708) most closely with *Raphidiopsis brookii* D9 and *Cylindrospermopsis raciborskii* CS 505, the two multicellular cyanobacteria with the smallest known genomes (Stucken et al. 2010). However, it shares the highest number of protein groups with *Nostoc* sp. PCC 7120, *Anabaena variabilis* ATCC 29413, and *N. punctiforme* PCC 73102 (Ran et al. 2010).

The taxonomy of the cyanobionts is generally in agreement with the taxonomy of the host plant (Plazinski 1990; Van Coppenolle et al. 1993; Zheng et al. 1999). These findings, taken in conjunction with the continuous maintenance of the symbiosis throughout the life cycle of the plant (see the section "The Infection Process" below), suggest coevolution of the cyanobionts and the host plant.

#### Developmental Profile Along the Main Stem Axis

The growth of the endophyte is coordinated with the growth of the plant. In the apical meristem and younger leaves, the cyanobacterial vegetative cells are smaller than in older leaves and undergo frequent cell divisions. Increases in leaf age are accompanied by a decrease in cell division and increased size of the cyanobacterial vegetative cells, as well as increased heterocyst frequencies (Hill 1975). The number of heterocysts and the nitrogen fixation rates vary in leaves of different ages, as well as in different *Azolla* species (Hill 1977). Heterocyst frequencies can reach up to 20–30 % of the cells within a filament in the symbiotically associated cyanobiont. These are much higher than the typical 10 % heterocyst frequency in free-living *Anabaena/Nostoc* species.

### Bacterial Symbionts

The presence of bacteria residing within the leaf cavity of *Azolla* has been recognized for many years (Carrapico 1991), yet still unclear is the specific function(s) of most of the bacteria in this symbiosis. Initial attempts to study the symbiotic bacteria employed traditional microbiological, biochemical, and physiological techniques for the identification of bacteria isolated from a variety of *Azolla* species. Utilizing these approaches, there were many reports of *Arthrobacter* spp. (most frequently *A. globiformis*) occurring in symbiotic association with *Azolla* (Gates et al. 1980; Wallace and Gates 1986; Forni et al. 1989, 1990; Nierzwicki-Bauer and Aulfinger 1991; Shannon et al. 1993). *Agrobacterium* has also been reported to be isolated from different *Azolla* species (Plazinski et al. 1990a; Shannon et al. 1993; Serrano et al. 1999). Other bacteria, such as *Staphylococcus* sp., *Rhodococcus* spp., *Corynebacterium jeikeium*, and *Weeksella zoohelcum*, were identified by BIOLOG and API tests as being in association with *Azolla* (Serrano et al. 1999). A detailed review of the identification of bacteria isolated from *Azolla* species is provided in Lechno-Yossef and Nierzwicki-Bauer (2002). Molecular techniques, in particular 16S rDNA gene amplification, cloning, screening, sequencing, and phylogenetic analysis, have provided more detailed information on the identity of the symbiotic bacteria (Lechno-Yossef 2002; Milano 2003). In the accessions studied, sequence similarity found that the most abundant bacterial symbionts in *A. caroliniana* and *A. filiculoides* were *Frateuria aurantia* and *Agrobacterium albertimagni* and in *A. mexicana*, *Agrobacterium tumefaciens* (Lechno-Yossef 2002). More recent research studying the endophytic bacteria within *A. microphylla* using PCR-DDGE and electron microscopy revealed a complex and divergent bacterial community with *Bacillus cereus* as the dominant species (Zheng et al. 2008).

## Host Structures and the Infection Process

### The Leaf Cavity

In the association, the symbionts reside in a leaf cavity, an extracellular compartment in the dorsal lobe of the leaf. In mature leaves, the symbionts (cyanobacteria and bacteria) are located in the periphery of the leaf cavity in mucilaginous material between internal (Nierzwicki-Bauer et al. 1989) and external envelopes (Uheda and Kitoh 1991). Electron microscopic analysis combined with specific staining showed that the inner envelope does not have a tripartite structure typical of a membrane and is rich in lipids (Nierzwicki-Bauer et al. 1989). The external three-layered envelope is believed to contain cutinic and suberic substances, as revealed by response to chemical treatments of degradation using hot alkali methanol (de Roissart et al. 1994).

The adaxial epidermis of the leaf cavity contains a pore that is surrounded by two cell layers (Veys et al. 1999, 2000). One layer inside the pore is composed of teat-shaped cells that are extended from the adaxial epidermis. The other layer corresponds to the inner epidermis, which lines the inside of the cavity. Three to four tiers of teat cells form a cone-like pore with an average diameter at the base of 80 μm. The pore opening is larger in younger leaves, and the morphology of the teat cells suggests that their function is as a physical barrier to prevent particles and organisms from entering the cavity and the symbionts from exiting (Veys et al. 2002).

### The Infection Process

*Azolla* is a heterosporous water fern that is capable of both sexual and asexual reproduction. Unlike any of the other cyanobacterial symbioses, the host is in continual association with the symbionts, making this the only known permanent symbiosis. Thus, rather than reinfect *Azolla*, the symbionts retain coordinated growth in association with the host throughout its life cycle. Descriptions of the processes involved in maintaining the continual association during sexual and asexual reproduction are described briefly below.

#### Sexual Reproduction

Sporulation is the sexual reproduction process in *Azolla*. During sexual reproduction, the host produces both mega- and microsporocarps. The partitioning of the cyanobacterial filaments into the developing sporocarps and the reestablishment of the symbiosis following embryogenesis were originally described for *A. mexicana* (Perkins and Peters 1993; Peters and Perkins 1993). The symbionts that are used as inoculum to the

developing sporocarps come from the dorsal lobe of the same leaf in which the sporocarps are developing. Recently, a comprehensive study of cellular responses in the cyanobacterial symbionts during its vertical transfer via megasporocarps between plant generations in the *A. microphylla* symbiosis was reported (Zheng et al. 2009). During colonization of the megasporocarp, the cyanobacterium entered through pores at the top of the indusium as motile hormogonium filaments. Subsequently, the cells differentiated into akinetes in a synchronized manner. Also discovered was that this process was accompanied by cytoplasmic reorganizations within the cyanobionts and the release of numerous membrane vesicles, most of which contained DNA, and the formation of a highly structured biofilm (Zheng et al. 2009). These data revealed complex adaptations in the cyanobacterium during transition between plant generations that merit further investigation.

The cyanobiont akinetes (which function as spores) and the bacterial symbionts (which do not always show ultrastructural characteristics of spore envelopes; Aulfinger et al. 1991) found in the megasporocarps are transferred to the developing spores and sporelings. After separation of the megasporocarp from the plant, part of the indusium is shed, and the proximal half becomes the indusium cap. The symbionts reside in a space called "the inoculation chamber" (Peters and Perkins 1993), located between the indusium cap and the apical membrane of the megasporocarp. Following fertilization and the beginning of embryogenesis, the symbionts resume metabolic activity. With the assistance of cotyledonary hairs, the symbionts are introduced into the embryonic leaf before it displaces the indusium cap (Peters and Perkins 1993). Leaves, which grow from the meristem, are initially unlobed but contain a structure similar to the leaf cavity that contains the symbionts. As the frondling continues to grow, the symbionts are distributed into the developing leaf cavities by a mechanism similar to the transfer mechanism used during asexual reproduction via vegetative fragmentation (see next section).

#### Asexual Reproduction

The main form of reproduction in *Azolla* is vegetative fragmentation. The apical meristem of each branch contains a colony of undifferentiated cyanobacterial cells. Cyanobacterial filaments from the apical colony are introduced into the leaf primordium before the development of the leaf and leaf cavity are complete. The partitioning of the endophytes into the developing leaves is facilitated by entanglement around primary branched hair (PBH) cells of *Azolla* (Calvert and Peters 1981). The leaf cavity starts to develop and engulf the cyanobacterial colony in the fourth or fifth leaf along the stem axis. In this way, symbionts are inoculated into every leaf cavity that is formed. The development of the leaf cavity is also accompanied by the formation of simple hair cells by *Azolla* (Peters and Calvert 1983).

#### "Artificial" In Vitro Infection of Cyanobacteria

In sporulating *Azolla*, sexual hybridization between different *Azolla* species, as well as the formation of new combinations of *Azolla* and *Nostoc*, has been somewhat successful (Watanabe 1994; Watanabe and Van Hove 1996). For example, *Nostoc* from *A. microphylla* (MI4031) was successfully introduced into *A. filiculoides* (FI1034) by exchange of the indusium cap of the megaspore (Lin et al. 1989). Successful sexual hybridizations between *A. microphylla* (megasporocarp) and *A. filiculoides* (microsporocarp; Wei et al. 1988; Do et al. 1989), between *A. filiculoides* (megasporocarp) and *A. microphylla* (microsporocarp; Watanabe et al. 1993), and between *A. mexicana* and *A. microphylla* (Zimmerman et al. 1991) have also been reported. The key to these successes has been having the cyanobacteria at the appropriate stage of development (during akinete germination and vegetative cell growth) that mimics what naturally occurs in situ.

## Host-Symbiont Signal Exchange

The recognition between *Azolla* and *Nostoc azollae* is facilitated by lectins in both the plant (Mellor et al. 1981) and the cyanobionts (Kobiler et al. 1981, 1982). Additionally, bacteria isolated from *A. pinnata* and *A. filiculoides* have been shown to contain lectins (Serrano et al. 1999). The presence of Rhizobiaceae symbionts in association with different *Azolla* species and cultures examined would suggest that this group of bacteria has a role in the symbiosis. Plazinski et al. (1991) showed that the *nodL* and *nodABC* genes gave hybridization signals to a plasmid and the chromosome of the isolate AFSR-1 from *A. filiculoides*. These authors suggest that the *nod* genes, if active in the bacterial symbionts of *Azolla*, play a regulatory role in the development of the symbiosis or in the maintenance of bacterial association with the plant.

## Host-Cyanobiont Interactions Post Infection

### Morphological Modifications to Host and Cyanobacteria

The leaf cavity and inner and external envelopes of *Azolla* do not appear to be present only when it is symbiotically associated with the cyanobionts. These structures are present in both *Nostoc*-free and *Nostoc*-containing *Azolla* (Nierzwicki-Bauer et al. 1989). This evidence excludes the involvement of the cyanobionts in the formation of these structures. However, given that the cyanobiont-free plants examined still contained symbiotic bacteria, a possible role of bacteria in the synthesis of the leaf cavity envelopes cannot yet be excluded.

### Nitrogen Fixation and Transfer of Fixed Nitrogen

Nitrogen fixation is carried out by the heterocysts of the cyanobiont. In leaf cavities of different ages along the stem axis of *Azolla*, the heterocyst frequencies and nitrogen fixation

rates vary. Nitrogen fixation, as determined by the acetylene reduction assay, occurs in the apical (younger) leaves but not in the stem apex, increases and reaches a peak in leaves of middle age, and then decreases in the older leaves (Canini et al. 1990). Ammonium, the product of nitrogen fixation, is released from heterocysts and assimilated by *Azolla* into glutamate using the glutamine synthetase (GS)-glutamate synthase (GOGAT) system (Peters and Calvert 1983). Nitrogenous compounds in the form of glutamate, glutamine, ammonia, and other glutamate derivatives are transferred from the mature leaf cavities to the stem apex (Peters et al. 1985). In *Nostoc azollae*, the activity and protein content of GS are only 5–10 % of that of free-living *Anabaena* (Orr and Haselkorn 1982). However, the nitrogen fixation activity is much higher because of the increased number of heterocysts. Some of the bacteria found in this association can fix nitrogen. Immunoelectron microscopy studies using antibodies against the Fe and FeMo protein subunits of nitrogenase revealed that a subset of the bacteria in the *A. caroliniana* and *A. filiculoides* associations contained these nitrogenase subunits (Lindblad et al. 1991). The potential nitrogen-fixing contribution of the bacteria in the association separate from that of cyanobacterial symbionts could not be measured because they coexist in the leaf cavities and, once removed, are likely to have altered capabilities.

## Carbon Assimilation and Transfer of Fixed Carbon

The cyanobiont, *Nostoc azollae*, has photosynthetic capabilities; however, in the symbiotic state, it is believed to contribute less than 5 % of the total $CO_2$ fixed in the association (Kaplan and Peters 1988). Pulse-chase studies have shown that sucrose from the plant is supplied to and accumulated by the cyanobiont (Peters et al. 1985). Simple hair cells of *Azolla* are involved in the transport of sugars from the photosynthetic mesophyll cells to the leaf cavity. Simple hair cells have ATPase activity in their plasmalemma and some accumulation of starch in their chloroplasts (which do not possess ribulose 1, 5 bisphosphate carboxylase, RuBisCO), suggesting active transfer of sugars from the simple hairs to the leaf cavity (Carrapico and Tavares 1989). Additionally, primary branched hair cells, having the morphology of a transfer cell, are believed to be involved in nutrient transfer from the plant to the cyanobiont(s) (Peters et al. 1985).

## Ecological Importance: Friend or Foe?

The *Azolla* symbiosis is of tremendous ecological importance, having both positive and negative impacts. On the positive side, the association has been extensively used as a biofertilizer, providing a source of combined nitrogen in the form of ammonium, thereby reducing or eliminating the need for the addition of chemical fertilizers. This role has been most extensively used in conjunction with rice paddies or fertilization of fields. The growth of *Azolla* into thick mats also makes it effective in suppressing weed growth. Owing to its high protein content, *Azolla* is used as a fodder for sheep, pigs, ducks, etc. The ability of *Azolla* to remove nitrates and phosphorous from water has resulted in improvement of water quality. Additionally, *Azolla* has been used to remove heavy metals from water. Ten useful characteristics attributed to this association have been described (Van Hove and Lejeune 1996; Lejeune et al. 1999), with the capacity to fix atmospheric nitrogen, high productivity, high protein content, and a depressive influence on both aquatic weeds and $NH_3$ volatilization being considered unquestionably useful.

The same characteristic feature that makes *Azolla* useful for weed suppression and biofertilization of fields (namely, the ability to grow in thick mats) also results in a number of negative ecological impacts. For example, growth of *Azolla* mats in streams in Zimbabwe has been shown to have a negative impact on animal biodiversity (Gratwicke and Marshall 2001). In many regions where *Azolla* is an invasive species, it has overgrown many native species. In efforts to control *Azolla* growth, biological controls such as the introduction of a frond-feeding weevil (McConnachie et al. 2004) or the flea beetle (Hill and Oberholzer 2002) are being explored. Thus, the overall ecological impact of the *Azolla* association continues to expand and may reach even to Mars, since *Azolla* is currently being used in studies examining possible bioregenerative life support on Mars (http://www.highmars.org/niac/niac04.html, The Caves of Mars Project website).

## Cyanobionts Becoming a Nitrogen-Fixing "Organelle"?

The most important recent advancements related to the *Azolla* symbiosis have been proteomic (Ekman et al. 2008) and genomic analyses (Ran et al. 2010) of the cyanobacterium of the *A. filiculoides* symbiosis. In brief, proteomic analyses revealed that processes related to energy production, nitrogen, and carbon metabolism and stress-related functions (e.g., superoxide dismutase and peroxiredoxins) were upregulated in the cyanobiont compared with a free-living strain, whereas photosynthesis and metabolic turnover rates were downregulated (Ekman et al. 2008). Genome sequencing of the cyanobiont of *A. filiculoides* strongly suggests that these cyanobionts are at the initial phase of a transition from a free-living organism to a nitrogen-fixing plant entity. There has been coevolution between *Azolla* and the cyanobiont with genome degradation and signs of reductive genome evolution resulting in an organism devoted to nitrogen fixation and devoid of autonomous growth (Ran et al. 2010). Noteworthy is the loss of function within gene categories for basic metabolic processes such as glycolysis, replication, and nutrient uptake. This genomic analysis now opens the door for obtaining a much better understanding of this ecologically and evolutionarily important symbiosis.

## The Cycad Symbioses

### Introduction

First appearing in the Pennsylvanian era some 300 million years ago, cycads are the most primitive and longest-lived of present day seed plants (gymnosperms). They are also the only gymnosperms that enter into symbiotic associations with cyanobacteria. Once plentiful during the Jurassic, occupying far reaching habitats stretching from Alaska and Siberia to the Antarctic, they are now found in diminishing numbers in certain subtropical and tropical regions of mostly the southern hemisphere, including Australia, parts of Southern Asia, and South Africa (Brenner et al. 2003; Vessey et al. 2005). There are two or three extant families with some 300 species in 10 genera (Chaw et al. 2005; Vessey et al. 2005; Bergman et al. 2007; see also Lindblad 2009). Cycads are long-lived, cone-bearing evergreen palm-like plants which can reproduce either asexually (producing stem offshoots or suckers) or sexually. They grow terrestrially, with the exception of *Zamia pseudoparasitica* which is the only true epiphytic cycad and hangs from branches by the tap and lateral roots (Stevenson 1993).

In general, cycads have a stout trunk with a large crown of tough spiny leaves and can vary in height from a few tens of centimeters to almost 20 m at maturity. Most cycads produce different root types—a thick taproot that extends some 9–12 m beneath the soil surface, lateral roots, and coralloid roots which are highly specialized lateral roots named for their resemblance to coral. Coralloid roots exhibit negative geotropism, growing sideways and upward toward the soil surface, and are the sites in which symbiotic cyanobacteria can be found (Costa and Lindblad 2002; Lindblad and Costa 2002; Brenner et al. 2003; Vessey et al. 2005; Bergman et al. 2007, 2008).

Cyanobacteria living in association with cycads were first reported in the nineteenth century (Reinke 1872), and this partnership is still the only known example of a naturally occurring plant root-cyanobacterial symbiosis. All cycad species examined to date are able to form symbiosis with nitrogen-fixing cyanobacteria, visible as a dark blue-green band (the cyanobacterial zone) between the inner and outer coralloid root cortex (*Fig. 16.9*). The association between the cycad *Zamia furfuracea* and its native cyanobiont *Nostoc* sp. strain FUR 94201 has been separated and reconstituted successfully under axenic laboratory conditions (Ow et al. 1999). The same authors also showed for the first time that a cycad symbiosis could be established with the soil cyanobacterium *Nostoc* 2S9B, a strain previously shown to form loose associations with wheat roots. The ability of cycads to thrive in nutrient-poor soils is often attributed to the associations they form with cyanobacteria. Nitrogen fixation in cycads not only contributes to the nitrogen metabolism of the plant but also up to 18.8 kg N $ha^{-1}$ $year^{-1}$ to the local nitrogen economy (see: Rai et al. 2000; Vessey et al. 2005).

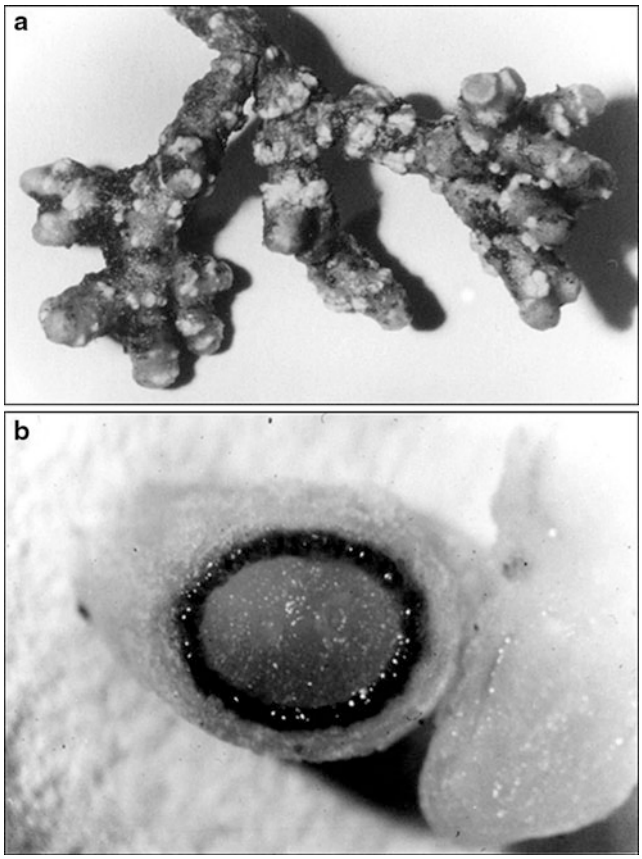



**Fig. 16.9**
**The cycad-*Nostoc* symbiosis. (a) A cycad coralloid root, the site of cyanobacterial infection. (b) Transverse section of the root showing the dark cyanobacterial band between the inner and outer cortical layers [(a) From Lindblad et al. (1985a) with permission. (b) From Rai et al. (2000) with permission]**

Heterotrophic bacteria have been found associated with cyanobacteria recovered from coralloid roots (Chang et al. 1988), although these bacteria have not been found in the cyanobacterial zone within the coralloid cortex (Grilli Caiola 1980). However, bacteria have been found inside the periderm of the coralloid roots of several different cycads (Joubert et al. 1989). It has been suggested that phenolic substances, detected in the mucilaginous material of the cyanobacterial zone and in the cortical cells surrounding the cyanobacterial zone, have antimicrobial properties that exclude organisms other than cyanobacteria (Grilli Caiola 1980; Obukowicz et al. 1981). Phenolics are among the most widespread plant secondary metabolites and have been shown to function as signaling molecules in the establishment of legume-rhizobial symbiosis and vesicular-arbuscular mycorrhiza, and there is some evidence that phenolic compounds may also influence the formation of symbiosis between cyanobacteria and cycads, as well as their metabolism (see Lobakova et al. 2004).

## Cycads and Toxicity

The toxic properties of cycads have been noted for centuries, with the azoxyglycosides, cycasin and macrozamine, and the neurotoxic nonprotein amino acid β-methylamino-L-alanine (BMAA), receiving the most documentation. BMAA synthesis has been associated with the cyanobionts of cycads (Cox et al. 2003) as well as the host plant (e.g., Vega and Bell 1967; Polsky et al. 1972; Marler et al. 2010) and has since been found in all known groups of free-living cyanobacteria (Cox et al. 2005; Banack et al. 2007). BMAA has been linked to the high incidence of the progressive neurodegenerative disease amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS-PDC) in the Chamorro people on the island of Guam. They were thought to have acquired damaging levels of the toxin through the ingestion of cycad seed-eating flying foxes in which the toxin is believed to have been "biomagnified" (Banack and Cox 2003; Cox et al. 2003; Banack et al. 2006). However, this hypothesis is controversial (see Marler et al. 2010; Snyder and Marler 2011) not least because of difficulties in reliable separation and detection of BMAA. Some groups have confirmed the presence of BMAA in cyanobacteria and cycad seeds (Esterhuizen and Downing 2008; Spáčil et al. 2010), whereas contradictory results have been obtained following analysis of underivatized samples (e.g., Rosén and Hellenäs 2008; Krüger et al. 2010). These latter studies were, however, able to detect 2, 4-diaminobutyric acid (DAB), a neurotoxic isomer of BMAA (Rosén and Hellenäs 2008; Krüger et al. 2010). The recent work by Banack et al. (2010) addressed the issues concerning effective BMAA analysis, and they were able to reliably and consistently separate BMAA from 2,4 DAB as well as distinguish it from other compounds previously mistaken for BMAA during chloroformate derivitization for GC analysis (Banack et al. 2010). They concluded that cyanobacteria do indeed produce BMAA and its neurotoxic structural isomer 2, 4-DAB.

The biological significance of the toxins remains unclear, although possible roles include protection from herbivory, competition with other plants, or antibacterial and antifungal defense mechanisms (Castillo-Guevara and Rico-Gray 2003). BMAA is, however, heavily concentrated in the coralloid roots, raising the suggestion that the primary function of this toxin is not anti-herbivory (Marler et al. 2010). An alternative role might be in communication involved in the initiation and maintenance of the symbiotic association (Marler et al. 2010; Snyder and Marler 2011).

## The Symbionts

The cyanobionts of cycads are filamentous, heterocystous species, largely restricted to the genus *Nostoc*, although *Calothrix* spp. have occasionally been found (Grobbelaar et al. 1987; Costa and Lindblad 2002; Rasmussen and Nilsson 2002; Gehringer et al. 2010; Thajuddin et al. 2010). Cycads can host multiple cyanobacterial strains in single plants as well as in single roots (Zheng et al. 2002; Thajuddin et al. 2010). However, taxonomic studies have revealed little if any specificity between cycads and their cyanobionts (Lindblad et al. 1989; Lotti et al. 1996; Costa et al. 1999; Zheng et al. 2002; Costa et al. 2004; Gehringer et al. 2010); the cyanobiont species found within a host plant is probably determined by the predominant symbiotically competent species available within the immediate rhizosphere (Gehringer et al. 2010).

## Establishment of Symbiosis

Coralloid root development begins with the initiation of precoralloid root formation in the seedling (Rai et al. 2000; Lindblad 2009). Cyanobacteria are not found in precoralloids, and their presence is not necessary for the initiation of precoralloid development (Staff and Ahern 1993), although exposure to light is considered significant in many cycad genera (Webb 1983a, b). Infection by cyanobacteria can occur at any stage of precoralloid root maturation, and their presence triggers further growth and the developmental changes required to transform precoralloids into coralloids. The mode of entry of the cyanobacteria is still unclear, although suggested access points have included lenticels, breaks in the dermal tissue, or via the papillose sheath (see Bergman et al. 2007; Lindblad 2009). In addition, bacteria and fungi in the cycad rhizosphere may cause local degradation of the cell wall, enabling the cyanobacteria to penetrate the root (Lobakova et al. 2003). Following entry into the root, the cyanobiont migrates toward the cyanobacterial zone, between the inner and outer cortex, through a channel created through the outer cortex, believed to be caused by the separation, distortion, and destruction of cortical cells (Nathanielsz and Staff 1975).

The process of coralloid formation is irreversible, with one of the most significant changes being a conversion from negative to positive geotropism, resulting in growth sideways and upward toward the soil surface. Other changes include the loss of the papillose sheath, proliferation of apical lenticels, and early differentiation of the conspicuous cyanobacterial zone (Ahern and Staff 1994; see also Rai et al. 2000). Some cycad cells within the cyanobacterial zone undergo a distinct differentiation process to interconnect the two adjacent cortical layers, and this may facilitate the transfer of nutrients between the partners (Lindblad et al. 1985a; Lindblad et al. 1991; Pate et al. 1988; Costa and Lindblad 2002; Vessey et al. 2005; see also Lindblad 2009). Although the cyanobiont location is extracellular, there have been reports of intracellular cyanobionts in *Cycas revoluta* Thunb. and *Macrozamia communis* L. (see Lindblad 2009 and references therein).

## Symbiotic Competence: Fit to Infect?

So, how do cyanobacteria living in the soil reach the sites of infection within the coralloid roots of cycads? As is the case with other plant hosts, cyanobacteria entering into functional symbiosis with cycads produce transient motile filaments

known as hormogonia that are highly adapted to sense and respond to environmental stimuli, including chemicals released by potential host plants. Water extracts from macerated seeds of the cycad *Zamia furfuracea* induce the development of motile hormogonia (Ow et al. 1999) and exhibit some chemoattractive properties. Chemotaxis of hormogonia in response to plant-derived attractants is likely to be of particular importance in the infection of cycad tissue, where the sites of infection receive little or no light, because the plant-derived signals must be able to override the natural phototactic response of the hormogonia.

## Other Symbiotic Competence Factors

Laboratory attempts to reconstitute a functional symbiosis between *Nostoc* PCC 73102 (originally isolated from the cycad *Macrozamia* sp.) and *Nostoc* ATCC 29133 (believed to be the same isolate as PCC 73102 but with a different laboratory history and morphology; see Ow et al. 1999 and references there in) have been unsuccessful (Ow et al. 1999). This is surprising because, as described elsewhere in this chapter, *Nostoc* ATCC 29133 readily enters into functional symbiosis with a wide range of plants such as liverworts, hornworts, and the angiosperm *Gunnera*. This implies the existence of a recognition and compatibility selection process that is able to select certain cyanobacteria and exclude others. Alternatively, successful cyanobionts may have evolved mechanisms to protect against or disguise themselves from the host's natural defense system.

## Carbon Metabolism

As the coralloid roots of most cycad species are located beneath the soil surface, their cyanobionts receive little or no light and are assumed to have a heterotrophic metabolism, possibly using fixed carbon supplied by the host and/or by their own dark $CO_2$ fixation (Lindblad 2009). Freshly isolated cyanobionts from *Cycas revoluta* coralloid roots fail to fix $CO_2$ in vivo under light or dark conditions (Lindblad et al. 1987), but their crude extracts have similar activities of RuBisCo and phosphoribulokinase to those in free-living cultures (Lindblad et al. 1987). The lack of $CO_2$ fixation by the cyanobiont might be due to a reversible inhibitor of RuBisCo, which is lost by dilution in the in vitro assay (see Meeks and Elhai 2002). Alternatively, the photosystems of the cyanobiont may be nonfunctional, or other enzymes of the Calvin cycle may be lacking (Lindblad et al. 1987).

In a study of the *Nostoc*-like cyanobiont of *Zamia skinneri*, a fully developed photosynthetic apparatus containing thylakoids with distinct phycobilisomes, harboring phycobiliproteins, was revealed (Lindblad et al. 1985a). Carboxysomes were also noted, although photosynthesis is unlikely to occur as the coralloid roots were collected from below the soil surface. However, high rates of photosynthetic oxygen evolution have been found in the cyanobacteria isolated from the roots of *Cycas circinalis* which are known to develop at or close to the soil surface where the cyanobacteria may be exposed to light (Perraju et al. 1986). Nevertheless, it is unclear why the dark-associated cycad cyanobionts retain a full photosynthetic apparatus, associated pigments, and carbon-fixing potential (cellular levels of RuBisCo are comparable with those in free-living counterparts).

## Nitrogen Metabolism

Nitrogenase protein is restricted to the heterocysts, including contiguous heterocysts, in both free-living heterocystous cyanobacteria and those in symbiosis with cycads (Bergman et al. 1986). Nitrogenase activity is some three- to fivefold higher in cycad-associated *Nostoc* than in the free-living cultures (Lindblad et al. 1985b). Although nitrogenase activity parallels increasing heterocyst frequency, it reaches a maximum at heterocyst frequencies of around 25–35 % (Lindblad et al. 1985b) at a location where single heterocysts predominate, and declines thereafter, although heterocyst frequency continues to increase and contiguous heterocysts become common (Lindblad et al. 1985b). The possibility that some of the heterocysts within these clusters are metabolically inactive cannot be ruled out (Bergman et al. 1986). Indeed, the decrease in nitrogenase activity observed in older parts of the coralloid roots of *Cycas* and *Zamia* is believed to be in part due to the aging of the cyanobacteria located there (Lindblad 1990).

Cyanobacteria freshly isolated from older coralloid root sections, as well as those located close to the apex of *Macrozamia riedlei*, an Australian cycad in which the coralloid roots can develop up to 0.5 m below the soil, show marked light-stimulated nitrogenase activity (measured by both acetylene reduction and $^{15}N_2$ fixation), providing they are maintained under low (<1 %) $O_2$ levels (Lindblad et al. 1991). The low level of nitrogenase activity recorded in freshly isolated cyanobionts incubated in darkness probably results from loss or damage of heterotrophic mechanisms that previously functioned to provide the necessary ATP to support nitrogenase activity within the intact coralloid roots (Lindblad et al. 1991; see also Lindblad 2009). The authors suggested that damage caused by separation of the cyanobacteria from the host plant disrupts the intercellular microenvironment and any biochemical interactions provided by the intact cyanobiont-coralloid root association (Lindblad et al. 1991).

In free-living cyanobacteria, the ammonia derived from nitrogen fixation is primarily assimilated via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (Muro-Pastor et al. 2005; Flores and Herrero 2005). In many cyanobacteria-plant symbioses, the release, to the host, of ammonia that would otherwise be assimilated by the cyanobiont is achieved partly by a host-mediated decrease in GS activity in the cyanobiont. However, xylem sap analysis of freshly detached *Nostoc*-colonized coralloid roots has revealed that the nitrogen fixed by the cyanobiont is instead translocated to the host as either a combination of the amino

acids citrulline and glutamine (in Zamiaceae) or glutamine alone (although in the Boweniaceae and Cycadaceae glutamic acid is also present; Pate et al. 1988; Costa and Lindblad 2002; Bergman et al. 2007). The possibility that ammonium is not the transferred N-solute in cycad associations is supported by the findings that cyanobionts of *Cycas revoluta*, *Ceratozamia mexicana*, and *Zamia skinneri* all have high in vitro GS activity and GS protein levels similar to those found in free-living cyanobacteria, including strains originally isolated from cycads (Lindblad and Bergman 1986).

Despite supplying the host with most of the nitrogen they fix, the cyanobionts do not show signs of nitrogen starvation, as they retain abundant sources of combined nitrogen. These include cyanophycin, which is a specialized nitrogen reserve (consisting of a copolymer of arginine and aspartic acid), carboxysomes, and phycobiliproteins (accessory photopigments), all of which can be degraded under conditions of nitrogen starvation (Meeks and Elhai 2002).

### Other Adaptations to Life in Symbiosis

Cycad cyanobionts generally show little morphological change compared with their free-living counterparts, apart from an increased heterocyst frequency. Baulina and Lobakova (2003a, b) observed cyanobionts with vegetative cells and heterocysts showing considerable degradation of the peptidoglycan layer. However, it is not clear if such cells are functional or are in various states of senescence. In free-living cyanobacteria, heterocysts largely occur singly at almost regularly spaced intervals within a filament of photosynthetic vegetative cells (reviewed by Zhang et al. 2006). In return for the fixed carbon (possibly sucrose; see Meeks and Elhai 2002 for further discussion) they receive from neighboring vegetative cells, the heterocyst provides fixed nitrogen. In symbiosis, there is a developmental gradient of heterocysts from low (a heterocyst frequency of 16.7 % of total cells) in the growing tips of the coralloid roots to high (46 %) in the base (older parts) of the roots. Indeed, at the very growing tips of the coralloid roots of some cycad species are short "free-living" type filaments that resemble hormogonia. There are few heterocysts, those present being restricted mostly to the filament poles (Grilli Caiola 1980). In various cycad species, multiple contiguous heterocysts (double to quadruple) are found frequently in older root tissue but rarely at the tip (see Lindblad et al. 1985a, 1985b; Lindblad et al. 1991: also reviewed by Lindblad 2009). However, these contiguous heterocysts in older tissue may not be metabolically active (Bergman et al. 1986).

## Cyanolichens

### Introduction

Lichens are associations of symbiotic fungi and green algae (bipartite lichens) or symbiotic fungi, green algae, and cyanobacteria (tripartite lichens). A lichen thallus is quite distinct in appearance from either of its symbionts, and its name refers to the dominating fungal partner (the mycobiont). Lichen thalli represent an integration of the mycobiont's heterotrophic metabolism and the autotrophic metabolism of the photosynthetic partners (the photobionts: green algae and cyanobacteria). In tripartite lichens, the cyanobacterial partner (the cyanobiont) is also referred to as the "secondary photobiont," whereas the green algal partner is referred to as the "primary photobiont." All lichens having a cyanobiont, either as the sole photobiont or as a secondary photobiont, are called "cyanolichens." For lack of space, this review on cyanolichens is brief. The reader can find further details in books and reviews elsewhere (Galun 1988; Ahmadjian 1993; Nash 1996; Rai et al. 2002; Rikkinen 2002). The journal Bryologist regularly lists recent literature on lichens, and a literature search is also possible at Mattick's Literature Index website ([{http://www.toyen.uio.no/botanisk/bot-mus/lav/sok_rll.htm}]).

There are approximately 1,550 known species of cyanolichens, representing roughly 12–13 % of all known lichens; among these, two-thirds are bipartite and the rest tripartite species. Lichen symbioses are thought to have arisen independently on several occasions. An estimated 100 lichenization events have occurred during diversification of extant fungi (Aptroot 1998; see also Rikkinen 2002).

### The Symbionts

#### Mycobionts

The current classification of fungi is in transition, and molecular approaches are being used to fine-tune it (Tehler et al. 2000; see also Rikkinen 2002). Approximately 13,500 species of lichen-forming fungi presently belong mostly to the Ascomycetes (98 %) and very few to the Basidiomycetes (1.6 %) and fungi imperfecti (0.4 %). About 15–18 orders of Ascomycetes (nearly 130 genera from 50 families) include lichen-forming taxa (see Rikkinen 2002). Most are from two orders, the Lecanorales and Lichinales. Nearly 1,700 species of fungi associate with different types of cyanobacteria. A fairly comprehensive list of these has been provided earlier (Rikkinen 2002).

#### Cyanobionts

A variety of heterocyst-producing and unicellular cyanobacteria occur as cyanobionts in cyanolichens where the mycobiont is an ascomycete. Among heterocystous forms, *Nostoc* is the most common. Others are *Scytonema*, *Calothrix*, *Dichothrix*, and *Fischerella* (including *Hypomorpha*, *Stigonema*, and *Mastigocladus*). Unicellular forms that occur as cyanobionts in cyanolichens include *Gloeocapsa* (also *Chroococcus*), *Gloeothece*, *Synechocystis* (also *Aphanocapsa*), *Chroococcidiopsis*, *Hyella*, and *Myxosarcina* (see Rai et al. 2000; Rikkinen 2002). The range of cyanobionts in cyanolichens where the mycobiont is

a basidiomycete is rather limited. Only two cyanobacteria (*Chroococcus* and *Scytonema*) are reported as cyanobionts in basidiolichens (see Schenk 1992).

Analyses of $tRNA^{Leu}$ (UAA) introns and 16S rDNA sequences have been used as genetic markers to study the diversity of *Nostoc* cyanobionts (Paulsrud and Lindblad 1998; Paulsrud et al. 1998, 2000, 2001; Lohtander et al. 2002; Rikkinen et al. 2002). These studies have shown that genetic variation among lichen-forming *Nostoc* strains is considerable. Within symbiotic *Nostoc* strains, there seem to be several subgroups. For example, one subgroup of *Nostoc* strains seems to occur only in epiphytic cyanolichens, whereas another includes strains that occur as cyanobionts in terricolous cyanolichens and other symbiotic systems (Rikkinen 2002; Rikkinen et al. 2002). Miura and Yokoto (2006) have reported the occurrence of two cyanobionts in the same lichen. Based on morphological observations and 16 s rDNA sequences of cyanobacterial isolates from lichens, they reported the occurrence of *Nostoc*, *Calothrix*, *Cylindrospermum*, *Phormidium*, *Leptolyngbya*, *Microcystis*, and *Chroococcidiopsis*.

## The Lichen Thallus

Lichen thalli have a stable and organized structure quite distinct from any of their symbionts. The thalli appear to be crustose (small lobes and scales; e.g., *Collema*), foliose (flat and dorsiventral lobes; e.g., *Peltigera*), or fruiticose (round or flat thalli, upright, or hanging down from the substratum; e.g., *Stereocaulon*). In foliose or fruticose thalli, the fungal hyphae form an outer pseudoparenchymatous zone (the cortex) that covers or encloses a more loosely interwoven medulla. Within the thallus, the partners remain extracellular to each other and can be isolated and grown in culture, but the symbiosis is fairly stable in nature because of the balanced and synchronized growth and development of the symbionts. Thinner cell walls (less sheath material) and specialized hyphae and haustoria, showing transfer cell ultrastructure, enable close contact between the mycobiont and the cyanobiont. Since the bulk of the thallus consists of the heterotrophic mycobiont, the thallus interior is microaerobic (see Rai et al. 2000).

In bipartite lichens, cyanobionts either are dispersed throughout the thallus (e.g., *Collema*) or occupy a distinct layer below the upper cortex (e.g., *P. canina*). In tripartite lichens, the cyanobiont is located in cephalodia, which occur at the upper surface of the thallus (external cephalodia; e.g., in *P. aphthosa*) or inside the medulla (internal cephalodia; e.g., in *Nephroma arcticum*). In some cases, internal cephalodia are found close to the lower surface of the thallus (e.g., in *P. venosa*). In tripartite lichens, direct contact between the cyanobiont and the phycobiont (green algal partner) is never direct.

Lichen symbioses perpetuate by direct transmission of the cyanobiont from one generation to the next and, as a result of the acquisition, by the mycobiont of fresh cyanobiont from the environment. For example, a lichen thallus can develop from propagules (phyllidia, isidia, soredia, and hormocystangia) of a preexisting thallus (direct transmission) or from fresh synthesis (fresh acquisition of cyanobiont from the environment). The former mode of transmission allows prolonged continuity of the partners. Similar modes of cyanobiont acquisition also apply to the development of cephalodia (see Rai et al. 2000). Cyanobionts are essential for the formation of thalli or cephalodia in cyanolichens. They may stimulate thallus morphogenesis but do not determine the kind of thallus formed; the mycobiont determines the structure and chemistry of a cyanolichen. Different lichen fungi form different lichen thalli even if associating with the same cyanobiont (see Rai 1990; Rai et al. 2000, 2002).

Because they are slow growing, the initiation and development of lichens is difficult to study in nature. Development of a lichen thallus afresh involves germination of the mycobiont spore, development of the hyphal mat, contact, recognition, and acquisition of the cyanobiont, and structural-functional integration of the symbionts. While a thallus may result within months when starting from propagules, it takes years when starting from isolated partners. During laboratory synthesis of lichens, the partners initially form undifferentiated aggregates that later differentiate into thalli (see Rai et al. 2000). Fresh synthesis in nature may also start from mycobiont hyphae that become detached and acquire a fresh cyanobiont (Smith and Douglas 1987).

Development of each cephalodium is a new event. External cephalodia develop on the main thallus by entrapment of a cyanobiont by hairs on the thallus surface, followed by involvement of medullary hyphae immediately below. Internal cephalodia may develop in a similar fashion starting with cyanobiont entrapment by cortical hyphae or rhizines. The cyanobiont, enmeshed by a thick layer of mycobiont, is pressed into the thallus where the cephalodium eventually develops. New cephalodia may develop from hormogonia released by earlier cephalodia (Stocker-Wörgötter 1995), ensuring cyanobiont homogeneity among cephalodia of a thallus. In laboratory synthesis, however, cephalodia developed by attachment of hyphae from primordia (containing cyanobiont and mycobiont) to the green thallus (Stocker-Wörgötter and Turk 1994). The latter mode of cephalodia development, if prevalent in nature, should cause considerable heterogeneity among symbiont populations within a single thallus, but this is not the case. Occasional reports of different cyanobionts (see Rai 1990) or different strains of a cyanobiont (Paulsrud et al. 2000) among cephalodia of a single thallus may, however, indicate instances of cephalodia development by capture of a fresh cyanobiont in some lichens. Entry of the cyanobiont for development of internal cephalodia is from the lower surface of the thallus, but occasionally, when the cyanobiont enters from above, the phycobiont layer is pressed deep into the medulla.

## Recognition and Signal Exchange Between Partners

For the right symbionts to enter into a lichen symbiosis, signal exchange must occur between the partners. Transformation of

*Nostoc* colonies into the symbiotic state occurred without the necessity for direct contact with the mycobiont during resynthesis of *Peltigera praetextata* (Yoshimura and Yamamoto 1991). This suggests that the substance responsible for *Nostoc* transformation may be a diffusible soluble substance from the mycobiont. The exact identity of such a substance is not known, but lichen-forming fungi do produce a large number of unique secondary metabolites and compounds, and their possible roles in signal exchange need to be investigated. Lectins (glycoproteins) of mycobiont origin have been implicated in the recognition of the cyanobiont by a mycobiont (Rai 1990; Kardish et al. 1991; Lehr et al. 1995, 2000). Cyanobiont cell surfaces possess specific sugars, fimbriae (pili), and in some cases, lectins, which may have a role in recognition and adherence (Stewart et al. 1983; Kardish et al. 1991; see Rai et al. 2000).

Direct observations, lectin-binding experiments, and $tRNA^{Leu}$ intron analysis all indicate a broader cyanobiont-mycobiont specificity in lichens than that in other cyanobacterial symbioses. Different lichen species can have the same cyanobiont, and different cyanobionts have been reported among cephalodia of a single lichen thallus. Different *Nostoc* strains have been found in different lichen species from the same site, while different lichen species from distant places had the same *Nostoc* strain. In chimeroid thalli, both bipartite and tripartite morphotypes are reported to have the same cyanobiont strain (Paulsrud et al. 1998, 2000, 2001). Overall, there is a great deal of cyanobiont diversity among the lichens, and much of it might be contributed by the mode of cyanobiont acquisition during the development of the lichen thallus and cephalodia (Rai et al. 2000).

Many cyanolichens share similar environmental requirements and may depend on a common pool of cyanobionts. Many cyanolichen species having identical cyanobiont strains co-occur in a particular habitat, forming characteristic communities or "guilds" (Rikkinen et al. 2002). Within a guild, the cyanobionts of all lichens are closely related, but the mycobionts are not. While some guilds include different mycobiont genera or even families, some closely related mycobionts belong to different guilds (associate with different types of cyanobionts).

## Structural-Functional Changes

Cyanobionts undergo structural-functional changes in the symbiosis that permit a close interaction and development of nutrient exchange between the partners. These changes include increased cell size, altered cell shape, lack of polyphosphate reserves, fewer carboxysomes, less sheath material, and slower growth and cell division (Rai et al. 2000).

The cyanobionts are photosynthetically active and fix $CO_2$ via the $C_3$ pathway. In addition, there is a significant level of dark $CO_2$ fixation (15–20 % of that in the light) via the $C_4$ pathway (Rai et al. 2000; Palmqvist 2002). However, $CO_2$ fixation by cyanobionts in internal cephalodia, particularly those on the lower surface (e.g., *Peltigera venosa*), may be minimal due to low light and RuBisCo. In the tripartite *Nephroma arcticum*, the *Nostoc* cyanobiont has 70 % fewer carboxysomes compared with that in the bipartite *P. canina* (Bergman and Rai 1989).

In free-living cyanobacteria, heterocysts are regularly spaced and represent about 5–10 % of the cell population. There is a change in the spacing pattern of heterocysts and an increase in their frequency in the cyanobionts in tripartite lichens (heterocyst frequency 15–35 %) but not in bipartite lichens. Heterocyst frequency correlates with the status of fixed carbon in the cyanobiont; in bipartite lichens, the cyanobiont bears the burden of providing both fixed nitrogen and fixed carbon to the mycobiont, whereas in tripartite lichens, it provides fixed nitrogen only. Indeed heterocyst frequency increases when *Nostoc* isolates are grown in the dark with sugars. In many cyanobacterial-plant symbioses, where the cyanobiont receives fixed carbon from the plant host, heterocyst frequencies of up to 80 % can occur (see Rai et al. 2002).

In free-living cyanobacteria, glutamine synthetase (GS) is the primary ammonia assimilating enzyme, and GS levels in heterocysts are twofold higher than those in vegetative cells (Bergman et al. 1985). In cyanobionts, the GS activity and protein levels decrease by over 90 %, and the remaining GS is uniformly distributed among heterocysts and vegetative cells (Bergman and Rai 1989; Rai 2002). GS activity is undetectable in the mycobiont, but mycobiont hyphae in contact with cyanobiont cells show high levels of nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent glutamate dehydrogenase (GDH) activity.

Nitrogen fixation occurs in all lichens containing heterocystous cyanobionts. The rates are higher in tripartite lichens owing to the higher heterocyst frequency of the cyanobiont (see Rai et al. 2000; Rai 2002). In contrast to free-living forms, cyanobionts in bipartite lichens and in excised cephalodia continue to fix $N_2$ even in the presence of nitrate or ammonia (Stewart and Rowell 1977; Rai et al. 1980). However, nitrogen fixation by the cyanobiont in cephalodia attached to the main thallus of the tripartite lichen *P. aphthosa* was repressed by nitrate and ammonia. The effect was obviously mediated via the phycobiont. Significant levels of $N_2$ fixation have also been reported in darkness, and under these conditions ammonia has an inhibitory effect (Rai et al. 1981a, 1983b). As in the free-living forms, nitrogenase is located only in the heterocysts, despite the microaerobic conditions in lichen thalli (Bergman et al. 1986).

The extent of the changes described above varies from young to older, more mature parts of the thallus. While growth rate gradually declines, cell division and GS levels, the levels of $N_2$ fixation, $CO_2$ fixation, and heterocyst frequency of the cyanobiont increase. There is a parallel increase in the GDH activity in the mycobiont (Rowell et al. 1985; Hill 1989; Rai 2002). Still undetermined is whether these changes are caused by the mycobiont or by endogenous regulation due to special environmental conditions offered by the host in the symbiosis.

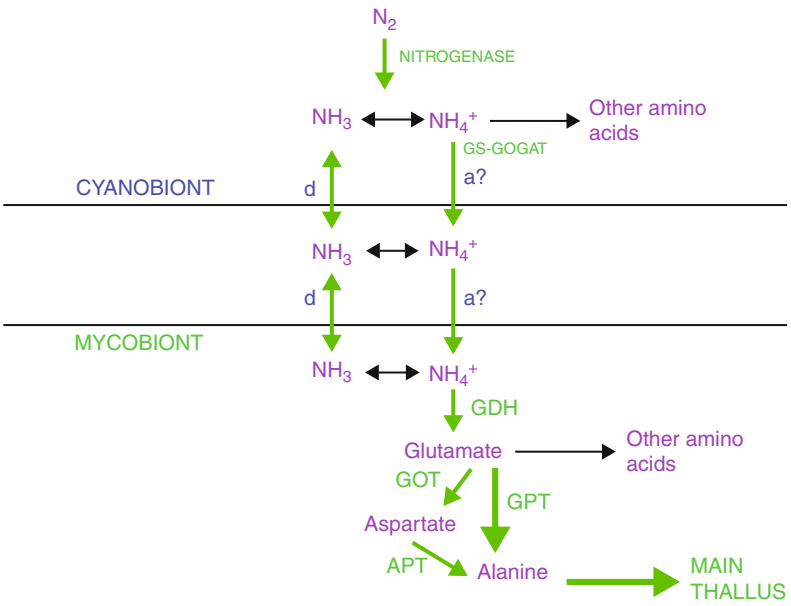



**Fig. 16.10**
**Pathways of N-metabolism in *P. aphthosa*. a, active transport; d, diffusion. *GS* glutamine synthetase, *GOGAT* glutamate synthase, *GOT* glutamate-oxaloacetate transaminase, *GPT* glutamate-pyruvate transaminase, *GDH* glutamate dehydrogenase, *APT* aspartate-pyruvate transaminase**

## Nutrient Exchange

Most studies on nutrient exchange relate to carbon and nitrogen transfer from the cyanobiont to the mycobiont in foliose lichens, particularly *Peltigera* species. Such nutrient transfer is biotrophic in nature and varies along the lichen thallus. From young to mature parts of the lichen thallus, the cyanobiont increases fixation and release of nitrogen and carbon. Specialized mycobiont hyphae and haustoria showing transfer cell ultrastructure (TCU) may play an important role in the nutrient exchange (see Rai et al. 2000).

In bipartite cyanolichens, 70–80 % of the $CO_2$ fixed is released by the cyanobiont to the mycobiont. The transfer of fixed carbon occurs mostly in the light and in the form of glucose. Cyanobionts in tripartite lichens transfer little (<5 % of $CO_2$ fixed) or no fixed C to the mycobiont. Their primary role seems to be the provision of fixed nitrogen. It would be interesting to know whether the cyanobionts in internal cephalodia occurring deep in the medulla or on the undersurface of a lichen thallus actually receive any fixed carbon from the phycobiont (either directly or via the mycobiont). The glucose transferred to the mycobiont is converted to mannitol, which serves as both a C source and a physiological buffer. Mannitol production by lichenized fungi could be an effective way of sequestering the fixed carbon since the other partners cannot use it. The mechanism underlying glucose transfer is not fully understood, but the glucose is thought to originate from a glucan pool rather than directly from $CO_2$ fixation. Altered cell wall synthesis may lead to a diversion of sugars from cell wall synthesis to simple release. Release of glucose declines sharply and stops soon after the isolation of the cyanobiont, indicating the influence of mycobiont and symbiotic conditions in the thallus on this process (Smith and Douglas 1987; Meindl and Loos 1990; Rai 1990; Palmqvist 2002).

$^{15}N$ tracer studies in *P. aphthosa* (tripartite) and *P. canina* (bipartite) have concluded that fixed N is transferred from cyanobiont to the mycobiont as ammonia (Rai et al. 1981b, 1983a). Over 90 % of the $N_2$ fixed in *P. aphthosa* (and about 50 % in *P. canina*) is released by the cyanobiont because GS in heterocysts is repressed. The partitioning of fixed N among the partners is proportionate to their contribution to the thallus composition. In *P. aphthosa*, the ammonium released by the cyanobiont is primarily assimilated by the mycobiont in cephalodia, and the phycobiont receives fixed N via the mycobiont. The mechanism of ammonia release by the cyanobiont and its uptake by the mycobiont at the cyanobiont-mycobiont interface have not been investigated. However, diffusion of $NH_3$ from heterocysts can occur in the absence of ammonia assimilation by GS. Ammonia assimilation in the mycobiont occurs via GDH followed by aminotransferases. In pulse-chase experiments, much of the $^{15}N$ label accumulated as alanine in the mycobiont of *P. aphthosa* cephalodia. Alanine could be the principal compound transferred to the rest of thallus (*Fig. 16.10*).

## Ecological Significance

Lichens are ubiquitous, occurring in terrestrial as well as aquatic habitats from the equator to the highest latitudes, at sea level to

9,000-m altitude, and in the wettest to driest habitats. They are excellent colonizers of nutrient-poor habitats (sand dunes, rocks, forest floors, and the surfaces of other vegetation), form dominant vegetation in tundra and arctic-alpine regions, and contribute significantly to the N economy of these ecosystems. Lichens are good bioindicators of air pollution.

Cyanobionts endow mycobionts with N and C autotrophy and thereby widen their potential habitats. In a lichen thallus, cyanobionts gain a safe habitat and protection from uncertainty of fluctuating nutrient availability and climatic conditions in nature.

### Outlook

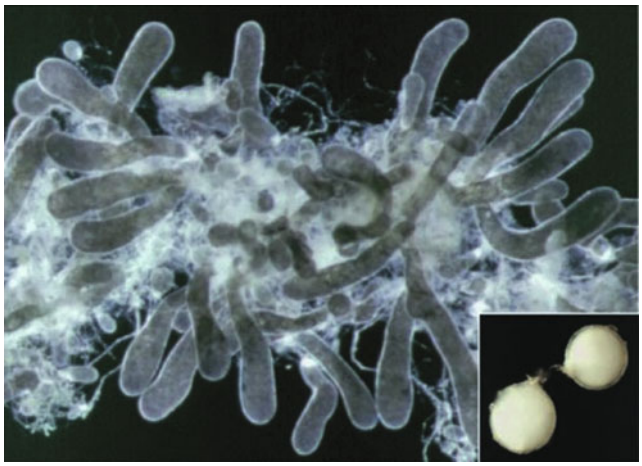
Many interesting aspects of the lichen symbioses remain to be elucidated. These include release and uptake of nutrients at the cyanobiont-mycobiont interface, cyanobiont acquisition, and regulatory mechanisms enforcing synchronized growth and development of the partners. Furthermore, whether the structural-functional changes in symbionts are a result of endogenous regulation due to the symbiotic environment (e.g., microaerobiosis, restricted growth, and cell division) or whether they are directly caused by the mycobiont will need to be resolved.

## The *Geosiphon pyriformis* - *Nostoc* Endocyanosis and Its Relationship to the Arbuscular Mycorrhiza (AM)

### The *Geosiphon pyriformis* Symbiosis

The fungus *Geosiphon pyriformis* (Kütz.) v. Wettstein (von Wettstein 1915) forms the only known fungal endocyanosis (endocytobiotic association with cyanobacteria). The coenocytic fungus forms unicellular, multinucleated cells ("bladders") of up to 2 mm in size (◉ *Fig. 16.11*), harboring endosymbiotic, filamentous cyanobacteria of the genus *Nostoc*. There have been only six reports describing this symbiosis in nature at locations ranging from eastern Germany to Austria. Probably, the symbiosis is geographically widespread in Central Europe but, due to its small size, rarely reported. Presently, field sites around the small village of Bieber in the Spessart Mountains (Germany) are the only known stable natural habitats worldwide (Mollenhauer 1992; Schüßler and Wolf 2005).

The species name "*Geosiphon pyriforme*" was sometimes used for the fungus as well as for the symbiosis because the latter was often regarded as a "phycomycetous lichen." Nowadays endosymbiotic associations are usually excluded from lichen definitions (Hawksworth and Honegger 1994). Thus, the species name should be used for the fungus only, also because phylogenetically the *Geosiphon* fungus belongs to the arbuscular mycorrhiza (AM)-forming and related fungi, the *Glomeromycota* (◉ *Fig. 16.12*). Here, the association between the fungus and cyanobacteria is referred to as the *Geosiphon-Nostoc* symbiosis, or simply the *Geosiphon* symbiosis, and the species name of the fungus is used in its orthographically correct form, *Geosiphon pyriformis* (Schüßler 2002).



◉ **Fig. 16.11**
**The *Geosiphon-Nostoc* symbiosis, isolated from a laboratory culture on natural substrate, incubated in liquid medium. The *dark* bladders are about 1.5 mm in length. The *insert* shows *Geosiphon pyriformis* spores, which have a diameter of about 250 µm**

### The Symbionts

#### *Geosiphon pyriformis*

After its original description as *Botrydium pyriforme*, a siphonal alga (Kützing 1849), *Geosiphon pyriformis* (as *G. pyriforme*) was recognized as a phycomycete (fungus with aseptate hyphae; Knapp 1933). Sixty years later, based on suggestions by Walter Gams, it was suggested that *Geosiphon* could be related to *Glomus*-like fungi (Mollenhauer 1992). Such fungi form the arbuscular mycorrhiza (AM) symbiosis with land plants which is extremely important ecologically and economically, therefore verification of the phylogenetic relationship of *Geosiphon* would make it conceivable that *Geosiphon* may also be capable of such association.

Because the systematics of AM fungi in the last century was based mainly on the characteristics of their spore structure, morphological and ultrastructural criteria of *Geosiphon* spores were compared with those of some AM fungi (Schüßler et al. 1994). This indeed revealed similarities between *G. pyriformis* and AM fungi like *Diversispora epigaea* BEG47 (at that time named *Glomus versiforme*; see Schüßler et al. 2011). Final evidence showing that *Geosiphon* is closely related to AM fungi was based on small subunit ribosomal RNA (SSU rRNA) gene sequences (Gehrig et al. 1996). The AM fungi, together with *Geosiphon*, formed a distinct clade not closely related to any other group of the zygomycetes. Further sequence analyses (Schüßler 1999; Redecker et al. 2000b) showed that *Geosiphon* is closely related to an AM fungus forming two different spore morphs, at that time named *Acaulospora gerdemannii*.

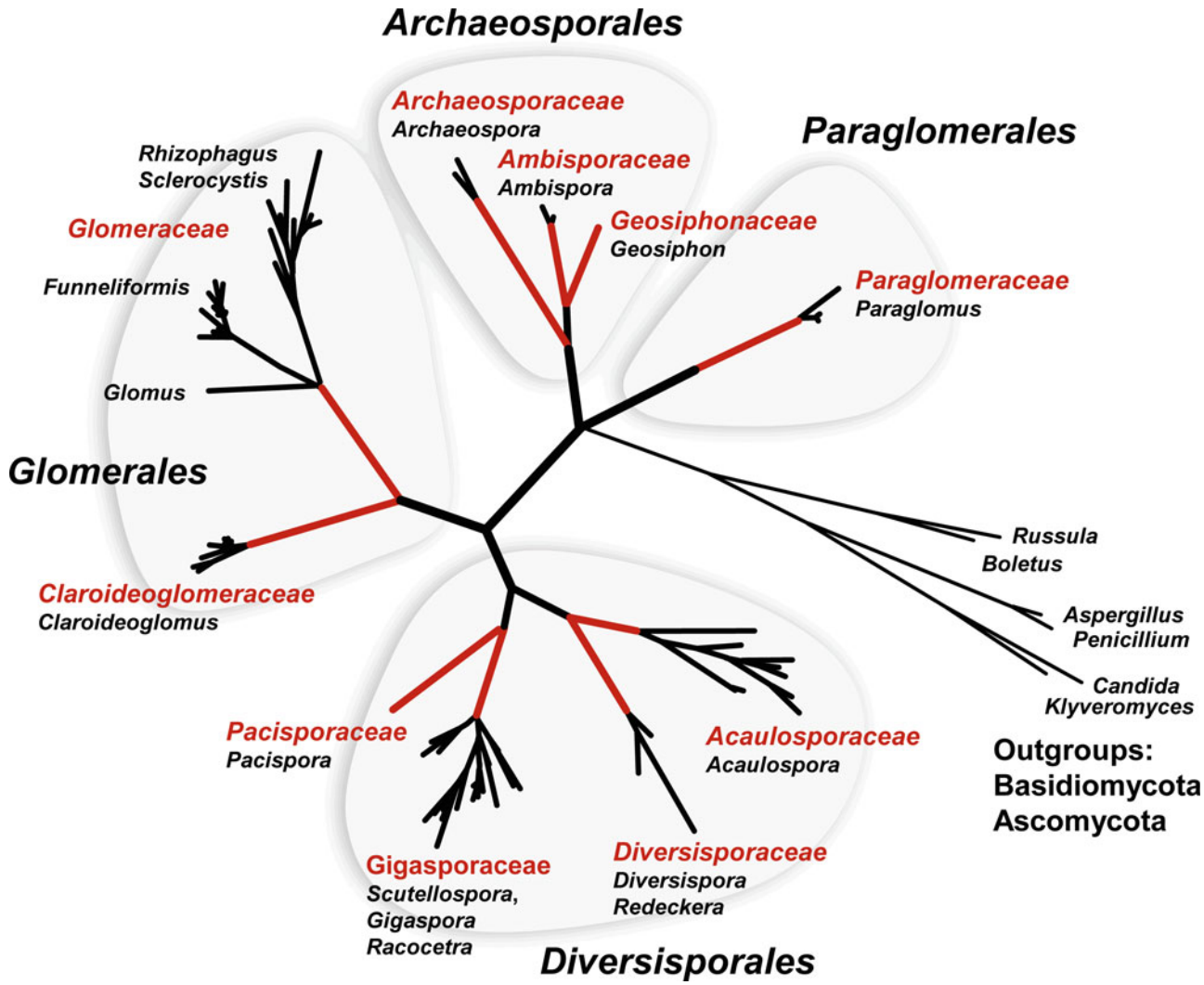



**Fig. 16.12**
**Phylogenetic tree of AM fungi (*Glomeromycota*), including *Geosiphon* (Modified and updated from Schüßler et al. 2001; Schüßler and Walker 2010, http://www.amf-phylogeny.com). Families (represented by *red* branches) and genera are given, except for *Entrophospora* and *Otospora*, for which placement is yet unclear**

Nowadays, the clade containing these lineages is defined as the order *Archaeosporales*, which represents one of the basal main phylogenetic lineages in the phylum containing the AM fungi and *Geosiphon*, the *Glomeromycota* (Schüßler et al. 2001). In this order, the *Geosiphonaceae* clusters as sister to the *Ambisporaceae*, thus appearing to be more derived than the *Archaeosporaceae*, which branch earlier. This means that *Geosiphon* does not represent a sister lineage to the AM fungi, as was sometimes wrongly suggested. It was the analysis of the phylogeny of *Geosiphon* that eventually led to the erection of the *Glomeromycota*, a widely accepted fungal phylum and, eventually to the phylogenetically based, revised classification of the *Glomeromycota* (Schüßler and Walker 2010).

The *Geosiphon-Nostoc* symbiosis attracted interest from the field of AM research. The AM symbiosis is formed by ~80 % of all vascular plants studied (Brundrett 2009) and moreover also by lower plants (Read et al. 2000; Schüßler 2000), despite their lack of roots. Considering this huge number of plants that form AM, it is obvious that the AM must be one of the most important factors in land ecosystems (Smith and Read 2008).

### *Nostoc punctiforme*

The endosymbiont in the *Geosiphon* symbiosis (*Fig. 16.13*) is *N. punctiforme*, which belongs to a clade of cyanobacteria containing many symbiosis-forming members. In laboratory cultures (Schüßler and Wolf 2005), a strain that originally was isolated from the *Geosiphon* symbiosis was used (Mollenhauer

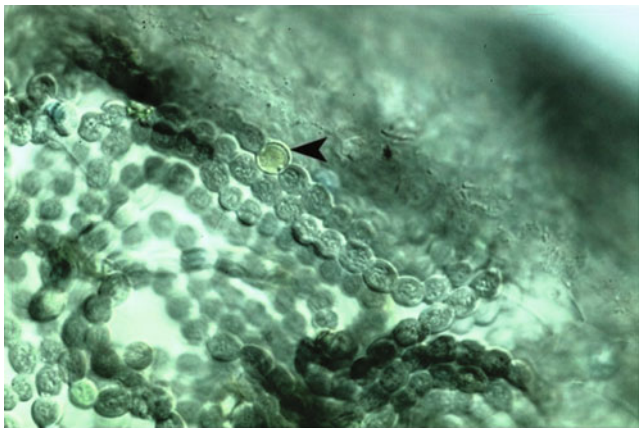


**Fig. 16.13**
**Endosymbiotic *Nostoc* cells (about 7 × 6 μm in size), within a *Geosiphon* bladder. One heterocyst is in focus (*arrowhead*)**

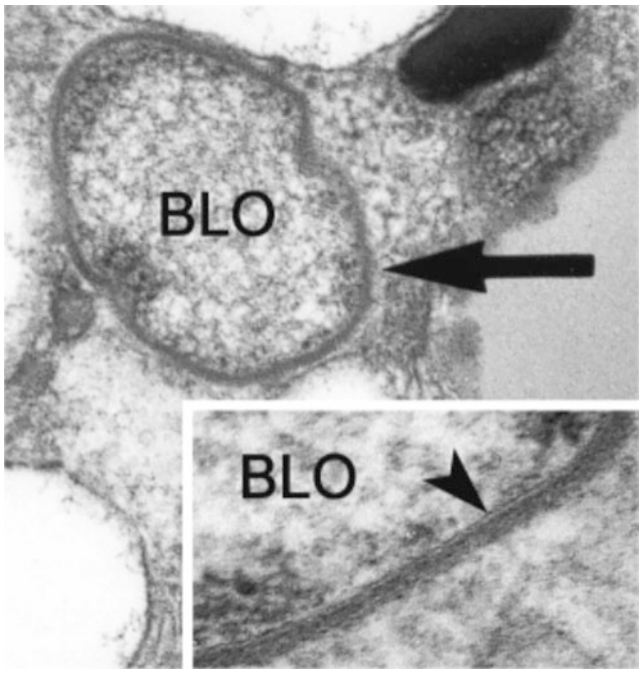



**Fig. 16.14**
**Electron micrograph of a "bacteria like organism" (BLO) in *Geosiphon pyriformis*. BLOs have a diameter of about 0.5 μm and are not enclosed by a host membrane (*arrow*). The *insert* shows the plasma membrane of the BLO (*arrowhead*), as well as the thick murein sacculus. Recent studies show them to be *Mycoplasma*-related, despite the Gram-positive appearance**

1992). However, various other strains of *N. punctiforme* from other symbiotic systems (e.g., *Anthoceros*, *Blasia*, *Gunnera*) are also capable of forming symbiosis with *G. pyriformis*. In the field, *G. pyriformis* was usually found together with *Anthoceros*, and the cyanobionts of *G. pyriformis* associate in symbioses with *Anthoceros* and *Blasia* (Mollenhauer 1992).

It has to be noted that *Geosiphon* harbors another prokaryotic endosymbiont, the so-called BLOs (bacteria-like organisms; *Fig. 16.14*), which are not enclosed by a fungal host membrane but live freely in the cytoplasm (Schüßler et al. 1996; Schüßler 2012). These endosymbiotic bacteria, and those living in most of the AM fungi that were studied for their occurrence, have the same typical ultrastructure. Because they are found in very diverse branches of the *Glomeromycota*, they were considered to be widespread, Gram-positive glomeromycotan symbionts (Schüßler et al. 1994).

The BLOs are indeed ancestral and typical endobacteria in AM fungi. New findings regarding their phylogeny and occurrence in very diverse AM fungal lineages (Naumann et al. 2010) showed that the BLOs are related to the cell-wall-lacking *Mollicutes*. We now know that they are monophyletic and laterally transferred within the AM fungi for more than 450 million years. Their phylogeny and biotrophic lifestyle are shared with the related mycoplasmas, despite the obvious difference of possessing a murein sacculus.

The *Geosiphon* symbiosis is facultative for one of the partners (*Nostoc* can be cultivated without the fungus) and obligate for the other one (*Geosiphon* is obligatory symbiotic). It is conceivable that the fungus is not restricted to the cyanobacteria as symbiotic partner but also forms symbioses with land plants (see below). However, this assumption is still speculative. Regardless, *Geosiphon* belongs to the *Glomeromycota*, and the *Nostoc* symbiosis bears functional and structural similarities to the AM. Thus, the *Geosiphon-Nostoc* symbiosis can play a role as a model symbiosis (Schüßler 2012) for the AM, which is difficult to investigate but extremely important. For example, the characterization of symbiosis-related genes is facilitated by use of this symbiosis (e.g., Schüßler et al. 2006).

## Infection Process, Development, and Structure of the Symbiosis

### Infection Process

Both symbiosis partners live in the upper layer and on the surface of humid soil, where they make contact. The interaction is considered to be specific for two reasons: (1) Only certain *Nostoc punctiforme* strains can form this symbiosis. (2) For a successful interaction with the fungus, *Nostoc* has to be differentiated into a specific stage represented by an early immobile stage of the cyanobacterial developmental cycle, the so-called primordium (Mollenhauer et al. 1996). The motile filaments (hormogonia) and late primordial, as well as vegetative stages of *Nostoc*, are not recognized by the fungus. When contacting *Nostoc*, the tip of the fungal hypha bulges out and surrounds part of a cyanobacterial filament, thus incorporating the *Nostoc* cells (*Fig. 16.15*). Usually, 5–15 *Nostoc* cells are taken up during this process, whereas the heterocysts are never incorporated but "cut off" by the fungus (see below). These events are documented in a scientific film available in German and English (Mollenhauer and Mollenhauer 1997).

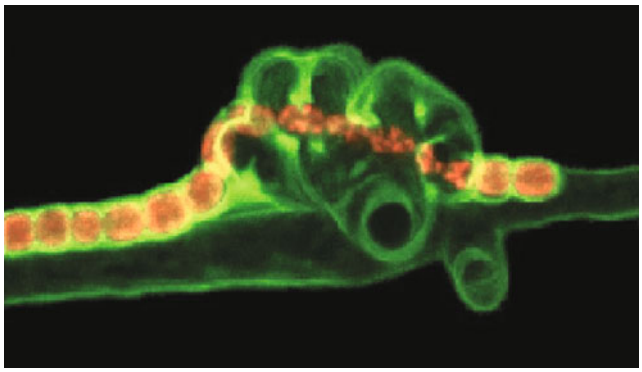


**Fig. 16.15**
**Confocal laser scanning microscopy (CLSM) projection of a short hypha branching from a main hypha (horizontally oriented, 4–6 μm in diameter) and "bulging out" to enclose a part of a *Nostoc* filament. The extracellular polysaccharides of *Nostoc* and the outer layer of the fungal cell wall are labeled by the fluorescence-coupled lectin ConA (*green*). The *Nostoc* cells (*red* autofluorescence, ~4 × 3 μm in size) that are taken up by the fungal structure show strong deformations and irregular and reduced pigment fluorescence**

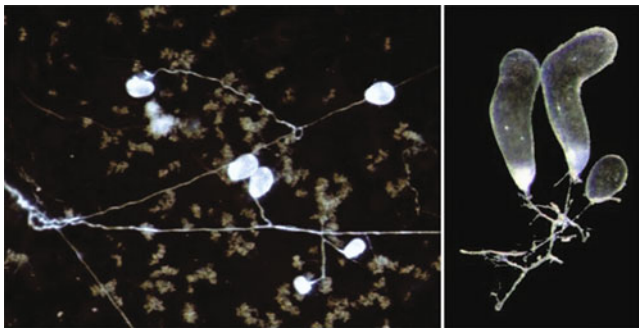


**Fig. 16.16**
**Young *Geosiphon* bladders, 100–150 μm in size, formed on the fungal mycelium 7–10 days after initial uptake of the cyanobacteria (*left*). The irregular structures in the background are vegetatively growing *Nostoc* colonies. At the *right*, two mature bladders of about 1 mm length, together with a young bladder, are shown**

## Development of the Symbiosis

Studies on the development of the *Geosiphon-Nostoc* symbiosis showed that a successful interaction depends on the appropriate developmental stage of the cyanobacterium (Mollenhauer et al. 1996; Wolf and Schüßler 2005). The life cycle of *Nostoc* starts from akinetes (spore-like resting stages) leading to vegetative colonies. These colonies release motile trichomes (hormogonia) which are positively phototactic in dim light and negatively in strong light. As a consequence, the hormogonia often congregate just below the soil surface where they spread and meet their symbiotic partners. They eventually undergo a transformation into an aseriate stage called a primordium. This then differentiates into so-called vegetative cells, which divide and form gelatinous colonies ("thalli"). Only the very early primordial stage of *Nostoc* can interact with the fungal partner to give rise to the symbiotic consortium.

The life cycle of the fungal partner starts from resting spores formed in the upper soil layer. The spores (Schüßler et al. 1994) germinate by the outgrowth of a hypha (sometimes more than one), which branches to form a small mycelium of up to 2–3 cm in the soil. When a hyphal tip contacts a compatible early *Nostoc* primordium, the fungal hypha bulges out just below the apex. This bulging process is repeated several times so that eventually the hyphal tip forms an irregularly shaped structure surrounding a part of a *Nostoc* primordium. After this incorporation into the fungal hypha, large amounts of cytoplasm stream into this *Nostoc*-containing structure, which then starts swelling and develops the fungal bladder (*Fig. 16.16*).

Each individual incorporation event results in the formation of a single pear-shaped aboveground bladder (Knapp 1933). Each bladder represents a polyenergid cell, coenocytic with the fungal mycelium, in which the symbiotic *Nostoc* cells divide and become physiologically active. Laboratory culturing experiments have shown that, as for AM, phosphate limitation (1–2 μM) of the nutrient solution triggers the stable establishment of the symbiosis. N limitation seems not to be a crucial factor. The same situation is found in the natural habitat, so P limitation seems to be a driving factor for the establishment of this symbiosis.

Within the first hours after incorporation into the fungal cytoplasm, the *Nostoc* filaments become heavily deformed, and some cells may die during this process. The photosynthetic pigments degrade considerably (*Fig. 16.15*; Mollenhauer et al. 1996; Schüßler and Wolf 2005). These alterations and significant changes in ultrastructure suggest that during the initial state of endocytotic life, the incorporated cyanobacteria suffer severe stress. Within 2–3 days, the enclosed *Nostoc* cells recover and begin to multiply and grow to reach as much as six times the volume of free-living cells (Schüßler et al. 1996; Mollenhauer and Mollenhauer 1997). Under phosphate limitation, the endosymbiotic cyanobacteria divide much faster and form a much higher biomass compared with the free-living ones (unpublished). In the symbiosis, the *Nostoc* cells arrange in filaments in which heterocysts are formed with the same frequency as in the filaments outside the bladders (if cultured under nitrogen limitation). Mature *Geosiphon* bladders can then reach more than 2 mm in length and up to 6 months in lab cultures. They possess a turgor pressure of about 0.6 MPa (=6 bar) (Schüßler et al. 1995).

## Structure and Compartmentation of the *Geosiphon* Bladder

The *Geosiphon* bladder is effectively a multikaryotic cell, coenocytic with the fungal mycelium in the soil. It shows a strong

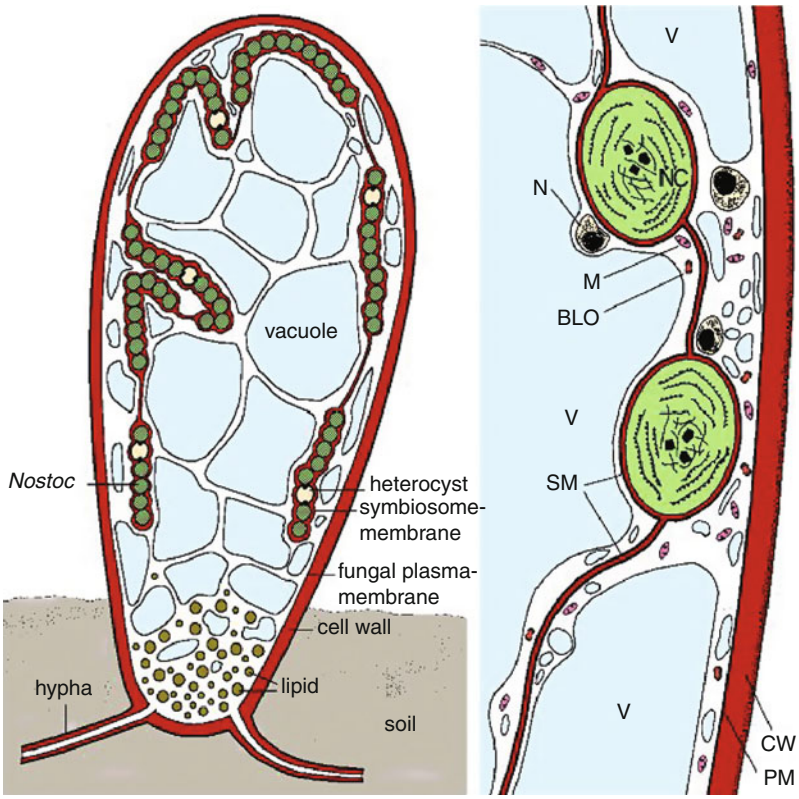



**Fig. 16.17**

**Schematic representation of the compartmentation of the *Geosiphon-Nostoc* symbiosis (*left*). At the *right*, a magnification of the peripheral part of a bladder is shown. The *Nostoc* cells are about 6 μm in diameter. Drawings are based on electron microscopical observations. *BLO* bacteria-like organism, *CW* cell wall, *M* mitochondrion, *N* nucleus, *NC*, *Nostoc* cell, *PM* plasma membrane, *SM* symbiosome membrane, *V* vacuole**

polarity and has a photosynthetically active region in the apical part of the cell exposed to light and air and a whitish-appearing storage region, containing many lipid droplets, in the basal part embedded in the soil surface. The center of the bladder is highly vacuolated. Schematic drawings of the compartmentation of *Geosiphon* are shown in *Fig. 16.17*. Ultrastructural observations show the *G. pyriformis* symbiosis as a system with very close contact between the partners. In fact, it is a symbiotic consortium of three organisms: (1) the fungus, supplying the consortium with inorganic nutrients like phosphate, trace elements, and water; (2) the cyanobacteria, supplying the consortium with carbohydrates by photosynthesis and, at least under some conditions, nitrogen compounds by $N_2$ fixation; (3) the "bacteria-like organisms" (BLOs), which are *Mollicutes*-related endobacteria, with yet unknown function.

Within the bladders, the cyanobacteria are located peripherally in a single, cup-shaped (often invaginated) compartment, the symbiosome. The *Nostoc* cells divide and are physiologically active as endosymbionts in this compartment. Within the cytoplasm of the fungus, glycogen granules exist as storage compounds. No dictyosomes are found; microtubules can rarely be observed. Fixation of the bladders during preparation for electron microscopy is often inadequate, probably due to the low cell wall permeability, but can be improved by using microwave acceleration.

Preparation of the *G. pyriformis* spores for electron microscopy (Schüßler et al. 1994) is even more difficult. This problem, caused by the thick spore wall being only slowly permeable to fixatives, also exists with other glomeromycotan species (Maia et al. 1993). Two main storage compounds occur inside the spores: lipid droplets of different sizes and "structured granules" that occupy about 25 % of the volume. The latter are discussed below with respect to element analysis. They show paracrystalline inclusions, as are also found in spores of some other glomeromycotan fungi. Small vacuoles are found in germinating spores and hyphae, often containing dark deposits. These are similar to the deposits in AM fungi and probably polyphosphate precipitates. The ultrastructure of the *Geosiphon* symbiosis was first studied by Schnepf (1964), and this was the crucial investigation leading to the theory of the compartmentation of the eukaryotic cell. The space between the symbiosome membrane and the wall of the enclosed *Nostoc* cells is only 30–40 nm wide and contains a layer of electron microscopically opaque and amorphous-appearing material which was originally assumed to be slime produced by the endosymbiont (Schnepf 1964). Later ultrastructural and confocal laser

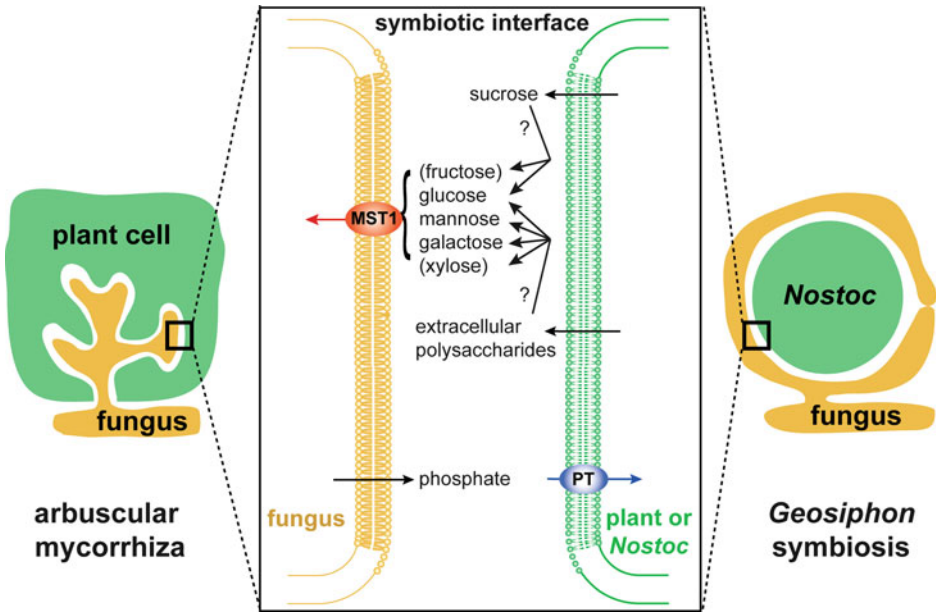



**Fig. 16.18**
**The symbiotic interface and bidirectional nutrient flows in the *Geosiphon* symbiosis, in comparison with those in the arbuscular mycorrhiza (AM) (From Schüßler et al. 2006)**

scanning microscopical (CLSM) studies by means of affinity techniques revealed that this amorphous layer inside the symbiosome contains chitin (Schüßler et al. 1996), confirmed by labeling with wheat germ agglutinin (WGA)-gold conjugates. Thus, the electron opaque layer within the symbiosome represents a "rudimentary" fungal cell wall, showing that the symbiosome membrane surrounding the *Nostoc* cells is homologous with a fungal plasma membrane.

Clear similarities exist between the fungal cell wall material present in the symbiosome space of the *Geosiphon* symbiosis and the thin arbuscular cell wall bordering the symbiotic AM fungus from the colonized plant cell: Both are electron dense after $OsO_4$ fixation, about 30–40 nm thick, and show the same amorphous structure and appearance. In general, the ultrastructural appearance of *G. pyriformis* is similar to that of AM fungi. Considering also the phylogenetic position of *G. pyriformis* and the known or proposed nutrient flows between the symbiotic partners, it has been suggested that the symbiotic interface in the AM and the *Geosiphon* symbiosis are homologous (Schüßler et al. 1996). The main difference between the symbioses is the relation of macro- and microbiont. In the *Geosiphon* symbiosis, the photoautotrophic partner (cyanobacterium) is the microsymbiont, whereas in the AM, it is the macrosymbiont (plant) (*Figs. 16.17*, *16.18*).

## Element Composition and Distribution

It is not yet known why AM fungi cannot be cultured axenically. Also, there is little information available about their trace element requirements and general element composition. Considering the fact that these fungi supply the majority of land plants with inorganic elements, studies on the element composition and transport processes are interesting topics. We have used PIXE (proton induced X-ray emission) measurements to obtain the first indications of the macro- and microelement composition of the spores and symbiotic bladders. The element content of some subcellular compartments could be quantitatively measured and, by a differential approach, calculated. PIXE, combined with STIM (scanning transmission ion microscopy), allowed elemental concentrations to be absolutely quantified with a lateral resolution in the 1 µm range and with high accuracy and precision (Maetz et al. 1999a).

Studies on the *G. pyriformis* symbiotic bladders (Maetz et al. 1999b) showed that the fungal partner of the symbiosis while grown on a nutrient-poor solution (e.g., containing 1 µM phosphate) accumulated P in high concentrations (about 2 %), but not in the symbiosome. The P is probably stored as polyphosphate in the vacuoles, as for AM (and many other) fungi. High amounts of Cl (about 2.5 %) and K (about 8 %), which appear to play major roles in osmoregulation of the fungus, are found (all values given here are related to dry weight, ppm = µg/g DW). The symbiosome (including the cyanobacteria) contains only small amounts of these elements. This is in line with a presumed high concentration of monovalent ions in the fungal vacuoles. The macroelements Mg, S, and Ca and the microelements Fe, Mn, Cu, and Zn occur in concentrations comparable with those found in plants. The Se concentration is below 1 ppm. Mo is present within the symbiosome in very low amounts, compared with the rest of the bladder,

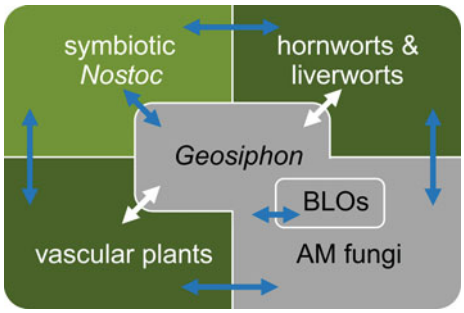



**Fig. 16.19**
**A "symbiosis network" between cyanobacteria, fungi and plants. Associations or interactions which are highlighted with *white arrows* are hypothetical. The BLOs in glomeromycotan fungi play an unknown but probably important role in this network of intimate associations**

although Mo is a constituent of nitrogenase, required for $N_2$ fixation of the cyanobacteria. Reasons for this might be that other Mo enzymes (e.g., nitrate reductase, sulfite oxidase) occur in sufficient amounts in the fungal cytoplasm or that Mo is located in the fungal vacuole. Mn and Ni, by contrast, are present in the symbiosome in much higher concentrations than in the rest of the bladder. Much of the Mn (approximately 50 ppm, which is comparable to values found in plant leaves) is probably contained in the water-cleaving Mn protein of photosystem II. Some may be from other enzymes, e.g., Mn-superoxide dismutase (SOD). A likely candidate for enzymes containing ~50 ppm Ni is cyanobacterial (or secreted fungal) urease; other Ni-containing bacterial enzymes are Ni-SOD and NiFe-hydrogenases.

Unpublished results on the element composition of the *Geosiphon* spores show that the structured granules (SGs), which are 4–6 µm in diameter, located each within a vesicle, together occupy about 25 % of the spore volume and contain most of the total P, K, and S. The S concentration of the spore cell wall is ~0.25 %, probably because of high protein content, as shown for an AM fungus (Bonfante and Grippiolo 1984). Compared with the bladders, Cl and K are concentrated within the spores in much lower amounts.

## Signal Exchange Between Host and Cyanobacterium

It is not known what triggers the recognition process and the morphological changes during the establishment of the symbiosis. Microscopical studies give no hints for any chemotactic or otherwise directed growth toward the respective symbiosis partner, but the symbiosis-compatible *Nostoc* stage can be synchronized (Schüßler and Wolf 2005) to study this in more detail. Cells of particular strains of *N. punctiforme* can be incorporated by *Geosiphon*, resulting in the formation of functional symbioses. For other strains, although incorporated, the formation of symbiotic bladders is blocked at an early stage of development. Yet other *N. punctiforme* strains are not incorporated at all. Further evidence for a specific recognition process is the fact that, among the various developmental stages of *Nostoc*, only the early primordia, existing for ~3–12 h during the life cycle, are incorporated by the fungus. Not only is the physiological activity of the primordia different from the other stages of the *Nostoc* life cycle (Bilger et al. 1994) but so is the composition of the gelatinous envelope. When differentiating into "symbiosis-compatible" primordia, a mannose-containing slime is produced by the cells, whereas other sugars within the extracellular glycoconjugates can be detected only in earlier or later stages of the life cycle (Schüßler et al. 1997). The heterocysts (specialized $N_2$-fixing cells), differentiating at regular spacing along the filaments of the *Nostoc* primordia when grown under nitrogen limitation, always remain outside the fungal hypha during the incorporation process (Mollenhauer et al. 1996). They are not surrounded by a newly appearing mannose-containing glycoconjugate (Schüßler et al. 1997), also indicating a specific recognition of the early primordial cell surface by the fungus. Thus, alterations of extracellular glycoconjugates could be involved in partner recognition. Some unpublished data further indicate a lectin-mediated process.

# Host-Cyanobiont Interactions Post Infection

## Morphological Modifications of the Symbiosis Partners

The most obvious morphological change taking place after partner recognition is the formation of the *Geosiphon* bladder. Mature bladders represent large cells, which are coenocytic with the mycelium. They show a clear polarity, with the photosynthetically active symbiotic compartment (symbiosome) located in the apical part of the bladder (*Figs. 16.16*, *16.17*).

The symbiosome is derived from the invaginated plasma membrane of the fungus and contains the cyanobacteria, which are morphologically modified by increasing in volume about six- to eightfold compared with free-living vegetative cells. This is probably caused by the high osmotic pressure inside the bladders. In many plant symbioses, cyanobacteria are known to increase in size (Bergman et al. 1992a; Grilli Caiola 1992), probably as a reaction to the higher osmotic pressure of the surrounding medium. High NaCl concentrations are also known to cause an increase in volume of cyanobacteria (Erdmann and Schiewer 1984). For *Geosiphon* bladders, the iso-osmolar concentration of sorbitol was measured with oil-filled microcapillaries and determined to be 220–230 mM, corresponding to a turgor pressure (P) of about 0.6 MPa (Schüßler et al. 1995).

However, despite the increase in size, the *Nostoc* cells inside the *Geosiphon* bladder have an almost normal ultrastructure. They contain a high number of thylakoids and carboxysomes; one alteration is that the outer membrane is hardly recognizable

electron microscopically. Heterocysts are formed with the same frequency as in free-living colonies, but their cell wall is thinner in the symbiosis, possibly indicating a lower surrounding $O_2$ concentration.

### $N_2$ and $CO_2$ Fixation and Transfer

$^{14}$C-tracer studies have shown that the *Geosiphon* bladders fix $CO_2$ both in light and in darkness, whereas the rate of $CO_2$ fixation in light is much higher (Kluge et al. 1991). In light, largely phosphate esters, poly-glucans, free sugars (including trehalose and raffinose), some amino acids, and organic acids trap $^{14}$C. In darkness only malic and fumaric acids together with some amino acids appear as labeled products. High photosynthetic activity of the endosymbiotic *Nostoc* cells is also shown by photosystem II chlorophyll-fluorescence kinetics (Bilger et al. 1994). The symbiotic *Nostoc* cells achieve much higher steady-state quantum yields and electron transport rates when compared with free-living *Nostoc*.

The capability of $N_2$ fixation is indicated by the occurrence of heterocysts, and considerable nitrogenase activity is shown for the bladders (Kluge et al. 1992). In contrast to symbioses of *Nostoc* with plants, where usually a great increase of the heterocyst frequency indicates $N_2$ fixation as the major role of the cyanobacteria, in *Geosiphon* the relative heterocyst number does not change. Here, the major role of the endosymbiotic *Nostoc* is photosynthesis. However, matter exchange between the partners is still poorly investigated, and it is even possible that the second bacterial endosymbiont (BLO; ❯ *Fig. 16.14*), which is typical for many glomeromycotan fungi, may contribute to $N_2$ fixation.

For the endosymbiotic *Nostoc* cyanobacteria, all inorganic nutrients except N have to be provided by the fungus, as the cyanobacteria live intracellularly. As shown by electrophysiological experiments (unpublished), inorganic ions (nitrate, chloride) and small organic molecules (e.g., glycine, cysteine) lead to rapid, transient depolarization of the plasma membrane potential of the *G. pyriformis* bladders, indicating that these substances are actively taken up from the outside. By contrast, there are no changes in membrane potential if hexoses and larger amino acids are applied. In addition, metabolism of radioactively labeled hexoses by the bladders cannot be detected after the usual incubation times. Low cell wall permeability was considered the likely reason for the lack of uptake of monosaccharides. This theory is supported by observations showing that the presence of solutes with large molecule radii leads to irreversible cytorrhysis, i.e., collapse of the whole bladder including the cell wall, whereas in the presence of small solutes, plasmolysis occurred (or cytorrhysis was quickly reversed). This different transport behavior is presumably due to the selective permeability of the bladder wall.

By systematically using solutes with known molecular radii, it was shown that the limiting pore radius of the *G. pyriformis* bladder wall is approximately 0.5 nm, which, compared with other cell wall types, is very small (Schüßler et al. 1995). Such a pore size is too small for an efficient permeation by, e.g., hexose molecules from the outside, but it allows permeation of inorganic hydrated ions like phosphate. If the hyphal cell wall also has such a small pore size, the fungus would not be capable of saprobic acquisition of organic molecules such as glucose, sucrose, and larger amino acids. However, cell wall permeability is a complex topic, and the thin hyphae formed by AM fungi known as "branching absorbing structures" might possess different cell wall permeability.

Because AM fungi obtain up to 20 % of the plant-fixed $CO_2$, putatively as monosaccharides, the study of glomeromycotan sugar transporters that could play a role in C transfer from plants to AM fungi is important. Only one such glomeromycotan monosaccharide transporter had been characterized, and this (GpMST1) was from the *Geosiphon* symbiosis (Schüßler et al. 2006). This putatively symbiosome membrane-located transporter was demonstrated also to transport sugars potentially deriving from plant-cell-wall material (❯ *Fig. 16.18*). The GpMST1 sequence moreover provided valuable data for the isolation of homologues from other AM fungi and could eventually lead to the better understanding of C flows in the AM.

## Ecological Importance

### Why the Symbiosis Is Mutualistic

The fungus in the *Geosiphon* symbiosis belongs to the *Glomeromycota* (❯ *Fig. 16.12*) and is, like these, obligatorily symbiotic. It is not yet known why glomeromycotan fungi are not capable of nonsymbiotic life. Possibly it will be feasible in the future to develop special culture methods for in vitro growth of AM fungi, including *Geosiphon*. Generally, in nature, glomeromycotan fungi seem incapable of saprotrophic existence but are dependent on their symbiosis partners for C delivery. For *Geosiphon* bladders, it was shown that only small molecules can pass the cell wall (Schüßler et al. 1995). The narrow pores do not allow the uptake of hexoses or disaccharides from the outside, but permeation of inorganic ions like phosphate can occur. This might reflect the situation in nature. However, it is also very possible that the fine hyphae growing into the substrate show higher permeability. In any case, by incorporating *Nostoc*, the fungus obtains the required organic compounds.

*Nostoc* also benefits from the cooperation with the fungal host, which probably facilitates the supply of the endosymbiont with water, phosphate, and also $CO_2$. It is interesting that all inorganic nutrients, except N, have to be delivered by the fungus, since the cyanobacteria live intracellularly. It should also be kept in mind that the establishment of the *Geosiphon* symbiosis, as is usually true for AM, is strongly promoted by P limitation, which is a severe stress for the photobiont. The endosymbiotic *Nostoc* cells thus divide and grow faster and bigger than their free-living sisters. Preliminary studies moreover show that the intracellular cyanobacteria are protected against heavy metals, which

accumulate in the fungus (Scheloske et al. 2001). Therefore, as in the AM, the photobiont seems to be protected against abiotic stress factors in the *Geosiphon* symbiosis.

## Evolutionary Implications with Ecological Meaning

Most vascular plant species form AM (Smith and Read 2008), including gametophytes and sporophytes of many ferns (Peterson et al. 1981) and *Lycopodiaceae* (Schmid and Oberwinkler 1993). Also, except for mosses, all groups of bryophytes contain species with AM associations (Ligrone 1988; Ligrone and Lopes 1989; Stahl 1949; Fonseca et al. 2009), indicating an early origin of the AM symbiosis.

In fact, the AM fungi have an ancient fossil record. Many of the oldest and best preserved ~400 MY old AM fungal fossils in association with plants are known from the Rhynie chert, radiometrically dated to the early Devonian (e.g., Remy et al. 1994; Dotzler et al. 2009). The oldest known fossils of what appear to be AM fungal spores and hyphae are from ~460 MY old Ordovician dolomite rock of Wisconsin (Redecker et al. 2000a), and it was concluded that terrestrial AM fungi already existed at a time when the land flora most likely consisted only of bryophyte-like "lower" plants.

From fossil cryptospore assemblages sharing characters with those of extant liverworts (found in what was eastern Gondwana; Rubinstein et al. 2010), it is estimated that land plants are more than 470 MY old (Early Middle Ordovician). The diversity of these assemblages implies an earlier, perhaps even Cambrian, origin of embryophytes. Early vascular plants already existed ~420 MY ago (Middle Silurian; Cai et al. 1996). A recent molecular clock study (Smith et al. 2010) suggested an origin of land plants around ~477 MY, but this dating in fact refers to the split between bryophytes and the remaining lineages, not the (presumably earlier) origin of the land plant lineage itself. Therefore, a minimum age of 420 MY for the liverwort-vascular plant divergence must be assumed, and bryophyte-like land plants were already present 510–470 MY ago.

Altogether, these data provide support for the hypothesis (Pirozynski and Malloch 1975) that AM fungi symbioses played a crucial role in the colonization of the land by plants, evolving from a partnership between two aquatic types of organisms, algae, and "oomycetous" fungi (the authors recognized the difference between AM fungi and other "phycomycetes," and thus interpreted them as "oomycetes," which are nowadays known not to be fungi), as the initial step of land plant evolution. A mycotrophic lifestyle could have been essential for an efficient supply of plants with water and nutrients from the soil (Malloch et al. 1980; Marschner and Dell 1994). However, molecular clock estimates always date the origin of the AM fungal lineage to be at least 50, possibly more than 200 MY earlier than that of land plants. If this holds true, it implies that there were other types of associations formed by AM fungi before land plants existed, whether saprobically, parasitically, or already mutualistically. *Geosiphon pyriformis*, representing a symbiotic association between a glomeromycotan fungus and a photoautotrophic prokaryote, may reflect such an ancestral partnership, and thus, indirectly but substantially, supports the hypothesis regarding pre-*Embryophyta* associations of AM fungal predecessors (Pirozynski and Malloch 1975). It is very plausible to assume that at the beginning of terrestrial plant life, also other associations between glomeromycotan fungi and photoautotrophic organisms (like the ubiquitous cyanobacteria) existed. The present knowledge regarding AM fungi and AM symbiosis evolution was recently discussed and reviewed (Schüßler and Walker 2011).

In summary, glomeromycotan fungi may have adapted to symbiotic life more than 500 MY ago. Without fossil support, this is speculative, but *G. pyriformis* clearly confirms the ability of glomeromycotan fungi to form symbioses with even prokaryotic photoautotrophic organisms. Therefore, cyanobacterial symbioses formed by glomeromycotan fungi could have been an ecologically important step for the colonization of the land habitat.

Arbuscular mycorrhizal fungi form symbioses with most land plants, and individual AM fungi can be symbiotic with widely divergent photoautotrophs such as hornworts and vascular plants (Schüßler 2000). The genetic base for these interactions is highly conserved (Wang et al. 2010). One can speculate that there might even be very ancestral symbiotic mechanisms in the AM that can be found in the *Geosiphon-Nostoc* symbiosis. There are some very fundamental and conserved mechanisms of plant-microorganism interactions present among the different AM(−like) associations. When conducting ecophysiological studies involving plants, it is important to consider that in nature the mycorrhizal fungal partners are the main facilitators of nutrient uptake, rather than the plant roots alone. If, as is thought, the mechanisms of nutrient acquisition by land plants coevolved since their origin with the AM fungi, ecologically and economically important questions might be answered by using the *Geosiphon* symbiosis as a model.

## A Network Between Fungi, Cyanobacteria, and Plants?

Against the above described evolutionary background, the interesting question arises as to whether *G. pyriformis* itself can act as a fungal partner to form AM. Unpublished results showed that *Geosiphon* rDNA can be PCR amplified from plant roots and bryophytes growing in the natural habitat of *Geosiphon*. However, it cannot be completely ruled out that the sensitive nested PCR approach is detecting tiny amounts of DNA from externally attached hyphae. Future studies at sites where the cyanobacteria symbiosis of *Geosiphon* never occurs will have to show whether *Geosiphon* is indeed forming an AM. If this were to be the case, a complex network of ecological importance may be imagined (*Fig. 16.19*).

The molecular probes to screen for the occurrence of *Geosiphon* in the soil and plant roots have been developed.

If *Geosiphon* indeed forms AM with plants, a complex network of biotic interactions would exist in the natural habitat. Within such a network, symbiotic *Nostoc* could be exchanged between *Geosiphon* and bryophytes, and *Geosiphon* could simultaneously form endosymbiosis with *Nostoc* and AM with plants, thus, e.g., transporting and delivering $N_2$ fixed by the cyanobacteria to the plants. Unfortunately, funding applications have been rejected, with the consequence that laboratory cultures are no longer available.

## References


Adams DG (2000) Symbiotic interactions. In: Whitton B, Potts M (eds) Ecology of cyanobacteria: their diversity in time and space. Kluwer, Dordrecht, pp 523–561

Adams DG (2002a) Cyanobacteria in symbiosis with hornworts and liverworts. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 117–135

Adams DG (2002b) The liverwort-cyanobacterial symbiosis. Biol Environ Proc R Ir Acad 102B:27–30

Adams DG, Duggan P (1999) Heterocyst and akinete differentiation in cyanobacteria: Tansley Review No. 107. New Phytol 144:3–33

Adams DG, Duggan PS (2008) Cyanobacteria-bryophyte symbioses. J Exp Bot 59:1047–1058

Adams DG, Duggan PS (2012) Signalling in cyanobacteria-plant symbioses. In: S. Perotto S, Baluska F (eds) Signaling and communication in plant symbiosis. Springer, Heidelberg, pp 93–121

Adams DG, Duggan PS, Jackson O (2012) Cyanobacterial symbioses. In: Whitton B (ed) Ecology of cyanobacteria II: their diversity in time and space. Springer, Heidelberg, pp 593–647

Adams DG, Bergman B, Nierzwicki-Bauer SA, Rai AN, Schussler A (2006) Cyanobacterial-plant symbioses. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The prokaryotes. A handbook on the biology of bacteria, vol 1, 3rd edn, Symbiotic associations, biotechnology, applied microbiology. Springer, New York, pp 331–363

Ahern CP, Staff IA (1994) Symbiosis in cycads: the origin and development of coralloid roots in *Macrozamia communis* (Cycadaceae). Am J Bot 81:1559–1570

Ahmadjian V (1993) The lichen symbiosis. Wiley, New York

Anbudurai PR, Mor TS, Ohad I, Shestakov SV, Pakrasi HB (1994) The *ctpA* gene encodes the C-terminal processing protease for the D1 protein of the photosystem II reaction center complex. Proc Natl Acad Sci USA 91:8082–8086

Aptroot A (1998) Aspects of the integration of the taxonomy of lichenized and non-lichenized pyrenocarpoous ascomycetes. Lichenologist 30:501–514

Aulfinger H, Braun-Howland EB, Kannaiyan S, Nierzwicki-Bauer SA (1991) Ultrastructural changes of the endosymbionts of *Azolla microphylla* during megaspore germination and early plantlet development. Can J Bot 69:2489–2496

Babic S (1996) Hormogonia formation and the establishment of symbiotic associations between cyanobacteria and the bryophytes *Blasia* and *Phaeoceros*. PhD thesis, University of Leeds, Leeds

Baker J, Entsch B, McKay DB (2003) The cyanobiont in an *Azolla* fern is neither *Anabaena* nor *Nostoc*. FEMS Microbiol Lett 229:43–47

Banack SA, Cox PA (2003) Biomagnification of cycad neurotoxins in flying foxes: implications for ALS-PDC in Guam. Neurology 61:387–389

Banack SA, Murch SJ, Cox PA (2006) Neurotoxic flying foxes as dietary items for the Chamorro people, Marianas Islands. J Ethnopharmacol 106:97–104

Banack SA, Johnson HE, Cheng R, Cox PA (2007) Production of the neurotoxin BMAA by a marine cyanobacterium. Mar Drugs 5:180–196

Banack SA, Downing TG, Spácil Z, Purdie EL, Metcalf JS, Downing S, Esterhuizen M, Codd GA, Cox PA (2010) Distinguishing the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) from its structural isomer 2, 4-diaminobutyric acid (2, 4-DAB). Toxicon 56:868–879

Basile DV (1990) Morphological role of hydroxyproline containing proteins in liverworts. In: Chopra RN, Bhatia SC (eds) Bryophyte development: physiology and biochemistry. CRC Press, Boca Raton, pp 225–243

Baulina OI, Lobakova ES (2003a) Atypical cell forms overproducing extracellular substances in populations of cycad cyanobionts. Microbiology 72:701–712

Baulina OI, Lobakova ES (2003b) Heterocysts with reduced cell walls in populations of cycad cyanobionts. Microbiology 72:713–722

Bergman B (2002) The *Nostoc-Gunnera* symbiosis. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 207–232

Bergman B, Osborne B (2002) The *Gunnera-Nostoc* symbiosis. In: Osborne B (ed) Commentaries on cyanobacterial symbioses. Royal Irish Academy, Dublin, pp 35–39

Bergman B, Rai AN (1989) The *Nostoc-Nephroma* symbiosis: localization, distribution pattern and levels of key proteins involved in nitrogen and carbon metabolism of the cyanobiont. Physiol Plant 77:216–224

Bergman B, Lindblad P, Pettersson A, Renström E, Tiberg E (1985) Immuno-gold localization of glutamine synthetase in a nitrogen-fixing cyanobacterium (*Anabaena cylindrica*). Planta 166:329–334

Bergman B, Lindblad P, Rai AN (1986) Nitrogenase in free-living and symbiotic cyanobacteria: immunoelectron microscopic localization. FEMS Microbiol Lett 35:75–78

Bergman B, Rai AN, Johansson C, Söderbäck E (1992a) Cyanobacterial-plant symbioses. Symbiosis 14:61–81

Bergman B, Johansson C, Söderbäck E (1992b) The *Nostoc-Gunnera* symbiosis. New Phytol 122:379–400

Bergman B, Matveyev A, Rasmussen U (1996) Chemical signalling in cyanobacterial-plant symbioses. Trends Plant Sci 1:191–197

Bergman B, Rasmussen U, Rai AN (2003) Cyanobacterial associations. In: Elmerich C, Newton WE (eds) Associative nitrogen-fixing bacteria and cyanobacterial associations. Kluwer, Dordrecht

Bergman B, Rasmussen U, Rai AN (2007) Cyanobacterial associations. In: Elmerich C, Newton WE (eds) Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations. Kluwer, Dordrecht, pp 257–301

Bergman B, Ran L, Adams DG (2008) Cyanobacterial-plant symbioses: signaling and development. In: Herrero A, Flores E (eds) The cyanobacteria: molecular biology, Genomics and evolution. Caister Academic Press, Norfolk, pp 447–473

Bhaya D (2004) Light matters: phototaxis and signal transduction in unicellular cyanobacteria. Mol Microbiol 53:745–754

Bilger W, Büdel B, Mollenhauer R, Mollenhauer D (1994) Photosynthetic activity of two developmental stages of a *Nostoc* strain (Cyanobacteria) isolated from *Geosiphon pyriforme* (Mycota). J Phycol 30:225–230

Black KG, Parsons R, Osborne BA (2002) Uptake and metabolism of glucose in the *Nostoc-Gunnera* symbiosis. New Phytol 153:297–305

Bonfante P, Grippiolo R (1984) Cytochemical and biochemical observations on the cell wall of the spore of *Glomus epigaeum*. Protoplasma 123:140–151

Bonnett HT, Silvester WB (1981) Specificity in the *Nostoc-Gunnera* endosymbiosis. New Phytol 89:121–128

Brasell HM, Davies SK, Mattay JP (1986) Nitrogen fixation associated with bryophytes colonizing burnt sites in Southern Tasmania, Australia. J Bryol 14:139–149

Braun-Howland EB, Nierzwicki-Bauer SA (1990) *Azolla-Anabaena* symbiosis: biochemistry, physiology, ultrastructure and molecular biology. In: Rai AN (ed) CRC handbook of symbiotic cyanobacteria. CRC Press, Boca Raton, pp 65–117

Brenner ED, Stevenson DW, Twigg RW (2003) Cycads: evolutionary innovations and the role of plant-derived neurotoxins. Trends Plant Sci 8:446–452

Brundrett MC (2009) Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant Soil 320:37–77

Burrows LL (2005) Weapons of mass retraction. Mol Microbiol 57:878–888

Bushnell T (1998) Molecular identification of endosymbionts present in the leaf cavities of *Azolla caroliniana* and *Azolla mexicana*. PhD thesis, Rensselaer Polytechnic Institute, Troy, pp 1–222

Cai CY, Ouyang S, Wang Y, Fang ZJ, Rong JY, Geng LY, Li XX (1996) An Early Silurian vascular plant. Nature 379:592

Calvert HE, Peters GA (1981) The *Azolla-Anabaena* relationship. IX: Morphological analysis of leaf cavity hair populations. New Phytol 89:327–335

Campbell EL, Meeks JC (1989) Characteristics of hormogonia formation by symbiotic *Nostoc* spp. in response to the presence of *Anthoceros punctatus* or its extracellular products. Appl Environ Microbiol 55:125–131

Campbell EL, Meeks JC (1992) Evidence for plant-mediated regulation of nitrogenase expression in the *Anthoceros-Nostoc* symbiotic association. J Gen Microbiol 138:473–480

Campbell EL, Hagen KD, Cohen MF, Summers ML, Meeks JC (1996) The *devR* gene product is characteristic of receivers of two-component regulatory systems and is essential for heterocyst development in the filamentous cyanobacterium *Nostoc* sp. strain ATCC 29133. J Bacteriol 178:2037–2043

Campbell EL, Brahamsha B, Meeks JC (1998) Mutation of an alternative sigma factor in the cyanobacterium *Nostoc punctiforme* results in increased infection of its symbiotic plant partner, *Anthoceros punctatus*. J Bacteriol 180:4938–4941

Campbell EL, Wong FCY, Meeks JC (2003) DNA binding properties of the HrmR protein of *Nostoc punctiforme* responsible for transcriptional regulation of genes involved in the differentiation of hormogonia. Mol Microbiol 47:573–582

Campbell EL, Summers ML, Christman H, Martin ME, Meeks JC (2007) Global gene expression patterns of *Nostoc punctiforme* in steady state dinitrogen-grown heterocyst-containing cultures, and at single time points during the differentiation of akinetes and hormogonia. J Bacteriol 189:5247–5256

Campbell EL, Christman H, Meeks JC (2008) DNA microarray comparisons of plant factor- and nitrogen deprivation-induced hormogonia reveal decision-making transcriptional regulation patterns in *Nostoc punctiforme*. J Bacteriol 190:7382–7391

Canini A, Caiola MG, Mascini M (1990) Ammonium content, nitrogenase activity and heterocyst frequency within the leaf cavities of *Azolla filiculoides* Lam. FEMS Microbiol Lett 71:205–210

Carrapico F (1991) Are bacteria the 3rd partner of the *Azolla-Anabaena* symbiosis? Plant Soil 137:157–160

Carrapico F, Tavares R (1989) New data on the *Azolla-Anabaena* symbiosis II: cytochemical and immunocytochemical aspects. In: Skinner FA, Boddey RM, Fendrik I (eds) Nitrogen fixation with non-legumes. Kluwer, Dordrecht, pp 95–100

Castillo-Guevara C, Rico-Gray V (2003) The role of macrozamin and cycasin in cycads (Cycadales) as antiherbivore defenses. J Torrey Bot Soc 130: 206–217

Chang DCN, Grobbelaar N, Coetzee J (1988) SEM observations on cyanobacteria-infected cycad coralloid roots. S Afr J Bot 54:491–495

Chapman KE, Duggan PS, Billington NA, Adams DG (2008) Mutation at different sites in the *Nostoc punctiforme cyaC* gene, encoding the multiple-domain enzyme adenylate cyclase, results in different levels of infection of the host plant *Blasia pusilla*. J Bacteriol 190:1843–1847

Chaw S-M, Walters TW, Chang C-C, Hu S-H, Chen S-H (2005) A phylogeny of cycads (Cycadales) inferred from chloroplast *matK* gene, *trnK* intron, and nuclear rDNA ITS region. Mol Phylogenet Evol 37:214–234

Chiu WL, Peters GA, Levielle G, Still PC, Cousins S, Osborne B, Elhai J (2005) Nitrogen deprivation stimulates symbiotic gland development in *Gunnera manicata*. Plant Physiol 139:224–230

Cohen MF, Meeks JC (1997) A hormogonium regulating locus, *hrmUA*, of the cyanobacterium *Nostoc punctiforme* strain ATCC 29133 and its response to an extract of a symbiotic plant partner *Anthoceros punctatus*. Mol Plant Microbe Interact 10:280–289

Cohen MF, Yamasaki H (2000) Flavonoid-induced expression of a symbiosis-related gene in the cyanobacterium *Nostoc punctiforme*. J Bacteriol 182:4644–4646

Cohen MF, Wallis JG, Campbell EL, Meeks JC (1994) Transposon mutagenesis of *Nostoc* sp. strain ATCC 29133, a filamentous cyanobacterium with multiple cellular differentiation alternatives. Microbiology 140:3233–3240

Cohen MF, Meeks JC, Cai Y, Wolk CP (1998) Transposon mutagenesis of heterocyst-forming filamentous cyanobacteria. Methods Enzymol 297:3–17

Cohen MF, Sakihama Y, Takagi YC, Ichiba T, Yamasaki H (2002) Synergistic effect of doexyanthocyanins from symbiotic fern *Azolla* spp. on *hrmA* gene induction in the cyanobacterium *Nostoc punctiforme*. Mol Plant Microbe Interact 15:875–882

Costa J-L, Lindblad P (2002) Cyanobacteria in symbiosis with cycads. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 195–205

Costa JL, Paulsrud P, Lindblad P (1999) Cyanobiont diversity within coralloid roots of selected cycad species. FEMS Microbiol Ecol 28:85–91

Costa J-L, Paulsrud P, Rikkinen J, Lindblad P (2001) Genetic diversity of *Nostoc* symbionts endophytically associated with two bryophyte species. Appl Environ Microbiol 67:4393–4396

Costa JL, Romero EM, Lindblad P (2004) Sequence based data supports a single *Nostoc* strain in individual coralloid roots of cycads. FEMS Microbiol Ecol 49:481–487

Cox PA, Banack SA, Murch SJ (2003) Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam. Proc Natl Acad Sci USA 100:13380–13383

Cox PA, Banack SA, Murch SJ, Rasmussen U, Tien G, Bidigare RR, Metcalf JS, Morrison LF, Codd GA, Bergman B (2005) Diverse taxa of cyanobacteria produce beta-*N*-methylamino-L-alanine, a neurotoxic amino acid. Proc Natl Acad Sci USA 102:5074–5078

Dalton DA, Chatfield JM (1985) A new nitrogen-fixing cyanophyte-hepatic association: *Nostoc* and *Porella*. Am J Bot 72:781–784

de Roissart P, Jucqued C, Watetkeyn L, Berghmans P, Van Hove C (1994) First evidence for the cutinic nature of the envelope at the interface of *Azolla* and its endophytes. In: Hegazi NA (ed) Nitrogen fixation with non-legumes: the sixth international symposium. The American University in Cairo Press, Cairo, pp 133–138

DeLuca TH, Zackrisson O, Nilsson MC, Sellstedt A (2002) Quantifying nitrogen-fixation in feather moss carpets of boreal forests. Nature 419:917–920

DeLuca TH, Zackrisson O, Gentili F, Sellstedt A, Nilsson MC (2007) Ecosystem controls on nitrogen fixation in boreal feather moss communities. Oecologia 152:121–130

DeLuca TH, Zackrisson O, Gundale MJ, Nilsson M-C (2008) Ecosystem feedbacks and nitrogen fixation in boreal forests. Science 320:1181

Deusch O, Landan G, Roettger M, Gruenheit N, Kowallik KV et al (2008) Genes of cyanobacterial origin in plant nuclear genomes point to a heterocyst forming plastid ancestor. Mol Biol Evol 25:748–761

Do VC, Watanabe I, Zimmerman WJ, Lumpkin TA, de Waha Gaillonville T (1989) Sexual hybridization among *Azolla* species. Can J Bot 67:3482–3485

Dotzler N, Walker C, Krings M, Hass H, Kerp H, Taylor TN, Agerer R (2009) Acaulosporoid glomeromycotan spores with a germination shield from the 400-million-year-old Rhynie chert. Mycol Prog 8:9–18

Duckett JG, Prasad AKSK, Davies DA, Walker S (1977) A cytological analysis of the *Nostoc*-bryophyte relationship. New Phytol 79:349–362

Duckett JG, Burch J, Fletcher PW, Matcham HW, Read DJ, Russell AJ, Pressel S (2004) In vitro cultivation of bryophytes: a review of practicalities, problems, progress and promise. J Bryol 26:3–20

Duggan PS, Gottardello P, Adams DG (2007) Molecular analysis of genes in *Nostoc punctiforme* involved in pilus biogenesis and plant infection. J Bacteriol 189:4547–4551

Ekman M, Tollbäck P, Klint J, Bergman B (2006) Protein expression profiles in an endosymbiotic cyanobacterium revealed by a proteomic approach. Mol Plant Microbe Interact 19:1251–1261

Ekman M, Tollback P, Bergman B (2008) Proteomic analysis of the cyanobacterium of the *Azolla* symbiosis: identity, adaptation, and NifH modification. J Exp Bot 59:1023–1034

Elifio SL, Da Silva M, Iacomini M, Gorin PAJ (2000) A lectin from the lichenized basidiomycete *Dictyonema glabratum*. New Phytol 148:327–334

Enderlin CS, Meeks JC (1983) Pure culture and reconstitution of the *Anthoceros-Nostoc* symbiotic association. Planta 158:157–165

Erdmann N, Schiewer U (1984) Cell size changes as indicator of salt resistance of blue green algae. Arch Hydrobiol Suppl (Algol Stud) 67:431–439

Esterhuizen M, Downing T (2008) β-*N*-methylamino-L-alanine (BMAA) in novel South African cyanobacterial isolates. Ecotoxicol Environ Saf 71:309–313

Evrard C, Van Hove C (2004) Taxonomy of the American *Azolla* species (Azollaceae): a critical review. Syst Geogr Plant 74:301–318

Fiedler G, Muro-Pastor A, Flores E, Maldener I (2001) NtcA-dependent expression of the *devBCA* operon, encoding a heterocyst-specific ATP-binding cassette transporter in *Anabaena* spp. J Bacteriol 183:3795–3799

Fisher RF, Long SR (1992) *Rhizobium*-plant signal exchange. Nature 357:655–660

Flores E, Herrero A (2005) Nitrogen assimilation and nitrogen control in cyanobacteria. Biochem Soc Trans 33:164–167

Fonseca HMAC, Ferreira JIL, Berbara RLL, Zatorre NP (2009) Dominance of Paris-type morphology on mycothallus of *Lunularia cruciata* colonised by *Glomus proliferum*. Braz J Microbiol 40:96–101

Forni C, Grilli Caiola M, Gentili S (1989) Bacteria in the *Azolla-Anabaena* symbiosis. In: Skinner FA, Boddey RM, Fendrik I (eds) Nitrogen fixation with non-legumes. Kluwer, Dordrecht, pp 83–88

Forni C, Gentili S, Van Hove C, Grilli Caiola M (1990) Isolation and characterization of the bacteria living in the sporocarps of *Azolla filiculoides* Lam. Ann Microbiol 40:235–243

Galun M (1988) Handbook of lichenology. CRC Press, Boca Raton

Gantar M, Kerby NW, Rowell P (1993) Colonization of wheat (*Triticum vulgare* L.) by $N_2$-fixing cyanobacteria. III: the role of a hormogonia-promoting factor. New Phytol 124:505–513

Gates JE, Fisher RW, Candler RA (1980) The occurrence of corynoform bacteria in the leaf cavity of *Azolla*. Arch Microbiol 127:163–165

Gebhardt JS, Nierzwicki-Bauer SA (1991) Identification of a common cyanobacterial symbiont associated with *Azolla* spp. through molecular and morphological characterization of free-living and symbiotic cyanobacteria. Appl Environ Microbiol 57:2141–2146

Gehrig H, Schüßler A, Kluge M (1996) *Geosiphon pyriforme*, a fungus forming endocytobiosis with *Nostoc* (Cyanobacteria), is an ancestral member of the Glomales: evidence by SSU rRNA analysis. J Mol Evol 43:71–81

Gehringer MM, Pengelly JJL, Cuddy WS, Fieker C, Forster PI, Neilan BA (2010) Host selection of symbiotic cyanobacteria in 31 species of the Australian cycad genus: *Macrozamia* (Zamiaceae). Mol Plant Microbe Interact 23:811–822

Gentili F, Nilsson MC, Zackrisson O, DeLuca TH, Sellstedt A (2005) Physiological and molecular diversity of feather moss associative $N_2$-fixing cyanobacteria. J Exp Bot 56:3121–3127

Golden JW, Yoon HS (2003) Heterocyst development in *Anabaena*. Curr Opin Microbiol 6:557–563

Gorelova OA, Baulina OI, Shchelmanova AG, Korzhenevskaya TG, Gusev MV (1996) Heteromorphism of the cyanobacterium *Nostoc* sp., a microsymbiont of the *Blasia pusilla* moss. Microbiology 65:719–726

Gratwicke B, Marshall BE (2001) The impact of *Azolla filiculoides* Lam. on animal biodiversity in streams in Zimbabwe. Afr J Ecol 39:216–218

Grilli Caiola M (1980) On the phycobionts of the cycad coralloid roots. New Phytol 85:537–544

Grilli Caiola M (1992) Cyanobacteria in symbiosis with bryophytes and tracheophytes. Biopress, Bristol

Grobbelaar N, Scott WE, Hattingh W, Marshall J (1987) The identification of the coralloid endophytes of the southern African cycads and the ability of the isolates to fix dinitrogen. S Afr J Bot 53:111–118

Guevara R, Armesto JJ, Caru M (2002) Genetic diversity of *Nostoc* microsymbionts from *Gunnera tinctoria* revealed by PCR-STRR fingerprinting. Microb Ecol 44:127–136

Gusev MV, Baulina OI, Gorelova OA, Lobakova ES, Korzhenevshaya TG (2002) Artificial cyanobacterium-plant symbioses. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 253–312

Hawksworth D, Honegger R (1994) The lichen thallus: a symbiotic phenotype of nutritionally specialized fungi and its response to gall producers. In: Williams MAJ (ed) Plant galls: organisms, interactions, populations. Clarendon, Oxford, The Systematics Association, Special Volume No. 4977–98

Herdman M, Rippka R (1988) Cellular differentiation: hormogonia and baeocytes. Methods Enzymol 167:232–242

Herrero A, Muro-Pastor AM, Flores E (2001) Nitrogen control in cyanobacteria. J Bacteriol 183:411–425

Herrero A, Muro-Pastor AM, Valladares A, Flores E (2004) Cellular differentiation and the NtcA transcription factor in filamentous cyanobacteria. FEMS Microbiol Rev 28:469–487

Hill DJ (1975) The pattern of development of *Anabaena* in the *Azolla-Anabaena* symbiosis. Planta 122:178–184

Hill DJ (1977) The role of *Anabaena* in the *Azolla-Anabaena* symbiosis. New Phytol 78:611–616

Hill DJ (1989) The control of cell cycle in microbial symbionts. New Phytol 112:175–184

Hill MP, Oberholzer IG (2002) Laboratory host range testing of the flea beetle, *Pseudolampsis guttata* (Leconte) (Coleoptera: Chrysomelidae), a potential natural enemy for red water fern, *Azolla filiculoides* Lamarck (Pteridophyta: Azollaceae) in South Africa. Coleopt Bull 56:79–83

Houle D, Gauthier SB, Paquet S, Planas D, Warren A (2006) Identification of two genera of $N_2$-fixing cyanobacteria growing on three feather moss species in boreal forests of Quebec, Canada. Can J Bot 84:1025–1029

Jackson O, Taylor O, Adams DG, Knox JP (2012) Arabinogalactan proteins occur in the free-living cyanobacterium genus *Nostoc* and in plant–*Nostoc* symbioses. Mol Plant-Microbe Interactions 25:1338–1349

Janes R (1998a) Growth and survival of *Azolla filiculoides* in Britain. I. Vegetative reproduction. New Phytol 138:367–375

Janes R (1998b) Growth and survival of *Azolla filiculoides* in Britain. II. Sexual reproduction. New Phytol 138:377–384

Johansson C, Bergman B (1992) Early events during the establishment of *Gunnera*/*Nostoc* symbiosis. Planta 188:403–413

Johansson C, Bergman B (1994) Reconstruction of the symbiosis of *Gunnera manicata* Linden: cyanobacterial specificity. New Phytol 126:643–652

Joseph CM, Meeks JC (1987) Regulation of expression of glutamine synthetase in a symbiotic *Nostoc* strain associated with *Anthoceros punctatus*. J Bacteriol 169:2471–2475

Joubert L, Grobbelaar N, Coetzee J (1989) In situ studies of the ultrastructure of the cyanobacteria in the coralloid roots of *Encephalartos arenarius*, *E. transvenosus* and *E. woodii* (Cycadales). Phycologia 28:197–205

Kaplan D, Peters GA (1988) Interaction of carbon metabolism in the *Azolla-Anabaena* symbiosis. Symbiosis 6:53–68

Kardish N, Silberstein L, Fleminger G, Galun M (1991) Lectins from the lichen *Nephroma laevigatum* Ach: localization and function. Symbiosis 11:47–62

Khamar HJ, Breathwaite EK, Prasse CE, Fraley ER, Secor CR, Chibane FL, Elhai J, Chiu WL (2010) Multiple roles of soluble sugars in the establishment of *Gunnera-Nostoc* endosymbiosis. Plant Physiol 154:1381–1389

Kimura J, Nakano T (1990) Reconstitution of a *Blasia-Nostoc* symbiotic association under axenic conditions. Nova Hedwig 50:191–200

Klint J, Ran L, Rasmussen U, Bergman B (2006) Identification of developmentally regulated proteins in cyanobacterial hormogonia using a proteomic approach. Symbiosis 41:87–95

Kluge M, Mollenhauer D, Mollenhauer R (1991) Photosynthetic carbon assimilation in *Geosiphon pyriforme* (Kützing) F.v. Wettstein, an endosymbiotic association of fungus and cyanobacterium. Planta 185:311–315

Kluge M, Mollenhauer D, Mollenhauer R, Kape R (1992) *Geosiphon pyriforme*, an endosymbiotic consortium of a fungus and a cyanobacterium (*Nostoc*), fixes nitrogen. Bot Acta 105:343–344

Knapp E (1933) Über *Geosiphon pyriformis* Fr.v. Wettst., eine intrazelluläre Pilz-Algen-Symbiose. Ber Dtsch Bot Ges 51:210–217

Knight CD, Adams DG (1996) A method for studying chemotaxis in nitrogen-fixing cyanobacterium-plant symbioses. Physiol Mol Plant Pathol 49:73–77

Kobiler D, Cohen-Sharon A, Tel-Or E (1981) Recognition between the $N_2$-fixing *Anabaena* and the water fern *Azolla*. FEBS Lett 133:157–160

Kobiler D, Cohen-Sharon A, Tel-Or E (1982) Lectins are involved in the recognition between *Anabaena* and *Azolla* Israel. J Bot 31:324–328

Komarek J, Anagnostidis K (1989) *Trichormus azollae* (Strasb.): modern approaches to the classification system of cyanophytes 4-nostocales. Arch Hydrobiol Algol Stud 56:303–345

Krüger T, Mönch B, Oppenhäuser S, Luckas B (2010) LC-MS/MS determination of the isomeric neurotoxins BMAA (β-*N*-methylamino-L-alanine) and DAB (2,4-diaminobutyric acid) in cyanobacteria and seeds of *Cycas revolute* and *Lathyrus latifolius*. Toxicon 55:547–557

Kützing FT (1849) Species *algarum*. F.A. Brockhaus, Leipzig

Lamb JR, Tugendreich S, Hieter P (1995) Tetratrico peptide repeat interactions: to TPR or not to TPR? Trends Biochem Sci 20:257–259

Large MF, Braggins JE (1993) Spore morphology of New Zealand *Azolla filiculoides* Lam. (Salviniaceae) New Zealand. J Bot 31:419–423

Lechno-Yossef S (2002) Identification and characterization of bacteria associated with the water fern *Azolla* sp. PhD thesis, Rensselaer Polytechnic Institute, Troy, pp 1–132

Lechno-Yossef S, Nierzwicki-Bauer SA (2002) *Azolla-Anabaena* symbiosis. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 153–178

Lee KY, Joseph CM, Meeks JC (1988) Glutamine synthetase specific activity and protein concentration in symbiotic *Anabaena* associated with *Azolla caroliniana*. Antonie Van Leeuwenhoek 54:345–355

Legaz M-E, Fontaniella B, Millanes A-M, Vicente C (2004) Secreted arginases from phylogenetically far-related species act as cross-recognition factors for two different algal cells. Eur J Cell Biol 83:435–446

Lehr H, Fleminger G, Galun M (1995) Lectin from the lichen *Peltigera membranacea* (Ach.) Nyl.: characterization and function. Symbiosis 18:1–13

Lehr H, Galun M, Ott S, Jahns HM, Fleminger G (2000) Cephalodia of the lichen *Peltigera aphthosa* (L.) Willd.: specific recognition of the compatible photobiont. Symbiosis 29:357–365

Lejeune A, Cagauan A, Van Hove C (1999) *Azolla* research and development: recent trends and priorities. Symbiosis 27:333–351

Liaimer A, Bergman B (2003) Phytohormones in cyanobacteria: occurrence and perspectives. In: Proceedings from plant-microbe interaction conference, St. Petersburg

Liaimer A, Matveyev A, Bergman B (2001) Isolation of host plant induced cDNAs from *Nostoc* sp. PCC 9229 forming symbiosis with the angiosperm *Gunnera* spp. Symbiosis 31:293–307

Ligrone R (1988) Ultrastructure of a fungal endophyte in *Phaeoceros laevis* (L.) Prosk. (Anthocerophyta). Bot Gaz 149:92–100

Ligrone R, Lopes C (1989) Cytology and development of mycorrhiza-like infection in the gametophyte of *Conocephalum conicum* (L.) Dum. (Marchantiales, Hepatophyta). New Phytol 111:423–433

Lin C, Liu ZZ, Zheng DY, Tang LF, Watanabe I (1989) Re-establishment of symbiosis to *Anabaena* free *Azolla*. Sci China Ser B Chem 32:551–559

Lindblad P (1990) Nitrogen and carbon metabolism in coralloid roots of cycads. Advances in cycad research I. Mem N Y Bot Gard 57:104–113

Lindblad P (2009) Cyanobacteria in symbiosis with cycads. In: Pawlovski K (ed) Prokaryotic endosymbionts in plants, vol 8, Microbiology monographs. Springer, Berlin/Heidelberg, pp 225–233

Lindblad P, Bergman B (1986) Glutamine synthetases: activity and localization in cyanobacteria of the cycads *Cycas revolute* and *Zamia skinneri*. Planta 169:1–7

Lindblad P, Costa J-L (2002) The cyanobacterial-cycad symbiosis. Biol Environ Proc R Ir Acad 102B:31–33

Lindblad P, Hällblom L, Bergman B (1985a) The cyanobacterium-*Zamia* symbiosis: $C_2H_2$ reduction and heterocyst frequency. Symbiosis 1:19–28

Lindblad P, Bergman B, Hofsten AV, Hallbom L, Nylund JE (1985b) The cyanobacterium-*Zamia* symbiosis: an ultrastructural study. New Phytol 101:707–716

Lindblad P, Rai AN, Bergman B (1987) The *Cycas revoluta-Nostoc* symbiosis: enzyme activities of nitrogen and carbon metabolism in the cyanobiont. J Gen Microbiol 133:1695–1699

Lindblad P, Haselkorn R, Bergman B, Nierzwicki-Bauer SA (1989) Comparison of DNA restriction fragment length polymorphisms of *Nostoc* strains in and from cycads. Arch Microbiol 152:20–24

Lindblad P, Atkins CA, Pate JS (1991) $N_2$-fixation by freshly isolated *Nostoc* from coralloid roots of the cycad *Macrozamia riedlei* (Firsch. ex Gand.) Garnd. Plant Physiol 96:753–759

Lobakova ES, Orazova MK, Dobrovol'skaya TG (2003) Microbial complexes occurring on the apogeotropic roots and in the rhizosphere of cycad plants. Microbiology 72:628–633

Lobakova ES, Dubravina GA, Zagoskina NV (2004) Formation of phenolic compounds in apogeotrophic roots of cycad plants. Russ J Plant Physiol 51:486–493

Lohtander K, Oksanen I, Rikkinen J (2002) A phylogenetic study of *Nephroma* (lichen-forming Ascomycota). Mycol Res 106:777–787

Lotti F, Giovanetti L, Margheri MC, Ventura S, Materassi R (1996) Diversity of DNA methylation pattern and total DNA restriction pattern in symbiotic *Nostoc*. World Microbiol Biotechnol 12:38–42

Maetz M, Przybylowicz WJ, Mesjasz-Przybylowicz J, Schüßler A, Traxel K (1999a) Low-dose nuclear microscopy as a necessity for accurate quantitative microanalysis of biological samples. Nucl Instrum Methods Phys Res B 158:292–298

Maetz M, Schüßler A, Wallianos A, Traxel K (1999b) Subcellular trace element distribution in *Geosiphon pyriforme*. Nucl Instrum Methods Phys Res B 150:200–207

Maia LC, Kimbrough JW, Erdos G (1993) Problems with fixation and embedding of arbuscular mycorrhizal fungi (Glomales). Mycologia 85:323–330

Malloch DW, Pirozynski KA, Raven PH (1980) Ecological and evolutionary significance of mycorrhizal symbiosis in vascular plants (a review). Proc Natl Acad Sci USA 77:2113–2118

Marler TE, Snyder LR, Shaw CA (2010) *Cycas micronesica* (Cycadales) plants devoid of endophytic cyanobacteria increase in beta-methylamino-L-alanine. Toxicon 56:563–568

Marschner H, Dell B (1994) Nutrient uptake in mycorrhizal symbiosis. Plant Soil 159:89–102

Martin W, Rujan T, Richly E, Hansen A, Cornelsen S, Lins T, Leister D, Stoebe B, Hasegawa M, Penny D (2002) Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. Proc Natl Acad Sci USA 99:12246–12251

Mattick JS (2002) Type IV pili and twitching motility. Annu Rev Microbiol 56:289–314

McConnachie AJ, Hill MP, Byrne MJ (2004) Field assessment of a frond-feeding weevil, a successful biological control agent of red waterfern, *Azolla filiculoides*, in southern Africa. Biol Control 29:326–331

Meeks JC (1988) Symbiotic associations. Methods Enzymol 167:113–121

Meeks JC (1990) Cyanobacterial-bryophyte associations. In: Rai AN (ed) CRC handbook of symbiotic cyanobacteria. CRC Press, Boca Raton, pp 43–63

Meeks JC (1998) Symbiosis between nitrogen-fixing cyanobacteria and plants. Bioscience 48:266–276

Meeks JC (2003) Symbiotic interactions between *Nostoc punctiforme*, a multicellular cyanobacterium, and the hornwort *Anthoceros punctatus*. Symbiosis 35:55–71

Meeks JC (2009) Physiological adaptations in nitrogen-fixing *Nostoc*-plant symbiotic associations. In: Pawlowski K (ed) Prokaryotic symbionts in plants, vol 8, Microbiol monograph. Springer, Berlin, pp 181–205

Meeks JC, Elhai J (2002) Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. Microbiol Mol Biol Rev 66:94–121

Meeks JC, Enderlin CS, Wycoff KL, Chapman JS, Joseph CM (1983) Assimilation of $^{13}NH_4^+$ by Anthoceros grown with and without symbiotic *Nostoc*. Planta 158:384–391

Meeks JC, Enderlin CS, Joesph CM, Chapman JS, Lollar MWL (1985a) Fixation of [$^{13}N$]; $N_2$ and transfer of fixed nitrogen in the *Anthoceros-Nostoc* symbiotic association. Planta 164:406–414

Meeks JC, Steinberg N, Joseph CM, Enderlin CS, Jorgensen PA, Peters GA (1985b) Assimilation of exogenous and dinitrogen-derived $^{13}NH_4^+$ by *Anabaena azollae* separated from *Azolla caroliniana* Willd. Arch Microbiol 142:229–233

Meeks JC, Campbell E, Hagen K, Hanson T, Hitzeman N, Wong F (1999) Developmental alternatives of symbiotic *Nostoc punctiforme* in response to its symbiotic partner *Anthoceros punctatus*. In: Peschek GA, Löffelhardt W, Schmetterer G (eds) The phototrophic prokaryotes. Kluwer/Plenum, New York, pp 665–678

Meeks JC, Elhai J, Thiel T, Potts M, Larimer F, Lamerdin J, Predki P, Atlas R (2001) An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Photosynth Res 70:85–106

Meeks JC, Campbell EL, Summers ML, Wong FC (2002) Cellular differentiation in the cyanobacterium *Nostoc punctiforme*. Arch Microbiol 178:395–403

Meindl D, Loos E (1990) Release of glucose by *Nostoc* species isolated from the lichen *Peltigera horizontalis*. Plant Sci 72:283–288

Mellor RB, Gadd GM, Rowell P, Stewart WDP (1981) A phytohaemagglutinin from the *Azolla-Anabaena* symbiosis. Biochem Biophys Res Commun 99:1348–1353

Milano V (2003) Identification of microbionts associated with the water fern *Azolla*. Masters thesis, Rensselaer Polytechnic Institute, Troy, pp 1–49

Miura S, Yokoto A (2006) Isolation and characterization of cyanobacteria from lichen. J Gen Appl Microbiol 52:365–375

Mollenhauer D (1992) *Geosiphon pyriformis*. In: Reisser W (ed) Algae and symbiosis: plants, animals, fungi, viruses, interactions explored. Biopress, Bristol, pp 339–351

Mollenhauer D, Mollenhauer R (1997) Endosymbiosis between *Nostoc* and *Geosiphon pyriforme*. Institut für den Wissenschaftlichen Film (IWF), Göttingen, Film-no. C1955

Mollenhauer D, Mollenhauer R, Kluge M (1996) Studies on initiation and development of the partner association in *Geosiphon pyriforme* (Kütz.) v. Wettstein, a unique endocytobiotic system of a fungus (Glomales) and the cyanobacterium *Nostoc punctiforme* (Kütz.) Hariot. Protoplasma 193:3–9

Muro-Pastor AM, Reyes JC, Florencio FJ (2005) Ammonium assimilation in cyanobacteria. Photosynth Res 83:135–150

Nash TH (1996) Lichen biology. Cambridge University Press, Cambridge, UK

Nathanielsz CP, Staff IA (1975) A mode of entry of blue-green algae into the apogeotrophic roots of *Macrozamia communis*. Am J Bot 62:232–235

Naumann M, Schüßler A, Bonfante P (2010) The obligate endobacteria of arbuscular mycorrhizal fungi are ancient heritable components related to the *Mollicutes*. ISME J 4:862–871

Newton JW, Herman AI (1979) Isolation of cyanobacteria from the aquatic fern, *Azolla*. Arch Microbiol 120:161–165

Nierzwicki-Bauer SA, Aulfinger H (1991) Occurrence and ultrastructural characterization of bacteria in association with and isolated from *Azolla caroliniana*. Appl Environ Microbiol 57:3629–3636

Nierzwicki-Bauer SA, Aulfinger H, Braun-Howland EB (1989) Ultrastructural characterization of an inner envelope that confines *Azolla* endosymbionts to the leaf cavity periphery. Can J Bot 67:2711–2719

Nilsson MC, Wardle DA (2005) Understory vegetation as a forest ecosystem driver: evidence from the northern Swedish boreal forest. Front Ecol Environ 3:421–428

Nilsson M, Bergman B, Rasmussen U (2000) Cyanobacterial diversity in geographically related and distant host plants of the genus *Gunnera*. Arch Microbiol 173:97–102

Nilsson M, Rasmussen U, Bergman B (2006) Cyanobacterial chemotaxis to extracts of host and nonhost plants. FEMS Microbiol Ecol 55:382–390

Nudleman E, Kaiser D (2004) Pulling together with type IV pili. J Mol Microbiol Biotechnol 7:52–62

Obukowicz M, Schaller M, Kennedy GS (1981) Ultrastructure and phenolic histochemistry of the *Cycas revoluta-Anabaena* symbiosis. New Phytol 87:751–759

Orr J, Haselkorn R (1982) Regulation of glutamine synthetase activity and synthesis in free-living and symbiotic *Anabaena* spp. J Bacteriol 152:626–635

Osborne B, Bergman B (2009) Why does *Gunnera* do it and other angiosperms don't? In: Pawlowski K (ed) Prokaryotic symbionts in plants, vol 8, Microbiology monographs. Springer, Heidelberg, pp 207–224

Osborne BA, Sprent JI (2002) Ecology of *Nostoc-Gunnera* symbiosis. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 207–232

Osborne B, Doris F, Cullen A, McDonald R, Campbell G, Steer M (1991) *Gunnera tinctoria*: an unusual invader. Bioscience 41:224–234

Osborne BA, Cullen A, Jones PW, Campbell GJ (1992) Use of nitrogen by the *Nostoc-Gunnera tinctoria* (Molina) Mirbel symbiosis. New Phytol 120:481–487

Ow MC, Gantar M, Elhai J (1999) Reconstitution of a cycad-cyanobacterial association. Symbiosis 27:125–134

Palmqvist K (2002) Cyanolichens: carbon metabolism. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 73–96

Papaefthimiou D, Hrouzek P, Mugnai MA, Lukesova A, Turicchia S, Rasmussen U, Ventura S (2008) Differential patterns of evolution and distribution of the symbiotic behaviour in nostocacean cyanobacteria. Int J Syst Evol Microbiol 58:553–564

Pate JS, Lindblad P, Atkins CA (1988) Pathways of assimilation and transfer of the fixed nitrogen in coralloid roots of cycad-*Nostoc* symbioses. Planta 176:461–471

Paulsrud P, Lindblad P (1998) Sequence variation of the $tRNA^{Leu}$ intron as a marker for genetic diversity and specificity of symbiotic cyanobacteria in some lichens. Appl Environ Microbiol 64:310–315

Paulsrud P, Rikkinen J, Lindblad P (1998) Cyanobiont specificity in some *Nostoc*-containing lichens and in a *Peltigera aphthosa* photosymbiodeme. New Phytol 139:517–528

Paulsrud P, Rikkinen J, Lindblad P (2000) Spatial patterns of photobiont diversity in some *Nostoc*-containing lichens. New Phytol 146:291–299

Paulsrud P, Rikkinen J, Lindblad P (2001) Field experiments on cyanobacterial specificity in *Peltigera aphthosa*. New Phytol 152:117–123

Pennell RI (1992) Cell surface arabinogalactan proteins, arabinogalactans and plant development. In: Collow JA, Green JR (eds) Perspectives in plant cell recognition. Cambridge University Press, Cambridge, UK, pp 105–121

Perkins SK, Peters GA (1993) The *Azolla-Anabaena* symbiosis: endophyte continuity in the *Azolla* life-cycle is facilitated by epidermal trichomes I: partitioning of the endophytic *Anabaena* into developing sporocarps. New Phytol 123:53–64

Perraju BTVV, Rai AN, Kumar AR, Singh HN (1986) *Cycas circinalis-Anabaena cycadeae* symbiosis: photosynthesis and the enzymes of nitrogen and hydrogen metabolism in symbiotic and cultured *Anabaena cycadeae*. Symbiosis 1:239–251

Peters GA, Calvert HE (1983) The *Azolla-Anabaena azollae* symbiosis. A continuum of interaction strategies. In: Goff LJ (ed) Algal symbiosis. Cambridge University Press, Cambridge, UK, pp 109–145

Peters GA, Perkins SK (1993) The *Azolla-Anabaena* symbiosis: endophyte continuity in the *Azolla* life-cycle is facilitated by epidermal trichomes II: reestablishment of the symbiosis following gametogenesis and embryogenesis. New Phytol 123:65–75

Peters GA, Kaplan D, Meeks JC, Buzby KM, Marsh BH, Corbin JL (1985) Aspects of nitrogen and carbon interchange in the *Azolla-Anabaena* symbiosis. In: Ludden PW, Burris JE (eds) Nitrogen fixation and $CO_2$ metabolism. Elsevier, New York, pp 213–222

Peterson RL, Howarth MJ, Whittier DP (1981) Interactions between a fungal endophyte and gametophyte cells in *Psilotum nudum*. Can J Bot 59:711–720

Pirozynski KA, Malloch DW (1975) The origin of land plants: a matter of mycotrophism. Biosystems 6:153–164

Plazinski J (1990) The *Azolla-Anabaena* symbiosis. In: Gresshoff PM (ed) Molecular biology of symbiotic nitrogen fixation. CRC Press, Boca Raton, pp 51–75

Plazinski J, Taylor R, Shaw W, Croft L, Rolfe BG, Gunning BES (1990a) Isolation of *Agrobacterium* sp. strain from the *Azolla* leaf cavity. FEMS Microbiol Lett 70:55–59

Plazinski J, Zheng Q, Taylor R, Croft L, Rolfe BG, Gunning BES (1990b) DNA probes show genetic variation in cyanobacterial symbionts of the *Azolla* fern and a closer relationship to free-living *Nostoc* strains than to free-living *Anabaena* strains. Appl Environ Microbiol 56:1263–1270

Plazinski J, Croft L, Taylor R, Zheng Q, Rolfe BG, Gunning BES (1991) Indigenous plasmids in *Anabaena azollae*: their taxonomic distribution and existence of regions of homology with symbiotic genes of rhizobium. Can J Microbiol 37:171–181

Polsky FI, Nunn PB, Bell EA (1972) Distribution and toxicity of α-amino-β-methylaminopropionic acid. Fed Proc 31:1473–1475

Rai AN (1990) Cyanobacterial-fungal symbioses: the cyanolichens. In: Rai AN (ed) Handbook of symbiotic cyanobacteria. CRC Press, Boca Raton, pp 9–41

Rai AN (2002) Cyanolichens: nitrogen metabolism. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 97–115

Rai AN, Rowell P, Stewart WDP (1980) $NH_4^+$ assimilation and nitrogenase regulation in the lichen *Peltigera aphthosa* Willd. New Phytol 85:545–555

Rai AN, Rowell P, Stewart WDP (1981a) Nitrogenase activity and dark $CO_2$ fixation in the lichen *Peltigera aphthosa* Willd. Planta 151:256–264

Rai AN, Rowell P, Stewart WDP (1981b) $^{15}N_2$ incorporation and metabolism in the lichen *Peltigera aphthosa* Willd. Planta 152:544–552

Rai AN, Rowell P, Stewart WDP (1983a) Interactions between cyanobacterium and fungus during $^{15}N_2$-incorporation and metabolism in the lichen *Peltigera aphthosa* Willd. Arch Microbiol 134:136–142

Rai AN, Rowell P, Stewart WDP (1983b) Mycobiont-cyanobiont interactions during dark nitrogen fixation by the lichen *Peltigera aphthosa*. Physiol Plant 57:285–290

Rai AN, Borthakur M, Singh S, Bergman B (1989) *Anthoceros-Nostoc* symbiosis: immunoelectronmicroscopic localization of nitrogenase, glutamine synthetase, phycoerythrin and ribulose-1,5-bisphosphate carboxylase/oxygenase in the cyanobiont and the cultured (free-living) isolate *Nostoc* 7801. J Gen Microbiol 135:385–395

Rai AN, Söderbäck E, Bergman B (2000) Tansley Review No. 116, cyanobacterium-plant symbioses. New Phytol 147:449–481

Rai AN, Bergman B, Rasmussen U (eds) (2002) Cyanobacteria in symbiosis. Kluwer, Dordrecht

Ran L, Larsson J, Vigil-Stenman T, Nylander J, Ininbergs K, Zheng W, Lapidus A, Lowry S, Haselkorn R, Bergman B (2010) Genome erosion in a nitrogen-fixing vertically transmitted endosymbiotic multicellular cyanobacterium. PLoS One 5:e11486

Rasmussen U, Nilsson M (2002) Cyanobacterial diversity and specificity in plant symbioses. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 313–328

Rasmussen U, Svenning M (2001) Characterization by genotypic methods of symbiotic *Nostoc* strains isolated from five species of *Gunnera*. Arch Microbiol 176:204–210

Rasmussen U, Johansson C, Bergman B (1994) Early communication in the *Gunnera-Nostoc* symbiosis: plant-induced cell differentiation and protein synthesis in the cyanobacterium. Mol Plant Microbe Interact 7:696–702

Rasmussen U, Johansson C, Renglin A, Petersson C, Bergman B (1996) A molecular characterization of the *Gunnera-Nostoc* symbiosis: comparison with *Rhizobium*-and *Agrobacterium*-plant interactions. New Phytol 133:391–398

Read DJ, Duckett JG, Francis R, Ligrone R, Russel A (2000) Symbiotic fungal associations in "lower" land plants. Philos Trans R Soc Lond B 355:815–831

Redecker D, Kodner R, Graham LE (2000a) Glomalean fungi from the Ordovician. Science 289:1920–1921

Redecker D, Morton JB, Bruns TD (2000b) Ancestral lineages of arbuscular mycorrhizal fungi. Mol Phylogenet Evol 14:276–284

Reinke J (1872) Ueber die anatomische Verhaltnisse einiger Arten von Gunnera L. Nachrichten der. Konigliche Gesellschaft der Wissenschaften Universitat, Gottingen, pp 100–108

Reinke J (1873) Untersuchungen über die Morphologie der Vegetationsorgane von Gunnera. In: Reinke J (ed) Morphologische Abhandlungen. Verlag W. Engelman, Leipzig, pp 45–123

Remy W, Taylor TN, Hass H, Kerp H (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci USA 91:11841–11843

Renzaglia KS (1982) A comparative developmental investigation of the gametophyte generation in the Metzgeriales (Hepatophyta). Bryophyt Bibl 24:1–238

Renzaglia KS, Duff RJ, Nickrent DL, Garbary D (2000) Vegetative and reproductive innovations of early land plants: implications for a unified phylogeny. Trans R Soc Lond 355:769–793

Rikkinen J (2002) Cyanolichens: an evolutionary overview. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 31–72

Rikkinen J, Oksanen I, Lohtander K (2002) Lichen guilds share related cyanobacterial symbionts. Science 297:357

Rodgers GA, Stewart WDP (1974) Physiological interrelations of the blue-green alga *Nostoc* with the liverworts *Anthoceros* and *Blasia*. Br Phycol J 9:223

Rodgers GA, Stewart WDP (1977) The cyanophyte-hepatic symbiosis. I: morphology and physiology. New Phytol 78:441–458

Rosén J, Hellenäs K (2008) Determination of the neurotoxin BMAA (β-*N*-methylamino-L-alanine) to cycad seed and cyanobacteria by LC-MS/MS (liquid chromatography tandem mass spectrometry). Analyst 133:1785–1789

Rowell P, Rai AN, Stewart WDP (1985) Studies on the nitrogen metabolism of the lichen *Peltigera aphthosa* and *Peltigera canina*. In: Brown DH (ed) Lichen physiology and cell biology. Plenum, New York, pp 145–160

Rubinstein CV, Gerrienne P, De La Puente GS, Astini RA, Steemans P (2010) Early Middle Ordovician evidence for land plants in Argentina (eastern Gondwana). New Phytol 188:365–369

Sacristan M, Millanes A-M, Legaz M-E, Vicente C (2006) A lichen lectin specifically binds to the alpha-1,4-polygalactoside moiety of urease located in the cell wall of homologous algae. Plant Signal Behav 1:23–27

Saunders RMK, Fowler K (1993) The supraspecific taxonomy and evolution of the fern genus *Azolla* (Azollaceae). Plant Syst Evol 184:175–193

Schaede R (1951) Über die Blaualgensymbiose von Gunnera. Planta 39:154–170

Scheloske S, Maetz M, Schüßler A (2001) Heavy metal uptake of *Geosiphon pyriforme*. Nucl Instrum Methods Phys Res Sect B 181:659–663

Schenk HEA (1992) Cyanobacterial symbioses. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The prokaryotes, vol 4. Springer, Berlin, pp 3819–3854

Schmid E, Oberwinkler F (1993) Mycorrhiza-like interactions between the achlorophyllous gametophyte of *Lycopodium clavatum* L. and its fungal endophyte studied by light and electron microscopy. New Phytol 124:69–81

Schnepf E (1964) Zur Feinstruktur von Geosiphon pyriforme. Arch Mikrobiol 49:112–131

Schüßler A (1999) Glomales SSU rRNA gene diversity. New Phytol 144:205–207

Schüßler A (2000) *Glomus claroideum* forms an arbuscular mycorrhiza-like symbiosis with the hornwort *Anthoceros punctatus*. Mycorrhiza 10:15–21

Schüßler A (2002) Molecular phylogeny, taxonomy, and evolution of *Geosiphon pyriformis* and arbuscular mycorrhizal fungi. Plant Soil 244:75–83

Schüßler A (2012) The *Geosiphon–Nostoc* endosymbiosis and its role as a model for arbuscular mycorrhiza research. In: Hock B (ed) The mycota IX—fungal associations, 2nd edn. Springer, Berlin/Heidelberg (in press)

Schüßler A, Walker C (2010) The Glomeromycota: a species list with new families and new genera published in libraries at The Royal Botanic Garden Edinburgh, The Royal Botanic Garden Kew, Botanische Staatssammlung Munich, and Oregon State University. Electronic version freely available online at www.amf-phylogeny.com. Gloucester

Schüßler A, Walker C (2011) Evolution of the 'plant-symbiotic' fungal phylum, Glomeromycota. In: Pöggeler S, Wöstemeyer J (eds) Evolution of fungi and fungal-like organisms. Springer, Berlin Heidelberg, pp 163–185

Schüßler A, Wolf E (2005) *Geosiphon pyriformis*—a glomeromycotan soil fungus forming endosymbiosis with cyanobacteria. In: Declerck S, Strullu D-G, Fortin A (eds) In vitro culture of mycorrhizas. Springer, Berlin/Heidelberg, pp 271–289

Schüßler A, Mollenhauer D, Schnepf E, Kluge M (1994) *Geosiphon pyriforme*, an endosymbiotic association of fungus and cyanobacteria: the spore structure resembles that of arbuscular mycorrhizal (AM) fungi. Bot Acta 107:36–45

Schüßler A, Schnepf E, Mollenhauer D, Kluge M (1995) The fungal bladders of the endocyanosis *Geosiphon pyriforme*, a *Glomus*-related fungus: cell wall permeability indicates a limiting pore radius of only 0.5 nm. Protoplasma 185:131–139

Schüßler A, Bonfante P, Schnepf E, Mollenhauer D, Kluge M (1996) Characterization of the *Geosiphon pyriforme* symbiosome by affinity techniques: confocal laser scanning microscopy (CLSM) and electron microscopy. Protoplasma 190:53–67

Schüßler A, Meyer T, Gehrig H, Kluge M (1997) Variations of lectin binding sites in extracellular glycoconjugates during the life cycle of *Nostoc punctiforme*, a potentially endosymbiotic cyanobacterium. Eur J Phycol 32:233–239

Schüßler A, Schwarzott D, Walker C (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105:1413–1421

Schüßler A, Martin H, Cohen D, Fitz M, Wipf D (2006) Characterization of a carbohydrate transporter from symbiotic glomeromycotan fungi. Nature 444:933–936

Schüßler A, Krüger M, Walker C (2011) Revealing natural relationships among arbuscular mycorrhizal fungi: culture line BEG47 represents *Diversispora epigaea*, not *Glomus versiforme*. PLoS One 6:e23333

Sergeeva E, Liaimer A, Bergman B (2002) Evidence for biosynthesis and release of the phytohormone indole-3-acetic acid by cyanobacteria. Planta 215:229–238

Serrano R, Carrapico F, Vidal R (1999) The presence of lectins in bacteria associated with the *Azolla-Anabaena* symbiosis. Symbiosis 27:169–178

Shannon BT, Gates JE, McCowen SM (1993) DNA base composition of eubacteria isolated from 4 species of *Azolla*. Symbiosis 15:165–175

Silvester WB (1976) Endophyte adaptation in *Gunnera-Nostoc* symbiosis. In: Nutman PS (ed) Symbiotic nitrogen fixation in plants. Cambridge University Press, Cambridge, UK, pp 521–541

Silvester WB, McNamara PJ (1976) The infection process and ultrastructure of the *Gunnera-Nostoc* symbiosis. New Phytol 77:135–141

Silvester WB, Smith DR (1969) Nitrogen fixation by *Gunnera-Nostoc* symbiosis. Nature 224:1231

Silvester WB, Parsons R, Watt PW (1996) Direct measurement of release and assimilation of ammonia in the *Gunnera-Nostoc* symbiosis. New Phytol 132:617–625

Singh P, Singh D (1997) *Azolla-Anabaena* symbiosis. In: Ddarwal K (ed) Biotechnological approaches in soil microorganisms for sustainable crop production. Scientific Press, Jodhpur, pp 93–107

Smith DC, Douglas AE (1987) The biology of symbiosis. Edward Arnold, London

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, London

Smith SA, Beaulieu JM, Donoghue MJ (2010) An uncorrelated relaxed-clock analysis suggests an earlier origin for flowering plants. Proc Natl Acad Sci 107:5897–5902

Snyder LR, Marler TE (2011) Rethinking cycad metabolite research. Commun Integr Biol 4:86–88

Söderbäck E (1992) Developmental patterns in the *Nostoc-Gunnera* symbiosis. Doctoral thesis, Stockholm University, Stockholm

Söderbäck E, Bergman B (1992) The *Nostoc-Gunnera magellanica* symbiosis: phycobiliproteins, carboxysomes and Rubisco in the microsymbiont. Physiol Plant 8:425–432

Söderbäck E, Bergman B (1993) The *Nostoc-Gunnera* symbiosis: carbon fixation and translocation. Physiol Plant 89:125–132

Söderbäck E, Lindblad P, Bergman B (1990) Developmental patterns related to nitrogen fixation in the *Nostoc-Gunnera magellanica* Lam symbiosis. Planta 182:355–362

Solheim B, Zielke M (2002) Associations between cyanobacteria and mosses. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer, Dordrecht, pp 137–152

Solheim B, Wiggen H, Roberg S, Spaink HP (2004) Associations between arctic cyanobacteria and mosses. Symbiosis 37:169–187

Sood A, Prasanna R, Prasanna BM, Singh PK (2008) Genetic diversity among and within cultured cyanobionts of diverse species of *Azolla*. Folia Microbiol 53:35–43

Spáčil Z, Eriksson J, Jonasson S, Rasmussen U, Ilag LL, Bergman B (2010) Analytical protocol for identification of BMAA and DAB in biological samples. Analyst 135:127–132

Staff IA, Ahern CP (1993) Symbiosis in cycads with special reference to *Macrozamia communis*. In: Stevenson DW, Norstog KJ (eds) The biology, structure and systematics of the Cycadales. Palm and Cycad Societies of Australia, Queensland, pp 200–210

Stahl M (1949) Die Mycorrhiza der Lebermoose mit besonderer Berücksichtigung der thallösen Formen. Planta 37:103–148

Steinberg NA, Meeks JC (1989) Photosynthetic $CO_2$ fixation and ribulose bisphosphate carboxylase/oxygenase activity of *Nostoc* sp. strain UCD 7801 in symbiotic association with *Anthoceros punctatus*. J Bacteriol 171:6227–6233

Steinberg NA, Meeks JC (1991) Physiological sources of reductant for nitrogen fixation activity in *Nostoc* sp. strain UCD 7801 in symbiotic association with *Anthoceros punctatus*. J Bacteriol 173:7324–7329

Stevenson DW (1993) The Zamiaceae in Panama with comments on phytogeography and species relationships. Brittonia 45:1–16

Stewart WDP, Rogers GA (1977) The cyanophyte-hepatic symbiosis. II: nitrogen fixation and the interchange of nitrogen and carbon. New Phytol 78:459–471

Stewart WDP, Rowell P (1977) Modifications of nitrogen-fixing algae in lichen symbiosis. Nature (London) 265:371–372

Stewart WDP, Rowell P, Rai AN (1983) Cyanobacteria-eukaryotic plant symbioses. Ann Inst Pasteur Microbiol 134B:205–228

Stock PA, Silvester WB (1994) Phloem transport of recently fixed nitrogen in the *Gunnera-Nostoc* symbiosis. New Phytol 126:259–266

Stocker-Wörgötter E (1995) Experimental cultivation of lichens and lichen symbionts. Can J Bot 73S:579–589

Stocker-Wörgötter E, Turk R (1994) Artificial resynthesis of the photosymbiodeme *Peltigera leucophlebia* under laboratory conditions. Cryptogam Bot 4:300–308

Stucken K, John U, Cembella A, Murillo A, Soto-Liebe K et al (2010) The smallest known genomes of multicellular and toxic cyanobacteria: comparison, minimal gene sets for linked traits and the evolutionary implications. PLoS One 5:e9235

Svenning MM, Eriksson T, Rasmussen U (2005) Phylogeny of symbiotic cyanobacteria within the genus *Nostoc* based on 16S rDNA sequence analyses. Arch Microbiol 183:19–26

Tabita FR (1994) The biochemistry and molecular regulation of carbon dioxide metabolism in cyanobacteria. In: Bryant DA (ed) The molecular biology of cyanobacteria. Kluwer, Dordrecht, pp 437–467

Tandeau de Marsac N (1994) Differentiation of hormogonia and relationships with other biological processes. In: Bryant DA (ed) The molecular biology of cyanobacteria. Kluwer, Amsterdam, pp 825–842

Tehler A, Farris JS, Lipscomb DL, Kallersjo M (2000) Phylogenetic analysis of the fungi based on large rDNA data sets. Mycologia 92:459–474

Tel-Or E, Sandovsky T, Kobiler D, Arad C, Weinberg R (1983) The unique symbiotic properties of *Anabaena* in the water fern *Azolla*. In: Papageorgiou GC, Packer L (eds) Prokaryotes: cell differentiation and function. Elsevier, New York, pp 303–314

Thajuddin N, Muralitharan G, Sundaramoorthy M, Ramamoorthy R, Ramachandran S, Akbarsha MA, Gunasekaran M (2010) Morphological and genetic diversity of symbiotic cyanobacteria from cycads. J Basic Microbiol 50:254–265

Towata EM (1985) Morphometric and cytochemical ultrastructural analyses of the *Gunnera kaalensis*/*Nostoc* symbiosis. Bot Gaz 146:293–301

Uheda E, Kitoh S (1991) Electron microscopic observations of the envelopes of isolated algal packets of *Azolla*. Can J Bot 69:1418–1419

Uheda E, Silvester WB (2001) The role of papillae during the infection process in the *Gunnera-Nostoc* symbiosis. Plant Cell Physiol 42:780–783

Usher K, Bergman B, Raven J (2007) Exploring cyanobacterial mutualisms. Annu Rev Ecol Evol Syst 38:255–273

Van Coppenolle B, Watanabe I, Van-Hove C, Second G, Huang N, McCouch SR (1993) Genetic diversity and phylogeny analysis of *Azolla* based on DNA amplification by arbitrary primers. Genome 36:686–693

Van Coppenolle B, McCouch SR, Watanabe I, Huang N, Van Hove C (1995) Genetic diversity and phylogeny analysis of *Anabaena azollae* based on RFLP detected in *Azolla-Anabaena azollae* DNA complexes using *nif* gene probes. Theor Appl Genet 91:589–597

Van Hove C, Lejeune A (1996) Does *Azolla* have any future in agriculture? In: Rahman M (ed) Biological nitrogen fixation associated with rice production. Kluwer, Dordrecht, pp 83–94

Vega A, Bell EA (1967) α-Amino-β-methylaminopropionic acid, a new amino acid from seeds of *Cycas circinalis*. Phytochemistry 6:759–762

Vessey JK, Pawlowski K, Bergman B (2005) Root-based $N_2$-fixing symbioses: legumes, actinorhizal plants, *Parasponia* sp and cycads. Plant Soil 274:51–78

Veys P, Waterkeyn L, Lejeune A, Van Hove C (1999) The pore of the leaf cavity of *Azolla*: morphology, cytochemistry and possible functions. Symbiosis 27:33–57

Veys P, Lejeune A, Van Hove C (2000) The pore of the leaf cavity of *Azolla*: interspecific morphological differences and continuity between the cavity envelopes. Symbiosis 29:33–47

Veys P, Lejeune A, Van Hove C (2002) The pore of the leaf cavity of *Azolla*: teat-cell differentiation and cell wall projections. Protoplasma 219:31–42

Villarreal JC, Renzaglia KS (2006) Structure and development of *Nostoc* strands in *Leiosporoceros dussii* (Anthocerotophyta): a novel symbiosis in land plants. Am J Bot 93:693–705

Von Wettstein F (1915) Geosiphon Fr. v. Wettst., eine neue, interessante Siphonee sterr. Bot Z 65:145–156

Wallace WH, Gates JE (1986) Identification of eubacteria isolated from leaf cavities of 4 species of the N-fixing *Azolla* fern as *Arthrobacter* Conn and Dimmick. Appl Environ Microbiol 52:425–429

Wang C-M, Ekman M, Bergman B (2004) Expression of cyanobacterial genes involved in heterocyst differentiation and dinitrogen fixation along a plant symbiosis development profile. Mol Plant Microbe Interact 17(4): 436–443

Wang B, Yeun LH, Xue J-Y, Liu Y, Ane J-M, Qiu Y-L (2010) Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants. New Phytol 186:514–525

Wanntorp L, Wanntorp H-E, Oxelman B, Källersjö M (2001) Phylogeny of *Gunnera*. Plant Syst Evol 226:85–107

Watanabe I (1994) Genetic enhancement and *Azolla* collection-problems in applying *Azolla-Anabaena* symbiosis. In: Hegazi NA, Fayez M, Monib M (eds) Nitrogen fixation with non-legumes. The American University in Cairo Press, Cairo, pp 437–450

Watanabe I, Van Hove C (1996) Phylogenetic, molecular, and breeding aspects of *Azolla-Anabaena* symbiosis. In: Camus JM, Gibby M, Jones RJ (eds) Pteridology in perspective. Royal Botanic Gardens, Kew, pp 611–619

Watanabe I, Lapis-Tenorio MT, Ventura TS, Padre BC (1993) Sexual hybrids of *Azolla filiculoides* with *A. microphylla*. Soil Sci Plant Nutr 39:669–676

Watts S (2000) Signalling in the *Nostoc*-plant symbiosis. PhD thesis, University of Leeds, Leeds

Watts SD, Knight CD, Adams DG (1999) Characterisation of plant exudates inducing chemotaxis in nitrogen-fixing cyanobacteria. In: Peschek GA, Löffelhardt W, Schmetterer G (eds) The phototrophic prokaryotes. Kluwer/Plenum, New York, pp 679–684

Webb DT (1983a) Nodulation in light- and dark-grown *Macrozamia communis* L. Johnson seedlings in sterile culture. Ann Bot 52:545–547

Webb DT (1983b) Developmental anatomy of light-induced root nodulation by *Zamia pumila* L. seedlings in sterile culture. Am J Bot 70:1109–1117

Wei WX, Jin GY, Zhang N, Chen J (1988) Studies of hybridization in *Azolla*. In: Singh KH, Kramer KU (eds) Proceedings of the international symposium on systematic pteriodology. China Sciientific and Technical Press, Beijing, pp 135–139

West N, Adams DG (1997) Phenotypic and genotypic comparison of symbiotic and free-living cyanobacteria from a single field site. Appl Environ Microbiol 63:4479–4484

West NJ, Adams DG, Sisson PR, Freeman R, Hawkey PM (1999) Pyrolysis mass spectrometry analysis of free-living and symbiotic cyanobacteria. Antonie Van Leeuwenhoek 75:201–206

Wolf E, Schüßler A (2005) Phycobiliprotein fluorescence of *Nostoc punctiforme* changes during the life cycle and chromatic adaptation: characterization by spectral confocal laser scanning microscopy and spectral unmixing. Plant Cell Environ 28:480–491

Wolk CP (2000) Heterocyst formation in *Anabaena*. In: Brun YV, Shimkets LJ (eds) Prokaryotic development. ASM Press, Washington, DC, pp 83–104

Wong FCY, Meeks JC (2001) The *hetF* gene product is essential to heterocyst differentiation and affects HetR function in the cyanobacterium *Nostoc punctiforme*. J Bacteriol 183:2654–2661

Wong FCY, Meeks JC (2002) Establishment of a functional symbiosis between the cyanobacterium *Nostoc punctiforme* and the bryophyte *Anthoceros punctatus* requires genes involved in nitrogen control and initiation of heterocyst differentiation. Microbiology 148:315–323

Wouters J, Janson S, Bergman B (2000) The effect of exogenous carbohydrates on nitrogen fixation and *hetR* expression in *Nostoc* PCC 9229 forming symbiosis with Gunnera. Symbiosis 28:63–76

Yoshimura I, Yamamoto Y (1991) Development of *Peltigera praetextata* lichen thalli in culture. Symbiosis 11:109–117

Zhang C-C, Laurent S, Sakr S, Peng L, Bedu S (2006) Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. Mol Microbiol 59:367–375

Zheng WW, Nilsson M, Bergman B, Rasmussen U (1999) Genetic diversity and classification of cyanobacteria in different *Azolla* species by the use of PCR fingerprinting. Theor Appl Genet 99:1187–1193

Zheng WW, Song TY, Bao XD, Bergman B, Rasmussen U (2002) High cyanobacterial diversity in coralloid roots of cycads revealed by PCR fingerprinting. FEMS Microbiol Ecol 40:215–222

Zheng S-P, Chen B, Guan X, Zheng W (2008) Diversity analysis of endophytic bacteria within *Azolla microphylla* using PCR-DGGE and electron microscopy. Chin J Agric Biotechnol 16:508–514

Zheng W, Bergman B, Chen B, Zheng S, Xiang G, Rasmussen U (2009) Cellular responses in the cyanobacterial symbiont during its vertical transfer between plant generations in the *Azolla microphylla* symbiosis. New Phytol 181:53–61

Zielke M, Ekker AS, Olsen RA, Spjelkavik S, Solheim B (2002) The influence of abiotic factors on biological nitrogen fixation in different types of vegetation in the High Arctic, Svalbard Arctic. Arctic Antarct Alpine Res 34:293–299

Zielke M, Solheim B, Spjelkavik S, Olsen RA (2005) Nitrogen fixation in the high arctic: role of vegetation and environmental conditions. Arctic Antarct Alpine Res 37:372–378

Zimmerman WJ, Watanabe I, Lumpkin TA (1991) The *Anabaena-Azolla* symbiosis: diversity and relatedness of neotropical host taxa. Plant Soil 137:167–170

# 17 Root and Stem Nodule Bacteria of Legumes

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## Introduction

The root and stem nodule bacteria of legumes (collectively called the rhizobia) comprise a genetically diverse group of microorganisms characterized by the ability to produce hypertrophies (swellings) or nodules on the stems or roots of most, but not all, leguminous plants (peas, beans, clover, etc.). Not all legumes form nodules with rhizobia.

Within the nodules, rhizobia convert atmospheric dinitrogen ($N_2$) gas into ammonia. This fixed nitrogen (N) is subsequently assimilated by the host and improves plant growth and productivity. Approximately 300 million hectares (Mha) of legumes are grown worldwide, and they collectively fix about 60 Tg ($6 \times 10^7$ metric tons) of N each year (Kinzig and Socolow 1994). Overall, $N_2$ fixation supplies about 50 % of the N used in agriculture, and because the fixed N is used directly by the host plant without exogenous addition to the soil, the process is generally considered environmentally friendly (Vance 1998). Fixation rates vary with plant species, length of the growing season, presence of a suitable microsymbiont, and environmental conditions, but rates commonly are in the range of 100–200 kg of $N_2$ fixed $ha^{-1}$ $year^{-1}$ (Sparrow et al. 1995; Unkovich et al. 1997). Because of the practical benefits of nodulation and $N_2$ fixation, the rhizobia have been extensively studied, particularly with respect to the genetic basis for their symbiotic interactions, their host specificity, and their nitrogen-fixation potential. However, the rhizobia are also good *saprophytes*, with soil populations of $10^3$–$10^4$ rhizobia $g^{-1}$ being common in soils previously used for legume growth. Thus, the ecological attributes of these organisms also have been studied extensively.

## Phylogeny

The taxonomy of the organisms producing root and stem nodules on legumes is in a state of flux. Currently 92 species of nodule bacteria in 12 genera have been defined (Table 17.1). Although this ever-changing taxonomy affects what the organisms are called and how they are distinguished, it has little impact on their phylogenetic relationships. Small subunit rRNA sequence analysis (SSU rRNA) supports division of these organisms into three major groups (*Rhizobium* [including *Agrobacterium*, *Allorhizobium*, *Ensifer*, and *Mesorhizobium*], *Bradyrhizobium*, and *Azorhizobium*) and *Devosia and Methylobacterium* within the α subclass of the Proteobacteria (Martinez-Romero and Caballero-Mellado 1996; Young and Haukka 1996). More recently, however, several β-proteobacteria, including *Burkholderia*, *Ralstonia*, and *Cupriavidus*, have been shown to nodulate some legumes (Chen et al. 2001, 2003, 2005;

*This chapter is dedicated to the memory of Peter H. Graham (1937–2009).

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**Table 17.1**
**List of current, validly published species of root and stem nodule bacteria**

| Species | References |
|---|---|
| **Alpha-proteobacteria** | |
| ***Rhizobium*** | Frank (1889) |
| *R. alamii* | Berge et al. (2009) |
| *R. alkalisoli* | Li et al. (2009) |
| *R. cellulosilyticum* | Garcia-Fraile et al. (2007) |
| *R. daejeonense* | Quan et al. (2005) |
| *R. etli* | Segovia et al. (1993) |
| *R. galegae* | Lindstrom (1989) |
| *R. gallicum* | Amarger et al. (1997) |
| *R. giardinii* | Amarger et al. (1997) |
| *R. hainanense* | Chen et al. (1997) |
| *R. huautlense* | Wang et al. (1998) |
| *R. indigoferae* | Wei et al. (2002) |
| *R. leguminosarum* | Frank (1889) |
| *R. loessense* | Wei et al. (2003) |
| *R. lusitanum* | Valverde et al. (2006) |
| *R. mesosinicum* | Lin et al. (2009) |
| *R. miluonense* | Gu et al. (2008) |
| *R. mongolense* | van Berkum et al. (1998) |
| *R. multihospitium* | Han et al. (2008b) |
| *R. oryzae* | Peng et al. (2008) |
| *R. phaseoli* | Ramirez-Bahena et al. (2008) |
| *R. pisi* | Ramirez-Bahena et al. (2008) |
| *R. tibeticum* | Hou et al. (2009) |
| *R. sullae* | Squartini et al. (2002) |
| *R. tropici* | Martinez-Romero et al. (1991) |
| *R. tubonense* | Zhang et al. (2011) |
| *R. undicola* | de Lajudie et al. (1998a), Young et al. (2001) |
| *R. vignae* | Ren et al. (2011) |
| *R. yanglingense* | Tan et al. (2001) |
| ***Mesorhizobium*** | Jarvis et al. (1997) |
| *M. albiziae* | Wang et al. (2007) |
| *M. alhagi* | Chen et al. (2010) |
| *M. amorphae* | Wang et al. (1999) |
| *M. australicum* | Nandasena et al. (2009) |
| *M. camelthorni* | Chen et al. (2011) |
| *M. caraganae* | Guan et al. (2008) |
| *M. chacoense* | Velazquez et al. (2001) |
| *M. ciceri* | Nour et al. (1994), Jarvis et al. (1997) |
| *M. gobiense* | Han et al. (2008a) |
| *M. huakuii* | Chen et al. (1991), Jarvis et al. (1997) |
| *M. loti* | Jarvis et al. (1982, 1997) |
| *M. mediterraneum* | Nour et al. (1994), Jarvis et al. (1997) |
| *M. metallidurans* | Vidal et al. (2009) |

**Table 17.1 (continued)**

| Species | References |
|---|---|
| *M. opportunistum* | Nandasena et al. (2009) |
| *M. plurifarium* | de Lajudie et al. (1998b) |
| *M. robiniae* | Zhou et al. (2010) |
| *M. shangrilense* | Lu et al. (2009) |
| *M. septentrionale* | Gao et al. (2004) |
| *M. tarimense* | Han et al. (2008a) |
| *M. temperatum* | Gao et al. (2004) |
| *M. tianshanense* | Chen et al. (1995), Jarvis et al. (1997) |
| ***Ensifer (formerly Sinorhizobium)*** | Casida (1982), de Lajudie et al. (1994), Young (2003) |
| *E. abri* | Ogasawara et al. (2003) |
| *E. adhaerens* | Casida (1982) |
| *E. arboris* | Nick et al. (1999) |
| *E. fredii* | Scholla and Elkan (1984), de Lajudie et al. (1994) |
| *E. garamanticus* | Merabet et al. (2010) |
| *E. indiaense* | Ogasawara et al. (2003) |
| *E. kostiensis* | Nick et al. (1999) |
| *E. kummerowiae* | Wei et al. (2002) |
| *E. medicae* | Rome et al. (1996) |
| *E. meliloti* | Dangeard (1926), de Lajudie et al. (1994) |
| *E. mexicanus* | Lloret et al. (2007) |
| *E. numidicus* | Merabet et al. (2010) |
| *E. saheli* | de Lajudie et al. (1994) |
| *E. sojae* | Li et al. (2010) |
| *E. teranga* | de Lajudie et al. (1994) |
| *Sinorhizobium americanum*[a] | Toledo et al. (2003) |
| *Sinorhizobium morelense*[a] | Wang et al. (2002) |
| ***Bradyrhizobium*** | Jordan (1982) |
| *B. canariense* | Vinuesa et al. (2005) |
| *B. elkanii* | Kuykendall et al. (1992) |
| *B. iriomotense* | Islam et al. (2008) |
| *B. japonicum* | Kirchner (1896), Jordan (1982) |
| *B. jicama* | Ramirez-Bahena et al. (2009) |
| *B. liaoningense* | Xu et al. (1995) |
| *B. pachyrhizi* | Ramirez-Bahena et al. (2009) |
| *B. yuanmingense* | Yao et al. (2002) |
| ***Azorhizobium*** | Dreyfus et al. (1988) |
| *A. caulinodans* | Dreyfus et al. (1988) |
| *A. doebereinerae* | Moreira et al. (2006) |
| ***Methylobacterium*** | Patt et al. (1976) |
| *Met. nodulans* | Jourand et al. (2004) |
| ***Devosia*** | Nakagawa et al. (1996) |
| *D. neptuniae* | Rivas et al. (2003) |
| ***Ochrobactrum*** | Holmes et al. (1988) |
| *O. cytisi* | Zurdo-Piñeiro et al. (2007) |

**Table 17.1 (continued)**

| Species | References |
|---|---|
| *O. lupini* | Trujillo et al. (2005) |
| ***Phyllobacterium*** | Knosel (1962) |
| *P. trifolii* | Valverde et al. (2005) |
| *P. ifriqiyense* | Mantelin et al. (2006) |
| *P. leguminum* | Mantelin et al. (2006) |
| ***Shinella*** | An et al. (2006) |
| *S. kummerowiae* | Lin et al. (2008) |
| **Beta-proteobacteria** | |
| ***Burkholderia*** | Yabuuchi et al. (1992) |
| *Bu. caribensis* | Vandamme et al. (2002) |
| *Bu. cepacia* | Rasolomampianina et al. (2005) |
| *Bu. mimosarum* | Chen et al. (2006) |
| *Bu. nodosa* | Chen et al. (2007) |
| *Bu. phymatum* | Vandamme et al. (2002) |
| *Bu. sabiae* | Chen et al. (2008) |
| *Bu. tuberum* | Vandamme et al. (2002) |
| ***Cupriavidus*** | Makkar and Casida (1987) |
| *C. taiwanensis* | Chen et al. (2001) |

[a]Not formally renamed as *Ensifer*

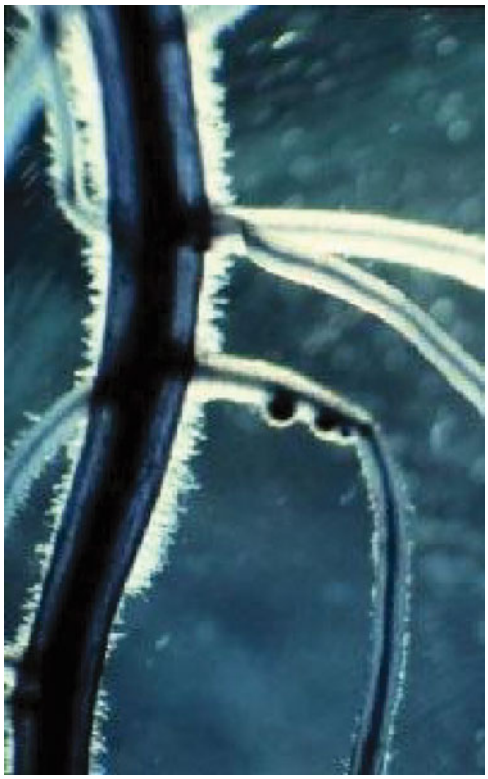

**Fig. 17.1**
**Initiation of nodule formation on the roots of *Phaseolus vulgaris* (L.) by *Rhizobium etli* 8 days after inoculation (Photo by permission of M. H. Chaverra)**

Moulin et al. 2001). Regardless of which taxonomic class these microorganisms belong to, the root/stem nodule bacteria are collectively referred to as the rhizobia.

Wang et al. (1998) showed that *Bradyrhizobium* and *Azorhizobium* are only distantly related to fast-growing *Rhizobium* and their relatives. Also amalgamation of *Rhizobium* and *Agrobacterium* has been proposed on a number of occasions (Graham 1964; Heberlein et al. 1967; De Ley 1968; Sawada et al. 1993; Parker 1957), suggesting that the rhizobia may have evolved from plant pathogenic bacteria. Nonpathogenic *Agrobacterium* are well-known as nodule contaminants (Hofer 1941; Graham 1976; de Lajudie et al. 1999) and often are confused with the nodule-forming rhizobia. Relative to the large number of species of *Rhizobium* that have been described, only a limited number of *Bradyrhizobium* and *Azorhizobium* species have been distinguished, although nine *Bradyrhizobium* species are now recognized (Risal et al. 2010). This is likely to change as additional tropical legume species are studied. Additional groups of bradyrhizobia have already been identified, but not detailed phylogenetically (So et al. 1994; Graham et al. 1995). Moreover, links between *Rhizobium* and related root-nodule bacteria (*Phyllobacterium*, *Devosia*, *Methylobacterium*, *Brucella*, and *Bartonella*) and between *Bradyrhizobium* and *Rhodopseudomonas*, *Blastobacter*, and *Afipia* have been described but need additional study (*Fig. 17.1*).

The ability to form nodules is restricted to a clade of plants including both legume and actinorhizal species. However, not all legumes bear nodules. The percentage of plant species with nodules increased from only 23 % in the more primitive *Caesalpinioideae* to 97 % in the *Papilionoideae*. Because these groups of legumes differ in the frequency of nodulation (and because *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* are so different), it has been suggested that the ability to nodulate and fix $N_2$ could have arisen on more than one occasion (Parker 1968; Doyle 1994; Doyle and Doyle 1997). Doyle (1994) suggested that nodulation arose on at least three previous occasions, including in the genus *Chamaecrista*. Species within *Chamaecrista* can be distinguished from the non-nodulated, but closely related, *Cassia* on the basis of randomly amplified polymorphic DNA (RAPD) analysis (Whitty et al. 1994). Within *Chamaecrista*, species differ in the retention and release of rhizobia from infection threads during differentiation (Naisbitt et al. 1992). It seems unlikely that legumes as different as *Phaseolus* and *Acacia* could nodulate with both *Rhizobium* and *Bradyrhizobium* (Lange 1961; Michiels et al. 1998).

The rhizobia associated with a particular legume host can show significant diversity (Pinero et al. 1988; Souza et al. 1994). However, some caution in interpreting results from biodiversity studies is advisable because a number of studies predate recent phylogenetic advances and changes in rhizobial taxonomy and could have included more than one species of rhizobia. As new legumes are commercialized and exploited, more studies examining the extent of legume/microsymbiont biodiversity near the legume's center of origin and exploration of the consequences of founder effects are warranted.

## Taxonomy

Rhizobia have traditionally been a difficult group to classify. Early researchers considered all rhizobia part of a single species that could nodulate any legume. Subsequently, each rhizobial strain was shown to only nodulate certain specific legumes. This led to the concept of cross-inoculation groups, with rhizobia being distinguished according to the legumes each could nodulate. Thus, rhizobia from alfalfa would generally nodulate medic species and vice versa, but neither would nodulate clover. Using this approach, more than 20 different cross-inoculation groups were identified, and a number of these were raised to species status within the genus *Rhizobium* (Fred et al. 1932). Fred et al. (1932) stated, "It seems true that the ability of an organism to infect certain plants and not others is as fixed and definite as any phase of the physiology of the organism. . .we feel justified in regarding it as the prime character in species differentiation." While host specificity is still important in the identification of rhizobia, it is often at odds with results from numerical and phylogenetic studies (Graham 1964; De Ley and Rassell 1965; Heberlein et al. 1967; Moffett and Colwell 1968). The demonstration that the nodulation genes in *Rhizobium* are plasmid borne (Nuti et al. 1979; Brewin et al. 1980), or located on chromosomal symbiotic islands, and move between soil microorganisms has further weakened infection-based taxonomic analyses.

The 1984 edition of (http://www.cme.msu.edu/bergeys/) *Bergey's Manual of Systematic Bacteriology* divides the *Rhizobiaceae* into four groups, including three genera of nodule- or gall-forming bacteria, *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* (Jordan 1984). The reduced emphasis on host range and the availability of several new phenotypic and phylogenetic techniques have resulted in the proliferation of new species and genera of nodule bacteria. Excellent review article on the taxonomy of rhizobia are provided by Sawada et al. (2003), Willems (2006), and Weir (2011) provides a great up-to-date website (http://www.rhizobia.co.nz/taxonomy/rhizobia.html) on the current taxonomy of rhizobia.

As of March 2011, 12 genera and 92 species of rhizobia were recognized (Weir 2011). Most of these rhizobial species belong to the α-proteobacteria and are members of the genera *Allorhizobium, Azorhizobium, Rhizobium, Mesorhizobium, Ensifer* (formerly *Sinorhizobium*), or *Bradyrhizobium* (Martinez-Romero and Caballero-Mellado 1996; Young and Haukka 1996). Although the genus *Sinorhizobium* was described by Chen et al. in 1988, some recent studies show that *Sinorhizobium* and the genus *Ensifer* (Casida 1982) belong to a single taxon. *Ensifer* is the earlier heterotypic synonym (it was named first) and thus takes priority (Young 2003). This means that all *Sinorhizobium* spp. must be renamed as *Ensifer* spp. according to the Bacteriological Code. This has not been universally agreed upon there has been some discussion in the literature if *Ensifer* or *Sinorhizobium* is correct (Young 2010).

In addition to these divisions, recent research has shown that some root-nodule bacteria, including *Devosia neptuniae* (Rivas et al. 2002), *Methylobacterium nodulans* (Sy et al. 2001), *Ochrobactrum lupini* (Trujillo et al. 2005), *Ochrobactrum cytisi* (Zurdo-Piñeiro et al. 2007), and *Shinella kummerowiae* (Lin et al. 2008), are members of the α-proteobacteria, whereas several beta-proteobacterial species, including *Cupriavidus* (formerly *Ralstonia*; Chen et al. 2001) and *Burkholderia* (Moulin et al. 2001; Vandamme et al. 2002), also have the ability to form nitrogen-fixing root nodules on some legumes (*Fig. 17.2*).

**Fig. 17.2**
**A *Bradyrhizobium japonicum* cell showing significant polyhydroxybutyrate accumulation (Photo by T. McDermott, used with permission)**

With new species of root-nodule bacteria now justified using a polyphasic approach that includes both phenotypic, and phylogenetic traits (Graham et al. 1991), and genomic analyses (MacLean et al. 2007), the further description of new species of rhizobia based solely on simple characteristics has become increasingly problematic. In the second edition of (http://www.prokaryotes.com) *The Prokaryotes*, Elkan and Bunn (1994) listed phenotypic traits useful in the distinction of *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium*. To do so again is a daunting and perhaps not a useful task because older species descriptions may include more than one organism and because differences in the tests applied and methods used can impact results and their interpretation.

*Table 17.2* lists carbon source utilization differences for many species of root and stem nodule bacteria in the α-proteobacteria. It was compiled from a number of different studies (Jarvis et al. 1997; de Lajudie et al. 1998b; Wang et al. 1999) and will likely change as new species are identified. Additional distinctive phenotypic differences are urgently needed.

**Table 17.2**

**Differences in the carbon compounds used for growth among genera of root-nodule bacteria in the α-proteobacteria[a]**

| Carbon source | Genus of nodule bacteria[a] | | | | | |
|---|---|---|---|---|---|---|
| | *Rhizobium* | *Ensifer (Sinorhizobium)* | *Mesorhizobium* | *Allorhizobium* | *Bradyrhizobium* | *Azorhizobium* |
| Adonitol | +[b] | + | + | − | +/− | − |
| D-Arabinose | + | + | + | − | + | − |
| L-Arabinose | + | + | (+) | + | + | − |
| D-Cellobiose | + | + | | + | − | − |
| L-Fucose | + | +/− | +/− | − | − | − |
| Inositol | + | + | +/− | + | − | − |
| Gluconate | + | (+) | | − | + | + |
| Lactose | + | + | (+) | + | − | − |
| L-ysine | | +/− | (−) | − | − | − |
| DL-Malate | (+) | (+) | +/− | + | (+) | + |
| D-Maltose | + | + | + | + | − | − |
| D-Mannose | + | + | (+) | + | + | − |
| Mannitol | + | (+) | + | + | (+) | − |
| D-Mellibiose | + | + | (−) | − | | − |
| D-Raffinose | + | + | +/− | − | − | − |
| Ribose | + | + | | + | + | − |
| L-Rhamnose | + | + | + | + | (+) | − |
| Sucrose | + | + | + | (−) | + | − |
| Trehalose | + | + | + | (−) | (+) | − |
| D-Xylose | + | + | + | − | + | − |

[a]Includes data from Elkan and Bunn (1994), de Lajudie et al. (1994, 1998a), Rome et al. (1996), Jarvis et al. (1997), and Wang et al. (1999).
[b]Symbols: + positive reaction, − negative reaction, +/− discriminatory within the genus, (+) mainly positive reaction, (−) mainly negative reaction

Analysis of rhizobial fatty acid methyl esters (FAME), using gas chromatography (Jarvis and Tighe 1994; Jarvis et al. 1996), has been recommended as a relatively simple and inexpensive method for the identification of fast-growing rhizobia. Rhizobial FAME profiles correctly identified nearly 95 % of almost 200 strains evaluated by Jarvis and Tighe (1994) and Jarvis et al. (1996). These studies only erred in identifying some *Ensifer fredii* as *E. meliloti* and *Rhizobium etli* as *R. leguminosarum*, and vice versa (Graham et al. 1999). Ballen and Graham (unpublished observations) have also shown that *R. etli*, *R. gallicum*, and strains from Dalea and *Onobrychis* overlap by FAME analysis. Similarly, FAME profiles have been used to distinguish slow-growing *Bradyrhizobium japonicum* and *B. elkanii* (Kuykendall et al. 1992; So et al. 1994; Graham et al. 1995); although in each case additional isolates were identified that did not group with these species.

## Habitat

Rhizobia, through their ability to fix $N_2$ in symbiosis with legumes, play a central role in the N supply of most natural terrestrial and aquatic ecosystems. The American tall grass prairie is but one ecosystem in which plant diversity and productivity is controlled, in large part, by N availability (Collins et al. 1998). Although they are thought to be solely soil saprophytes, rhizobia can also be found in aquatic systems associated with water-growing leguminous plants. Owing to cultural and agricultural practices, the migration of birds and animals, and atmospheric deposition of soil particles, there are relatively few soils in the world that do not contain some rhizobia. Moreover, rhizobia have been shown to exist in soils for a relatively long time in the absence of a host plant (Bottomley 1992; Brunel et al. 1988; Kucey and Hynes 1989; Sanginga et al. 1994; Slattery and Coventry 1993; Weaver et al. 1972), indicating that they are also efficient autochthonous soil bacteria.

Rhizobia have been recognized as being important for the functioning of soil ecosystems for centuries (Fred et al. 1932). Shortly, after legume root nodules were shown conclusively to assimilate atmospheric $N_2$ (Hellriegel and Wilfarth 1888), Nodbe and Hiltner were granted a patent for the use of these microorganisms as legume inoculants (Elkan and Bunn 1994). This and subsequent farming and cultural practices have led to the dissemination of rhizobia on a global basis.

Rhizobia in soils may be introduced by application of commercial inoculants or, as in many cases, be the normal flora

present as microsymbionts of an indigenous legume. Inoculants applied to seed, as recommended by their manufacturer, achieve inoculation rates of $10^3$–$10^6$ rhizobia seed$^{-1}$ (Somasegaran and Hoben 1994). This corresponds to application rates of up to $8 \times 10^{10}$ rhizobia ha$^{-1}$ (Brockwell and Bottomley 1995). At these rates, inoculant strains often dominate in nodulation in the first year of a newly introduced crop (Brockwell et al. 1982; Gibson et al. 1976; Singleton and Tavares 1986). Moreover, inoculant strains contribute to the rapid buildup of rhizobia in the soil once nodules senesce and release large numbers of viable rhizobia into the soil system (McDermott et al. 1987; Sutton 1983). Several studies have documented that inoculant strains dominate in nodules 5–15 years after initial inoculation (Brunel et al. 1988; Diatloff 1977; Lindstrom et al. 1990). It should be noted, however, that not all introduced legumes receive inoculation, and in such situations, seed, soil, or aerial contamination will usually lead to some initial nodule formation. A buildup of soil rhizobial populations typically occurs over a period of 4–5 years (Sadowsky and Graham 1998). Moreover, diverse rhizobial populations can develop in association with species that are not initially indigenous to a particular region (Leung et al. 1994). Although it is thought that rhizobia in soil have a clonal population structure, genetic recombination between groups of soil rhizobia may be contributing to diversity in soils (Demezas et al. 1995; Sullivan et al. 1995). It has been demonstrated that soil rhizobia can transfer plasmids (Jarvis et al. 1989; Kinkle and Schmidt 1991; Thomas et al. 1994; Young and Wexler 1988) and chromosomal symbiotic genes (Sullivan et al. 1995).

The rhizobia obtained from any given soil habitat are drastically influenced by the common method of isolation. This usually involves the use of serial dilutions of soil and inoculation on a trap host, followed by recovery from nodules (Somasegaran and Hoben 1994). This procedure often underestimates the numbers of rhizobia in the soil and biases diversity determinations (Dye et al. 1995). Numerous studies have documented the influence of a trap host on the recovery of particular groups of rhizobia from soils (Bottomley et al. 1994; Bromfield et al. 1995; Brunel et al. 1996; Keatinge et al. 1995; Kumar Rao et al. 1982; van Berkum et al. 1995). Selective culture media, when available, will most likely prove useful in determining the identity of natural populations of rhizobia in soil (Gault and Schwinghamer 1993; Tong and Sadowsky 1993).

Lastly, the legume host itself has been shown to strongly influence the prevalence and type of rhizobia in soils (Bottomley 1992). How this occurs is not known, but it is thought to be due to nonspecific, root-exudate-enhanced growth of rhizobia in the rhizosphere, multiplication and release of rhizobia from the nodule, and selection by the trap host of particular groups of rhizobia from mixed soil populations (Sadowsky and Graham 1998).

## Strain Isolation

Several studies have detailed methods for the collection, sampling, isolation, authentication, and maintenance of rhizobia (Date 1982; Date and Halliday 1987; Somasegaran and Hoben 1994). Extensive collection and conservation is necessary because many isolates will prove to be ineffective in symbiosis or host/strain interactions will be significant. For example, in the case of *Stylosanthes scabra*, more than 1,000 isolates were evaluated before a strain suitable for use in commercial legume inoculation was identified (Date 1997).

### Collection

The collection of rhizobia is most commonly undertaken as part of a plant introduction program to supply suitable host germplasm with the rhizobia they need for symbiosis (see The National Plant Germplasm Collection System [http://www.ars-grin.gov]). Ideally, the collection of nodules should coincide with early season growth and well-watered conditions. However, where collection involves remote geographic regions, sample acquisition may be delayed until plant maturity when most nodules may have senesced. Nodule collection may also be limited where the plant species in question is endangered, and therefore, no plant harvest is permitted. In both of these cases, soil may be used as a source of rhizobia, using surface-sterilized seed of an appropriate host to "trap" nodule bacteria. As discussed above, however, this may bias the type and efficacy of rhizobia identified.

Collection of rhizobia also may be undertaken to study the biodiversity of indigenous microorganisms and macroorganisms, the success in nodulation, or the soil establishment of bioengineered organisms. In some cases, the culture of rhizobia from nodules may be unnecessary because enough cell material may be present in soils or plant tissue to directly characterize nodule occupants using serological or genetic methods (Sadowsky and Graham 1998). Somasegaran and Hoben (1994) list several culture collections of rhizobia throughout the world. In addition, the USDA-ARS National Rhizobium Resource Collection (http://bldg6.arsusda.gov/pberkum/Public/collection/index.htm) provides a searchable database of rhizobia grouped by legume host, and several culture collections that specialize in legume inoculants can be found at the WFCC-MIRCEN World Data Centre for Microorganisms (http://www.wfcc.nig.ac.jp/datacenter.html).

### Sampling

Sampling of plants and nodules should be done from undisturbed locations and, where possible, from healthy plants. Accurate site description and record keeping are essential. The number of nodules needed to be sampled can vary with the reason for collection. Where the aim is to identify inoculant-quality rhizobia, 15–20 nodules per plant, taken from the crown region of the host root system, are usually sufficient. In contrast, if the goal is to evaluate strain biodiversity in soil, a large number of nodules should be collected from as much of the root system as possible. Ease of collection may vary; stoloniferous species

may have nodules on adventitious roots within 1–2 cm of the surface (Date 1982), while nodules on tree species may be at a great depth in the soil at some distance from the trunk of the tree. Collected nodules should be protected in *Vacutainers* or in vials containing a desiccant (e.g., silica gel) overlain by cotton wool (Somasegaran and Hoben 1994).

## Isolation

Successful isolation of rhizobia from nodules depends on the quality of nodules recovered. When nodules have been stored dry over silica gel or $CaCl_2$, they must first be allowed to imbibe (sterile) water fully before being surface sterilized. Rhizobia also can be frequently recovered from nodules obtained from intact root system frozen at −20 °C. Sodium hypochlorite (3 %), hydrogen peroxide (3 %), and acidified mercuric chloride (0.1 %) are all effective surface sterilants. The former is usually preferred due to its low-cost, ready availability, and ease of disposal. Surface sterilization procedures are described in detail by Vincent (1970) and Somasegaran and Hoben (1994).

Yeast extract mannitol (YEM) medium is commonly used in the routine isolation and subculture of rhizobia. Many different formulations for this medium exist (Vincent 1970; Somasegaran and Hoben 1994). That used in our laboratory contains:

| | |
|---|---|
| Mannitol | 10.0 g |
| $MgSO_4 \cdot 7H_2O$ | 0.2 g |
| NaCl | 0.1 g |
| $K_2HPO_4$ | 0.5 g |
| $CaCl_2 \cdot 2H_2O$ | 0.2 g |
| $FeCl_3 \cdot 6H_2O$ | 0.01 g |
| Yeast extract | 1.0 g |
| Agar | 20.0 g |
| Distilled water | 1 l |
| pH | 6.7–7.0 |

Sterilize by autoclaving 20 min at 103 H $10_3$ Pa (15 lb/$in_2$) pressure.

Since many rhizobia produce copious quantities of extracellular polysaccharides on this medium, which makes colony purification difficult, many researchers prefer to use AG (Sadowsky et al. 1987b) and TY media (Beringer et al. 1978) for isolating slow- and fast-growing rhizobia from nodules.

The YEM medium may be amended with cycloheximide (20 µg/ml) to reduce fungal contamination and bromothymol blue (BTB; 25 µg/ml) or Congo red (25 µg/ml) to facilitate identification of rhizobia. These should be filter-sterilized separately and added to autoclaved, molten YEM medium before plates are poured (Vincent 1970). Tong and Sadowsky (1993) described a selective medium specific for *Bradyrhizobium*, based primarily on the heavy metal tolerance of these organisms. Media selective for fast-growing rhizobia have been described (Barber 1979; Louvrier et al. 1995), but they have not proven generally effective.

*Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Allorhizobium* strains will generally produce moist, gummy colonies on YEM medium that are 4–6 mm in diameter after 7 days incubation. On medium containing BTB, the colonies and surrounding medium are yellow due to acid production by the microorganisms. The slower-growing bradyrhizobia produce smaller colonies, usually only 1–2 mm diameter after 7–10 days incubation, which are raised and mucoid. The colonies and surrounding medium are blue in color on YEM containing BTB. Most nodule isolates will produce white or cream-colored colonies, though some isolates produce melanin (Cubo et al. 1988), or in the case of bradyrhizobia, a rust red pigmentation in older colonies. Since some rhizobia have recently been shown to be members of β-proteobacteria, care must be taken in making any assessment of taxonomic identity based on morphological criteria.

## Authentication

Authentication of rhizobia usually involves completion of Koch's postulates with the host from which strains were originally isolated. Somasegaran and Hoben (1994) provide details of this methodology. Inoculated seedlings produced from surface-sterilized seed of a suitable legume host are typically grown in sterile low N plant growth medium or on seedling agar in large test tubes, growth pouches, or in Leonard jars (Vincent 1970). Plants are examined for nodulation after 25–30 days of incubation under lights. The presence of nodules on uninoculated control plants invalidates the experiment.

# Identification

The identity of rhizobia or bradyrhizobia often requires a multiphasic approach using many of the techniques employed in naming new genera, species, and strains of rhizobia (Graham et al. 1991). Members of the International Subcommittee on *Rhizobium* and *Agrobacterium*, a subcommittee of the International Committee on Systematic Bacteriology of the International Union of Microbiological Societies, have recommended a minimal set of criteria for naming new species and genera of nodule bacteria (Graham et al. 1995). These criteria are also useful for identifying the genus and species status of unknown rhizobia isolated from nodules. In addition to biochemical, cultural, and symbiotic data, 16S rRNA (rDNA) sequencing (Young 1996; Young and Haukka 1996), DNA-DNA hybridization (Scholla and Elkan 1984), FAME (Graham et al. 1995), and multilocus enzyme electrophoresis (MLEE) (Strain et al. 1995) data are of primary importance for identifying rhizobia isolated from newly surveyed legumes.

Following strain authentication, it is often useful to genetically mark these isolates to facilitate identification in subsequent ecological, genetic, or plant studies. This can be done using a variety of techniques. These include intrinsic resistance to

a series of different antibiotics (Josey et al. 1979) or the selection of mutants resistant to high levels of antibiotics. In the latter case, selected mutants must be evaluated to show that the acquisition of antibiotic resistance has not influenced nodulation, $N_2$ fixation or competitive abilities.

Strains can also be identified using strain or group-specific antibodies (Sadowsky et al. 1987a; Schmidt et al. 1968). Antibodies, which are typically produced in rabbits to somatic whole-cell antigens, are useful in strain identification because they do not require genetic modification of strains. Agglutination, immunodiffusion, immunofluorescence, and ELISA techniques all have found wide acceptance in serological identification of rhizobia (Dudman 1977; Humphrey and Vincent 1965; Kishinevsky and Jones 1987; Schmidt et al. 1968). The fluorescent antibody technique is especially useful because it allows for the direct in situ examination of rhizobia in soil and nodules (Bohlool and Schmidt 1973), using direct or sandwich labeling procedures.

Strains also can be genetically modified with $\beta$-glucuronidase (GUS) (Wilson et al. 1995, 1999), green fluorescent protein (GFP) (Gage et al. 1996), and lux (Chabot et al. 1996) genes, and these strains have proven especially useful in ecological studies. Again, however, it is essential that such genetically marked strains be plant tested before use in ecological, symbiotic, or field studies.

DNA fingerprinting techniques have been used to identify and study biodiversity of rhizobial strains (Sadowsky 1994; Versalovic et al. 1998; Sadowsky and Hur 1998; Demezas 1998). Initially, DNA fingerprints of strains were generated following restriction enzyme digestion of total genomic DNA (Glynn et al. 1985; Demezas 1998). More recently, however, restriction fragment polymorphism (RFLP) analysis techniques, DNA hybridization probes, and DNA primers corresponding to repetitive elements, coupled to the polymerase chain reaction (PCR) technique, have been used in strain identification, competition, and biodiversity studies (de Bruijn 1992; Judd et al. 1993; Sadowsky 1994; Sadowsky et al. 1990; Wheatcroft and Watson 1988).

## Cultivation

Rhizobia are relatively robust, ubiquitous, aerobic bacteria with the ability to utilize many different substrates (carbon [C] and nitrogen [N] sources) for growth (Parke and Ornston 1984). Consequently, rhizobia can be cultivated on a large variety of complex and defined culture media. Only a limited number of rhizobia grow on highly enriched media, such as nutrient broth or LB medium. Medium used in the cultivation of rhizobia depends on the species of nodule bacteria, growth characteristics desired, and the method of cultivation. Most rhizobia are mesophiles and can grow in shake cultures at 25–30 °C. However, rhizobia isolated from legumes grown in the Canadian High Arctic grow well at 5 °C (Prévost et al. 1987), and high temperature tolerant strains have been isolated in Africa and Brazil.

As stated earlier, most rhizobia grow well in YEM medium (Vincent 1970), though most produce copious quantities of capsular- and exopolysaccharides in this medium, limiting its use in biochemical and genetic studies. The bradyrhizobia grow fairly slowly in this medium, with generation times greater than 6 h. Rhizobia and bradyrhizobia shift the pH of this medium; the rhizobia produce acid and the bradyrhizobia, alkaline by-products, from growth. Polysaccharide production can be drastically reduced in fast-growing rhizobia by cultivation in TY medium (Beringer et al. 1978), containing (g/l) tryptone (5.0), yeast extract (3.0), and $CaCl_2{\cdot}2H_2O$ (0.87), pH 6.9. In this medium, turbid cultures, up to $10^9$ cells $ml^{-1}$, can be obtained after overnight incubation at 28 °C. The slow-growing bradyrhizobia do not grow in TY medium. A growth medium useful for near polysaccharide-free growth by bradyrhizobia is AG medium (Sadowsky et al. 1987b). This medium, which promotes rapid growth of *B. japonicum* and *B. elkanii* strains, contains (g/l) HEPES (0.13), MES (0.11), $FeCl_3{\cdot}6H_2O$ (0.0067), $MgSO_4{\cdot}7H_2O$ (0.18), $CaCl_2{\cdot}2H_2O$ (0.013), $Na_2SO_4$ (0.25), $NH_4Cl$ (0.32), $Na_2HPO_4$ (0.125), arabinose (1.0), Na-gluconate (1.0), and yeast extract (1.0), pH 6.9. Several defined, minimal, media are also used for the growth of rhizobia (Vincent 1970; Somasegaran and Hoben 1994), especially for biochemical and molecular biological studies. We also use AG medium without arabinose, gluconate, and yeast extract as a minimal medium for the cultivation of prototrophic rhizobia and in genetic mating studies.

Rhizobia for legume inoculants can be grown in shake flasks or in fermentors (Somasegaran and Hoben 1994). Lorda and Balatii (1996) described a glycerol-based culture medium capable of producing approximately $10^{10}$ cells/ml, even in shake flask culture. In contrast, Stephens and Rask (2000) suggest that carbon-limited media be used to produce legume inoculants, to condition rhizobia to the less favorable conditions found in soil.

## Preservation

*Rhizobium* strains in frequent use are usually maintained on YMA, TY, or AG agar slants in screw-capped test tubes stored at 6–10 °C. Longer-term storage is achieved by lyophilization with 10 % glycerol or 10 % sucrose and 5 % peptone as cryoprotectants or by storage at −70 °C in 15 % glycerol. Gherna (1994) details methodologies for lyophilization and storage at −70 °C. Changes in *Rhizobium* characteristics, especially symbiosis, with repeated growth on laboratory media have been reported (Herridge and Roughley 1975) and must be of concern. For this reason, it is strongly suggested that repetitive subculturing or colony restreaking not be done for the fast-growing rhizobia. Since symbiosis-related genes are chromosome-borne in the slow-growing bradyrhizobia, subculturing does not appear to be a problem with this group. Some inoculant companies maintain large numbers of ampoules of each *Rhizobium* strain in the freeze-dried state *Rhizobium* and routinely replace all working cultures at 3-month to 1-year intervals.

### The Nodulation Process

The nodulation process requires molecular communication between both symbiotic partners and involves the induction and repression of a large number of bacterial and plant genes. Free-living rhizobia infect and form $N_2$-fixing symbioses with legumes in a series of discrete stages or steps. Stages in the process include proliferation of rhizobia in the rhizosphere, recognition of host by rhizobia, attachment of rhizobia to susceptible root-hair cells, root-hair curling and infection-thread formation, initiation of nodule primordium, and transformation of free-living rhizobia into $N_2$-fixing bacteroids.

Rhizobia infect their respective host plants and induce root or stem nodules using several different mechanisms. Infection through root hairs is commonly seen with most legumes (Hadri et al. 1998). Rhizobia can also invade the host plant by entry through wounds, cracks, or lesions caused by emergence of secondary roots (Boogerd and van Rossum 1997), as occurs in peanut and *Stylosanthes*. In these cases, rhizobia spread intercellularly. There are instances where the same rhizobia infect one legume through root hairs and another via cracks or wounds (Sen and Weaver 1988). Lastly, rhizobia may initiate infection of the host via cavities surrounding adventitious root primordia on the stems of *Sesbania*, *Aeschynomene*, *Neptunia*, and *Discolobium* (Boivin et al. 1997; Giraud et al. 2007). As above, one bacterium may produce both stem and root nodules on different legume plants.

In root-hair infection, rhizobia attach to susceptible root hairs within minutes of inoculation or contact with the host plant. Rhizobial cells often attach perpendicular to the root-hair cell. It has been suggested that adhesion is initially mediated by the calcium (Ca)-binding protein rhicadhesin, or by plant lectins, and subsequent bonding via production of cellulose fibrils (Kijne 1992). It is hypothesized that rhizobia produce localized hydrolysis of the root-hair cell wall. Subsequent penetration of rhizobia through the cell wall leads to root-hair curling, which may be visible 6–18 h after inoculation. The proportion of root hairs infected is low, the percentage of these giving rise to nodules is low and highly variable, and aborted root hairs can frequently be found.

Within the root hair, rhizobia are enclosed within a plant-derived infection thread and move down the root hair in the direction of the root cortex (Jones et al. 2007). Cell division in the root cortex, in advance of the approaching infection thread, leads to the production of nodule primordia (Kijne 1992). Spread of the infection thread among cells of the nodule primordium follows, with the release of rhizobia into host cortex by an endocytotic process. Rhizobia are never free in the cytoplasm but rather are surrounded by a host-derived peribacteroid membrane, which serves to compartmentalize the rhizobia into a symbiosome. One to several rhizobia can be confined to a single symbiosome. Nodulation is usually visible 6–18 days after inoculation, but this varies considerably with the selection of bacterial strain and host cultivar, the inoculant density and placement, and the temperature. Initially, nodulation is heaviest in the crown of the root, with secondary nodules appearing on lateral roots as the first-formed nodules senesce. The number of nodules produced on each legume host is tightly controlled by the host and rhizobial genotype, the efficiency of the symbiotic interaction, and by environmental factors such as soil N level and the presence of existing nodules (Caetano-Annoles 1997; Sagan and Gresshoff 1996; Singleton and Stockinger 1983).

Nodule shape in legumes is determined by the host plant and is regulated by the pattern of cortical cell divisions. There are two basic types of nodules that are formed on legumes: determinant and indeterminant (Franssen et al. 1992). Indeterminant nodules are most commonly formed in symbioses between the fast-growing root-nodule bacteria, such as members of the genera *Ensifer* or *Rhizobium*, and temperate legumes (pea, clover, and alfalfa). In contrast, determinate nodules are typically induced by the bradyrhizobia and some rhizobial species strains and are more common on tropical legumes, such as soybean and bean. Morphologically, indeterminate nodules have defined, persistent apical meristems and are elongated and sometimes lobed, whereas determinant nodules do not have persistent meristems and are usually round (Hadri et al. 1998).

## Genetics

Understanding the genetics of the rhizobia has, in most cases, centered on the genetics of nodulation and symbiotic $N_2$-fixation, key characters that set the rhizobia apart from other soil bacteria. Recent advances in molecular biology and genetics have elucidated a large number of genes with symbiotic functions. Though many of these genes are clustered together (on the chromosome in some organisms and on symbiotic plasmids in others), additional genes may be dispersed or located on different replicons. Consequently, all symbiotically related genes will most likely not be found until total sequencing and functional genomic efforts are completed. Because the scope of this chapter is broad, more detailed information on the genetics of nodulation and $N_2$ fixation can be found in several reviews (Boivin et al. 1997; Denarie et al. 1996; Jones et al. 2007; Niner and Hirsch 1998; Pueppke 1996; Spaink 1995; Schultze and Kondorosi 1998; van der Drift et al. 1998).

### Nodulation Genes

In the last several years, a large number of bacterial genes have been identified which are involved in the formation of nodules on leguminous plants. Collectively, more than 65 nodulation genes have been identified in rhizobia, although each strain may only have a subset of these. Niner and Hirsch (1998), Pueppke (1996), and Bladergroen and Spaink (1998) provide a more complete description of the function of a majority of these genes.

Several studies have shown that relatively few genes are required for nodulation of legumes (Göttfert 1993; Long et al.

1985; Long 1989; van Rhijn and Vanderleyden 1995). In the case of the fast-growing rhizobia, a majority of nodulation genes are located on large, indigenous, symbiotic (Sym), and often self-transmissible, plasmids (Broughton et al. 1984; Hombrecher et al. 1981; Kondorosi et al. 1989). The complete genomic sequence of the symbiotic plasmid from *Rhizobium* sp. strain NGR234, a *Rhizobium* strain with broad nodulation ability (Pueppke and Broughton 1999), is currently available. In *E. meliloti*, the symbiont of alfalfa, nodulation genes (located on an 8.5 kb fragment of the Sym plasmid) contain sequences necessary for the nodulation of a wide variety of legume hosts (Kondorosi et al. 1989; Truchet et al. 1991). These genes, referred to as "common nodulation" genes and designated *nodA*, *nodB*, and *nodC*, have homologues in other fast- and slow-growing species. The common nodulation genes are involved in biosynthesis of the chitin backbone of Nod factor (see below) and organized in a similar cluster in most rhizobia (Long et al. 1985; Long 1989; van Rhijn and Vanderleyden 1995). A fourth gene, *nodD*, is regulatory and together with plant flavonoid signals (see below) activates transcription of other inducible *nod* genes (Long 1989; Martinez et al. 1990; van Brussel et al. 1990). *R. leguminosarum* bv. *viceae* and *trifolii* have single copies of *nodD*. The symbionts *E. meliloti, B. japonicum*, and *M. loti* have multiple copies of *nodD* (Göttfert et al. 1986, 1990; Honma and Ausubel 1987; Kaneko et al. 2000). In some instances, *nodD* also appears to impart host-specificity functions (Spaink et al. 1987). Another nodulation gene cluster, originally designated *hsn* (for host-specific nodulation), is closely linked to the common nodulation region in *E. meliloti* and controls nodulation of specific legume genera (Bachem et al. 1986; Horvath et al. 1986). Mutations in the *hsn* genes (designated *nodFEGH*) cannot be complemented with Sym plasmids from other species of *Rhizobium*. Analogous *hsn* genes also have been isolated from *R. leguminosarum* bv. *trifolii* (Djordjevic et al. 1985; Rolfe et al. 1985), *leguminosarum* bv. *viceae* (Wijffelman et al. 1985), and from *Rhizobium* strain MPIK3030 (Bachem et al. 1986; Bassam et al. 1986; Broughton et al. 1984; Lewin et al. 1987). An *hsn* gene linked to the common nodulation region in *B. japonicum* strain USDA 110 was also reported (Nieuwkoop et al. 1987). This sequence, subsequently called *nodZ* (Dockendorff et al. 1994), was shown to be involved in the host-specific nodulation of siratro, but not soybean. Hahn and Hennecke (1988) and Göttfert et al. (1990) have identified another *hsn* locus in *B. japonicum* strain USDA 110, *nodVW*, which is essential for the nodulation of siratro, mungbean, and cowpea, but not soybean. In B. japonicum strain USDA 110, the essential nodulation genes are located on the chromosome in several transcriptional units in the order: *nolZ*, *nolA*, $nodD_2$, $nodD_1$, *nodYABCSUIJmolMNO* (Dockendorff et al. 1994). Unlike other rhizobial *nodD* genes, the *B. japonicum* $nodD_1$ is induced by the flavonoids genistein and daidzein (Banfalvi et al. 1988; Kosslak et al. 1987) and by xanthones (Zaat et al. 1987).

Intriguingly, *Bradyrhizobium* strains BTAi1 and ORS278, which induce nodules on both the root and stem of the aquatic legume *Aeschynomene*, do not possess functional *nodABC* genes (Giraud et al. 2007). This indicates that canonical *nodABC* genes and Nod factor are not required for symbiosis for these strains and implies that an alternative nodulation strategy exists in the *Bradyrhizobium-Aeschynomene* symbiotic interaction (Giraud et al. 2007; Bonaldi et al. 2010).

## Genotype-Specific Nodulation Genes

Although many *hsn* genes have been identified in *Rhizobium* and *Bradyrhizobium*, there are only limited reports on the identification of genotype-specific nodulation (GSN) genes in the rhizobia (Sadowsky et al. 1991). The GSN genes specifically refer to those bacterial genes that allow nodulation of specific plant genotypes within a given legume species. For example, strain TOM nodulates the pea genotype *Pisum sativum* cv. *Afghanistan* (Lie 1978; Lie et al. 1978), but European *R. leguminosarum* bv. *viceae* strains fail to nodulate this host. Some GSN-like genes have been found on plasmid pRL5JI of strain TOM (Gotz et al. 1985; Hombrecher et al. 1984). Davis et al. (1988) have identified a single gene on this plasmid, *nodX*, mediating the O-acetylation of Nod factors (Firmin et al. 1993), which is necessary for the nodulation of "Afghanistan" peas. In *E. fredii* strain USDA 257, two other GSN-like loci, *nolC* (Krishnan and Pueppke 1991) and *nolBTUVW* (Meinhardt et al. 1993), allow this strain to nodulate primitive lines of soybean, but not improved soybean varieties, such as "McCall" (Heron et al. 1989). In each case, Tn5 insertions in the gene regions allow *E. fredii* to nodulate commercial soybean cultivars. Phenotypically, these regions are similar to that reported by Djordjevic et al. (1985) and Innes et al. (1985) for clover rhizobia. More recently, however, Lewis-Henderson and Djordjevic (1991a) reported that *nodM* in *R. leguminosarum* bv. *trifolii* is a GSN which prevents effective nodulation of subterraneum cv. Woogenellup (Lewis-Henderson and Djordjevic 1991b). Analysis of *R. leguminosarum* bv. *trifolii* strain TA1 demonstrated that this strain also lacks *nodT* and that introduction of *nodT* from *R. leguminosarum* bv. *viceae* strain ANU843 into TA1 allows effective nodulation of "Woogenellup" (Lewis-Henderson and Djordjevic 1991a). The GSN genes can act in either a positive or negative manner (Djordjevic et al. 1987a; Sadowsky et al. 1990), insertions in a negatively acting nodulation gene extending host range and insertions in a positively acting GSN gene limiting host range. *B. japonicum* serogroup 123 strains are restricted for nodulation by PI 377578 (Cregan and Keyser 1986). The *B. japonicum nolA* gene, identified in strain USDA 110, is a positively acting gene that allows serogroup 123 strains to nodulate PI 377578 (Sadowsky et al. 1991). We recently identified a mutant of *B. japonicum* strain USDA 110 that has the ability to overcome nodulation restriction conditioned by soybean PI 417566 (Lohrke et al. 1995). More recently, the soybean genes Rj2 and Rfg1, responsible for restricting nodulation by specific strains of *B. japonicum* and *E. fredii*, respectively, were positionally cloned and found to be members of the Toll-interleukin receptor/nucleotide-binding site/leucine-rich repeat class of plant resistance proteins (Yang et al. 2010).

## Signal Exchange and Induction of Nod Genes

Although the regulation of nodulation genes in rhizobia is still not fully understood, we know a lot about communication between rhizobia and susceptible legume hosts. Flavonoid signal molecules present in root and seed exudates are necessary for *nod* gene expression (Banfalvi et al. 1988; Boundy-Mills et al. 1994; Djordjevic et al. 1987b; Fellay et al. 1995; Göttfert et al. 1988; Innes et al. 1985; Kosslak et al. 1987; Long 1989; Mulligan and Long 1985; Olson et al. 1985; Peters et al. 1986; Price et al. 1992; Sadowsky et al. 1988; van Brussel et al. 1990; Zaat et al. 1987). Other Sym plasmid-borne genes are also induced by root exudates in *E. fredii* and *Rhizobium* sp. strain NGR234 (Boundy-Mills et al. 1994; Fellay et al. 1995; Olson et al. 1985; Sadowsky et al. 1988). Flavones, isoflavones, flavanols, flavanones, and closely related compounds have been identified as *nod* gene inducers, and each is specific for a particular legume-*Rhizobium* interaction (Schlaman et al. 1998). Flavonoid compounds are only one of several determinants of host specificity. Spaink et al. (1991) reported differential induction of *nodD* in various fast-growing rhizobia by a range of flavonoids and exudates. Induction of nodulation genes requires the regulatory *nodD* gene product (Long 1989; Mulligan and Long 1985; Shearman et al. 1986). The inducer apparently binds NodD, causing a change in conformation (Kondorosi et al. 1988; Fisher and Long 1989). Activated NodD then binds to a regulatory, promoter-like sequence, found upstream of rhizobial *nod* genes, the Nod box (Hong et al. 1987; Horvath et al. 1987; Kondorosi et al. 1988; Rostas et al. 1986; Shearman et al. 1986). Repressor proteins have also been suggested to play a role in *nod* gene regulation (Kondorosi et al. 1988), a repressor encoded by the *nolR* gene has been identified in *E. meliloti* strain 41 (Kondorosi et al. 1989, 1991), and a repressor encoded by the *nolA* and *nodD*$_2$ has been identified in *B. japonicum* strain USDA 110 (Sadowsky et al. 1991; Göttfert et al. 1992; Loh and Stacey 2003). This section has recently been reviewed by Downie (2010) and the reader is directed here.

## Extracellular Nodulation Factors

One of the primary functions of *nod* genes is the production of extracellular lipochitooligosaccharide (LCO) molecules, also known as Nod factors (Carlson et al. 1993, 1994). These molecules, acting at $10^{-8}$ to $10^{-9}$ M, can (1) stimulate the plant to produce more *nod* gene inducers (van Brussel et al. 1990), (2) deform root hairs on homologous hosts (Banfalvi and Kondorosi 1989; Faucher et al. 1989), and (3) initiate cell division in the root cortex (Lerouge et al. 1990; Price et al. 1992; Sanjuan et al. 1992; Schultze et al. 1992; Relic et al. 1993; Spaink et al. 1991). In *E. meliloti*, these signal molecules are acetylated and sulfated glucosamine oligosaccharides (Lerouge et al. 1990). Similar molecules have been identified in other legume symbiotic systems (Pueppke (1996) and Downie (1998, 2010) for a review). Numerous observations support the theory that *hsn* genes control host specificity by decorating Nod factors with various substituents. For example, the *E. meliloti* genes *nodP*, *nodQ*, and *nodH* are involved in the sulfation of the Nod factor reducing sugar (Faucher et al. 1989; Roche et al. 1991). Disruption of any of these genes affects host specificity. *Rhizobium* spp. NGR234, which can nodulate over 125 different legume species (Pueppke and Broughton 1999), produce diverse (more than 18) Nod factors, which vary in the substituents attached to a similar backbone structure (Price et al. 1992).

Purified Nod factors, which are structurally similar to those produced by the appropriate rhizobial symbiont, can induce nodules on the specific host plant in the absence of a bacterium (Downie 1998; Mergaert et al. 1993; Relic et al. 1993; Schultze et al. 1992; Truchet et al. 1991). Nod factors from several strains of *B. japonicum* have been characterized (Carlson et al. 1993; Sanjuan et al. 1992). The functions of *nod* genes and the basic structure of Nod factors for *B. japonicum* and several species of the genus *Rhizobium* can be found in Downie (1998), and recent review can be found in Murray (2011).

## Nitrogen Fixation Genes

Nitrogen fixation is the natural process, either biological or abiotic, by which nitrogen gas ($N_2$) in the atmosphere is converted into ammonium ($NH_4$). Only bacteria containing the enzyme nitrogenase can reduce $N_2$ to ammonium. This is the only known enzyme that can carry out this energetically unfavorable reaction. As described above, legume plants enter into a symbiotic interaction with nitrogen-fixing rhizobia resulting in the formation of root or stem nodules. Nitrogenase is very sensitive to inactivation by oxygen, and within the nodules, leghemoglobin maintains a low-oxygen concentration to protect enzyme function (nanomolar range) (Downie 2005).

Two major types of $N_2$ fixation genes have been described, *nif* genes and *fix* genes. The *nif* refer to genes involved in the $N_2$-fixation process and have structurally and functionally related genes in the free-living diazotrophic microorganism, *Klebsiella pneumoniae*. *K. pneumoniae* was the first $N_2$-fixing microorganism studied in detail (Kennedy 1989). As with the nodulation genes, a majority of the *nif* genes are plasmid borne and contiguous in the rhizobia. In contrast, the *nif* genes are chromosomally located in the bradyrhizobia. The $N_2$-fixation process is catalyzed by the enzyme complex nitrogenase, encoded by the *nifDK* and *nifH* genes. The *fix* genes are also involved in the $N_2$-fixation process but have no similar structural or functional homologues in *K. pneumoniae*. The organization of *nif* genes varies in the rhizobia (Kaminski et al. 1998).

Nitrogenase consists of two protein subunits, a molybdenum-iron (MoFe) protein and an iron-containing (Fe) protein. These structural components of the nitrogenase enzyme complex are often referred to as subunits I and II, respectively. The *nifK* and *nifD* genes encode the MoFe protein subunits. A FeMo cofactor (FeMo-Co) is required for activation of the MoFe protein and is assembled from the *nifB*, *nifV*, *nifN*, and *nifE* genes. The *nifH* gene encodes the Fe subunit protein. In *K. pneumoniae*, there are at least 20 *nif*-specific genes that are

**Table 17.3**
**Other bacterial genes involved in symbiotic nitrogen fixation**

| Gene designation | Phenotype or function | References |
|---|---|---|
| *exo* | Exopolysaccharide | Becker and Puhler (1998), Glazebrook and Walker (1989) |
| *hup* | Hydrogen uptake | Maier (1986) |
| *gln* | Glutamine synthase | Carlson et al. (1987) |
| *dct* | Dicarboxylate transport | Finan et al. (1983), Jiang et al. (1989) |
| *nfe* | Nodulation efficiency | Sanjuan and Olivares (1989) |
| *ndv* | *β*-1,2 Glucans | Breedveld and Miller (1998) |
| *lps* | Lipopolysaccharide | Carlson et al. (1987) |
| *bacA* | Bacteroid development | Glazebrook et al. (1993) |
| *tts* | Type III secretion system | Krause et al. (2002) |
| *virB* | Type IV secretion system | Hubber et al. (2004) |
| *acdS, rtx* | Inhibition of plant ethylene biosynthesis | Ma et al. (2003), Duodu et al. (1999) |
| *pur* | Purine biosynthesis | Noel et al. (1988), Giraud et al. (2007) |

localized in about 8 operons (Dean and Jacobson 1992). Though the organization of *nif* genes in other organisms varies tremendously (Downie 1998), *nifHD* and *nifK* are conserved in disparate $N_2$-fixing organisms and rhizobia (Ruvkin and Ausubel 1980). The gene products NifA and NifL control the regulation of all other *nif* genes. Whereas NifA is positive activator of transcription of *nif* operons, NifL is involved in negative control. In *K. pneumoniae* and several other free-living diazotrophic microbes, *nif* gene expression is regulated by oxygen and nitrogen levels (Merrick 1992). Ammonia ($NH_3$) causes NifL to act as a negative control and prevents the activator function of NifA. This has been referred to as the N control system and has been shown to regulate several enzymes that are capable of producing $NH_3$. Merrick (1992) and Dean and Jacobson (1992) give excellent in-depth reviews of the structure and regulation of $N_2$ fixation in free-living and symbiotic bacteria.

### Other Genes Involved in Symbiotic Nitrogen Fixation

Other plasmid- and chromosomally borne bacterial genes also have been found to function indirectly in nodulation and symbiotic $N_2$-fixation in rhizobia (*Table 17.3*). Recent review articles on the structure and function of these and other symbiosis-related genes are provided by Pueppke (1996), Spaink (1995), Long et al. (1999), Sugawara et al. (2006), and Kobayashi and Broughton (2008).

## Genomics

Several approaches have been used to define and study the involvement of whole bacterial genomes in the symbiotic process. As of this date, about 15 rhizobial genome sequences have been completed and are currently available online (*Table 17.4*), and approximately 150 more are currently being sequenced. In most of the genome-sequenced rhizobia, the genes involved in nodulation and nitrogen-fixation genes are clustered on large and often self-transmissible, megaplasmids (pSyms) or are located within large genomic islands, referred to as symbiotic islands (SIs). These features in many ways emphasize the accessory nature of the symbiosis-related genes and their ability to be acquired by microorganisms via horizontal gene transfer (MacLean et al. 2007).

A SI present in *Mesorhizobium loti* strain ICMP3153 was found capable of transforming nonsymbiotic strains of *M. loti* into symbiotic counterparts (Sullivan and Ronson 1998). Large symbiotic islands of 611 and 681 kb have been found in *M. loti* MAFF303099 and *B. japonicum* strain USDA 110, respectively (Kaneko et al. 2000, 2002). Although the widespread transmissibility of these islands has yet to be confirmed, the integration of symbiotic islands into the *M. loti* and *B. japonicum* genomes occurs within phe-tRNA and val-tRNA genes, respectively (Kaneko et al. 2000, 2002). The association of symbiotic islands, in both rhizobial species, with a phage-related integrase implies the symbiotic islands may have originated from the ancient integration of a bacteriophage. Beside the SI, 14 smaller genomic islands, with lower GC content, were also found in the genome of *B. japonicum* USDA 110 (Kaneko et al. 2002). Since some of these genomic islands were found to be missing in several strains of *B. japonicum*, the 14 genomic islands were thought to be likely inserted into the ancestor genome of USDA 110 via horizontal gene transfer events (Itakura et al. 2009). In contrast to what has been found in the slower-growing bradyrhizobia, most of the genes involved in symbioses in genome-sequenced *Ensifer* and *Rhizobium* strains are located on symbiotic plasmids, pSyms. In many cases, the pSyms have been shown to be transferred among bacteria via conjugation (Rao et al. 1994; Freiberg et al. 1997; Brom et al. 2004; Pérez-Mendoza et al. 2004).

**Table 17.4**
**Architecture of sequenced rhizobial genomes**

| Rhizobial strain | No. replicons | Total size (bp) | Genbank accession no. | References |
|---|---|---|---|---|
| **Alpha-proteobacteria** | | | | |
| *A. caulinodans* ORS571 | 1 | 5,369,772 | AP009384 | Lee et al. (2008) |
| *B. japonicum* USDA 110 | 1 | 9,105,828 | BA000040 | Kaneko et al. (2002) |
| *Bradyrhizobium* sp. BTAi1 | 2 | 8,493,513 | CP000494, CP000495 | Giraud et al. (2007) |
| *Bradyrhizobium* sp. ORS278 | 1 | 7,456,587 | CU234118 | Giraud et al. (2007) |
| *Ensifer* (*Sinorhizobium*) *medicae* WSM419 | 4 | 6,817,576 | CP000738–CP000741 | Reeve et al. (2010) |
| *Ensifer* (*Sinorhizobium*) *meliloti* 1021 | 3 | 6,691,694 | AL591688, AL591985, AE006469 | Galibert et al. (2001) |
| *Mesorhizobium loti* MAFF303099 | 3 | 7,596,297 | AP002994–AP003017 | Kaneko et al. (2000) |
| *Methylobacterium nodulans* ORS2060 | 8 | 8,839,022 | CP001349–CP001356 | Unpublished |
| *Rhizobium etli* CFN42 | 7 | 6,530,228 | CP000133–CP000138 | González et al. (2006) |
| *Rhizobium etli* CIAT652 | 4 | 6,448,048 | CP001074–CP001077 | Unpublished |
| *Rhizobium leguminosarum* bv. *trifolii* WSM1325 | 6 | 7,418,122 | CP001622–CP001627 | Unpublished |
| *Rhizobium leguminosarum* bv. *trifolii* WSM2304 | 5 | 6,872,702 | CP001191–CP001195 | Unpublished |
| *Rhizobium leguminosarum* bv. *viciae* 3841 | 7 | 7,751,309 | AM236080–AM236086 | Young et al. (2006) |
| *Rhizobium* sp. NGR234 | 3 | 6,891,900 | CP000874, CP001389, U00090 | Schmeisser et al. (2009) |
| **Beta-proteobacteria** | | | | |
| *Burkholderia phymatum* STM815 | 4 | 8,676,562 | CP001043–CP001046 | Unpublished |
| *Cupriavidus* (*Ralstonia*) *taiwanensis* LMG19424 | 3 | 6,476,522 | CU633749–CU633751 | Amadou et al. (2008) |

## Ecology

Rhizobia are relatively unique among the majority of soil microorganisms in that they have an extensive soil phase as free-living, saprophytic, heterotrophic microorganisms, yet they have the ability to form species-specific, $N_2$-fixing symbiotic associations with legumes. The ability to form $N_2$-fixing nodules affords unique opportunities for the rhizobia. When a legume crop is grown in soil for the first time, few rhizobia are likely to be present, and in most instances, inoculation will most likely be needed for adequate nodulation and subsequent $N_2$ fixation (Date 1991; Diatloff 1977). In contrast, soils surrounding legumes that have been planted for several years usually contain relatively large numbers of rhizobia and do not require added strains. Numerous studies have documented that legume inoculants added to soils containing relatively small populations of rhizobia usually give rise to only a small percentage of the nodules formed (Thies et al. 1991; Date 1991; Ellis et al. 1984; Ham 1978). Despite intensive investigations over the last 30+ years, however, some of the factors that influence the survival and the persistence of rhizobia in the soil, and their ecology and competitiveness for nodulation sites on the host, are only now beginning to be understood. It is beyond the scope of this chapter to present all that is known about the ecology and soil biology of the rhizobia. The reader is directed to more extensive reviews by Bottomley (1992), McInnes et al. (2004) and Sadowsky and Graham (1998) on this material.

The establishment of the symbiotic state results in the production of nodule populations of more than $10^{10}$ rhizobia $g^{-1}$ nodule tissue (McDermott et al. 1987). When these nodules senesce at the end of the growing season, large numbers of rhizobia are released into the soil. Nodule bacteroids are subject to changes in surface chemistry (Roest et al. 1995) and are susceptible to osmotic and other soil stresses (Sutton 1983). However, many of the released organisms manage to persist as free-living, heterotrophic, saprophytes in the soil until a susceptible legume is again planted. As a consequence of this, most soils contain at least some rhizobia, and a dramatic buildup in their numbers occurs when a leguminous host is included in a crop rotation, pasture, or natural setting.

Ellis et al. (1984) reported soil populations of bradyrhizobia approaching $10^6$ cells $g^{-1}$ in soils of the American Midwest following cultivation of soybean, and rhizosphere populations can reach $10^8$ cells $g^{-1}$ (Bottomley 1992). The distribution of rhizobia in soil is not uniform. Postma et al. (1990) reported that the greatest numbers of rhizobia are associated with soil aggregates of larger than 50 µm, and Mendes and Bottomley (1998) noted that the percentage of *Rhizobium* recovered from aggregates of different sizes varied over the course of a growing season.

Rhizobia are excellent soil saprophytes and can persist for many years in the absence of their host (Brunel et al. 1988; Kucey and Hynes 1989; Bottomley 1992). Chatel et al. (1968) used the term saprophytic competence to describe this ability, but the factors involved have yet to be determined. Even though Bushby (1990) noted surface electrophoretic charge in bradyrhizobia correlated to the pH of soils from which they came, Rynne et al. (1994) found no correlation between catabolic ability and

strain persistence. More recently, Ratcliff et al. (2008) suggested that there is a relationship between strain survival and PHB accumulation in rhizobia. Inoculant strains used at the time a particular host was introduced may still occupy a large percentage of the nodules formed on that host 10–15 years after their introduction (Diatloff 1977; Brunel et al. 1988; Lindstrom et al. 1990). However, many studies have shown that inoculant strains may also decline in nodule representation over time or quite quickly disappear from soil.

The growth of rhizobia in the rhizosphere may also be stimulated by specific root exudates (Van Egaraat 1975). Rhizobia, in turn, also stimulate growth and respiration of leguminous plants (Phillips et al. 1999). Several research studies have sought to create biased rhizospheres, in which plants transformed to synthesize opines and favor the growth of rhizosphere bacteria utilizing this substrate (Rossbach et al. 1994; Oger et al. 1997; Savka and Farrand 1997). The inability of strains to compete for nodulation sites on the host legume does not necessarily mean their displacement from the soil population. Bromfield et al. (1995) compared populations of *E. meliloti* recovered from soil and nodules and found significant differences in the frequency with which particular genotypes were recovered. Similarly, Segovia et al. (1991) found the population of noninfective bean rhizobia in soil numerically superior to those capable of inducing nodule formation.

Environmental factors, particularly soil pH, temperature, and water availability, often affect rhizobial survival in soil and the balance between particular genotypes. In soils of pH >7.0, Brockwell et al. (1991) found an average of 89,000 *E. meliloti* $g^{-1}$ soil, whereas in soils of pH <6.0, the number was only 37 $g^{-1}$. Even more striking is the replacement of the normal bean microsymbiont etli by the acid-tolerant tropici to which beans were introduced (Anyango et al. 1995; Hungria et al. 1997). This change occurred in the relatively short time since Spanish and Portuguese colonization of Latin America and despite the fact that tropici is less competitive than etli in nodule formation with beans (Martinez-Romero and Rosenblueth 1990; Chaverra and Graham 1992). In contrast, Richardson and Simpson (1989) found that many rhizobia from acid soils are sensitive to acidity, suggesting that microniches in soils protect these microorganisms from extremes of soil pH.

Temperature also has a marked influence on survival and persistence of rhizobial strains. Eaglesham et al. (1981) found cowpea rhizobia from the hot, dry Sahelian savannah of West Africa to be temperature tolerant, with good growth at 37 °C. More than 90 % of the strains isolated from this region grew well to 40 °C, whereas rhizobia from the more humid Onne region of West Africa generally failed to grow at this temperature. Soil temperature might contribute to the number of noninfective rhizobia found in some soils (Segovia et al. 1991). Temperature effects appear to be both strain and soil dependent. Marshall (1964) studied a clover nodulation problem in which autumn-sown plants nodulated well but frequently failed to do so in subsequent regrowth. He found that *Bradyrhizobium* sp. (*Lupinus*) was less susceptible than *R. leguminosarum bv. trifolii* to high soil temperatures but also noted amelioration of this problem in sandy soils amended with montmorillonite and illite. Along with temperature, soil water stress can have a profound influence on the survival of rhizobia in soil, affecting the induction and repression of a large number of bacterial genes (Cytryn et al. 2007).

Although rhizobia comprise only 0.1–8.0 % of the total bacterial population in soil and 0.01–0.14 % of its biomass (Bottomley 1992; Schortemeyer et al. 1997), their biodiversity in soil and the factors which can affect it have been extensively studied. The development of improved techniques for strain fingerprinting, particularly restriction fragment length polymorphism (RFLP) and PCR analyses (de Bruijn 1992; Judd et al. 1993; Dye et al. 1995; Madrzak et al. 1995; Richardson et al. 1995; Brunel et al. 1996; Labes et al. 1996; Paffetti et al. 1996; Rome et al. 1996; Sadowsky and Hur 1998), multilocus enzyme electrophoresis (MEE) (Pinero et al. 1988; Eardly et al. 1990; Demezas et al. 1991, 1995; Bottomley et al. 1994; Dupuy et al. 1994; Souza et al. 1994; Strain et al. 1994, 1995), and SDS-PAGE analysis of total cell proteins (Roberts et al. 1980; Dupuy et al. 1994), has been used extensively to study strain biodiversity.

Population biodiversity among the rhizobia for a particular legume species tends to be greatest near the center of origin/domestication of that legume (Lie et al. 1987). Pinero et al. (1988) recorded a mean genetic distance per enzyme locus of 0.691 for 51 isolates of etli from the Mesoamerican center of origin for *Phaseolus vulgaris* (L.), while Souza et al. (1994) grouped 372 bean rhizobia into seven clusters comprising 95 electrophoretic types. In the later study, rhizobia isolated from wild bean populations grouped by location and *Phaseolus* species, whereas those from cultivated beans were very heterogeneous. An emerging consideration in data such as this is the promiscuity of *Phaseolus vulgaris*. This host is nodulated by at least five different species of rhizobia (Michiels et al. 1998), necessitating care in distinguishing between intra- and extraspecific diversity. At the other extreme, the movement of rhizobia as seed-borne contaminants (Perez-Ramirez et al. 1998) can give the impression of limited biodiversity, more properly identified as founder effects (Hagen and Hamrick 1996). The environmental stresses noted above also can have profound effects on the biodiversity of rhizobia in soil. In an extreme case (Hirsch 1996), application of manure containing heavy metals reduced the biodiversity of *R. leguminosarum* bv. *trifolii* in soil to a single biotype. Caballero-Mellado and Martinez-Romero (1999) also reported fertilizer effects on strain biodiversity in soil.

## Applications

It is unlikely that soils contain appropriate rhizobia when a legume species is planted in a new area for the first time. In these cases, inoculation is usually required for adequate nodulation and $N_2$ fixation. Yield increases following inoculation with appropriate inoculant-quality rhizobia can exceed 50 %, with clear differences between inoculated and uninoculated plants as shown in ❯ *Fig. 17.3*.

**Fig. 17.3**
**Response of soybean to inoculation in newly cultivated areas of Puerto Rico. The yellow-green plants in the center of the picture were not inoculated with *Rhizobium* (Courtesy of R. Stewart Smith)**


**Fig. 17.4**
**PulseR™: A presterilized-peat-based inoculant for soybean (Photograph: H. Mc Ives, Agribiotics)**

Where plants are not inoculated with rhizobia in the first year of introduction into a new area, nodulation will most likely be limited to that coming from seed-borne or aerial contaminants. It is common that these rhizobia are less than fully effective in symbiosis with their host (Guar and Lowther 1980). Over several years of cultivation, these rhizobia will increase in numbers in the soil, limiting subsequent inoculation response. It is important, therefore, that inoculation with an inoculant-quality organism lead to early establishment and persistence of effective rhizobia in the soil, ensuring long-term benefits. Where this is done, the original inoculant strain(s) may still dominate in nodulation 10–15 years later (Brunel et al. 1988; Kucey and Hynes 1989; Bottomley 1992). In Thailand, 80 % of farmers in new areas of soybean production are willing to inoculate, but only 30 % of farmers in older established areas do, and in many cases, the latter apply fertilizer N (Hall and Clark 1995).

Inoculation of legumes with suitable rhizobia has been practiced for more than 100 years. Initially, this involved the collection of soil from areas where particular legumes had established successfully and the mixing of soil and seed prior to planting. Inoculation with pure cultures of rhizobia followed, and today the provision of rhizobial inoculants to farmers, home gardeners, and others is a multimillion dollar industry with approximately 4 t peat culture sold annually in the United States alone (Burton 1980). More than 80 % of this is for two crops, soybean and alfalfa. In Brazil where there was an early commitment to $N_2$ fixation as the principal source of plant N, Dobereiner et al. (1995) estimate the benefits of symbiotic and associative symbioses at more than $1.8 billion each year. Most inoculant preparations are peat based (Smith 1992; Brockwell and Bottomley 1995), but frozen, granular, liquid, and other preparations also are used. Applied as recommended, these preparations will supply between $10^9$ and $10^{13}$ rhizobia $ha^{-1}$, equivalent to $10^3$–$10^6$ rhizobia $seed^{-1}$ (Lupwayi et al. 2000) (*Fig. 17.4*).

Many factors have to be considered if inoculation is to function properly. Most important in legume inoculation is use of the correct rhizobia. Specificity in nodule formation between host and rhizobia has been referred to already; specificities between host and *Rhizobium* in terms of $N_2$ fixation also exist (Burton 1967) and impact inoculant strain selection. EMD Crop Bioscience (Milwaukee, WI), formerly the Nitragin Company, provides more than 100 different strain preparations for legume inoculation (Smith 1988). This company now produces legume inoculants supplemented with LCOs (Optimize®) to enhance nodulation. Other legume inoculant companies also produce products with chemical additives to enhance nodulation.

Consistent quality in the inoculant material is also essential but can be surprisingly variable. A number of countries regulate inoculant quality. Thus, for example, inoculants sold in Canada, Australia, Uruguay, and France must contain in excess of $10^8$ or $10^9$ rhizobia $g^{-1}$ peat carrier and be essentially contaminant free (Lupwayi et al. 2000). Inoculant quality in these countries is often controlled by a government testing agency and subject to law. Best results have traditionally been with inoculant formulations that are sterilized before introduction of the rhizobia (D. J. Hume and J. A. Omelian, personal communication). These maintain higher numbers of rhizobia and have a longer shelf life than unsterile peat or granular inoculants. Whereas the large-scale production of inoculants is a relatively simple process, not all inoculants meet the quality standards mentioned above. For example, the 18 Argentine inoculants examined by Gomez et al. (1997) ranged in cell count from 0 to $10^9$ rhizobia $g^{-1}$, and 14 contained more contaminants than rhizobia. More recent liquid formulations have shown good survival of rhizobia in storage and on seeds and show great promise in delivering large numbers of effective strains under field conditions (Albareda et al. 2008; Singleton et al. 2002; Walley et al. 2004).

Environmental conditions also can affect inoculation success. The effect of soil acidity on rhizobia has already been mentioned and has necessitated both a search for more acid-tolerant rhizobia (Graham et al. 1982, 1994; Howieson et al. 1988; Howieson and Ewing 1989) or the use of pelleted, inoculated seed that provides a neutral environment prior to infection

(Somasegaran and Hoben 1994). Higher inoculation rates may be needed where high temperatures at seeding limit rhizobial survival (Smith et al. 1981; Smith and del Rio Escurra 1982). A recent trend is for mixed inoculants, for example, containing *Rhizobium* plus biocontrol, phosphate-solubilizing, or growth hormone–producing organisms (Rice et al. 1995; Burdmann et al. 1996; Xi et al. 1996; Zhang et al. 1997). Results of coinoculation experiments with *Azospirillum* are promising; more detailed field experimentation is needed to establish the value of the other combined formulations. A concern in all such preparations must be the compatibility and survival of the various inoculant organisms used.

## References


Albareda M, Rodríguez-Navarro DN, Camacho M, Temprano FJ (2008) Alternatives to peat as a carrier for rhizobia inoculants: solid and liquid formulations. Soil Biol Biochem 40:2771–2779

Amadou C, Pascal G, Mangenot S, Glew M, Bontemps C, Capela D, Carrère S, Cruveiller S, Dossat C, Lajus A, Marchetti M, Poinsot V, Rouy Z, Servin B, Saad M, Schenowitz C, Barbe V, Batut J, Médigue C, Masson-Boivin C (2008) Genome sequence of the beta-rhizobium *Cupriavidus taiwanensis* and comparative genomics of rhizobia. Genome Res 18:1472–1483

Amarger N, Macheret V, Laguerre G (1997) *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. Int J Syst Bacteriol 47:996–1006

An DS, Im WT, Yang HC, Lee ST (2006) *Shinella granuli* gen. nov., sp. nov., and proposal of the reclassification of *Zoogloea ramigera* ATCC 19623 as *Shinella zoogloeoides* sp. nov. Int J Syst Evol Microbiol 56:443–444

Anyango B, Wilson KJ, Beynon JL, Giller KE (1995) Diversity of rhizobia nodulating *Phaseolus vulgaris* L in two Kenyan soils with contrasting pHs. Appl Environ Microbiol 61:4016–4021

Bachem CW, Banfalvi Z, Kondorosi E, Schell J, Kondorosi A (1986) Identification of host range determinants in the *Rhizobium* species MPIK3030. Mol Gen Genet 203:42–48

Banfalvi Z, Kondorosi A (1989) Production of root hair deformation factors by *Rhizobium* meliloti nodulation genes in *Escherichia coli*: HsnD (*nodH*) is involved in plant host-specific modification of the *nodABC* factor. J Mol Biol 13:1–12

Banfalvi Z, Nieuwkoop A, Schell M, Best L, Stacey G (1988) Regulation of *nod* expression in *Bradyrhizobium japonicum*. Mol Gen Genet 214:420–424

Barber LE (1979) Use of selective agents for recovery of *Rhizobium meliloti*. Soil Sci Soc Am J 43:1145–1148

Bassam BJ, Rolfe BG, Djordjevic MA (1986) *Macroptilium atropurpureum* (siratro) host specificity genes are linked to a *nodD*-like gene in the broad host range *Rhizobium* strain NGR234. Mol Gen Genet 203:49–57

Becker A, Puhler A (1998) Production of exopolysaccharides. In: Spaink HP, Kondorosi A, Hooykaas PJJ (eds) The *Rhizobiaceae*. Kluwer, Dordrecht, pp 97–118

Berge O, Lodhi A, Brandelet G, Santaella C, Roncato M, Christen R, Heulin T, Achouak W (2009) *Rhizobium alamii* sp. nov., an exopolysaccharide-producing species isolated from legume and non-legume rhizospheres. Int J Syst Evol Microbiol 59:367–372

Beringer JE, Beynon JL, Buchanan-Wollaston AV, Johnston AWB (1978) Transfer of the drug-resistance transposon Tn5 to *Rhizobium*. Nature 276:633–634

Bladergroen MR, Spaink HP (1998) Genes and signal molecules involved in the Rhizobia-leguminosae symbiosis. Curr Opin Plant Biol 1:353–359

Bohlool BB, Schmidt EL (1973) Persistence and competition aspects of *Rhizobium japonicum* observed in soil by immunofluorescence microscopy. Soil Sci Soc Am Proc 37:561–564

Boivin C, Ndoye I, Molouba F, Delajudie P, Dupuy N, Dreyfus B (1997) Stem nodulation in legumes–diversity, mechanisms, and unusual characteristics. Crit Rev Plant Sci 16:1–30

Bonaldi K, Gourion B, Fardoux J, Hannibal L, Cartieaux F, Boursot M, Vallenet D, Chaintreuil C, Prin Y, Nouwen N, Giraud E (2010) Large-scale transposon mutagenesis of photosynthetic *Bradyrhizobium* sp. strain ORS278 reveals new genetic loci putatively important for Nod-independent symbiosis with *Aeschynomene indica*. Mol Plant Microbe Interact 23:760–770

Boogerd FC, van Rossum D (1997) Nodulation of groundnut by *Bradyrhizobium* – a simple infection process by crack entry. FEMS Microbiol Rev 21:5–27

Bottomley PJ (1992) Ecology of *Bradyrhizobium* and *Rhizobium*. In: Stacey G, Burris R, Evans HJ (eds) Biological nitrogen fixation. Chapman and Hall, New York, p 943

Bottomley PJ, Cheng HH, Strain SR (1994) Genetic structure and symbiotic characteristics of a *Bradyrhizobium* population recovered from a pasture soil. Appl Environ Microbiol 60:1754–1761

Boundy-Mills KL, Kosslak RM, Tully RE, Pueppke SG, Lohrke S, Sadowsky MJ (1994) Induction of the *Rhizobium fredii* nod box-independent nodulation gene *nolJ* requires a functional *nodD1* gene. Mol Plant Microbe Interact 7:305–308

Breedveld MW, Miller KJ (1998) Cell-surface γ-glycans. In: Spaink HP, Kondorosi A, Hooykaas PJJ (eds) The *Rhizobiaceae*. Kluwer, Dordrecht, pp 81–96

Brewin NJ, Beringer JE, Johnston AWB (1980) Plasmid mediated transfer of host range specificity between two strains of *Rhizobium leguminosarum*. J Gen Microbiol 120:413–420

Brockwell J, Bottomley PJ (1995) Recent advances in inoculant technology and prospects for the future. Soil Biol Biochem 27:683–697

Brockwell J, Gault RR, Zorin M, Roberts MJ (1982) Effects of environmental variables on the competition between inoculum strains and naturalized populations of *Rhizobium trifolii* for nodulation of *Trifolium subterraneum* L. and on rhizobia persistence in the soil. Aust J Agric Res 33:803–815

Brockwell J, Pilka A, Holliday RA (1991) Soil pH is a major determinant of the numbers of naturally-occurring *Rhizobium meliloti* in non-cultivated soils in central New South Wales, Australia. Aust J Exp Agric 31:211–220

Brom S, Girard L, Tun-Garrido C, Garcia SA de los, Bustos P, González V, Romero D (2004) Transfer of the symbiotic plasmid of *Rhizobium etli* CFN42 requires cointegration with p42a, which may be mediated by site-specific recombination. J Bacteriol 186:7538–7548

Bromfield ESP, Barran LR, Wheatcroft R (1995) Relative genetic structure of a population of *Rhizobium meliloti* isolated directly from soil and from nodules of alfalfa (*Medicago sativa*) and sweet clover (*Melilotus alba*). Mol Ecol 4:183–188

Broughton WJ, Heycke N, Meyer ZA, Pankhurst CE (1984) Plasmid linked *nif* and *nod* genes in fast-growing rhizobia that nodulate *Glycine max*, *Psophocarpus tetragonolobus* and *Vigna unguiculata*. Proc Natl Acad Sci USA 81:3093–3097

Brunel B, Cleyet-Marel JC, Normand P, Bardin R (1988) Stability of *Bradyrhizobium japonicum* inoculants after introduction into soil. Appl Environ Microbiol 54:2636–2642

Brunel B, Rome S, Ziani R, Cleyet-Marel JC (1996) Comparison of/nucleotide diversity and symbiotic diversity of *Rhizobium meliloti* populations from annual *Medicago* species. FEMS Microbiol Ecol 19:71–82

Burdmann S, Sarig S, Kigel J, Okon Y (1996) Field inoculation of common bean (*Phaseolus vulgaris* L.) and chickpea (*Cicer arietinum* L.) with *Azospirillum brasilense* strain CD. Symbiosis 212:41–48

Burton JC (1967) *Rhizobium* culture and use. In: Peppler HJ (ed) Microbial technology. Van Nostrand-Reinhold, New York, pp 1–33

Burton JC (1980) Modern concepts in legume inoculation. In: Graham PH, Harris SC (eds) BNF technology for tropical agriculture. CIAT, Colombia, pp 105–114

Bushby HVA (1990) The role of bacterial surface charge in the ecology of root nodule bacteria: An hypothesis. Soil Biol Biochem 22:1–9

Caballero-Mellado J, Martinez-Romero E (1999) Soil fertilization limits the genetic diversity of *Rhizobium* in bean nodules. Symbiosis 26:111–121

Caetano-Annoles G (1997) Molecular dissection and improvement of the nodule symbiosis in legumes. Field Crops Res 53:47–68

Carlson RW, Kalembasa S, Turowski D, Pachori P, Noel KD (1987) Characterization of the lipopolysaccharide from a *Rhizobium phaseoli* mutant that is defective in infection thread development. J Bacteriol 169:4923–4928

Carlson RW, Sanjuan J, Bhat UR, Glushka J, Spaink HP, Wijfjes AHM, van Brussel AAN, Stokkermans TJW, Peters NK, Stacey G (1993) The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type I and II strains of *Bradyrhizobium japonicum*. J Biol Chem 268:18372–18381

Carlson RW, Price NPJ, Stacey G (1994) The biosynthesis of rhizobial lipo-oligosaccharide nodulation signal molecules. Mol Plant-Microbe Interact 7:684–695

Casida LE Jr (1982) *Ensifer adhaerens* gen. nov., sp. nov.: A bacterial predator of bacteria in soil. Int J Syst Bacteriol 32:339–345

Chabot R, Antoun H, Kloepper JW, Beauchamp CJ (1996) Root colonization of maize and lettuce by bioluminescent *Rhizobium leguminosarum* biovar *phaseoli*. Appl Environ Microbiol 62:2767–2772

Chatel DL, Greenwood RM, Parker CA (1968) Saprophytic competence as an important characteristic in the selection of *Rhizobium* for inoculation. In: *Proceedings of the Ninth International Congress of Soil Science Transactions*, Vol. 2 (pp. 65–73). Adelaide.

Chaverra MH, Graham PH (1992) Cultivar variation in traits affecting early nodulation of common bean. Crop Sci 32:1432–1436

Chen WX, Yan GH, Li JL (1988) Numeric taxonomic study of the fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int J Syst Bacteriol 38:392–397

Chen WX, Li GS, Qi YL, Wang ET, Yuan HL, Li JL (1991) *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. Int J Syst Bacteriol 41:275–280

Chen W, Wang E, Wang S, Li Y, Chen X, Li Y (1995) Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. Int J Syst Bacteriol 45:153–159

Chen WX, Tan ZY, Gao JL, Li Y, Wang ET (1997) *Rhizobium hainanense* sp. nov., isolated from tropical legumes. Int J Syst Bacteriol 47:870–873

Chen WM, Laevens S, Lee TM, Coenye T, De Vos P, Mergeay M, Vandamme P (2001) *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. Int J Syst Evol Microbiol 51:1729–1735

Chen WM, Moulin L, Bontemps C, Vandamme P, Bena G, Boivin-Masson C (2003) Legume symbiotic nitrogen fixation by beta-proteobacteria is widespread in nature. J Bacteriol 185:7266–7272

Chen WM, de Faria SM, Straliotto R, Pitard RM, Simoes-Araujo JL, Chou JH, Chou YJ, Barrios E, Prescott AR, Elliot GN, Sprent JI, Young JPW, James EK (2005) Proof that *Burkholderia* forms effective symbioses with legumes: A study of novel Mimosa-nodulating strains from South America. Appl Environ Microbiol 71:7461–7471

Chen WM, James EK, Coenye T, Chou JH, Barrios E, de Faria SM, Elliott GN, Sheu SY, Sprent JI, Vandamme P (2006) *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America. Int J Syst Evol Microbiol 56:1847–1851

Chen WM, de Faria SM, James EK, Elliott GN, Lin KY, Chou JH, Sheu SY, Cnockaert M, Sprent JI, Vandamme P (2007) *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody Brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. Int J Syst Evol Microbiol 57:1055–1059

Chen WM, de Faria SM, Chou JH, James EK, Elliott GN, Sprent JI, Bontemps C, Young JPW, Vandamme P (2008) *Burkholderia sabiae* sp. nov., isolated from root nodules of *Mimosa caesalpiniifolia*. Int J Syst Evol Microbiol 58:2174–2179

Chen WM, Zhu WF, Bontemps C, Young JP, Wei GH (2010) *Mesorhizobium alhagi* sp. nov., isolated from root nodules of the wild legume *Alhagi sparsifolia*. Int J Syst Evol Microbiol 60:958–962

Chen WM, Zhu WF, Bontemps C, Young JPW, Wei GH (2011) *Mesorhizobium camelthorni* sp. nov., isolated from *Alhagi sparsifolia*. Int J Syst Evol Microbiol 61:574–579

Collins SL, Knapp AK, Briggs JM, Blair JM, Steinauer EM (1998) Modulation of diversity by grazing and mowing in native tallgrass prairie. Science 280:745–747

Cregan PB, Keyser HH (1986) Host restriction of nodulation by *Bradyrhizobium japonicum* strain USDA 123 in soybean. Crop Sci 26:911–916

Cubo MT, Buendia-Claveria AA, Beringer JE, Ruiz-Sainz JE (1988) Melanin production by *Rhizobium* strains. Appl Environ Microbiol 54:1812–1817

Cytryn EJ, Sangurdekar DP, Streeter JG, Franck WL, Chang W-S, Stacey G, Emerich DW, Joshi T, Xu D, Sadowsk MJ (2007) Transcriptional and physiological responses of *Bradyrhizobium japonicum* to desiccation-induced stress. J Bacteriol 189:6751–6762

Dangeard PA (1926) Recherches sur les tubercles radicaux des Le´gumineuses. Botaniste (Paris) 16:1–275

Date RA (1982) Collection, isolation, characterization and conservation of *Rhizobium*. In: Vincent JM (ed) Nitrogen fixation in legumes. Academic, Sydney, pp 95–109

Date RA (1991) Nodulation success and persistence of recommended inoculum strains for subtropical and tropical forage legumes in Northern Australia. Soil Biol Biochem 23:533–541

Date RA (1997) The contribution of R and D on root nodule bacteria to future cultivars of tropical forage legumes. Trop Grassl 31:350–354

Date RA, Halliday J (1987) Collection, isolation, cultivation and maintenance of rhizobia. In: Elkan GH (ed) Symbiotic nitrogen fixation technology. Marcel Dekker, New York, pp 1–27

Davis EO, Evans IJ, Johnston AWB (1988) Identification of *nodX*, a gene that allows *Rhizobium leguminosarum* biovar *viceae* strain TOM to nodulate Afghanistan peas. Mol Gen Genet 212:531–535

De Bruijn FJ (1992) Use of repetitive (repetitive extragenic element and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl Environ Microbiol 58:2180–2187

de Lajudie P, Willems A, Pot B, Dewettinck D, Maestrojuan G, Neyra M, Collins MD, Dreyfus B, Kersters K, Gillis M (1994) Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium* teranga sp. nov. Int J Syst Bacteriol 44:715–733

de Lajudie P, Laurent-Fulele E, Willems A, Torck U, Coopman R, Collins MD, Kersters K, Dreyfus B, Gillis M (1998a) *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. Int J Syst Bacteriol 48:1277–1290

de Lajudie P, Willems A, Nick G, Moreira F, Molouba F, Hoste B, Torck U, Neyra M, Collins MD, Lindstrom K, Dreyfus B, Gillis M (1998b) Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. Int J Syst Bacteriol 48:369–382

de Lajudie P, Willems A, Nick G, Mohamed SH, Torck U, Coopman R, Filali-Maltouf A, Kersters K, Dreyfus B, Lindstrom K, Gillis M (1999) *Agrobacterium* bv. 1 strains isolated from nodules of tropical legumes. Syst Appl Microbiol 22:119–132

De Ley J (1968) DNA base composition and hybridization in the taxonomy of phytopathogenic bacteria. Ann Rev Phytopathol 6:63–90

De Ley J, Rassell A (1965) DNA base composition, flagellation and taxonomy of the genus *Rhizobium*. J Gen Microbiol 41:85–91

Dean DR, Jacobson MR (1992) Biochemical genetics of nitrogenase. In: Stacey G, Burris B, Evans HJ (eds) Biological nitrogen fixation. Chapman and Hall, New York, pp 763–834

Demezas DH (1998) Fingerprinting bacterial genomes using restriction fragment length polymorphisms. In: de Bruijn FJ, Lupski JR, Weinstock GM (eds) Bacterial genomes: physical structure and analysis. Chapman and Hall, New York, pp 383–398

Demezas DH, Reardon TB, Watson JM, Gibson AH (1991) Genetic diversity among *Rhizobium leguminosarum* bv *trifolii* strains revealed by allozyme and restriction length polymorphisms analyses. Appl Environ Microbiol 59:1702–1708

Demezas DH, Reardon TB, Strain SR, Watson JM, Gibson AH (1995) Diversity and genetic structure of a natural population of *Rhizobium leguminosarum* bv *trifolii* isolated from *Trifolium subterraneum*. Mol Ecol 4:209–220

Denarie J, Debelle F, Prome JC (1996) *Rhizobium* lipo-chitooligosaccharide nodulation factors signaling molecules mediating recognition and morphogenesis. Annu Rev Biochem 65:503–535

Diatloff A (1977) Ecological studies of root nodule bacteria introduced into field environments. 6: antigenic and symbiotic stability in Lotononis rhizobia over a 12 year period. Soil Biol Biochem 9:85–88

Djordjevic MA, Sargent CL, Innes RW, Kuempel PL, Rolfe BG (1985) Host-range genes also affect competitiveness in *Rhizobium trifolii*. In: Evans HJ, Bottomley PJ, Newton WE (eds) Nitrogen fixation research progress. Martinus Nijhoff, Dordrecht, p 117

Djordjevic MA, Gabriel DW, Rolfe BG (1987a) *Rhizobium* – the refined parasite of legumes. Ann Rev Phytopathol 25:145–168

Djordjevic MA, Redmond JW, Batley M, Rolfe BG (1987b) Clovers secrete specific phenolic compounds which either stimulate or repress nod gene expression in *Rhizobium trifolii*. EMBO J 6:173–1179

Dobereiner J, Urquiaga S, Boddey RM (1995) Alternatives for nitrogen nutrition of crops in tropical agriculture. Fertilizer Res 42:339–346

Dockendorff TC, Sanjuan J, Grob P, Stacey G (1994) NolA represses *nod* gene expression in *Bradyrhizobium japonicum*. Mol Plant-Microbe Interact 7:596–602

Downie JA (1998) Functions of rhizobial nodulation genes. In: Spaink HP, Kondorosi A, Hooykaas PJJ (eds) The *Rhizobiaceae*. Kluwer, Dordrecht, pp 387–402

Downie JA (2005) Legume haemoglobins: Symbiotic nitrogen fixation needs bloody nodules. Curr Biol 15:196–198

Downie JA (2010) The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. FEMS Microbiol Rev 34:150–170

Doyle JJ (1994) Phylogeny of the legume family: An approach to understanding the origins of nodulation. Annu Rev Ecol Syst 25:325–349

Doyle JJ, Doyle JL (1997) In: Legocki A et al (eds) Biological fixation of nitrogen for ecology and sustainable agriculture, vol G39, NATO ASI series. Springer, New York, pp 307–312

Dreyfus B, Garcia JL, Gillis M (1988) Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. Int J Syst Bacteriol 38:89–98

Dudman WF (1977) Serological methods and their application to dinitrogen-fixing organisms. In: Treatise on dinitrogen-fixation, vol 4. Wiley, New York, pp 487–508

Duodu S, Bhuvaneswari TV, Stokkermans TJW, Peters NK (1999) A positive role for Rhizobitoxine in *Rhizobium*-legume symbiosis. Mol Plant Microbe Interact 12:1082–1089

Dupuy N, Willems A, Pot B, Dewettinck D, Vandenbruaene I, Maestrojuan G, Dreyfus B, Kersters K, Collins MD, Gillis M (1994) Phenotypic and genotypic characterization of bradyrhizobia nodulating the leguminous tree Acacia albida. Int J Syst Bacteriol 44:461–473

Dye M, Skot L, Mytton LR, Harrison SP, Dooley JJ, Cresswell A (1995) A study of *Rhizobium leguminosarum* biovar *trifolii* populations from soil extracts using randomly amplified polymorphic DNA profiles. Can J Microbiol 41:336–344

Eaglesham A, Seaman B, Ahmad H, Hassouna S, Ayanaba A, Mulongoy K (1981) High temperature tolerant "cowpea" rhizobia. In: Gibson AH, Newton WE (eds) Current perspectives in nitrogen fixation. Australian Academy of Science, Canberra, p 346

Eardly BD, Materon LA, Smith NH, Johnson DA, Rumbaugh MD, Selander RK (1990) Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. Appl Environ Microbiol 56:187–194

Elkan GH, Bunn CR (1994) The rhizobia. In: Balows A et al (eds) The prokaryotes. Springer, Berlin, pp 2197–2213, http://www.prokaryotes.com&rcub

Ellis WR, Ham GE, Schmidt EL (1984) Persistence and recovery of *Rhizobium japonicum* inoculum in a field soil. Agron J 76:573–576

Faucher C, Camut S, Denarie J, Truchet G (1989) The *nodH* and *nodQ* host range genes of *Rhizobium meliloti* behave as avirulence genes in *R. leguminosarum* bv *viceae* and determine changes in the production of plant-specific extracellular signals. Mol Plant-Microbe Interact 2:291–300

Fellay R, Perret X, Viprey V, Broughton WJ (1995) Organization of host-inducible transcripts on the symbiotic plasmid of *Rhizobium* sp. NGR234. Mol Microbiol 16:657–667

Finan TM, Wood JM, Jordon DC (1983) Symbiotic properties of C4-dicarboxylic acid transport mutants of *Rhizobium leguminosarum*. J Bacteriol 154:1403–1413

Firmin JL, Wilson KE, Carlson RW, Davies AE, Downie JA (1993) Resistance to nodulation of cv. Afghanistan peas is overcome by *nodX*, which mediates an O-acetylation of the *Rhizobium leguminosarum* lipo-oligosaccharide nodulation factor. Mol Microbiol 10:351–360

Fisher RF, Long SR (1989) DNA footprint analysis of the transcriptional activator proteins Nod-D1 and Nod-D3 on inducible nod gene promoters. J Bacteriol 171:5492–5502

Frank B (1889) Über die Pariseten in den Wurzelanschwellungen der Papilionaceen. Botanische Zeitung 37:377–388, 393–400

Franssen HJ, Nap J-P, Bisseling T (1992) Nodulins in root nodule development. In: Stacey G, Burris B, Evans HJ (eds) Biological nitrogen fixation. Chapman and Hall, New York, pp 598–624

Fred EB, Baldwin IL, McCoy E (1932) Root nodule bacteria and leguminous plants. University of Wisconsin, Madison, WI, p 343

Freiberg C, Fellay R, Bairoch A, Broughton WJ, Rosenthal A, Perret X (1997) Molecular basis of symbiosis between Rhizobium and legumes. Nature 387:394–401

Gage DJ, Bobo T, Long SR (1996) Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). J Bacteriol 178:7159–7166

Galibert F, Finan TM, Long SR, Puhler A, Abola P, Ampe F, Barloy-Hubler F, Barnett MJ, Becker A, Boistard P, Bothe G, Boutry M, Bowser L, Buhrmester J, Cadieu E, Capela D, Chain P, Cowie A, Davis RW, Dreano S, Federspiel NA, Fisher RF, Gloux S, Godrie T, Goffeau A, Golding B, Gouzy J, Gurjal M, Hernandez-Lucas I, Hong A, Huizar L, Hyman RW, Jones T, Kahn D, Kahn ML, Kalman S, Keating DH, Kiss E, Komp C, Lelaure V, Masuy D, Palm C, Peck MC, Pohl TM, Portetelle D, Purnelle B, Ramsperger U, Surzycki R, Thebault P, Vandenbol M, Vorholter FJ, Weidner S, Wells DH, Wong K, Yeh KC, Batut J (2001) The composite genome of the legume symbiont Sinorhizobium meliloti. Science 293:668–672

Gao JL, Turner SL, Kan FL, Wang ET, Tan ZY, Qiu YH, Terefework Z, Young JPW, Lindstrom K, Chen WX (2004) *Mesorhizobium septentrionale* sp. nov. and *Mesorhizobium temperatum* sp. nov., isolated from *Astragalus adsurgens* growing in the northern regions of China. Int J Syst Evol Microbiol 54:2003–2012

Garcia-Fraile P, Rivas R, Willems A, Peix A, Martens M, Martinez-Molina E, Mateos PF, Velazquez E (2007) *Rhizobium cellulosilyticum* sp. nov. isolated from sawdust of *Populus alba*. Int J Syst Evol Microbiol 57:844–848

Gault RR, Schwinghamer EA (1993) Direct isolation of *Bradyrhizobium japonicum* from soil. Soil Biol Biochem 25:1161–1166

Gherna RL (1994) Culture preservation. In: Gerhardt P et al (eds) Methods for general and molecular bacteriology. ASM Press, Washington, DC

Gibson AH, Date RH, Ireland JA, Brockwell J (1976) A comparison of competitiveness and persistence among five strains of *Rhizobium trifolii*. Soil Biol Biochem 8:395–401

Giraud E, Moulin L, Vallenet D, Barbe V, Cytryn E, Avarre JC, Jaubert M, Simon D, Cartieaux F, Prin Y, Bena G, Hannibal L, Fardoux J, Kojadinovic M, Vuillet L, Lajus A, Cruveiller S, Rouy Z, Mangenot S, Segurens B, Dossat C, Franck WL, Chang WS, Saunders E, Bruce D, Richardson P, Normand P, Dreyfus B, Pignol D, Stacey G, Emerich D, Verméglio A, Médigue C, Sadowsky M (2007) Legumes symbioses: Absence of Nod genes in photosynthetic bradyrhizobia. Science 316:1307–1312

Glazebrook J, Walker GC (1989) A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell 56:661–672

Glazebrook J, Ichige A, Walker GC (1993) A *Rhizobium meliloti* homolog of the *Escherichia coli* peptide-antibiotic transport protein SbmA is essential for bacteroid development. Genes Dev 7:1485–1497

Glynn P, Higgins P, Squartini A, O'Gara F (1985) Strain identification in *Rhizobium trifolii* using DNA restriction analysis, plasmid DNA profiles, and intrinsic antibiotic resistances. FEMS Microbiol Lett 30:177–182

Gomez M, Silva N, Hartman A, Sagardoy M, Catroux G (1997) Evaluation of commercial soybean inoculants from Argentina World. J Microbiol Biotechnol 13:167–173

González V, Santamaría RI, Bustos P, Hernández-González I, Medrano-Soto A, Moreno-Hagelsieb G, Janga SC, Ramírez MA, Jiménez-Jacinto V, Collado-Vides J, Dávila G (2006) The partitioned *Rhizobium etli* genome: Genetic and metabolic redundancy in seven interacting replicons. Proc Natl Acad Sci USA 103:3834–3839

Göttfert M (1993) Regulation and function of rhizobial nodulation genes. FEMS Microbiol Lett 104:39–64

Göttfert M, Horvath B, Kondorosi E, Putnoky P, Rodriguez-Quniones F, Kondorosi A (1986) At least two *nodD* genes are necessary for efficient nodulation of alfalfa by *Rhizobium* meliloti. J Mol Biol 191:411–420

Göttfert M, Webber J, Hennecke H (1988) Induction of a *nodA-lacZ* fusion in *Bradyrhizobium japonicum* by an isoflavone. J Plant Physiol 132:394–397

Göttfert M, Grob P, Hennecke H (1990) Proposed regulatory pathway encoded by the *nodV* and the *nodW* genes, determinants of host specificity in *Bradyrhizobium japonicum*. Proc Natl Acad Sci USA 87:2680–2684

Göttfert M, Holzhäuser D, Bäni D, Hennecke H (1992) Structural and functional analysis of two different *nodD* genes in *Bradyrhizobium japonicum* USDA110. Mol Plant Microbe Interact 5:257–265

Gotz R, Evans IJ, Downie JA, Johnston AWB (1985) Identification of host-range DNA which allows *Rhizobium leguminosarum* strain TOM to nodulate cv. Afghanistan peas. Mol Gen Genet 201:296–300

Graham PH (1964) The application of computer techniques to the taxonomy of the root-nodule bacteria of legumes. J Gen Microbiol 35:511–517

Graham PH (1976) Identification and classification of root nodule bacteria. In: Nutman PS (ed) Symbiotic nitrogen fixation in plants. Cambridge University Press, Cambridge, UK, pp 99–112

Graham PH, Viteri SE, Mackie F, Vargas AAT, Palacios A (1982) Variation in acid soil tolerance among strains of *Rhizobium phaseoli*. Field Crops Res 5:121–128

Graham PH, Sadowsky MJ, Keyser HH, Barnet YM, Bradley RS, Cooper JE, De Ley J, Jarvis BWD, Roslycky EB, Strijdom BW, Young JPW (1991) Proposed minimal standards for the description of new genera and species of root-and stem-nodulating bacteria. Int J Syst Bacteriol 41:582–587

Graham PH, Draeger KJ, Ferrey ML, Conroy MJ, Hammer BE, Martinez E, Aarons SR, Quinto C (1994) Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for pH tolerance of *Rhizobium tropici* UMR1899. Can J Microbiol 40:198–207

Graham PH, Sadowsky MJ, Tighe SW, Thompson JA, Date RA, Howieson JG, Thomas R (1995) Differences among strains of *Bradyrhizobium* in fatty acid–methyl ester analysis. Can J Microbiol 41:1038–1042

Graham PH, Ballen KL, Montealegre C, Jones R, Fischer B, Luque E (1999) Characterization of rhizobia associated with *Dalea* spp. in natural prairies and revegetation areas in Minnesota. In: Martinez E, Hernandez G (eds) Highlights in nitrogen fixation research. Plenum Press, New York

Gu CT, Wang ET, Tian CF, Han TX, Chen WF, Sui XH, Chen WX (2008) *Rhizobium miluonense* sp. nov., a symbiotic bacterium isolated from *Lespedeza* root nodules. Int J Syst Evol Microbiol 58:1364–1368

Guan SH, Chen WF, Wang ET, Lu YL, Yan XR, Zhang XX, Chen WX (2008) *Mesorhizobium caraganae* sp. nov., a novel rhizobial species nodulated with *Caragana* spp. in China. Int J Syst Evol Microbiol 58:2646–2653

Guar YD, Lowther WL (1980) Distribution, symbiotic effectiveness and fluorescent antibody reaction of naturalized populations of *Rhizobium trifolii* in Otago, New Zealand soils NZ. J Agric Res 23:529–532

Hadri A-E, Spaink HP, Bisseling T, Brewin NJ (1998) Diversity of root nodulation and rhizobial infection processes. In: Spaink HP, Kondorosi A, Hooykaas PJJ (eds) The *Rhizobiaceae*. Kluwer, Dordrecht, pp 348–360

Hagen MJ, Hamrick JL (1996) Population level processes in *Rhizobium leguminosarum* bv *trifolii*: The role of founder effects. Mol Ecol 5:707–714

Hahn M, Hennecke H (1988) Cloning and mapping of a novel nodulation region from *Bradyrhizobium japonicum* by genetic complementation of a deletion mutant. Appl Environ Microbiol 54:55–61

Hall A, Clark N (1995) Coping with change, complexity and diversity in agriculture-the case of rhizobial inoculants in Thailand. World Dev 23:1601–1614

Ham GE (1978) Interactions of *Glycine max* and *Rhizobium japonicum*. In: Summerfield RJ, Bunting AH (eds) Advances in legume science. Royal Botanic Gardens Kew, London, pp 289–296

Han TX, Han LL, Wu LJ, Chen WF, Sui XH, Gu JG, Wang ET, Chen WX (2008a) *Mesorhizobium gobiense* sp. nov. and *Mesorhizobium tarimense* sp. nov., isolated from wild legumes growing in desert soils of Xinjiang, China. Int J Syst Evol Microbiol 58:2610–2618

Han TX, Wang ET, Wu JL, Chen WF, Gu JG, Gu CT, Tian CF, Chen WX (2008b) *Rhizobium multihospitium* sp. nov., isolated from multiple legume species native of Xinjiang, China. Int J Syst Evol Microbiol 58:1693–1699

Heberlein GT, De Ley J, Tyjtgat R (1967) Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium* and *Chromobacterium*. J Bacteriol 94:116–124

Hellriegel H, Wilfarth H (1888) Untersuchungen ueber die stickstoffnahrung der Gramineen und Leguminsen. Beilageheft su der Zeitschrift des Verein Rubenzucker-Industrie Detchen Reichs Unknown, p 234

Heron DS, Ersek T, Krishan HB, Pueppke SG (1989) Nodulation mutants of *Rhizobium fredii* USDA 257. Mol Plant Microbe Interact 2:4–10

Herridge DF, Roughley RJ (1975) Variation in colony characteristics and symbiotic effectiveness of *Rhizobium*. J Appl Bacteriol 38:19–27

Hirsch PR (1996) Population dynamics of indigenous and genetically modified rhizobia in the field. New Phytol 133:159–171

Hofer AW (1941) A characterization of Bacterium radiobacter (Beijerinck and Van Delden) Lohnis. J Bacteriol 41:193–224

Holmes B, Popoff M, Kiredjian M, Kersters K (1988) *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimens and previously known as group Vd. Int J Syst Bacteriol 38:406–416

Hombrecher G, Brewin NJ, Johnston AWB (1981) Linkage of genes for nitrogenase and nodulation ability on plasmids in *Rhizobium leguminosarum* and *R. phaseoli*. Mol Gen Genet 182:133–136

Hombrecher G, Gotz R, Dibb NJ, Downie JA, Johnston AWB, Brewin NJ (1984) Cloning and mutagenesis of nodulation genes from *Rhizobium leguminosarum* TOM, a strain with extended host range. Mol Gen Genet 194:293–298

Hong G-F, Burn JE, Johnston AWB (1987) Evidence that DNA involved in the expression of nodulation (*nod*) genes in *Rhizobium* binds to the regulatory gene *nodD*. Nucleic Acids Res 15:9677–9690

Honma MA, Ausubel FM (1987) *Rhizobium meliloti* has three functional copies of the *nodD* symbiotic regulatory element. Proc Natl Acad Sci USA 84:8558

Horvath B, Kondorosi E, John M, Schmidt J, Torok I, Gyorgypal Z, Barabas I, Wieneke U, Schell J, Kondorosi A (1986) Organization, structure, and symbiotic function of *Rhizobium meliloti* nodulation genes determining host specificity for alfalfa. Cell 46:335–343

Horvath B, Bachem CW, Schell J, Kondorosi A (1987) Host-specific regulation of nodulation genes in *Rhizobium* is mediated by a plant-signal, interacting with the *nodD* gene products. EMBO J 6:841–848

Hou BC, Wang ET, Li Y, Jia RZ, Chen WF, Gao Y, Dong RJ, Chen WX (2009) *Rhizobium tibeticum* sp. nov., a symbiotic bacterium isolated from *Trigonella archiducis-nicolai* (Sirj.) Vassilcz. Int J Syst Evol Microbiol 59:3051–3057

Howieson JG, Ewing MA (1989) Annual species of *Medicago* differ greatly in their ability to nodulate on acid soils. Aust J Agric Res 40:843–850

Howieson JG, Ewing MA, Dantuono MF (1988) Selection for acid tolerance in *Rhizobium meliloti*. Plant Soil 105:179–188

Hubber A, Vergunst AC, Sullivan JT, Hooykaas PJ, Ronson CW (2004) Symbiotic phenotypes and translocated effector proteins of the *Mesorhizobium loti* strain R7A VirB/D4 type IV secretion system. Mol Microbiol 54:561–574

Humphrey BA, Vincent JM (1965) The effect of calcium nutrition on the production of diffusible antigens by *Rhizobium trifolii*. J Gen Microbiol 41:109–118

Hungria M, Andrade DD, Colozzi A, Balota EL (1997) Interactions among soil microorganisms and bean and maize grown in monoculture or intercropped. Pesq Agropec Brasil 32:807–818

Innes RW, Kuempel PL, Plazinski J, Canter-Cremers H, Rolfe BG, Djordjevic MA (1985) Plant factors induce expression of nodulation and host-range genes in *R. trifolii*. Mol Gen Genet 201:426–432

Islam MS, Kawasaki H, Muramatsu Y, Nakagawa Y, Seki T (2008) *Bradyrhizobium iriomotense* sp. nov., isolated from a tumor-like root of the legume *Entada koshunensis* from Iriomote Island in Japan. Biosci Biotechnol Biochem 72:1416–1429

Itakura M, Saeki K, Omori H, Yokoyama T, Kaneko T, Tabata S, Ohwada T, Tajima S, Uchiumi T, Honma K, Fujita K, Iwata H, Saeki Y, Hara Y, Ikeda S, Eda S, Mitsui H, Minamisawa K (2009) Genomic comparison of *Bradyrhizobium japonicum* strains with different symbiotic nitrogen-fixing capabilities and other Bradyrhizobiaceae members. ISME J 3:326–339

Jarvis BDW, Tighe SW (1994) Rapid identification of *Rhizobium* species based on cellular fatty acid analysis. Plant Soil 161:31–41

Jarvis BDW, Pankhurst CE, Patel JJ (1982) *Rhizobium loti*, a new species of legume root nodule bacteria. Int J Syst Bacteriol 32:378–380

Jarvis BWD, Ward LJH, Slade EA (1989) Expression by soil bacteria of nodulation genes from *Rhizobium leguminosarum* bv. *trifolii*. Appl Environ Microbiol 55:1426–1434

Jarvis BDW, Sivakumaran S, Tighe SW, Gillis M (1996) Identification of *Agrobacterium* and *Rhizobium* species based on cellular fatty acid composition. Plant Soil 184:143–158

Jarvis BDW, van Berkum P, Chen WX, Nour SM, Fernandez M, Cleyet-Marel JC, Gillis M (1997) Transfer of *Rhizobium loti, Rhizobium huakuii, Rhizobium cicer,i Rhizobium mediterraneum* and *Rhizobium tianshansense* to *Mesorhizobium* gen. nov. Int J Syst Bacteriol 47:895–898

Jiang J, Gu BH, Albright LM, Nixon BT (1989) Conservation between coding and regulatory elements of *Rhizobium meliloti* and *Rhizobium leguminosarum dct* genes. J Bacteriol 171:44–53

Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007) Invasion of the plant host by symbiotic rhizobial bacteria: molecular clues from *Sinorhizobium meliloti* and the plant *Medicago truncatula*. Nat Rev Microbiol 5:619–633

Jordan DC (1982) Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. Int J Syst Bacteriol 32:136–139

Jordan DC (1984) Family III, Rhizobiaceae Conn 1938. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, 1st edn. Williams and Wilkins, Baltimore, pp 234–242, http://www.cme.msu.edu/bergeys/&rcub

Josey DP, Beynon JL, Johnston AWB, Beringer JE (1979) Strain identification in *Rhizobium* using intrinsic antibiotic resistance. J Appl Bacteriol 46:343–350

Jourand P, Giraud E, Bena G, Sy A, Willems A, Gillis M, Dreyfus B, de Lajudie P (2004) *Methylobacterium nodulans* sp. nov., for a group of aerobic, facultatively methylotrophic, legume root-nodule-forming and nitrogen-fixing bacteria. Int J Syst Evol Microbiol 54:2269–2273

Judd AK, Schneider M, Sadowsky MJ, de Bruijn FJ (1993) Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. Appl Environ Microbiol 59:1702–1708

Kaminski PA, Batutu J, Boistard P (1998) A survey of symbiotic nitrogen fixation by Rhizobia. In: Spaink HP, Kondorosi A, Hooykaas PJJ (eds) The *Rhizobiaceae*. Kluwer, Dordrecht, pp 432–460

Kaneko T, Nakamura Y, Sato S, Asamizu E, Kato T, Sasamoto S, Watanabe A, Idesawa K, Ishikawa A, Kawashima K, Kimura T, Kishida Y, Kiyokawa C, Kohara M, Matsumoto M, Matsuno A, Mochizuki Y, Nakayama S, Nakazaki N, Shimpo S, Sugimoto M, Takeuchi C, Yamada M, Tabata S (2000) Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. DNA Res 7:331–338

Kaneko T, Nakamura Y, Sato S, Minamisawa K, Uchiumi T, Sasamoto S, Watanabe A, Idesawa K, Iriguchi M, Kawashima K, Kohara M, Matsumoto M, Shimpo S, Tsuruoka H, Wada T, Yamada M, Tabata S (2002) Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res 9:189–197

Keatinge JDH, Beck DP, Materon LA, Yurtsever N, Karuc K, Altuntas S (1995) The role of rhizobial diversity in legume crop productivity in the west Asian highlands. 4: *Rhizobium ciceri*. Expl Agric 31:501–507

Kennedy C (1989) The genetics of nitrogen fixation. In: Hopwood DA, Chater KE (eds) Genetics of bacterial diversity. Academic, New York, pp 107–127

Kijne JW (1992) The *Rhizobium* infection process. In: Stacey G, Burris B, Evans HJ (eds) Biological nitrogen fixation. Chapman and Hall, New York, pp 349–398

Kinkle BK, Schmidt EL (1991) Transfer of the pea symbiotic plasmid pJB5JI in non-sterile soil. Appl Environ Microbiol 57:3264–3269

Kinzig AP, Socolow RH (1994) Is nitrogen fertilizer use nearing a balance? Reply Physics Today 47:24–35

Kirchner O (1896) Die wurzelnollchen der sojabohne. Beitrage Biologie Pflanzen Cohn's 7:213–223

Kishinevsky BD, Jones DG (1987) Enzyme-linked immunosorbent assay (ELISA) for the detection and identification of *Rhizobium* strains. In: Elkan GH (ed) Symbiotic nitrogen fixation technology. Marcel Dekker, New York, pp 157–184

Knosel D (1962) Prufung von Bakterien auf Fahigkeit zur Sternbildung. Zentralbl Bakteriol Parasitenkd Infedtionskr Hyg II Abt 116:79–100

Kobayashi H, Broughton WJ (2008) Fine-tuning of symbiotic genes in rhizobia: flavonoid signal transduction cascade. In: Dilworth MJ, James EK, Sprent JI, Newton WE (eds) Nitrogen-fixing leguminous symbioses. Springer, Dordrecht, pp 117–152

Kondorosi E, Gyuris J, Schmidt J, John M, Duda E, Schell J, Kondorosi A (1988) Positive and negative control of nodulation genes in *Rhizobium meliloti* strain 41. In: Verma DPS, Palacios R (eds) Molecular microbe-plant interactions. APS Press, St. Paul, p 73

Kondorosi E, Gyuris J, Schmidt J, John M, Duda E, Hoffman B, Schell J, Kondorosi A (1989) Positive and negative control of nod gene expression in Rhizobium meliloti is required for optimal nodulation. EMBO J 8:1331–1340

Kondorosi E, Pierre M, Cren M, Haumann U, Buire M, Hoffman B, Schell J, Kondorosi A (1991) Identification of *nolR*, a negatively transacting factor controlling the nod regulon in *Rhizobium meliloti*. J Mol Biol 222:885–896

Kosslak RM, Bookland R, Barkei J, Paaren HE, Applebaum ER (1987) Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. Proc Natl Acad Sci USA 84:7428–7432

Krause A, Doerfel A, Göttfert M (2002) Mutational and transcriptional analysis of the type III secretion system of *Bradyrhizobium japonicum*. Mol Plant Microbe Interact 15:1228–1235

Krishnan HB, Pueppke SG (1991) *nolC*, a *Rhizobium fredii* gene involved in cultivar-specific nodulation of soybean, shares homology with a heat-shock gene. Mol Microbiol 5:737–745

Kucey RMN, Hynes MF (1989) Populations of *Rhizobium leguminosarum* biovars *phaseoli* and *viciae* in fields after bean or pea in rotation with nonlegumes. Can J Microbiol 35:661–667

Kumar Rao JVDK, Dart PJ, Usha Khan M (1982) Cowpea group *Rhizobium* in soils of the semiarid tropics. In: Graham PH, Harris SC (eds) BNF technology for tropical agriculture. CIAT, Cali. Colombia, pp 291–295

Kuykendall LD, Saxena B, Devine TE, Udell SE (1992) Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. Can J Microbiol 38:501–505

Labes G, Ulrich A, Lentzch P (1996) Influence of bovine slurry deposition on the structure of nodulating *Rhizobium leguminosarum* bv *viciae* soil populations in a natural habitat. Appl Environ Microbiol 62:1717–1722

Lange RT (1961) Nodule bacteria associated with the indigenous Leguminosae of South Western Australia. J Gen Microbiol 26:351–359

Lee KB, De Backer P, Aono T, Liu CT, Suzuki S, Suzuki T, Kaneko T, Yamada M, Tabata S, Kupfer DM, Najar FZ, Wiley GB, Roe B, Binnewies TT, Ussery DW, D'Haeze W, Herder JD, Gevers D, Vereecke D, Holsters M, Oyaizu H (2008) The genome of the versatile nitrogen fixer *Azorhizobium caulinodans* ORS571. BMC Genomics 9:271

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Prome JC, Denarie J (1990) Symbiotic host specificity of Rhizobium meliloti is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344:781–784

Leung K, Strain SR, de Bruijn FJ, Bottomley PJ (1994) Genotypic and phenotypic comparisons of chromosomal types within an indigenous soil population of *Rhizobium leguminosarum* bv. *trifolii*. Appl Environ Microbiol 60:416–426

Lewin A, Rossenberg C, Meyer H, Wong ACH, Nelson L, Manen JF, Stanley J, Dowling DN, Denarie J, Broughton WJ (1987) Multiple host-specificity loci of the broad host-range *Rhizobium* sp. NGR234 selected using the widely compatible legume *Vigna unguiculata*. Plant Mol Biol 8:447–459

Lewis-Henderson WR, Djordjevic MA (1991a) A cultivar specific interaction between *Rhizobium leguminosarum* bv. *trifolii* and subterranean clover is conditioned by *nodM*, other bacterial cultivar specificity genes and a single recessive host gene. J Bacteriol 173:2791–2799

Lewis-Henderson WR, Djordjevic MA (1991b) *nodT*, a positively-acting cultivar specificity determinant controlling nodulation of *Trifolium subterraneum* by *Rhizobium leguminosarum* bv. *trifolii*. Plant Mol Biol 16:515–526

Li LY, Chen WF, Li HL, Wang ET, Chen WX (2009) *Rhizobium alkalisoli* sp. nov., isolated from *Caragana intermedia* growing in saline-alkaline soils in the north of China. Int J Syst Evol Microbiol 59:3006–3011

Li QQ, Wang ET, Chang YL, Zhang YZ, Zhang YM, Sui XH, Chen WF, Chen WX (2010) *Ensifer* (*Sinorhizobium*) *sojae* sp. nov., isolated from root nodules of *Glycine max* grown in saline-alkaline soils in Hebei province of China. Int J Syst Evol Microbiol 61(Pt 8):1981–1988

Lie TA (1978) Symbiotic specialization in pea plants: The requirement of specific *Rhizobium* strains for peas from Afghanistan. Ann Appl Biol 88:462–465

Lie TA, Winarno R, Timmermans PCJM (1978) *Rhizobium* strains isolated from wild and cultivated legumes: suppression of nodulation by a non-nodulating *Rhizobium* strain. In: Loutit MW, Miles JAR (eds) Microbial ecology. Springer, Berlin

Lie TA, Goktan D, Pijnenborg J, Anlarsal E (1987) Co-evolution of the legume-Rhizobium association. Plant Soil 100:171–181

Lin DX, Chen WF, Wang FQ, Hu D, Wang ET, Sui XH, Chen WX (2009) *Rhizobium mesosinicum* sp. nov., isolated from root nodules of three different legumes. Int J Syst Evol Microbiol 59:1919–1923

Lin DX, Wang ET, Tang H, Han TX, He YR, Guan SH, Chen WX (2008) *Shinella kummerowiae* sp. nov., a symbiotic bacterium isolated from root nodules of the herbal legume *Kummerowia stipulacea*. Int J Syst Evol Microbiol 58:1409–1413

Lindstrom K (1989) *Rhizobium galegae*, a new species of legume root nodule bacteria. Int J Syst Bacteriol 39:365–367

Lindstrom K, Lipsanen P, Kaijalainen S (1990) Stability of markers used for identification of two *Rhizobium galegae* inoculant strains after five years in the field. Appl Environ Microbiol 56:444–450

Lloret L, Ormeno-Orrillo E, Rincon R, Martinez-Romero J, Rogel-Hernandez MA, Martinez-Romero E (2007) *Ensifer mexicanus* sp. nov. a new species nodulating *Acacia angustissima* (Mill.) Kuntze in Mexico. Syst Appl Microbiol 30:280–290

Loh J, Stacey G (2003) Nodulation gene regulation in *Bradyrhizobium japonicum*: A unique integration of global regulatory circuits. Appl Environ Microbiol 69:10–17

Lohrke SM, Orf JH, Martínez-Romero E, Sadowsky MJ (1995) Host-controlled restriction of nodulation by *Bradyrhizobium japonicum* strains in serogroup 110. Appl Environ Microbiol 61:2378–2383

Long SR (1989) *Rhizobium*-legume nodulation: Life together in the underground. Cell 56:203–214

Long SR, Egelhoff T, Fisher RF, Jacobs TW, Mulligan JT (1985) Fine structure studies of *R. meliloti nodDABC* genes. In: Evans HJ, Bottomley PJ, Newton WE (eds) Nitrogen fixation research progress. Martinus Nijhoff, Boston, pp 87–94

Long S, Reed JW, Himawan J, Walker GC (1999) Genetic analsysis of a clsuter of genes required for synthesis of the calcofluor-binding exopolysaccharide of *Rhizobium* meliloti. J Bacteriol 170:4239–4248

Lorda GS, Balatii A (1996) Production of high cell concentrations of *Rhizobium* and *Bradyrhizobium*. In: Balatti AP, Freire JR (eds) Legume inoculants: Selection and characterization of strains – production, use, and management. Editorial Kingraf Buenos Aires, Argentina

Louvrier P, Laguerre G, Amarger N (1995) Semi selective medium for isolation of *Rhizobium leguminosarum* from soils. Soil Biol Biochem 27:919–924

Lu YL, Chen WF, Han LL, Wang ET, Zhang XX, Chen WX, Han SZ (2009) *Mesorhizobium shangrilense* sp. nov., isolated from root nodules of *Caragana* spp. Int J Syst Evol Microbiol 59:3012–3018

Lupwayi NZ, Olsen PE, Sande ES, Keyser HH, Collins MM, Singleton PW, Rice WA (2000) Inoculant quality and its evaluation. Field Crops Res 65:259–270

Ma W, Guinel FC, Glick BR (2003) *Rhizobium leguminosarum* biovar *viciae* 1-aminocyclopropane-1-carboxylate deaminase promotes nodulation of pea plants. Appl Environ Microbiol 69:4396–4402

MacLean AM, Finan TM, Sadowsky MJ (2007) Genomes of the symbiotic nitrogen-fixing bacteria of legumes. Plant Physiol 144:615–622

Madrzak CJ, Golinska B, Kroliczak J, Pudelko K, Lazewska D, Lampka B, Sadowsky MJ (1995) Diversity among field populations of *Bradyrhizobium japonicum* in Poland. Appl Environ Microbiol 61:1194–1200

Maier RJ (1986) Biochemistry, regulation, and genetics of hydrogen oxidation in *Rhizobium japonicum* CRC. Crit Rev Biotechnol 3:17–38

Makkar NS, Casida LE (1987) *Cupriavidus necator* gen. nov., sp. nov. – A nonobligate bacterial predator of bacteria in soil. Int J Syst Bacteriol 37:323–326

Mantelin S, Fischer-Le Saux M, Zakhia F, Bena G, Bonneau S, Jeder H, de Lajudie P, Cleyet-Marel JC (2006) Emended description of the genus *Phyllobacterium* and description of four novel species associated with plant roots: *Phyllobacterium bourgognense* sp. nov., *Phyllobacterium ifriqiyense* sp. nov., *Phyllobacterium leguminum* sp. nov. and *Phyllobacterium brassicacearum* sp. nov. Int J Syst Evol Microbiol 56:827–839

Marshall KC (1964) Survival of root nodule bacteria in dry soils exposed to high temperatures. Aust J Agric Res 15:273–281

Martinez E, Romero D, Palacios R (1990) The *Rhizobium* genome. Crit Rev Plant Sci 9:59–93

Martinez-Romero E, Caballero-Mellado J (1996) *Rhizobium* phylogenies and bacterial genetic diversity. Crit Rev Plant Sci 15:113–140

Martinez-Romero E, Rosenblueth M (1990) Increased bean *Phaseolus vulgaris* L. nodulation competitiveness of genetically modified strains. Appl Environ Microbiol 56:2384–2388

Martinez-Romero E, Segovia L, Mercante FM, Franco AA, Graham P, Pardo MA (1991) *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int J Syst Bacteriol 41:417–426

McDermott TR, Graham PH, Brandwein DM (1987) Viability of *Bradyrhizobium japonicum* bacteroids. Arch Microbiol 148:100–106

McInnes A, Thies JE, Abbott LK, Howieson JG (2004) Structure and diversity among rhizobial strains, populations and communities–a review. Soil Biol Biochem 36:1295–1308

Meinhardt LW, Krishnan HB, Balatti PA, Pueppke SG (1993) Molecular cloning and characterization of a Sym-plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA 257. Mol Microbiol 9:17–27

Mendes IC, Bottomley PJ (1998) Distribution of a population of *Rhizobium leguminosarum* bv *trifolii* among different size classes of soil aggregates. Appl Environ Microbiol 64:970–975

Merabet C, Martens M, Mahdhi M, Zakhia F, Sy A, Le Roux C, Domergue O, Coopman R, Bekki A, Mars M, Willems A, de Lajudie P (2010) Multilocus sequence analysis of root nodule isolates from *Lotus arabicus* (Senegal), *Lotus creticus*, *Argyrolobium uniflorum* and *Medicago sativa* (Tunisia) and description of *Ensifer numidicus* sp. nov. and *Ensifer garamanticus* sp. nov. Int J Syst Evol Microbiol 60:664–674

Mergaert P, van Montagu M, Prome J-C, Holsters M (1993) Three unusual modifications, a D-arabinosyl, an N-methyl, and a carbamoyl group, are present on the Nod factors of *Azorhizobium caulinodans* strain ORS571. Proc Natl Acad Sci USA 90:1551–1555

Merrick MJ (1992) Regulation of nitrogen fixation genes in free-living and symbiotic bacteria. In: Stacey G, Burris RH, Evans HJ (eds) Biological nitrogen fixation. Chapman and Hall, New York, pp 835–876

Michiels J, Dombrecht B, Vermeiren N, Xi CW, Luyten E, Vanderleyden J (1998) *Phaseolus vulgaris* L. is a non-selective host for nodulation. FEMS Microbiol Ecol 26:193–205

Moffett ML, Colwell RR (1968) Adansonian analysis of the *Rhizobiaceae*. J Gen Microbiol 51:245–266

Moreira FMS, Cruz L, de Faria SM, Marsh T, Martinez-Romero E, de Oliveira Pedrosa F, Pitard RM, Young JPW (2006) *Azorhizobium doebereinerae* sp. nov. microsymbiont of *Sesbania virgata* (Caz.) Pers. Syst Appl Microbiol 29:197–206

Moulin L, Munive A, Dreyfus B, Boivin-Masson C (2001) Nodulation of legumes by members of the β-subclass of Proteobacteria. Nature 411:948–950

Mulligan JT, Long SR (1985) Induction of *Rhizobium meliloti nodC* expression by plant exudate requires *nodD*. Proc Natl Acad Sci USA 82:6609–6613

Murray JD (2011) Invasion by invitation: Rhizobial infection in legumes. Mol Plant Microbe Interact 24:631–639

Naisbitt T, James E, Sprent JI (1992) The evolutionary significance of the legume genus *Chamaecrista*, as determined by nodule structure. New Phytol 122:487–492

Nakagawa Y, Sakane T, Yokota A (1996) Transfer of 'Pseudomonas riboflavina' (Foster 1944), a gram-negative, motile rod with long-chain 3-hydroxy fatty acids, to *Devosia riboflavina* gen. nov., sp. nov., nom. rev. Int J Syst Bacteriol 46:16–22

Nandasena KG, O'Hara GW, Tiwari RP, Willems A, Howieson JG (2009) *Mesorhizobium australicum* sp. nov. and *Mesorhizobium opportunistum* sp. nov. isolated from *Biserrula pelecinus* L. growing in Australia. Int J Syst Evol Microbiol 59:2140–2157

Nick G, de Lajudie P, Eardly BD, Suomalainen S, Paulin L, Zhang X, Gillis M, Lindstrom K (1999) *Sinorhizobium arboris* sp. nov. and *Sinorhizobium kostiense* sp. nov., isolated from leguminous trees in Sudan and Kenya. Int J Syst Bacteriol 49:1359–1368

Nieuwkoop AJ, Banfalvi Z, Deshmane N, Gerhold D, Schell M, Sirotkin K, Stacey G (1987) A locus encoding host range is linked to the common nodulation genes of *Bradyrhizobium japonicum*. J Bacteriol 169:2631–2638

Niner BM, Hirsch AM (1998) How many *Rhizobium* genes, in addition to *nod*, *nif/fix*, and *exo*, are needed for nodule development and function. Symbiosis 24:51–102

Noel KD, Diebold RJ, Cava JR, Brink BA (1988) Rhizobial purine and pyrimidine auxotrophs: nutrient supplementation, genetic analysis, and the symbiotic requirement for the novo purine biosynthesis. Arch Micobiol 149:499–506

Nour SM, Fernandez MP, Normand P, Cleyet-Marel JC (1994) *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). Int J Syst Bacteriol 44:511–522

Nuti MP, Lepidi AA, Prakash RK, Shilperoot RA, Cannon FC (1979) Evidence for nitrogen fixation (*nif*) genes on indigenous *Rhizobium* plasmids. Nature (London) 282:533–535

Ogasawara M, Suzuki T, Mutoh I, Annapurna K, Arora NK, Nishimura Y, Maheshwari DK (2003) *Sinorhizobium indiaense* sp. nov. and *Sinorhizobium abri* sp. nov. isolated from tropical legumes, *Sesbania rostrata* and *Abrus precatorius*, respectively. Symbiosis 34:53–68

Oger P, Petit A, Dessaux Y (1997) Genetically engineered plants producing opines alter their biological environment. Nat Biotechnol 15:369–372

Olson ER, Sadowsky MJ, Verma DPS (1985) Identification of genes involved in the *Rhizobium*-legume symbiosis by Mu-dI(kan, lac)-generated transcription fusions. Nat Biotechnol 3:143–149

Paffetti G, Scotti C, Gnocchi S, Francelli S, Bazzicalupo M (1996) Genetic diversity of an Italian *Rhizobium meliloti* population from different *Medicago sativa* varieties. Appl Environ Microbiol 62:2279–2285

Parke D, Ornston LN (1984) Nutritional diversity of Rhizobiaceae revealed by auxanography. J Gen Microbiol 130:1743–1750

Parker CA (1957) Evolution of nitrogen-fixing symbiosis in higher plants. Nature 179:593–594

Parker CA (1968) On the evolution of symbiosis in legumes. In: Jensen HL (ed) Festskrift til Hans Laurits Jensen. Gadgaard Nielsens Bogtrykkeri, Lemvig, pp 107–115

Patt TE, Cole GC, Hanson RS (1976) *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. Int J Syst Bacteriol 26:226–229

Peng G, Yuan Q, Li H, Zhang W, Tan Z (2008) *Rhizobium oryzae* sp. nov., isolated from the wild rice *Oryza alta*. Int J Syst Evol Microbiol 58:2158–2163

Pérez-Mendoza D, Domínguez-Ferreras A, Muñoz S, Soto MJ, Olivares J, Brom S, Girard L, Herrera-Cervera JA, Sanjuán J (2004) Identification of functional *mob* regions in *Rhizobium etli*: evidence for self-transmissibility of the symbiotic plasmid pRetCFN42d. J Bacteriol 186:5753–5761

Perez-Ramirez NO, Rogel MA, Wang E, Castellanos JZ, Martinez-Romero E (1998) Seeds of *Phaseolus vulgaris* bean carry *Rhizobium etli*. FEMS Microbiol Ecol 26:289–296

Peters NK, Frost JW, Long SR (1986) A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science 233:977–979

Phillips DA, Joseph CM, Yang GP, Martinez-Romero E, Sanborn JR, Volpin H (1999) Identification of lumichrome as a *Sinorhizobium* enhancer of alfalfa root respiration and shoot growth. Proc Natl Acad Sci USA 96:12275–12280

Pinero D, Martinez E, Selander RK (1988) Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar *phaseoli*. Appl Environ Microbiol 54:2825–2832

Postma J, Hok-a-Hin CH, van Veen JA (1990) Role of microniches in protecting introduced *Rhizobium leguminosarum* biovar *trifolii* against competition and predation in soil. Appl Environ Microbiol 56:495–502

Prévost D, Bordeleau LM, Caudry-Reznick S, Schulman HM, Antoun H (1987) Characteristics of rhizobia isolated from three legumes indigenous to the high arctic: *Astragalus alpinus, Oxytropis maydelliana*, and *Oxytropis arctobia*. Plant Soil 98:313–324

Price NJ, Relic B, Talmont F, Lewin A, Prome D, Pueppke SG, Maillet F, Denarie J, Broughton WJ (1992) Broad host range *Rhizobium* species strain NGR234 secretes a family of carbamoylated and fucosylated nodulation signals that are O-acetylated or sulphated. Mol Microbiol 6:3575–3584

Pueppke SG (1996) The genetic and biochemical basis for nodulation of legumes by rhizobia. Crit Rev Biotechnol 16:1–51

Pueppke SG, Broughton WJ (1999) *Rhizobium* strain NGR234 and *R. fredii* USDA257 share exceptionally broad nested host ranges. Mol Plant Microbe Interact 12:293–318

Quan ZX, Bae HS, Baek JH, Chen WF, Im WT, Lee ST (2005) *Rhizobium daejeonense* sp. nov., isolated from a cyanide treatment bioreactor. Int J Syst Evol Microbiol 55:2543–2549

Ramirez-Bahena MH, Garcia-Fraile P, Peix A, Valverde A, Rivas R, Igual JM, Mateos PF, Martinez-Molina E, Velazquez E (2008) Revision of the taxonomic status of the species *Rhizobium leguminosarum* (Frank 1879) Frank 1889AL, *Rhizobium phaseoli* Dangeard 1926AL and *Rhizobium trifolii* Dangeard 1926AL. *R. trifolii* is a later synonym of *R. leguminosarum*. Reclassification of the strain *R. leguminosarum* DSM 30132 (=NCIMB 11478) as *Rhizobium pisi* sp. nov. Int J Syst Evol Microbiol 58:2484–2490

Ramirez-Bahena MH, Peix A, Rivas P, Camacho M, Rodriguez-Navarro DN, Camacho P, Mateos PF, Martinez-Molina E, Velazquez E (2009) *Bradyrhizobium pachyrhizi* sp. nov. and *Bradyrhizobium jicamae* sp. nov. isolated from effective nodules of *Pachyrhizus erosus*. Int J Syst Evol Microbiol 59:1929–1934

Rao JR, Fenton M, Jarvis BDW (1994) Symbiotic plasmid transfer in *Rhizobium leguminosarum* biovar *trifolii* and competition between the inoculant strain ICMP2163 and transconjugant soil bacteria. Soil Biol Biochem 26:339–351

Rasolomampianina R, Bailly X, Fetiarison R, Rabevohitra R, Béna G, Ramaroson L, Raherimandimby M, Moulin L, De Lajudie P, Dreyfus B, Avarre JC (2005) Nitrogen-fixing nodules from rose wood legume trees (*Dalbergia* spp.) endemic to Madagascar host seven different genera belonging to alpha- and beta-Proteobacteria. Mol Ecol 14:4135–4146

Ratcliff WC, Kadam SV, Denison RF (2008) Poly-3-hydroxybutyrate supports survival and reproduction in starving rhizobia. FEMS Microbiol Ecol 65:391–399

Reeve W, Chain P, O'Hara G, Ardley J, Nandesena K, Bräu L, Tiwari R, Malfatti S, Kiss H, Lapidus A, Copeland A, Nolan M, Land M, Hauser L, Chang YJ, Ivanova N, Mavromatis K, Markowitz V, Kyrpides N, Gollagher M, Yates R, Dilworth M, Howieson J (2010) Complete genome sequence of the *Medicago* microsymbiont *Ensifer* (*Sinorhizobium*) *medicae* strain WSM419. Stand Genomic Sci 28:77–86

Relic B, Talmont F, Kopcinska J, Golinowski W, Prome J-C, Broughton WJ (1993) Biological activity of *Rhizobium* sp. NGR234 Nod factors on *Macroptilium atropurpureum*. Mol Plant Microbe Interact 6:764–774

Ren DW, Chen WF, Sui XH, Wang ET, Chen WX (2011) *Rhizobium vignae* sp. nov., a symbiotic bacterium isolated from multiple legume species nov. Int J Syst Evol Microbiol 61:580–586

Rice WA, Olsen PE, Leggett ME (1995) Co-culture of *Rhizobium meliloti* and a phosphorus solubilizing fungus (*Penicillium bilaii*) in sterile peat. Soil Biol Biochem 27:703–705

Richardson AE, Simpson RJ (1989) Acid-tolerance and symbiotic effectiveness of *Rhizobium trifolii* associated with a *Trifolium subterraneum* L. based pasture growing in an acid soil. Soil Biol Biochem 21:87–95

Richardson AE, Viccars LA, Watson JM, Gibson AH (1995) Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. Soil Biol Biochem 27:515–524

Risal CP, Yokoyama T, Ohkama-Ohtsu N, Djedidi S, Sekimoto H (2010) Genetic diversity of native soybean bradyrhizobia from different topographical regions along the southern slopes of the Himalayan Mountains in Nepal. Syst Appl Mirobiol 33:416–425

Rivas R, Velázquez E, Willems A, Vizcaíno N, Subba-Rao NS, Mateos PF, Gillis M, Dazzo FB, Martínez-Molina E (2002) A new species of *Devosia* that forms a unique nitrogen-fixing root nodule symbiosis with the aquatic legume *Neptunia natans*. Appl Environ Microbiol 68:5217–5222

Rivas R, Willems A, Subba-Rao NS, Mateos PF, Dazzo FB, Kroppenstedt RM, Martinez-Molina E, Gillis M, Vizcaino N (2003) Description of *Devosia neptuniae* sp. nov. that nodulates and fixes nitrogen in symbiosis with *Neptunia natans*, an aquatic legume from India. Syst Appl Microbiol 26:47–53

Roberts GP, Leps WT, Silver LE, Brill WJ (1980) Use of two dimensional polyacrylamide gel electrophoresis to identify and classify *Rhizobium* strains. Appl Environ Microbiol 39:414–422

Roche P, Debelle F, Maillet F, Lerouge P, Faucher C, Truchet G, Dénarié J, Prome JC (1991) Molecular basis of symbiotic host specificity in *Rhizobium meliloti*: *nodH* and *nodPQ* genes encode the sulfation of lipo-oligosaccharides. Cell 67:1131–1143

Roest HP, Goosenderoo L, Wijffelman CA, de Maagd RA, Lugtenberg BJJ (1995) Outer membrane protein changes during bacteroid development are independent of nitrogen fixation and differ between indeterminate and determinate nodulating host plants of *Rhizobium leguminosarum*. Mol Plant Microbe Interact 8:14–22

Rolfe BG, Innes RW, Schofield PR, Watson JW, Sargent CL, Kuempel PL, Plazinski J, Canter-Cremers H, Djordjevic MA (1985) Plant-secreted factors influence the expression of *R. trifolii* nodulation and host-range genes. In: Evans HJ, Bottomly PJ, Newton WE (eds) Nitrogen fixation research progress. Martinus Nijhoff Publishers, Boston, pp 79–85

Rome S, Fernandez MP, Brunel B, Normand P, Cleyet-Marel JC (1996) *Sinorhizobium medicae* sp. nov. isolated from annual *Medicago* spp. Int J Syst Bacteriol 46:972–980

Rossbach S, Kulpa DA, Rossbach U, De Bruijn FJ (1994) Molecular and genetic characterization of the rhizopine catabolism (mocabrc) genes of *Rhizobium meliloti* l5–30. Mol Gen Genet 245:11–24

Rostas K, Kondorosi E, Horvath B, Simoncsits A, Kondorosi A (1986) Conservation of extended promoter regions of nodulation genes in *Rhizobium*. Proc Natl Acad Sci USA 83:1757–1761

Ruvkin GB, Ausubel FM (1980) Interspecies homology of nitrogenase genes. Proc Natl Acad Sci USA 77:191–195

Rynne FG, Glenn AR, Dilworth MJ (1994) Effect of mutations in aromatic catabolism on the persistence and competitiveness of *Rhizobium leguminosarum* bv. *trifolii*. Soil Biol Biochem 26:703–710

Sadowsky MJ (1994) Microbial DNA fingerprinting and restriction fragment length polymorphism analysis. In: Weaver RW, Angle JS, Bottomley P (eds) Methods of soil analysis, chemical and microbiological properties of soils. ASA-SSSA, Madison, pp 647–664

Sadowsky MJ, Graham PH (1998) Soil biology of the *Rhizobiaceae*. In: Spaink HP, Kondorosi A, Hooykaas PJJ (eds) The *Rhizobiacea*. Kluwer, Dordrecht, pp 155–172

Sadowsky MJ, Hur HG (1998) Use of endogenous repeated sequences to fingerprint bacterial genomic DNA. In: Lupski JR, Weinstock G, de Bruijn FJ (eds) Bacterial genomes: structure and analysis. Chapman and Hall, New York

Sadowsky MJ, Bohlool BB, Keyser HH (1987a) Serological relatedness of *Rhizobium fredii* to other rhizobia and to the bradyrhizobia. Appl Environ Microbiol 53:1785–1789

Sadowsky MJ, Tully RE, Cregan PB, Keyser HH (1987b) Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relation to genotype specific nodulation of soybeans. Appl Environ Microbiol 53:2624–2630

Sadowsky MJ, Olson ER, Foster VE, Kosslak RM, Verma DPS (1988) Two host-inducible genes of *Rhizobium fredii* and the characterization of the inducing compound. J Bacteriol 170:171–178

Sadowsky MJ, Cregan PB, Rodriguez-Quinones F, Keyser HH (1990) Microbial influence on gene-for-gene interactions in legume-*Rhizobium* symbioses. Plant and Soil 129:53–60

Sadowsky MJ, Cregan PB, Gottfert M, Sharma A, Gerhold D, Rodriguez-Quniones F, Keyser HH, Henneke H, Stacey G (1991) The *Bradyrhizobium japonicum nolA* gene and its involvement in the genotype-specific nodulation of soybeans. Proc Natl Acad Sci USA 88:637–641

Sagan M, Gresshoff PM (1996) Developmental mapping of nodulation events in pea (*Pisum sativum* L.) using supernodulating plant genotypes and bacterial variability reveals both plant and *Rhizobium* control of nodulation regulation. Plant Sci 117:167–179

Sanginga N, Danso SKA, Mulongoy K, Ojeifo AA (1994) Persistence and recovery of introduced *Rhizobium* 10 years after inoculation on *Leucaena leucocephala* grown on an alfisol in southwestern Nigeria. Plant Soil 159:199–204

Sanjuan J, Olivares J (1989) Implication of *nifA* in regulation of genes located a *Rhizobium meliloti* cryptic plasmid that effect nodulation efficiency. J Bacteriol 171:4154–4161

Sanjuan J, Carlson RW, Spaink HP, Bhat UP, Barbour WM, Glushka J, Stacey G (1992) A 2-O-methylfucose moiety is present in the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*. Proc Natl Acad Sci USA 89:8789–8793

Savka MA, Farrand SK (1997) Modification of rhizobacterial populations by engineering bacterium utilization of a novel plant source. Nat Biotechnol 15:363–368

Sawada H, Ieki H, Oyaizu H, Matsumoto S (1993) Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. Int J Syst Bacteriol 43:694–702

Sawada H, Kuykendall LD, Young JM (2003) Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. J Gen Appl Microbiol 49:155–179

Schlaman HRM, Phillips DA, Kondorosi E (1998) Genetic organization and transcriptional regulation of rhizobial nodulation genes. In: Spaink HP, Kondorosi A, Hooykaas PJJ (eds) The *Rhizobiacea*. Kluwer, Dordrecht, pp 351–386

Schmeisser C, Liesegang H, Krysciak D, Bakkou N, Le Quéré A, Wollherr A, Heinemeyer I, Morgenstern B, Pommerening-Röser A, Flores M, Palacios R, Brenner S, Gottschalk G, Schmitz RA, Broughton WJ, Perret X, Strittmatter AW, Streit WR (2009) *Rhizobium* sp. strain NGR234 possesses a remarkable number of secretion systems. Appl Environ Microbiol 75:4035–4045

Schmidt EL, Bankole RO, Bohlool BB (1968) Fluorescent antibody approach to study rhizobia in soil. J Bacteriol 95:1987–1992

Scholla MH, Elkan GH (1984) *Rhizobium fredii* sp. nov., a fast-growing species that effectively nodulates soybeans. Int J Syst Bacteriol 34:484–486

Schortemeyer M, Santruckova H, Sadowsky MJ (1997) Relationship between root length density and soil microorganisms in the rhizospheres of white clover and perennial ryegrass. Comm Soil Sci Plant Anal 28:1675–1682

Schultze M, Kondorosi A (1998) Regulation of symbiotic root nodule development. Ann Rev Genet 32:33–57

Schultze M, Quiclet-Sire B, Kondorosi E, Virelizier H, Glushka N, Endre G, Gero D, Kondorosi A (1992) *Rhizobium meliloti* produces a family of sulphated lipooligosaccharides exhibiting different degrees of plant host specificity. Proc Natl Acad Sci USA 89:192–196

Segovia L, Pinero D, Palacios R, Martinez-Romero E (1991) Genetic structure of a soil population of nonsymbiotic *Rhizobium leguminosarum*. Appl Environ Microbiol 57:426–433

Segovia L, Young JP, Martinez-Romero E (1993) Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp. nov. Int J Syst Bacteriol 43:374–377

Sen D, Weaver RW (1988) Nitrogenase acetylene activities of isolated peanut and cowpea bacteroids at optimal oxygen availability and comparison with whole nodule activities. J Exp Bot 35:785–789

Shearman CA, Rossen L, Johnston AWB, Downie JA (1986) The *Rhizobium leguminosarum* nodulation gene *nodF* encodes a polypeptide similar to acyl carrier protein and is regulated by *nodD* plus a factor in pea root exudate. EMBO J 5:647

Singleton PW, Stockinger KR (1983) Compensation against ineffective nodulation in soybean (*Glycine max*). Crop Sci 23:69–72

Singleton PW, Tavares JW (1986) Inoculation response of legumes in relation to the number and effectiveness of indigenous *Rhizobium* populations. Appl Environ Microbiol 51:1013–1018

Singleton P, Keyser HH, Sande E (2002) Development and evaluation of liquid inoculants. In: Herridge D (ed) Inoculants and nitrogen fixation of legumes in vietnam, ACIAR Proceedings 109e. ACIAR, Canberra, pp 52–66

Slattery JF, Coventry DR (1993) Variation of soil populations of *Rhizobium leguminosarum* bv. *trifolii* and the occurrence of inoculant rhizobia in nodules of subterranean clover after pasture renovation in north eastern Victoria. Soil Biol Biochem 25:1725–1730

Smith RS (1988) Nitragin bran inoculants technical bulletin. Lipha Tech, Milwaukee WI, p 9

Smith RS (1992) Legume inoculant formulation and application. Can J Microbiol 38:485–492

Smith RS, del Rio Escurra GA (1982) Soybean inoculant types and rates evaluated under dry and irrigated field conditions. J Agr Univ Puert Rico 66:241–249

Smith RS, Ellis MA, Smith RE (1981) Effect of *Rhizobium japonicum* inoculation rates on soybean nodulation in a tropical soil. Agron J 73:505–508

So RB, Ladha JK, Young JPW (1994) Photosynthetic symbionts of Aeschynomene form a cluster with bradyrhizobia on the basis of fatty acid and r RNA analysis. Int J Syst Bacteriol 44:392–403

Somasegaran P, Hoben HJ (1994) Handbook for rhizobia. Springer, New York, p 450

Souza V, Eguiarte L, Avila G, Cappello G, Gallardo C, Montoya J, Pinero D (1994) Genetic structure of *Rhizobium etli* biovar *phaseoli* associated with wild and cultivated bean plants (*Phaseolus vulgaris* and *Phaseolus coccineus* in Morelos Mexico). Appl Environ Microbiol 60:1260–1268

Spaink HP (1995) The molecular basis of infection and nodulation by rhizobia – the ins and outs of sympathogenesis. Annu Rev Phytopathol 33:345–368

Spaink HP, Wijffelman CA, Pees E, Okker RJH, Lugtenberg BJJ (1987) *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. Nature 328:337–340

Spaink HP, Sheeley DM, van Brussel AAN, Glushka J, York WS, Tak T, Geiger O, Kennedy EP, Reinholdt VN, Lugtenberg BJJ (1991) A novel highly unsaturated fatty acid moiety of lipooligosaccharide signals determines host specificity of *Rhizobium*. Nature 354:124–130

Sparrow SD, Cochran VL, Sparrow EB (1995) Dinitrogen fixation by seven legume crops in Alaska. Agron J 87:34–41

Squartini A, Struffi P, Doring H, Selenska-Pobell S, Tola E, Giacomini A, Vendramin E, Velazquez E, Mateos PF, Martínez-Molina E, Dazzo FB, Casella S, Nuti MP (2002) *Rhizobium sullae* sp. nov. (formerly '*Rhizobium hedysari*'), the root-nodule microsymbiont of *Hedysarum coronarium* L. Int J Syst Evol Microbiol 52:1267–1276

Stephens JHG, Rask H (2000) Inoculant production and formulation. Field Crops Res 65:249–258

Strain SR, Leung K, Whittam TS, de Bruijn FJ, Bottomley PJ (1994) Genetic structure of *Rhizobium leguminosarum* biovar *trifolii* and *viciae* populations found in two Oregon soils under different plant communities. Appl Environ Microbiol 60:2772–2778

Strain SR, Whittam TS, Bottomley PJ (1995) Analysis of genetic structure in soil populations of *Rhizobium leguminosarum* recovered from the USA and UK. Mol Ecol 4:105–114

Sugawara M, Okazaki S, Nukui N, Ezura H, Mitsui H, Minamisawa K (2006) Rhizobitoxine modulates plant-microbe interactions by ethylene inhibition. Biotechnol Adv 24:382–388

Sullivan JT, Ronson CW (1998) Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc Natl Acad Sci USA 95:5145–5149

Sullivan JT, Patrick HN, Lowther WL, Scott DB, Ronson CW (1995) Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. Proc Natl Acad Sci USA 92:8985–8989

Sutton WD (1983) Nodule development and senescence. In: Broughton WJ (ed) Nitrogen fixation, vol 3, Legumes. Oxford University Press, Oxford, pp 144–212

Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, Prin Y, Neyra M, Gillis M, Boivin-Masson C, Dreyfus B (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J Bacteriol 183:214–220

Tan ZY, Kan FL, Peng GX, Wang ET, Reinhold-Hurek B, Chen WX (2001) *Rhizobium yanglingense* sp. nov., isolated from arid and semi-arid regions in China. Int J Syst Evol Microbiol 51:909–914

Thies JE, Singleton PW, Bohlool BB (1991) Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Appl Environ Microbiol 57:19–28

Thomas PM, Golly KF, Zyskind JW, Virginia RA (1994) Variation of clonal, mesquite-associated rhizobial and bradyrhizobial populations from surface and deep soils by symbiotic gene region restriction fragment length polymorphism and plasmid profile analysis. Appl Environ Microbiol 60:1146–1153

Toledo I, Lloret L, Martinez-Romero E (2003) *Sinorhizobium americanus* sp. nov., a new *Sinorhizobium* species nodulating native *Acacia* spp. in Mexico. Syst Appl Microbiol 26:54–64

Tong Z, Sadowsky MJ (1993) A selective medium for the isolation and quantification of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains from soils and inoculants. Appl Environ Microbiol 60:581–586

Truchet G, Roche P, Lerouge P, Vasse J, Camut S (1991) Sulphated lipo-oligosaccharide signals of the symbiotic prokaryote *Rhizobium meliloti* elicit root nodule organogenesis on the host plant *Medicago sativa*. Nature 351:670–673

Trujillo ME, Willems A, Abril A, Planchuelo AM, Rivas R, Ludeña D, Mateos PF, Martínez-Molina E, Velázquez E (2005) Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. Appl Environ Microbiol 71:1318–1327

Unkovich MJ, Pate JS, Sandford P (1997) Nitrogen fixation by annual legumes in Australian Mediterranean agriculture. Aust J Agric Res 48:267–293

Valverde A, Velazquez E, Fernandez-Santos F, Vizcaino N, Rivas R, Mateos PF, Martinez-Molina E, Igual JM, Willems A (2005) *Phyllobacterium trifolii* sp. nov., nodulating *Trifolium* and *Lupinus* in Spanish soils. Int J Syst Evol Microbiol 55:1985–1989

Valverde A, Igual JM, Peix A, Cervantes E, Velazquez E (2006) *Rhizobium lusitanum* sp. nov. a bacterium that nodulates *Phaseolus vulgaris*. Int J Syst Evol Microbiol 56:2631–2637

van Berkum P, Beyene D, Vera FT, Keyser HH (1995) Variability among *Rhizobium* strains originating from nodules of *Vicia faba*. Appl Environ Microbiol 61:2649–2653

van Berkum P, Beyene D, Bao G, Campbell TA, Eardly BD (1998) *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. Int J Syst Bacteriol 48:13–22

van Brussel A, Recourt K, Pees E, Spaink HP, Tak T, Wijffelman C, Kijne J, Lugtenberg BB (1990) A biovar specific signal of *Rhizobium leguminosarum* bv. *viceae* induces increased nodulation gene-inducing activity in root exudate of *Vicia sativa* sub-sp. *nigra*. J Bacteriol 172:5394–5401

van der Drift KMGM, Olsthoorn MMA, Brull LP, Blok-Tip L, Thomas-Oates JE (1998) Mass spectrometric analysis of lipo-chitin oligosaccharides signal molecules mediating the host-specific legume-*Rhizobium* symbiosis. Mass Spectrom Rev 17:75–95

Van Egaraat AWSM (1975) The possible role of homoserine in the development of *Rhizobium leguminosarum* in the rhizosphere of pea seedlings. Plant Soil 42:381–386

van Rhijn P, Vanderleyden J (1995) The *Rhizobium*-plant symbiosis. Microbiol Rev 59:124–142

Vance CP (1998) Legume symbiotic nitrogen fixation: agronomic aspects. In: Spaink HP et al (eds) The *Rhizobiaceae*. Kluwer, Dordrecht, pp 509–530

Vandamme P, Goris J, Chen WM, de Vos P, Willems A (2002) *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. Syst Appl Microbiol 25:507–512

Velazquez E, Igual JM, Willems A, Fernandez MP, Munoz E, Mateos PF, Abril A, Toro N, Normand P, Cervantes E, Gillis M, Martinez-Molina E (2001) *Mesorhizobium chacoense* sp. nov. a novel species that nodulates *Prosopis alba* in the Chaco Arido region (Argentina). Int J Syst Evol Microbiol 51:1011–1021

Versalovic J, de Bruijn FJ, Lupksi JR (1998) Repetive sequence-based PCR (rep-PCR) DNA fingerprinting of bacterial genomes. In: de Bruijn FJ, Lupski JR, Weinstock GM (eds) Bacterial genomes: physical structure and analysis. Chapman and Hall, New York, pp 437–456

Vidal C, Chaintreuil C, Berge O, Maure L, Escarre J, Bena G, Brunel B, Cleyet-Marel JC (2009) *Mesorhizobium metallidurans* sp. nov., a metal-resistant symbiont of *Anthyllis vulneria* growing on metallicolous soil in Languedoc France. Int J Syst Evol Microbiol 59:850–855

Vincent JM (1970) A manual for the practical study of root-nodule bacteria, vol 15, IBP handbook. Blackwell, Oxford, p 164

Vinuesa P, Leon-Barrios M, Silva C, Willems A, Jarabo-Lorenzo A, Perez-Galdona R, Werner D, Martinez-Romero E (2005) *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (Papilionoideae: Genisteae) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. *genistearum*, *Bradyrhizobium* genospecies alpha and *Bradyrhizobium* genospecies beta. Int J Syst Evol Microbiol 55:569–575

Walley F, Clayton G, Gan Y, Lafond G (2004) Performance of rhizobial inoculant formulations in the field. Crop Manag. doi:10.1094/CM-2004-0301-03-RV

Wang ET, van Berkum P, Beyene D, Sui XH, Dorado O, Chen WX, Martinez-Romero E (1998) *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. Int J Syst Bacteriol 48:687–699

Wang ET, van Berkum P, Sui XH, Beyene D, Chen WX, Martinez-Romero E (1999) Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov. Int J Syst Bacteriol 49:51–65

Wang ET, Tan ZY, Willems A, Fernandez-Lopez M, Reinhold-Hurek B, Martinez-Romero E (2002) *Sinorhizobium morelense* sp. nov., a *Leucaena leucocephala*-associated bacterium that is highly resistant to multiple antibiotics. Int J Syst Evol Microbiol 52:1687–1693

Wang FQ, Wang ET, Liu J, Chen Q, Sui XH, Chen WF, Chen WX (2007) *Mesorhizobium albiziae* sp. nov., a novel bacterium that nodulates *Albizia kalkora* in a subtropical region of China. Int J Syst Evol Microbiol 57:1192–1199

Weaver RW, Frederick LR, Dumenil LC (1972) Effect of soybean cropping and soil properties on numbers of *Rhizobium japonicum* in Iowa soils. Soil Sci 114:137–141

Wei GH, Wang ET, Tan ZY, Zhu ME, Chen WX (2002) *Rhizobium indigoferae* sp. nov. and *Sinorhizobium kummerowiae* sp. nov., respectively isolated from *Indigofera* spp. and *Kummerowia stipulacea*. Int J Syst Evol Microbiol 52:2231–2239

Wei GH, Tan ZY, Zhu ME, Wang ET, Han SZ, Chen WX (2003) Characterization of rhizobia isolated from legume species within the genera *Astragalus* and *Lespedeza* grown in the Loess Plateau region of China and description of *Rhizobium loessense* sp. nov. Int J Syst Evol Microbiol 53:1575–1583

Weir BS (2011) The current taxonomy of rhizobia. New Zealand rhizobia website. http://www.rhizobia.co.nz/taxonomy/rhizobia.html. Last updated 13 March 2011

Wheatcroft R, Watson RJ (1988) A positive strain identification method for *Rhizobium meliloti*. Appl Environ Microbiol 54:574–576

Whitty PW, Powell W, Sprent JI (1994) Molecular separation of genera in Cassiinae (Leguminosae), and analysis of variation in the nodulating species of *Chamaecrista*. Mol Ecol 3:507–515

Wijffelman CA, Pees E, van Brussel AA, Priem M, Okker R, Lugtenberg BJ (1985) Analysis of the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. In: Evans HJ, Bottomly PJ, Newton WE (eds) Nitrogen fixation research progress. Martinus Nijhoff, Boston, p 127

Willems A (2006) The taxonomy of rhizobia: an overview. Plant Soil 287:3–14

Wilson KJ, Sessitsch A, Corbo JC, Giller KE, Akkermans ADL, Jefferson RA (1995) B-glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram-negative bacteria. Microbiology 141:1691–1705

Wilson KJ, Parra A, Botero L (1999) Application of the GUS marker gene technique to high-throughput screening of rhizobial competition. Can J Microbiol 45:678–685

Xi K, Stephens JHG, Verma PR (1996) Application of formulated rhizobacteria against root rot of field pea. Plant Path 45:1150–1158

Xu LM, Ge C, Cui Z, Li J, Fan H (1995) *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. Int J Syst Bacteriol 45:706–711

Yabuuchi E, Kosako Y, Oyaizu H, Yano I, Hotta H, Hashimoto Y, Ezaki T, Arakawa M (1992) Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol Immunol 36:1251–1275

Yang S, Tang F, Gao M, Krishnan HB, Zhu H (2010) R-gene-controlled host specificity in the legume-rhizobia symbiosis. Proc Natl Acad Sci USA 107:18735–18740

Yao ZY, Kan FL, Wang ET, Wei GH, Chen WX (2002) Characterization of rhizobia that nodulate legume species of the genus Lespedeza and description of *Bradyrhizobium yuanmingense* sp. nov. Int J Syst Evol Microbiol 52:2219–2230

Young JPW (1996) Phylogeny and taxonomy of rhizobia. Plant Soil 186:45–52

Young JM (2003) The genus name *Ensifer Casida* 1982 takes priority over *Sinorhizobium* Chen 1988, and *Sinorhizobium morelense* Wang et al. 2002 is a later synonym of *Ensifer adhaerens* Casida 1982. Is the combination '*Sinorhizobium adhaerens*' (Casida 1982) Willems et al. legitimate? Request for an opinion. Int J Syst Evol Microbiol 53:2107–2110

Young JM (2010) *Sinorhizobium* versus *Ensifer*: may a taxonomy subcommittee of the ICSP contradict the Judicial Commission? Int J Syst Evol Microbiol 60:1711–1713

Young JPW, Haukka KE (1996) Diversity and phylogeny of rhizobia. New Phytol 133:87–94

Young JPW, Wexler M (1988) Sym plasmid and chromosomal genotypes are correlated in field populations of *Rhizobium leguminosarum*. J Gen Microbiol 134:2731–2739

Young JM, Kuykendall LD, Martinez-Romero E, Kerr A, Sawada H (2001) A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudieet al. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. Int J Syst Evol Microbiol 51:89–103

Young JP, Crossman LC, Johnston AW, Thomson NR, Ghazoui ZF, Hull KH, Wexler M, Curson AR, Todd JD, Poole PS, Mauchline TH, East AK, Quail MA, Churcher C, Arrowsmith C, Cherevach I, Chillingworth T, Clarke K, Cronin A, Davis P, Fraser A, Hance Z, Hauser H, Jagels K, Moule S, Mungall K, Norbertczak H, Rabbinowitsch E, Sanders M, Simmonds M, Whitehead S, Parkhill J (2006) The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. Genome Biol 7:R34

Zaat SAJ, Wijffelman CA, Spaink HP, van Brussel AAN, Okker RJH, Lugtenberg BJJ (1987) Induction of the *nodA* promoter of *Rhizobium leguminosarum* Sym plasmid pRL1JI by plant flavanones and flavones. J Bacteriol 169:198–204

Zhang F, Dashti N, Hynes RK, Smith NL (1997) Plant growth promoting rhizobacteria and soybean (*Glycine max* (L.) Merr) growth and physiology at suboptimal root temperatures. Ann Bot 79:243–249

Zhang RJ, Hou BC, Wang ET, Li Y, Zhang XX, Chen WX (2011) *Rhizobium tubonense* sp. nov., isolated from root nodules of *Oxytropis glabra*. Int J Syst Evol Microbiol 61:512–517

Zhou PF, Chen WM, Wei GH (2010) *Mesorhizobium robiniae* sp. nov., isolated from root nodules of *Robinia pseudoacacia*. Int J Syst Evol Microbiol 60:2552–2556

Zurdo-Piñeiro JL, Rivas R, Trujillo ME, Vizcaíno N, Carrasco JA, Chamber M, Palomares A, Mateos PF, Martínez-Molina E, Velázquez E (2007) *Ochrobactrum cytisi* sp. nov., isolated from nodules of *Cytisus scoparius* in Spain. Int J Syst Evol Microbiol 57:784–788

# 18 Symbiotic Associations Between Ciliates and Prokaryotes

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## Introduction

Ciliates are frequently colonized by bacteria. This is partly due to the cytology and biology of these highly evolved heterotrophic protozoa (Hausmann and Bradbury 1996). As phagotrophic predators on microorganisms, they bear a high risk of microbial, namely, bacterial infections: Ingested bacteria may resist

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digestion, escape from the phagosomes, and persist in the cells as endocytobionts. Traditionally, intracellular bacteria in ciliates have been termed "symbionts" (Preer et al. 1974a). Phagocytosis appears to be the usual way infectious bacteria enter their host cells. In addition to intracellular bacteria, ectosymbionts may be intimately associated with ciliates. The most intriguing example is certainly the epixenosomes of *Euplotidium*, which even defend their host against predators (Petroni et al. 2000; see also ❯ "Terms in Symbiosis Research").

The vast majority of bacterial symbionts of ciliates comprise a variety of bacteria in many unrelated genera of different subgroups of proteobacteria. They are alike in that their habitat is the cytoplasm or the nucleus of a ciliate cell. Within the cytoplasm, some bacteria associate with organelles, the endomembrane system, or cytoskeletal elements specifically. Many of these symbionts appear to be well adapted to their environment; they are no longer free living and have genomes that are reduced in size, indicating a lengthy period of symbiont-host association. Most of the symbionts are not infectious, but a few are *Holospora*: infectious bacteria of the nuclei. These infectious symbionts have developed specific features that guarantee uptake and transport to the intracellular sites where they can multiply. In most cases, it is not clear whether the symbionts provide their hosts with a selective advantage; under laboratory conditions, most of the symbionts have proved to be dispensable. On the other hand, it is significant that the majority of cells of *Paramecium biaurelia* freshly collected from nature contain symbionts (Beale et al. 1969), and work by Landis (Landis 1981, 1987) and Kusch et al. (2001) gives evidence that paramecia that bear bacteria of the genus *Caedibacter* (formerly called "kappa particles") have a selective advantage over those that are symbiont-free, see ❯ "The Killer Trait in *Paramecium*." Moreover, *Polynucleobacter necessarius* (formerly called "omikron particles") and the closely related omikron-like symbionts occurring in several freshwater *Euplotes* species have been shown to be necessary for survival of their hosts (Heckmann 1975; Heckmann et al. 1983; See also ❯ "Prokaryotic Symbionts of *Euplotes*"). Since in these cases the hosts depend on their symbionts and those in turn depend upon their hosts, they are no longer free living—the distinction between "symbiont" on the one hand and "organelle" on the other hand becomes blurred. Furthermore, *Polynucleobacter necessarius* is the first documented example of a prokaryote, separated in an obligate endocytobiotic form and a free-living form within the same bacterial species (Hahn et al. 2009).

Ciliates can usually be handled easily, and some of them—particularly species of the *Paramecium aurelia* complex—have been investigated so thoroughly that they can be manipulated in a variety of ways. This has led to a wealth of information about their symbionts that has been reviewed extensively by Preer et al. (1974a), Soldo (1974), Quackenbush (1988), and Pond et al. (1989). It is not possible to list all the types of symbionts of ciliates encountered. Their number is very large, and certainly, many more will be added to this list. Few of them, however, have been deposited in stock cultures and have been investigated in such a way that they can easily be identified when found again. An even smaller number have been described with validated binomial names in keeping with the international rules of nomenclature (for prokaryotic endosymbionts, see Stackebrandt et al. 2002). Especially these validly described endosymbionts will be discussed in detail in this chapter. A lag phase in the description of new symbionts in ciliates for several years may be partly due to the fact that intracellular bacteria could not be investigated with classical microbiological methods, since they cannot be grown outside their host cells. New powerful techniques for detection and phylogenetic classification facilitate localization and identification of endosymbiotic bacteria. The increasing number of data in public databases allows to detect their distinct phycogenetic relationship.

## The Ciliate Cell as a Microcosm

Ciliates—phylum Ciliophora—are large protozoa that offer a variety of suitable habitats within their cells with plenty of space for bacteria (❯ *Fig. 18.1*). The ciliate cell is characterized by a highly organized cortex consisting of the plasma membrane, flat vesicles termed "alveoles" underneath the plasma membrane, and a network of microtubules and other cytoskeletal elements. Cilia, the motile organelles of ciliates, are anchored to the cytoskeleton.

Ciliates are heterotrophic. Because of their solid cortex of three membranes and multiple cytoskeletal systems, phagocytosis occurs only at a preformed structure termed the "oral apparatus." This is a complex metaorganelle consisting of a funnel-like pouch with batteries of cilia and a highly ordered cytoskeletal basket at the bottom of which new phagosomes are formed. Newly formed phagosomes are acidified and fuse with primary lysosomes (Fok and Allen 1988). As known from other professional phagocytes such as macrophages, ingested bacteria are attacked by acidification, oxidative burst, and lysosomal enzymes; see ❯ "The Nuclei of Ciliates." In spite of these attacks, certain bacteria with appropriate features may survive and even escape from the phagosomes. In the cytoplasm of ciliates, bacteria prevail in symbiontophorous vesicles or naked (i.e., not encircled by host membranes). Symbionts may also live in the large, somatic macronuclei as well as in the much smaller, generative micronuclei. Of those bacteria that are, however, nuclear specific, certain species are only found in micronuclei, and others only in macronuclei, of their specific hosts. Bacteria have even been found in the perinuclear space and in the endoplasmic reticulum (Fokin and Karpov 1995). The ciliate cell thus may be regarded as a microcosm that might contain even different symbionts at any given time. A striking example may be found in the giant ciliates of the genus *Spirostomum* (e.g., Fokin et al. 2005a).

## The Nuclei of Ciliates

Characteristically, ciliates have two types of nuclei: small generative micronuclei and large somatic macronuclei (Paulin 1996). Ciliates multiply by binary division and sexually propagate by

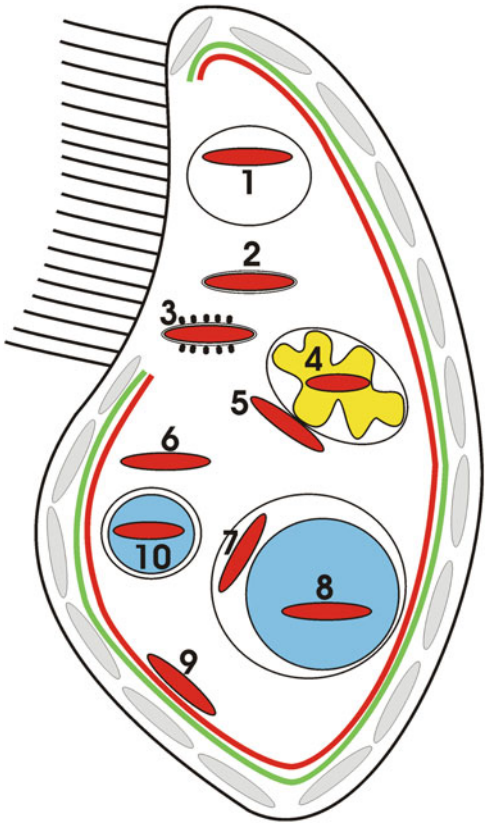



**Fig. 18.1**
**The ciliated cell. Both macronucleus and micronucleus (*8* and *10*) are *encircled* by the nuclear envelope with nuclear pores (not shown). Note that the nuclear envelopes have a perinuclear space that may be colonized by certain bacteria. The oral apparatus/cytostome is the only site where phagocytosis takes place. Intracellular compartments in a ciliate from where intracellular bacteria were reported: *1*: inside a food vacuole, *2*: embraced by a peribacterial membrane, *3*: inside the lumen of rER, *4*: inside mitochondria, *5*: associated with mitochondria, *6*: in the cytoplasm, *7*: in the lumen of the nuclear envelope, *8*: inside the macronucleus, *9*: associated with cell cortex structures, and *10*: *inside* the micronucleus, *outer line* = cytoskeleton, *inner line* = fibrillar skeletal elements (e.g., myonemes)**

conjugation. The latter includes meiosis of the micronucleus, resorption of the old macronucleus, and formation of a new macronucleus from the synkaryon that will also give rise to the new micronucleus. Whereas cytoplasmic symbionts may be simply distributed to daughter cells during binary division, endonuclear symbionts may even make use of the nuclear division machinery. Both micronuclear mitosis and macronuclear division are closed: The nuclear envelopes are maintained, keeping nucleoplasm and cytoplasm separated throughout the cell cycle. Endonuclear symbionts (see ❯ "Terms in Symbiosis Research") are therefore caught in the nuclei unless they have developed means of passing through the nuclear envelopes. During conjugation of the host cell, resorption of the old macronucleus may be deleterious to endonuclear symbionts that are not adapted to this process; bacteria are then digested in the process of resorption. New macronuclei developing after conjugation are free of bacteria unless they are infected anew.

In spite of the problems arising for bacteria colonizing nuclei, endonuclear symbionts are frequently found in ciliates because in the nuclei, symbionts should (1) have access to the most complete supply of metabolites, (2) be more assured of distribution/segregation to the daughter cells at cell division, and (3) be protected from cellular defense mechanisms. The last reason is more obvious when the fact that symbionts in nuclei are naked—not encircled by host membranes—and less vulnerable to attacks by lytic enzymes, which would be deleterious to nuclear structures such as chromatin, is considered.

## The History of Symbiont Research in Ciliates

Prokaryotes living in ciliates were first noticed over a century ago by Müller (1856). Rod-shaped structures were observed in the macronuclei and micronuclei of a number of ciliates and, less commonly, in their cytoplasm. In the beginning, it was not clear whether they were parasites or spermatozoa, because the micronucleus was considered to be a testis and the macronucleus an ovary, while chromosome filaments and endonuclear symbionts were mistaken for spermatozoa. This view was corrected by Bütschli (1876), who also wrote the first review on parasites in ciliates (Bütschli 1889). Accounts of early observations of bacteria in protozoa that followed this initial period were reviewed by Kirby (1941), Wichterman (1953), and Ball (1969).

Interest in prokaryotic symbionts of ciliates arose again in the 1950s when it was discovered that a killer phenotype is frequently associated with them. The possibility that certain ciliate strains kill other strains by liberating a toxic agent into the medium was first expressed by Sonneborn (1938), who noted this phenomenon in paramecia during experiments on mating types that involved mixing different strains (see ❯ "The Killer Trait in *Paramecium*"). He found that under certain conditions, conjugation could be brought about between killers and sensitive paramecia so that genetic analysis of these traits became technically feasible. Sonneborn (1943) demonstrated that the killer phenotype was an inherited trait transmitted via cytoplasmic particles, which he named "kappa." His findings aroused great interest among geneticists and other biologists because it furnished one of the first clear examples of a cytoplasmic genetic factor. That kappa was an endosymbiont not known at that time. From data obtained in studies using X-rays, Preer (1948b) determined that kappa was similar in size to bacteria. He subsequently demonstrated the presence of kappa in the cytoplasm of killer paramecia as Feulgen-staining bodies (Preer 1950). In the following years, cytological, biochemical, and physiological studies by a number of workers established that kappa was actually a Gram-negative bacterium. In 1974, it was given a binomial designation—*Caedobacter taeniospiralis*, which has since been changed to *Caedibacter taeniospiralis* (for a detailed review, see Preer et al. 1974, and Preer and Preer 1984).

After the initial discovery of the first killer paramecia, other types were found. Siegel (1953) described "mate killers," whose toxins act only after cell-to-cell contact is made during conjugation, and Schneller (1958) described "rapid-lysis" killers, which may injure sensitives in 10 min and kill them in 30 min, a process that is much more rapid than when kappa mediates killing. Over decades, bacterial symbionts had been studied most extensively in the *Paramecium aurelia* species complex (for reviews, see Beale et al. 1969; Preer et al. 1972, 1974; Preer and Preer 1984; Gibson 1974; and Soldo 1974). They have also been found and studied in other ciliates, particularly in *Paramecium caudatum* (for reviews; Görtz 1983, 2001 ; Görtz and Fokin 2009; Fokin and Görtz 2009; Fujishima 2009) and in *Euplotes* species (see ➲ "Prokaryotic Symbionts of *Euplotes*"). Many cases of simultaneous infections of ciliate cells by two or more different bacteria have been observed (e.g., Görtz 1992; Fokin et al. 2005). In each of the killer paramecia, particles were found that later proved to be prokaryotic symbionts. One only needed to wash the paramecia free of extracellular bacteria, crush the ciliates, and observe the resulting preparations in a phase contrast microscope to ascertain the presence of the symbionts (see ➲ "Identification of Symbionts in *Paramecium*"). This procedure also revealed, however, that some strains carry endosymbionts without showing any kind of killing ability. The symbionts of nonkiller paramecia were named "nu" (Sonneborn et al. 1959).

The presence of endo- and episymbionts of marine and freshwater ciliates living in anaerobic habitats has been generating increasing interest among ecologists and microbiologists. In these habitats, many of the symbionts were identified as methanogenic bacteria (Stumm and Vogels 1989; Fenchel and Finlay 1991a; Finlay and Fenchel 1992; see ➲ "Prokaryotic Symbionts of Ciliates from Anaerobic Environments").

In recent years, it became obvious to find many more prokaryotic symbionts of ciliates once further host species are studied. The description of episymbionts that have a defensive function for their host *Euplotidium* by Rosati, Verni, and colleagues (Rosati et al. 1999) is a fantastic example; see "Prokaryotic Ectosymbionts of *Euplotidium*." (Only recently, these episymbionts have been identified as belonging to the *Verrucomicrobia*.)

## Terms in Symbiosis Research

The following terms are used in this chapter or in the references cited:

Symbiosis—the living together of dissimilarly named organisms. Sometimes, the term is used in a restricted sense in the sense of mutualism.

Mutualism—associations which involve mutual benefit (symbiosis in its restricted sense).

Parasitism—associations in which there is overt exploitation of one associate.

Endosymbiosis—the symbiont lives within its host, intracellular symbiosis.

Episymbiosis—the symbiont lives attached to the surface of its host cell.

Episymbiont—microorganism living attached to the surface of a host cell.

Epibiont—synonymous with episymbiont.

Ectosymbiont—synonymous with episymbiont.

Xenosome—intracellular symbiont in a broad sense.

Endocytobiont—intracellular symbiont in a broad sense of symbiosis.

Epixenosome—episymbiont in a broad sense.

For definitions of the terms, see Henry (1966), Read (1970), Corliss (1985), Margulis and Fester (1991), Sitte (1993), and Rosati (1999).

## Prokaryotic Symbionts of *Paramecium*

With respect to endosymbionts, *Paramecium* is by far the best-studied ciliate genus. In the *Paramecium aurelia* species complex, consisting of 14 sibling species described by and named *P. primaurelia* to *P. quadecaurelia*, many different types of endosymbionts have been discovered. They have been thoroughly reviewed by and descriptions appeared in earlier editions of this book (Preer 1981; Heckmann and Görtz 1991). Valuable information on isolation and identification of symbionts is taken from these earlier editions. In the present edition, descriptions of further endosymbionts were added, and the information about many symbionts has increased owing to the availability of new techniques. Among the symbionts where a wealth of data has been presented are the *Holospora* bacteria. They differ from most of the *Paramecium* symbionts in being infectious and in their ability to invade nuclei for reproduction. The holosporas are being investigated to elucidate the mechanisms that allow a prokaryote to invade a eukaryote. Being relatively large, the holospora can be seen even at relatively low magnification and are therefore used to monitor their route of infection and the changes they undergo after entering a cell.

## Habitat and Biology of the Bacterial Symbionts of *Paramecium*

*Paramecia* are aerobic ciliates from freshwater and brackish water habitats. Many of the paramecia brought in from nature are found to contain symbionts. Usually, there are hundreds and sometimes even thousands of symbionts per paramecium (see TEM Bild Cc23 and 3). Endosymbionts may be found in the micronucleus, macronucleus, perinuclear space, and cytoplasm of paramecia. Different types of symbiont invade different parts of the ciliate cell, and they are, moreover, often adapted to one

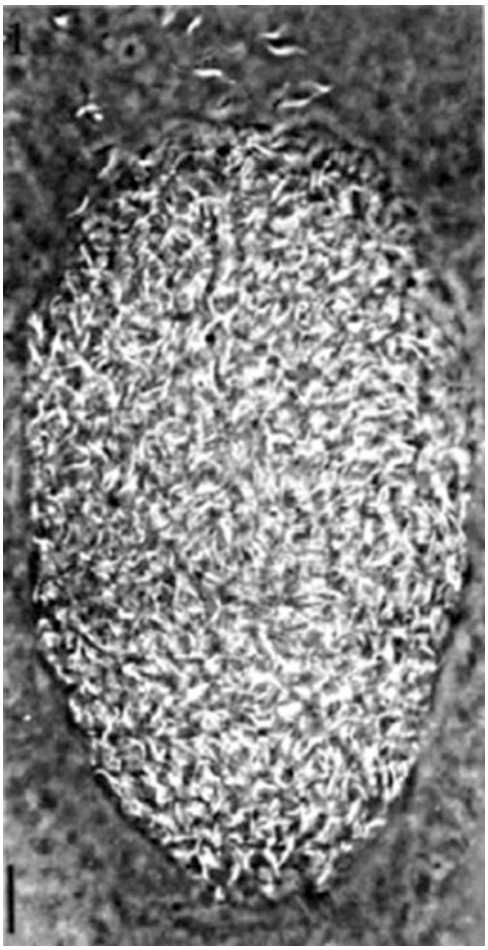


**Fig. 18.2**
**Vegetative macronucleus of *Paramecium biaurelia* stock 562. The spiral endosymbionts filling the macronucleus are cells of *Holospora caryophila*. A few symbionts are also visible in the cytoplasm. Osmium-lacto-orcein preparation. Whole mount, bright phase contrast. Bar = 10 μm (From Preer 1969)**

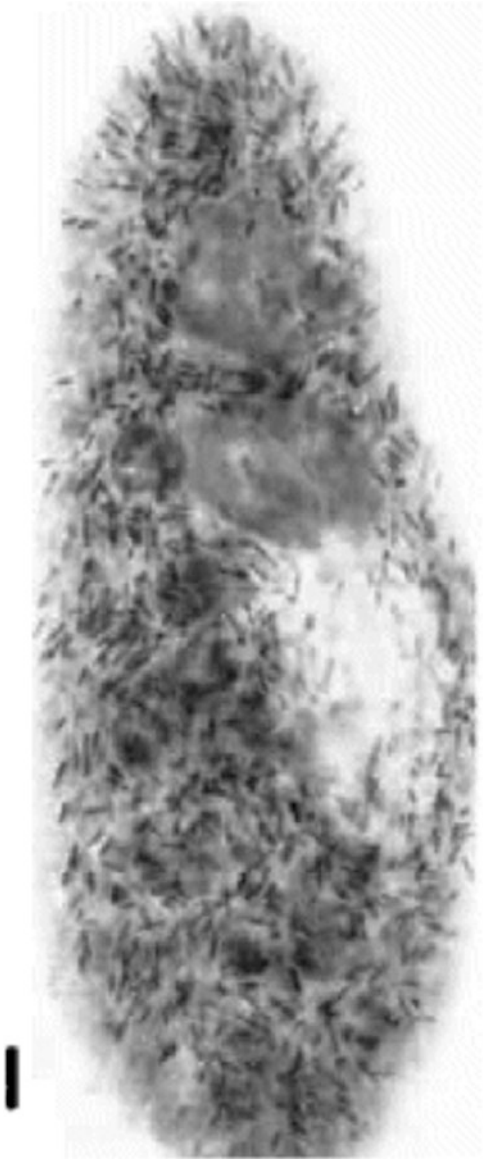


**Fig. 18.3**
***Paramecium tetraurelia* stock 239 bearing endosymbiont *Lyticum flagellatum*, seen as *dark-staining rods* in the cytoplasm. Osmium-lacto-orcein preparation, whole mount, dark phase contrast. Bar = 10 μm (From Preer, 1969)**

*Paramecium* species only. The cell compartment in which a symbiont multiplies and the *Paramecium* species in which multiplication occurs are therefore important taxonomic characters. Though it had been observed that bacterial endosymbionts may prevent secondary infections by another bacterium in laboratory cultures (Görtz 1983), double and even triple infections are found in cells of natural populations (Görtz 1992; Kusch et al. 2000).

Several symbionts have been shown to require the presence of specific *Paramecium* genes for their maintenance (Sonneborn 1943; Siegel 1953; Schneller et al. 1959; Gibson and Beale 1961; Fujishima and Fujita 1985). It is not known whether the genes that assure maintenance of the symbionts are active—e.g., providing the symbionts with some essential metabolite—or whether they are merely inactive alleles, the active ones preventing growth of an "invader." In this context, it should be mentioned that the *P. aurelia* species 3, 7, 9, 10, 11, 12, 13, and 14 have never been found to contain symbionts (Preer and Preer 1984); however, not all species of the *P. aurelia* complex have been studied with the same thoroughness. It has been argued that symbionts profit from living inside a paramecium by being better protected from predation, as compared with free-living species of bacteria, and that symbionts are provided with a convenient and abundant supply of nutrients (Beale et al. 1969). Which metabolites of the host are used, however, is known rarely.

Although many of the symbionts have a smaller genome size than free-living bacteria (Soldo and Godoy 1973a; Hahn et al. 2009) and some of the associations were suggested to be very ancient (Preer 1977), no indications for a transfer of genes from symbiont to host nucleus have been discovered as has been found in the case of mitochondria (Gellissen and Michaelis 1987). Schmidt (1984), studying the association of *Caedibacter varicaedens* with *P. biaurelia*, was unable to obtain evidence for a sharing of the translational systems of host and symbiont. His observations indicate that all major proteins found in *Caedibacter* are synthesized in the symbiont itself.

In only a few cases, it has been demonstrated that host cells may profit from bearing symbionts; Soldo (1963) and Soldo and Godoy (1973b) found that it was not necessary to provide a *Paramecium* stock bearing *Lyticum flagellatum* (formerly called "lambda particles") with folic acid, while the same stock freed of this symbiont required the vitamin. Holtzman (1959) observed that *P. pentaurelia* bearing *Pseudocaedibacter falsus* was more resistant to killer paramecia bearing *Lyticum flagellatum* than *P. pentaurelia* strains that were free of symbionts, and Landis (1981, 1987) showed that under natural conditions, paramecia with killer properties have a selective advantage over nonkillers; see ❯ "The Killer Trait in *Paramecium*." On the other hand, in the laboratory, the symbionts are all dispensable, and many of them are lost when paramecia are cultured for some time. The reason for such loss is in most cases a rapid multiplication of the paramecia, resulting in a dilution of the symbionts and then in their loss. Although there is little information about what occurs in nature, it appears unlikely that reinfection of paramecia that have lost their intracellular bacteria plays a major role for most of the symbionts (Kusch et al. 2001).

Most symbionts of ciliates are not infectious and are propagated in the host population with the divisions of host cells only. An exception to this rule is provided by symbionts of the Holosporaceae. In addition to being propagated with host cell division, they develop forms specialized for infection (infectious forms) that are released and infect new cells upon being taken up with food. Unlike most other endosymbionts, they tend to harm their hosts and can therefore be regarded as parasites, although it cannot be excluded that under certain conditions, the host cells may have advantages from the presence of these symbionts (Görtz 1983). Interactions between *Holospora* and its host *Paramecium* are described in more detail in the section entitled "❯ Biology of the Holosporaceae."

## The Killer Trait in *Paramecium*

Many of the symbionts of *Paramecium* confer on their hosts the ability to produce toxins capable of killing sensitive *Paramecium* strains of the same species and even of other species, if the toxins are liberated into the medium. The toxin producers are called "killers" and their victims "sensitives." If the toxins act only during the period of cell-to-cell contact at conjugation, the toxin producers are called "mate killers." Different killer stocks of *Paramecium* induce different prelethal symptoms in sensitives mixed with killers. These symptoms include spinning, vacuolization, paralysis, formation of aboral humps, and rapid lysis. The symptoms were taken as crucial characters for identification of a symbiont. The value of such symptoms for identification and determination of the symbionts must, however, be doubted; see ❯ "Identification of Symbionts in *Paramecium*." In addition to making their hosts capable of producing toxins, the symbionts also confer upon the host-specific resistance to the toxins produced. When a symbiont is lost from a killer strain, the paramecia lose both toxin production and toxin resistance (Sonneborn 1959).

Killer strains have been reported not only for species of the *P. aurelia* complex but also for *P. caudatum* (Schmidt et al. 1987b, 1988), *P. bursaria* (Chen 1955; Dorner 1957), and *P. polycaryum* (Takayanagi and Hayashi 1964). In the latter two species, however, symbionts were not observed, although it is likely that they were present and were responsible for the killing properties of the paramecia.

The most information on killer bacteria was collected from symbiotic bacterial genus *Caedibacter* (for review see Schrallhammer and Schweikert 2009). A feature unique to the killer symbionts of the genus *Caedibacter* is the ability to produce R bodies. These are proteinaceous ribbons, 20–30 µm long, coiled inside the bacterial cell to form a hollow cylindrical structure (❯ *Figs. 18.4*, ❯ *18.5*, and ❯ *18.6*). This structure has a diameter of about 0.4 µm in all species

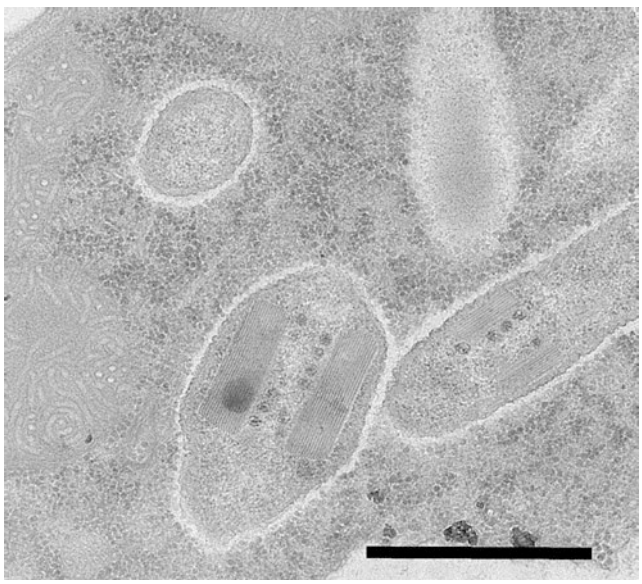


**Fig. 18.4**
**Electron micrograph of longitudinal and cross sections of *Caedibacter varicaedens*, endosymbiont of *Paramecium biaurelia* stock. Longitudinal sections display an R-body each. Note the numerous dark-stained phages inside the coiled R-body. Bar = 1µm. (in courtesy of Anna Steyer)**

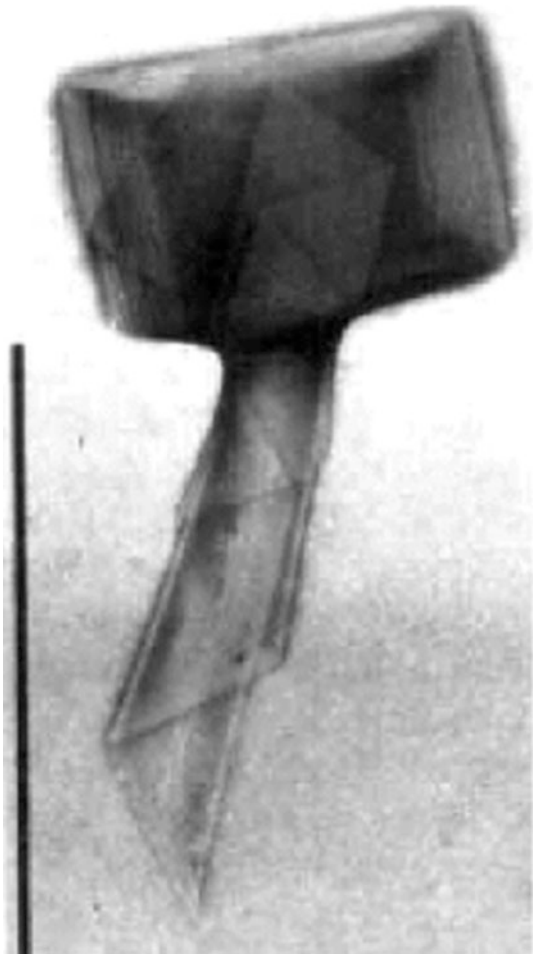


**Fig. 18.5**
**Electron micrograph of an R body isolated from *Caedibacter taeniospiralis* of *Paramecium tetraurelia* stock 51. The R body begins to unroll from the inside. Negative staining with phosphotungstic acid. Bar = 1 µm (From Preer et al. 1972)**

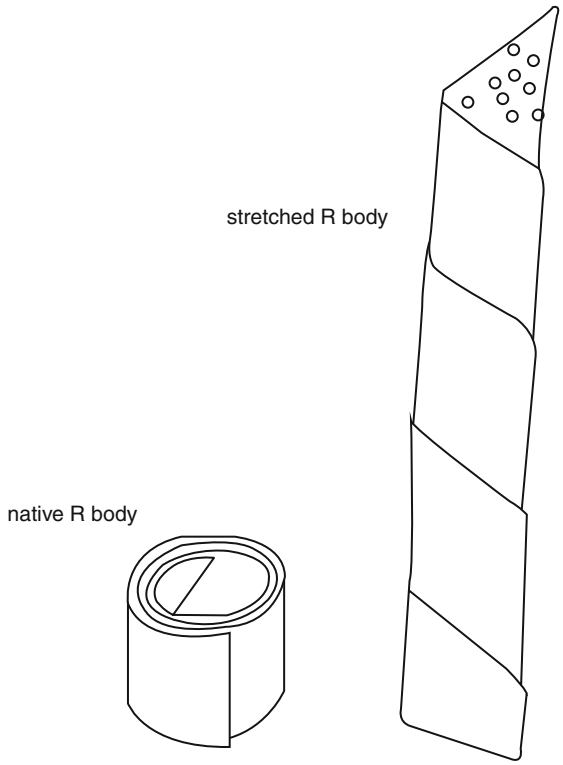



**Fig. 18.6**
**R bodies of *Caedibacter*. R bodies (refractile bodies) are coiled proteinaceous ribbons. By appropriated triggers, such as acid pH, they are induced to unroll**

and is about 0.4 µm long, except in *C. caryophilus* where the R bodies are approximately 0.8 µm in width and length. The R bodies unroll when ingested into a phagosome and also under certain in vitro conditions, e.g., when placed at low pH (Preer et al. 1966; Schrallhammer and Schweikert 2009).

It has been suggested that the R bodies play an important role in the killing mediated by *Caedibacter*. Mueller (1963) and Smith-Sonneborn and Van Wagtendonk (1964) demonstrated that only *Caedibacter* particles that contained R bodies were toxic to sensitive paramecia. In addition, R bodies purified from certain strains of *C. varicaedens* have been shown to be toxic to sensitive paramecia (Preer and Preer 1964; Preer et al. 1972). However, neither the toxin itself nor its mode of action have been identified nor is it known how paramecia are protected from the toxic action of their own symbionts.

Early observations suggested that the genetic determinants of R bodies are plasmids or bacteriophages that have lost the ability to lyse their host cells—the symbiotic bacteria—upon maturation of the virions (Preer and Preer 1967; Preer et al. 1974). Dilts (1976) isolated plasmid DNA from *C. taeniospiralis* 51 and suggested that the extrachromosomal DNA might be the determinant of the R bodies. Further investigation revealed that plasmids are present in all strains of *C. taeniospiralis* and that they are highly homologous as determined by restriction mapping (Quackenbush 1983). Further evidence that the genetic determinant for R-body synthesis resides on the plasmid was presented by Quackenbush and Burbach (1983), who cloned portions of a plasmid and obtained expression of the R-body-encoding sequences in *Escherichia coli*. Analysis of various subclones allowed them to determine the approximate location of the R-body-encoding sequence. The DNA required for type 51 R-body synthesis is about 1.8 kbp in size and has been completely sequenced (see Pond et al. 1989; Heruth et al. (1994)). However, none of the R-body-producing *E. coli* clones was found to be toxic to sensitive paramecia, although function of the R body was successfully tested. The DNA sequence required for toxin production has not yet been located. It is assumed to reside on the plasmid, too (Pond et al. 1989). The R bodies may unroll and destroy phagosomal membranes when ingested, and apparently they play a role in killing mediated by *Caedibacter* (Preer et al. 1974), but their presence is not required for the host cell to resist being killed.

R bodies of some *Caedibacter* species are associated with phage capsids (*Fig. 18.4*). The proteins of these R bodies and perhaps also the toxin are encoded on bacteriophage genomes

(Quackenbush 1988; Schmidt et al. 1988; Pond et al. 1989; Fokin and Görtz 1993). Only *Caedibacter* bearing phages or plasmids may confer the killer trait to their hosts. Heruth et al. (1994) have characterized and sequenced three genes (*rebA*, *rebB*, and *rebC*) for synthesis and assembly of R-body synthesis in *C. taeniospiralis*. These genes are independent transcriptional units on a plasmid. Two polymerization events were found to be involved in R-body assembly: One event requires RebB and RebC; the other requires all three proteins. The RebC protein is apparently involved in posttranslational modifications of RebA and RebB, both of them showing peptide species with different molecular weights. The role of a fourth protein whose gene has not been sequenced remains unknown (Kanabrocki et al. 1986; Heruth et al. 1994).

Dilts and Quackenbush (1986) have provided evidence that R bodies are required for the killing trait to be expressed but not for resistance of the ciliate host to killing mediated by *C. taeniospiralis*. They described a mutant strain of *C. taeniospiralis* 169 that simultaneously lost the ability to produce R bodies and to kill sensitive paramecia but still rendered its host resistant to killing. Investigations of the R-body-encoding plasmid isolated from the mutant revealed that a transposon-like element had been inserted within the R-body-encoding region, thereby eliminating R-body production. Two separate mutational events occurred in the same cell, one inactivating the R-body-encoding sequence and the other inactivating the toxin-encoding sequence. This shows that R bodies are crucial to expression of the killer trait. Their exact role remains, however, unknown. Evidence indicates that their action probably involves delivery of the toxin to the sensitive paramecium and to its target site by unrolling and penetrating the food vacuole membrane (Dilts 1986). Evidence that R bodies are required for killing but not for resistance of the host has also been obtained for *C. caryophilus*. Paramecia bearing mutant *C. caryophilus* with blocked R-body synthesis no longer showed a killer trait but were resistant to the toxin (Schmidt et al. 1988).

The R bodies of other species of the genus were shown to be associated with icosahedral viral capsids (Preer and Jurand 1968; Grimes and Preer 1971). The capsids were in most cases found to contain DNA (Preer et al. 1971). The relationships between genomes of different phages of kappa and between R-body-encoding plasmids and kappaphage genomes have been studied by restriction endonuclease analysis and by DNA-DNA hybridization (Quackenbush 1978; Quackenbush et al. 1986). These studies demonstrate that the R-body-encoding plasmids show little or no homology with kappaphages and that there is also considerable diversity among the kappaphages.

R bodies have also been reported in the free-living bacteria *Pseudomonas taeniospiralis* (Lalucat et al. 1979), *P. avenae* (Wells and Horne 1983), and *Marinomonas mediterranea* (Hernández-Romero et al. 2003). However, with respect to antigenicity, genetic determinants, and other features, these R bodies appear to be unrelated to the R bodies of *Caedibacter* species (Bedingfield et al. 1984; Gibson 1984; Meenaghan et al. 1984; Kanabrocki et al. 1986; Lalucat et al. 1986). Additionally, R bodies have been reported for *Rhodospirillum centenum*, a nonsulfur photosynthetic bacterium (Favinger et al. 1989). Since R bodies are often present at low frequencies, it appears likely that R-body-producing bacteria are more common than previously suspected. With respect to the shape of the ends and the mode of unrolling, the different types of R body vary (for details, see Preer et al. 1974; Quackenbush 1988, and Pond et al. 1989).

## Biology of Holosporaceae

All *Holospora* species known up to the present are residents of the micro- or macronuclei of *Paramecium*; see ❯ "The Ciliate Cell as a Microcosm." The bacteria grow and multiply exclusively in the host nuclei (❯ *Figs. 18.2* and ❯ *18.7*) and have evolved mechanisms to invade nuclei as well as to escape from intact nuclei (Hafkine 1890; Ossipov et al. 1975; Ossipov 1981; Görtz 1983; Fokin and Sabaneyeva 1997; Abamo et al. 2008; Fujishima 2009; Iwatani et al. 2005; Sabaneyeva et al. 2009; Eschbach et al. 2009). To date, two kinds of models for *Holospora*'s escape from the host digestive vacuole had been proposed: a transport vesicle model (❯ *Fig. 18.8*) and a disruption of the digestive vacuole model (Fujishima 2009). *Holospora* species are host specific, infecting only certain species of *Paramecium*, and nucleus specific, infecting only the micronucleus or the macronucleus. Host cells remain vital with *Holospora* infections, and it has been shown that *Holospora*-bearing paramecia are able to grow, divide, and even mate. However, conjugation is unsuccessful, as new macronuclei are apparently not functional (Görtz and Fujishima 1983), and growth and physiological conditions of infected cells may be affected, depending on culture conditions. In starving *Paramecium* or after inhibition of host protein synthesis, most bacteria differentiate into the infectious form (Fujishima 1993; Dohra and Fujishima 1999b). Paramecia seem to have mechanisms to cure themselves from endonuclear symbionts. Namely, in "wrong" hosts, infectious forms are digested having been phagocytosed or, if

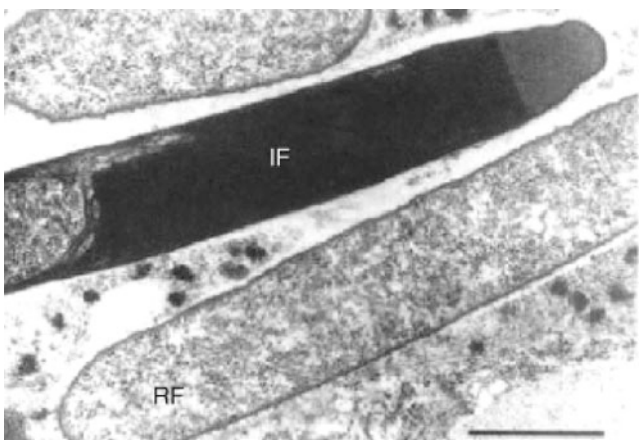



**Fig. 18.7**
***Holospora obtusa* in the macronucleus of *Paramecium caudatum*. *IF* infectious form and *RF* reproductive form. Bar 1 μm (From Görtz et al. 1988)**

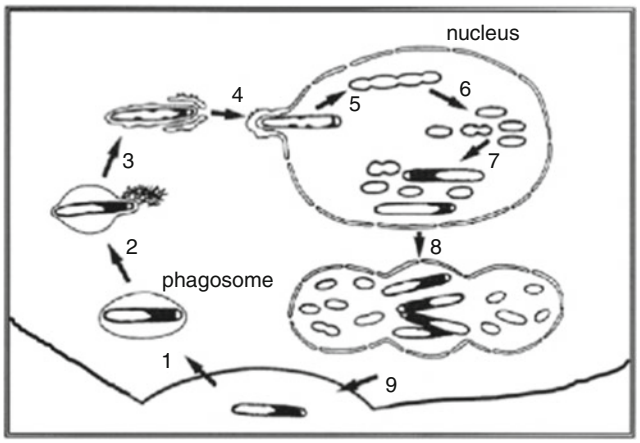



**Fig. 18.8**
**Infection cycle of *Holospora obtusa*. *1* The infectious form of the bacterium (long rod) is ingested by a *Paramecium caudatum* cell into a phagosome (food vacuole). *2* Microfilaments are nucleated at the phagosomal membrane covering the bacterium protruding from the phagosome. *3* The bacterium is sluiced out of the phagosome. It remains encircled by a host membrane that forms a transport vesicle. Later on, the transport vesicle is surrounded by vesicles of endoplasmic reticulum. These vesicles finally form a secondary transport vesicle. *4* While the inner (phagosome-derived) membrane disintegrates, the membranes originating from endoplasmic reticulum fuse with the membranes of the nucleus, and the bacterium is incorporated into the macronucleus. *5* The nucleus of the bacterium constricts at several sites. *6* The bacterium divides into 4–10 short rods. By this multiple division, the reproductive form is established. *7* The reproductive form multiplies by binary fission. Some of the short rods grow longer and differentiate into infectious forms, see "Development of *Holospora*." *8* At the division of the host cell, mature infectious forms are collected in the connecting piece of the dividing nucleus, and *9* are later released from the host cell (Ossipov 1981; Görtz and Wiemann 1989; Wiemann and Görtz 1989)**

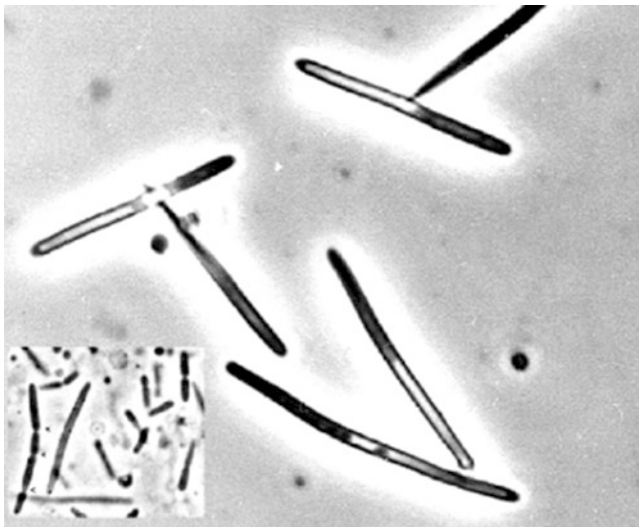


**Fig. 18.9**
**Phase contrast micrograph of *Holospora obtusa* isolated from *Paramecium caudatum*. Note the peculiar appearance of the infectious forms reflecting their fine structure (*Fig. 18.8*). *Inset*: developmental stages of *Holospora obtusa*. On the *left*, a bacterium with three constrictions (*arrowheads*) undergoing multiple divisions is seen**

they enter the nucleus, may later be lysed within the nucleus (Skoblo et al. 1990; Ossipov et al. 1993; Fokin and Skovorodkin 1991, 1997; Fokin et al. 2005). This lysis may be due to an unknown mechanism of cellular defense. After lysis of bacteria in the nucleus, host cells remain vital nevertheless.

Not only the infection cycle but also the development of the Holosporaceae is unique. The bacteria show a developmental cycle with a long infectious form and a short reproductive form (*Fig. 18.9*). It is, however, not just the length that characterizes infectious forms but also their specific organization. Even short forms are infectious (Kawai and Fujishima 2000). The infectious form is unique among bacteria. It is polarly organized and has a voluminous periplasm (*Figs. 18.7*, *18.10*, *18.11*, and *18.12*) that contains a number of stage-specific proteins, some of which appear to be released during the infection process (Görtz and Wiemann 1987; Dohra et al. 1994, 1997; Dohra and Fujishima 1999a; Fujishima et al. 1990a; Wiemann and Görtz 1991; Abamo et al. 2008). The cytoplasm is condensed and located toward one end of the symbiont, while a voluminous periplasmic area is located mainly at the other end. The periplasm consists of fine granular, strongly osmiophilic material. Some less osmiophilic material is located at the end, distal from the cytoplasm. Obviously, the infectious form is physiologically inactive. It is a resting stage that may remain vital and infectious outside host cells for days to weeks. Infectious forms were therefore regarded as spores ("holospores"; Hafkine 1890; Ossipov 1981). The mode of development and reactivation is, however, different from formation and hatching of endospores (Görtz and Wiemann 1989 and Wiemann 1989; *Fig. 18.11*).

When an infective form has been taken up by a paramecium and is on its way into the nucleus, the periplasmic material disappears while the cytoplasm expands (Dohra et al. 1994.; Fujishima et al. 1997) (*Fig. 18.11*). It is assumed that the periplasm of the infectious form contains substances that interact with host membranes during the infection process (Görtz et al. 1988; Görtz and Wiemann 1989). The further development of the infectious form into the short, reproductive form is completed in the nucleus. Induction of the development of infectious forms appears to be activated by specific triggers of host cells. Acidification of the phagosome and possibly also the actions of oxidative radicals and lysosomal enzymes may be such triggers for the development of infectious forms of *Holospora* and may initiate a recognition mechanism depending upon protein-protein interactions between bacterium and host cell (Wiemann and Görtz 1991; Fujishima et al. 1997; Iwatani et al. 2005). Apparently, there is a stepwise interaction between

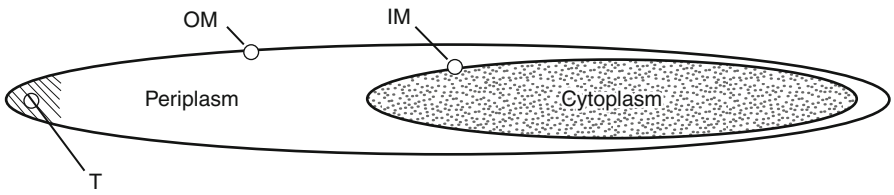



**Fig. 18.10**
**Diagram of infectious form of *Holospora*. The cytoplasm is condensed and occupies only half the volume of the bacterial cell. Accordingly, the periplasm is extremely voluminous. *IM* the inner membrane (cell membrane), *OM* the outer membrane, and *T*, the less electron dense, apically oriented material in the periplasm (special tip)**

invading bacterium and host cell. This is indicated by the observation that the equipment of the invading infectious form with surface proteins changes dramatically (Görtz et al. 1992; Iwatani et al. 2005).

Certain proteins were immunolocalized in the periplasm of the infectious form (◉ *Fig. 18.13*). During the invasion process, some of these proteins were found on the surface of the bacteria after their ingestion into the phagosome, and others were associated with the phagosomal membrane (Görtz et al. 1990; Fujishima et al. 1990b; Dohra et al. 1994, 1997; Wiemann and Görtz 1991). This is what would be expected if such proteins were used for communication with host membranes. Released periplasmic proteins could also protect the bacteria against lysosomal enzymes of the host or inactivate such enzymes. Dohra et al. (1997) have sequenced the gene of a small periplasmic protein of 5.4 kDa. The gene is highly expressed in the intermediate form, a transitional stage in the development from the reproductive into the infectious form of the bacterium. It has been suggested that the protein may function in the recognition process in the early phase of infection. Amino acid sequence similarities with other polypeptides have not been found. Binding experiments of biotinylated macronuclear proteins on blotted *Holospora* proteins had revealed binding to a 50-kDa polypeptide (Ehrsam and Görtz 1999). The 50-kDa protein was immunolocalized in the periplasm and on the surface of *H. obtusa* on sections for light and electron microscopy and may function in the recognition process during infection. Dohra et al. (1998) have identified a GroEL-like protein in *H. obtusa* that is selectively expressed in the reproductive form.

The infectious form differs from the reproductive form not only in morphology but also in the protein patterns produced on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; ◉ *Figs. 18.14* and ◉ *18.15*). Some proteins specific for the infectious form of *H. obtusa* are located in the periplasm, as shown by means of immune electron microscopy (◉ *Fig. 18.15*) using poly- and monoclonal antibodies as probes against these proteins (Wiemann and Görtz 1991; Dohra et al. 1994; Fujishima et al. 1997).

The fine structure of the reproductive forms of species of *Holospora* is that of Gram-negative bacteria. Freeze-fracture studies of *H. obtusa* revealed that the outer membrane of the reproductive form has a density of intramembrane particles (IMPs) that is similar to that of the inner membrane. In contrast to this, the outer membrane of the infectious form was found to contain only very few IMPs (Görtz et al. 1989), which corresponds to the observation that the infectious form shows only a limited number of surface proteins (Görtz et al. 1992). These differences between the two forms correlate with a difference in the behavior of the two forms during the division of the host nuclei. The infectious forms of *H. obtusa* are concentrated in the connecting piece around the separation spindle of the dividing nucleus and are later released from the host cell. Outer membrane of the reproductive forms has high affinity to the host chromatin so that the bacteria are transported to the poles and in this way, get into the daughter nuclei (Ossipov et al. 1975; Ossipov 1981; Görtz and Dieckmann 1980; Wiemann 1989; Wiemann and Görtz 1989). ). Apparently, *H. obtusa* makes use of the division apparatus of the host nucleus for their distribution to the daughter cells.

The physiology of *Holospora* and the triggers needed at different stages of its development are still enigmatic. It appears that during development of the infectious into the reproductive form, protein synthesis of the host cell is needed (Dohra and Fujishima 1999b). It has also been shown that induction of the development of the reproductive form into the infectious form depends upon protein synthesis of the host cell (Fujishima 1993). Freiburg (1985) presented evidence that RNA polymerase of infected macronuclei of *Paramecium* had a 5-fold higher activity than that of uninfected nuclei (Freiburg 1985). Unlike endosymbionts of *Euplotes* species (Fujishima and Heckmann 1983; Heckmann 1983), *Amoeba proteus* (Jeon and Jeon 1976), *Holospora* species is not a necessary endosymbiont for the host's survival. However, *Holospora*-bearing *Paramecium* cells can acquire heat-shock resistance (Hori and Fujishima 2003; Fujishima et al. 2005; Hori et al. 2008) and osmotic-shock resistance (Smurov and Fokin 1999); therefore, *Paramecium* cells become adapted to unsuitable environments from their habitats through *Holospora* species. Furthermore, differential display reverse transcribed PCR shows that *H. obtusa* alters multiple gene expression of the host after establishing endosymbiosis (Nakamura et al. 2004).

## Isolation of Symbiont-Bearing *Paramecium*

Prokaryotic symbionts of *Paramecium* are not free living. They can only be found in their host cells. To investigate the

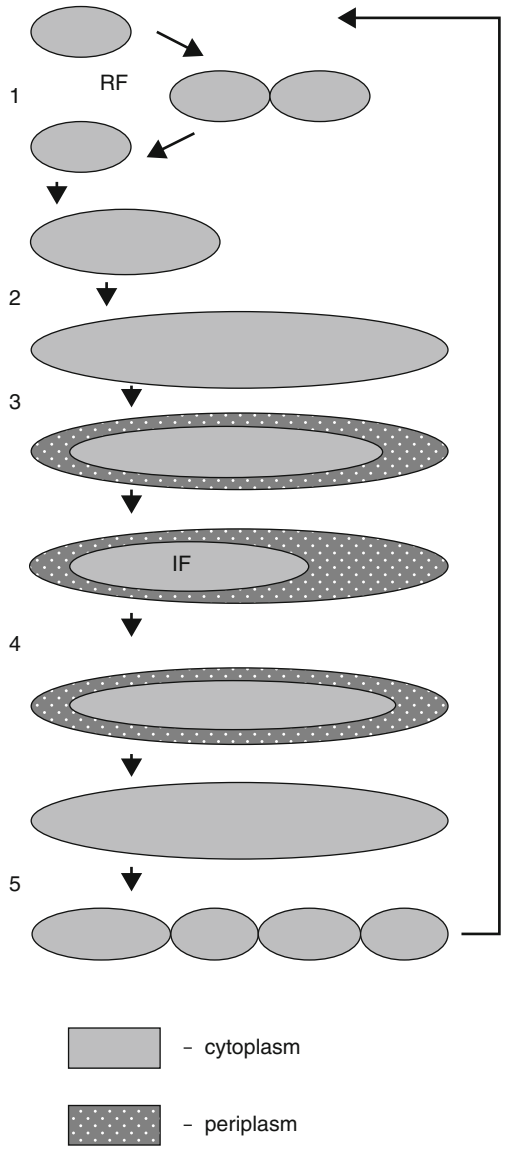



**Fig. 18.11**
**Developmental cycle of *Holospora*. *1* Reproductive forms (*RF*) multiply by binary fission. *2* Some reproductive forms grow longer. *3* After having reached their final length, the cytoplasm condenses and proteins are deposited in the periplasm until the periplasm makes up more than half of the total cell volume. At the end of this differentiation, the infectious form (*IF*) is established. *4* When an infectious form is ingested into a phagosome of a suitable host, it starts to develop into the reproductive form. The cytoplasm expands, while periplasmic proteins are assumed to be secreted. *5* Once the periplasm has almost completely disappeared, the bacterium constricts at several points and undergoes a multiple division. By this division, the reproductive form is established anew**

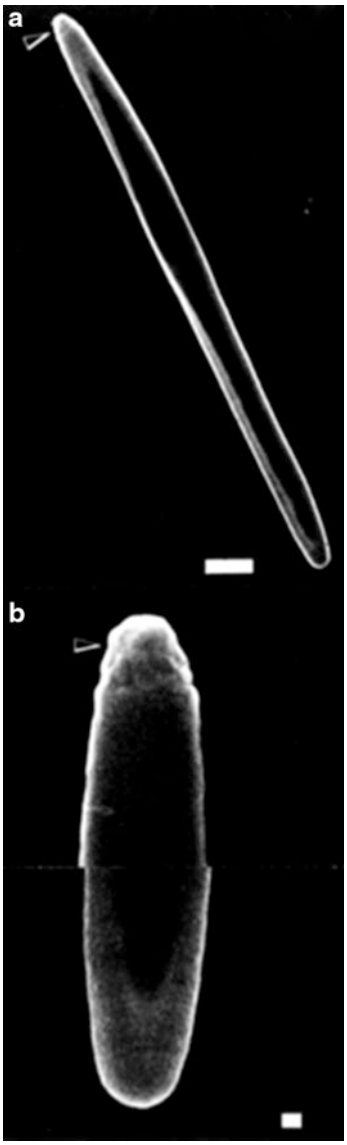



**Fig. 18.12**
**Scanning electron micrograph (SEM) of the isolated infectious form of *Holospora obtusa*. (a) One pole (*arrows*) of the infectious forms appears rough compared to the other pole. Bar = 1 μm. (b) Ends of the bacterium at higher magnification. Bar = 0.1 μm (From Fujishima et al. 1990b)**

symbionts, paramecia have to be isolated from natural habitats. The techniques used in collecting and cultivating paramecia have been described by Sonneborn (1950, 1970). Paramecia are easily detected in samples of pond or lake water and even in rivers with the help of a dissection microscope. For most species of *Paramecium*, the highest abundance is found in the eulittoral or in the microaerobic areas just above the anaerobic layer of detritus on the water level, where the abundance of bacteria and small eukaryotes is high. In the pelagial—the free water body of a lake—the abundance of paramecia may be low. Few species of *Paramecium* are found in brackish water, and no marine species are known (Wichterman 1953; Fokin and Chivilev 1999). *Paramecia* do not form cysts and, unless they have been introduced with the water added, cannot be obtained from infusions of straw or hay. When brought in from nature, the paramecia must be grown slowly at low temperature (about 16 °C) without

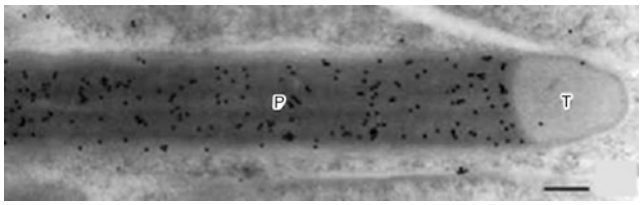



**Fig. 18.13**
**Immunoelectron micrograph of *Holospora obtusa* in the macronucleus of *Paramecium caudatum*, treated with a monoclonal antibody specific for a 39-kDa periplasmic protein of the infectious form. Secondary label with gold anti-mouse IgG. Bar = 0.2 μm (From Dohra et al. 1994)**

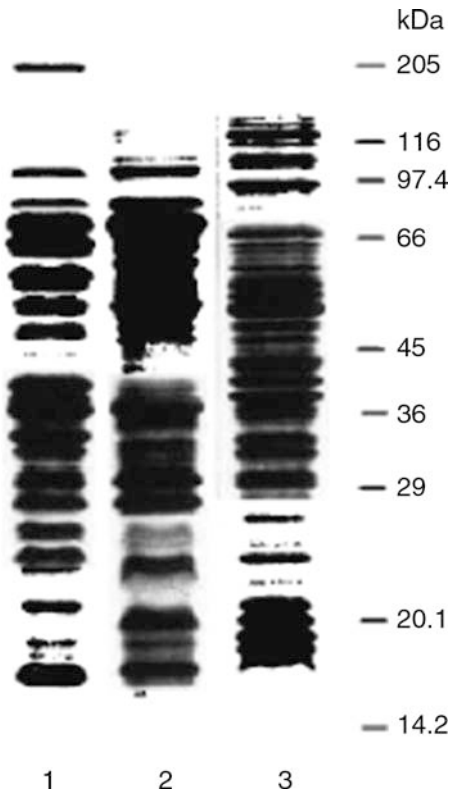



**Fig. 18.14**
**SDS-polyacrylamide gel electrophoresis of total protein of *Holospora obtusa* and *Escherichia coli*. *Lane 1*, *H. obtusa*, infectious form (from $2 \times 10^7$ cells); *lane 2*, *H. obtusa*, reproductive form (from $2 \times 10^8$ cells); and *lane 3*, *E. coli* (from $5 \times 10^7$ cells). The position and size (kDa) of marker proteins are indicated on the *right* (From Görtz et al. 1988)**

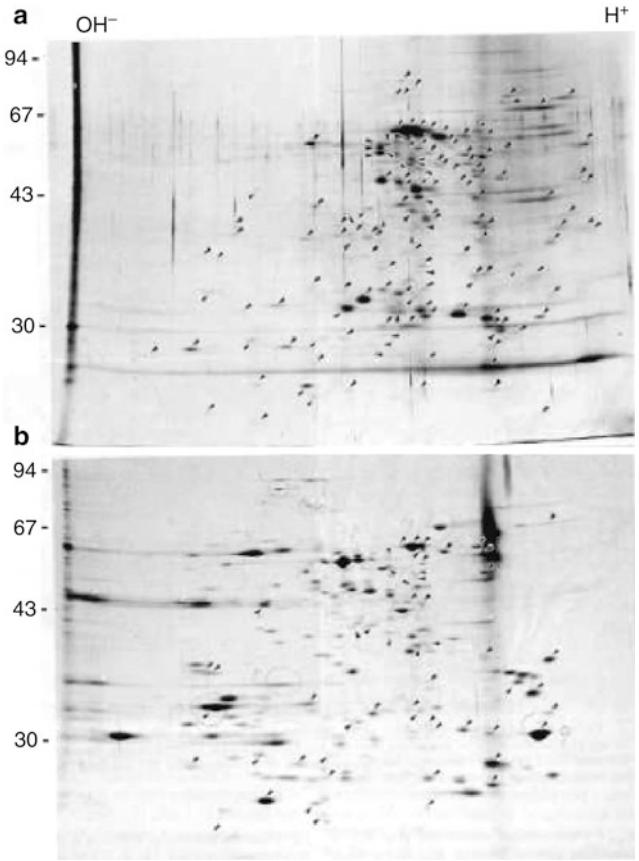



**Fig. 18.15**
**Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels of the reproductive (a) and the infectious (b) forms of *Holospora obtusa*. *Spots* being unique for either of the forms were labeled with *single arrowheads*, and those common to both of the forms were indicated with *white-rimmed arrowheads* (From Fujishima et al. 1990)**

adding any antibiotics, if the purpose is to look for symbionts. Their presence can be monitored as described in the section "Identification of Symbionts in *Paramecium*."

If the paramecia are grown at high fission rates, their symbionts often become diluted and may even be lost. Natural populations of *Paramecium* are infected with symbionts to varying degrees. Most strains carry only one kind of symbiont. A culture containing only one type of symbiont may be obtained, therefore, by isolating a single paramecium and growing it into a clone. If a strain of *Paramecium* does carry more than one type of symbiont, it is often possible to obtain pure cultures by growing the strain at a high fission rate until the symbionts are diluted down to no more than one symbiont per paramecium. Cells isolated at that time and cultured at a low fission rate will grow into populations containing one type of symbiont only. This technique has also been used to separate different types of symbionts (Preer Jr. 1948a).

## Culturing Paramecia with Symbionts

A method of culturing paramecia involves the use of bacterized cereal leaf medium (Sonneborn 1970). For a stock solution, 75 g of dried cereal leaves (a mixture of wheat and barley leaves that were harvested before growth of ears) are boiled for 15 min in 1 l of distilled water. The solution is then filtered, diluted with water (1: 30), buffered with $Na_2HPO_4$, and autoclaved. A day

before use, the autoclaved medium is inoculated with *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Micrococcus luteus*, or another bacterium suitable as food for *Paramecium*. The medium should have a pH of about 7. The growth rate of *Paramecium* can be regulated by varying the temperature and the amount of bacterized medium that is added to a culture.

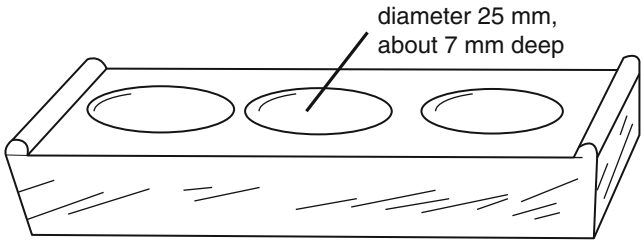
## Other Culture Methods

An even simpler method is to culture paramecia in autoclaved straw or hay infusions. However, this method is less reproducible than the one using cereal leaf medium. For preparing the straw or hay medium, 3–5 g of straw or hay per liter of distilled water is boiled, filtered, and buffered with $Na_2HPO_4$ (pH 6.8–7.0) and then autoclaved. After cooling and a day before use, the medium is inoculated with bacteria.

Unfiltered straw medium, with the straw remaining in the medium, may be used for maintaining stock cultures. In these cultures, paramecia with or without symbionts can be kept for months at low temperatures (6–16 °C) without special care. Another, even safer, method for maintaining stocks is to freeze the paramecia and to keep them in liquid nitrogen, from which they may be recovered with their symbionts on thawing (Simon and Schneller 1973). A great number of *Paramecium* stocks bearing symbionts are maintained in the frozen state at the American Type Culture Collection, Rockville, MD, USA. It appears, however, that some symbionts may be lost after prolonged maintenance in liquid nitrogen.

Some strains of *Paramecium* can also be grown axenically. For sterile growth, the paramecia must first be washed free of bacteria. This is achieved by allowing the paramecia to swim through sterile medium for a time sufficient to permit the bacteria in the food vacuoles to be eliminated (Sonneborn 1950; Van Wagtendonk and Soldo 1970). A simple technique modified from Heatherington (1934) is to place several paramecia at one edge of a hole of a depression slide filled with wash fluid, allow them to swim to the other side, and then to transfer them to a new depression. The procedure should be repeated four times before the cells are left in the fifth wash for an hour. They should then be transferred to a new depression, and this should be repeated hourly for another 4 h. A helpful tool for this method and for the growth of small numbers of paramecia is the so-called three-spot depression slide, also called the "Sonneborn slide." This is a thick slide with three depressions (◉ *Fig. 18.16*). For sterile washing, the paramecia must be taken up each time in a new sterile micropipette with as little fluid as possible; slides and wash fluid have to be sterile. The use of antibiotics to obtain bacteria-free cultures is to be avoided because such substances may harm the symbionts.

A method for culturing paramecia without bacteria involves use of the photoautotrophic alga *Chlamydomonas reinhardtii* as a food organism (Preer et al. 1974). The medium contains 1 g of yeast autolysate, 0.25 g sodium acetate, 0.625 g of cereal leaves, and 0. 125 g of $Na_2HPO_4$ in 1 l of double-distilled water. The medium is dispensed into test tubes, autoclaved, and stored.




three spot depression slide

**◘ Fig. 18.16**
**Three-spot depression slide. Each depression has the volume of about 1.5 ml. The depression slides may be labeled by pencil on the etched sides. Kept in a moist chamber, the slides may be covered by glass plates supported by the elevated edges of the slides**

Before it is used, the medium is inoculated with a small number of *Chlamydomonas* and incubated under light for 2 days. A single bacterium-free paramecium, put into a tube half-filled with this *Chlamydomonas* medium, usually will multiply and ingest most of the algae within 3–4 days.

Most of the axenic media used for growing paramecia are based on a recipe initially designed by Soldo et al. (1966). Recent modifications by Thiele et al. (1980) and Schönefeld et al. (1986) have proved particularly valuable for large-scale cultures of *P. tetraurelia*. A modification (Soldo 1987) that should be capable of supporting the axenic growth of paramecia and many other ciliates of freshwater and marine origin is shown below.

Medium for Axenic Growth of Marine and Freshwater Ciliates (Soldo 1987)

| | |
|---|---|
| Proteose peptone | 10.00 g |
| Trypticase | 5.0 g |
| Yeast nucleic acid | 1.0 g |
| Biopterin | 0.5 mg |
| Folic acid | 0.5 mg |
| Nicotinamide | 2.5 mg |
| D-Pantothenate, Ca | 7.5 mg |
| Pyridoxal hydrochloride | 2.5 mg |
| Riboflavin | 2.5 mg |
| Thiamine hydrochloride | 0.01 mg |
| DL-Thioctic acid | 0.01 mg |
| Phospholipid (oleate-containing) | 250 mg |
| Stigmasterol | 2 mg |
| Distilled water (or seawater) | 1 l |

The medium is prepared in distilled water for freshwater ciliates and in seawater (density 1.015–1.026 g/ml) for marine forms. The final pH is 7.2. Stigmasterol is added from a stock solution (0.5 g of stigmasterol dissolved in 100 ml of absolute ethanol, stored at 4 °C in a tightly capped plastic bottle) by injection into the culture medium from a syringe.

It is important to transfer paramecia gradually from a bacterized to an axenic medium; the ciliates need to be allowed to adapt slowly. Several procedures have been proposed for this transfer (see, e.g., Van Wagtendonk and Soldo 1970, and Fok and Allen 1979). An adapting medium (called "VS medium") based on that of Allen and Nerad (1978) is one containing all the vitamins of the axenic medium given above plus stigmasterol, but not the other components, the pH being adjusted to 7.0. In VS medium, bacteria (e.g., *Klebsiella pneumoniae* previously grown in a tryptone medium) are suspended and adjusted to $OD_{590}$ = 3.0 by dilution. The bacteria are then dispensed in 1 ml portions in screw-cap tubes and placed in a deep freeze. The final medium is prepared, about a week's supply at a time, by adding 1 ml of the frozen bacterized VS medium to 9 ml of unbacterized VS medium. This medium is autoclaved and inoculated with paramecia. The protozoa grow in this medium at a rate of 1/2–2 fissions per day. Not all stocks of paramecium can be adapted to an axenic medium. Furthermore, some media were found to support growth of paramecia, but the latter were not able to maintain their symbionts under these conditions.

## Purification of Symbionts from *Paramecium*

The purification of symbionts from paramecia (i.e., separation from host cell material) has been achieved in a number of ways: passage of cell homogenates through ion-exchange cellulose columns (Smith 1961; Mueller 1963), through filter paper columns (Preer et al. 1966), and centrifugation (Soldo et al. 1970) have all been used. For many symbionts, Percoll gradient centrifugation proved to be the method of choice (Görtz et al. 1988; Fujishima et al. 1990a). Depending on the type of symbionts, different methods of purification may be necessary. For example, the R-body-containing *Caedibacter caryophila* can best be isolated with the help of a discontinuous 70 % Percoll gradient, whereas isolation of *Caedibacter caryophila* containing no R bodies is better achieved by means of an ECTEOLA (anion exchanger) column (Schmidt et al. 1987c, 1988). For the isolation of holosporas, two different approaches have been followed. One method starts with homogenization of the host cells, and the other involves separation of the macronuclei from the cytoplasm and then isolation of the symbionts. The latter method involving prior isolation of nuclei (Freiburg 1985) proved especially useful for preparing clean reproductive forms of the symbionts because it avoids contamination of the bacteria with food vacuoles. For isolation of nuclei, the paramecia are lysed in a buffer containing 10-mM Tris, pH 7.9, 0.25-M sucrose, 3 mM-$CaCl_2$, 8 mM-$MgCl_2$ plus 0.1-mM phenylmethylsulfonyl fluoride, 0.1 % (w/v) spermidine, and 0.2 % Nonidet P-40 by gently stirring the cells in an ice bath and subsequently passing the suspension 5–10 times through a 20-ml pipette. The nuclei are concentrated on a cushion of 1.6 M sucrose by spinning for 10 min at 700*g*. The purified nuclei are then homogenized in a Mg-free buffer, and the bacteria are pelleted. Two different forms of the bacteria, the infectious and reproductive forms, can be separated by sedimentation. The infectious form of holosporas sediments at 350*g* within 10 min, while the reproductive form remains in the supernatant (Görtz et al. 1988).

Protocols for isolating holosporas directly from cell homogenates have been published by Fujishima and Nagahara (1984b), Schmidt et al. (1987a), Görtz et al. (1988), Fujishima et al. (1990a), and Kawai and Fujishima (2000). Infected cells are homogenized in sodium phosphate buffer with a hand homogenizer. The Teflon pestle should fit tightly. The homogenate is then centrifuged at about 3,000*g* for 10 min. The pellet is resuspended in buffer and centrifuged in a preformed continuous gradient of 70 % Percoll for 12 min at 40,000*g*. To maintain the gradient, it is advisable to use a centrifuge with an acceleration rate control. For *H. obtusa*, *H. elegans*, *H. undulata*, *and H. recra*, the infectious forms then concentrate in a sharp band that is usually uncontaminated with food bacteria and cell debris (microscopical control). The reproductive forms do not form a band in a continuous Percoll gradient. These forms can be obtained with the help of a discontinuous gradient (at 10,000*g*) where—depending on the species—they form a layer above ca. 60 % Percoll. Most of the cell organelles of *P. caudatum* do not enter at the 50 % Percoll step.

The isolation procedures described here have been helpful for studying ultrastructural details and protein composition of symbionts, their R bodies, and their DNA. Attempts to culture *Holospora* endosymbionts outside their hosts have been unsuccessful, as has been the case for other symbionts of aerobic ciliates.

## Identification of the *Paramecium* Symbionts

Most of the endosymbionts are easy to observe. Paramecia are collected from ponds, lakes, or rivers and brought into the laboratory. With the help of a micropipette, a few cells are placed on a microscope slide, covered with a cover glass, and then observed with a light microscope using phase contrast optics. Crushing of the paramecia is sometimes necessary (Preer and Stark 1953). The classification of the endosymbionts of *Paramecium* is traditionally based on the following characteristics: morphology and killing activity, the species in which they live, DNA base ratios calculated from the buoyant density (Bd) or from thermal denaturation profiles ($T_m$), and interstrain DNA/DNA hybridizations. Type strains, which have been deposited at the American Type Culture Collection (ATCC), are listed below by their ATCC numbers. Some type strains have also been deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, St. Petersburg State University, Russia, and can be obtained from that source.

Since endosymbionts do not multiply outside their hosts, characterization based on metabolism and growth, as is customary for bacteria, is not possible. Instead, morphological and biological features and nowadays, sequencing of ribosomal genes after PCR amplification and classification by fluorescence in situ hybridization (FISH) using specific oligonucleotide probes complementary to ribosomal sequences (Fig. 18.17) unique for different taxonomic levels (from species to universal for all bacteria) have become powerful tools for classification of

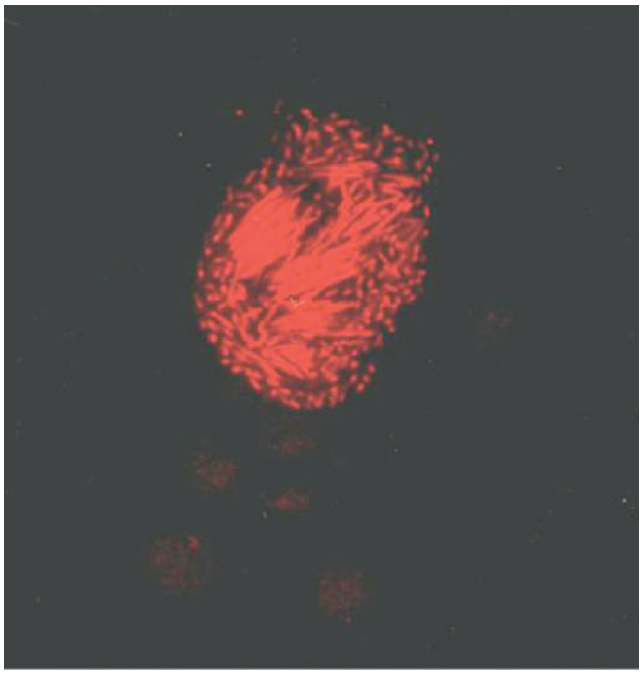


**Fig. 18.17**
**Fluorescence in situ hybridization (FISH) using an oligonucleotide probe specific for *Holospora obtusa*. *Short* and *long rods* of holosporas in the host macronucleus are labeled. Faintly, phagosomes with prey bacteria are visible (From Fokin et al. 1996)**

uncultured intracellular bacteria (Amann et al. 1991, 1995). Nonetheless, detection and sometimes a preliminary identification are still possible with the help of a light microscope equipped with phase contrast, namely, after staining with orcein (Beale and Jurand 1966; see "Orcein Staining of Intracellular Bacteria").

In addition to microscopic observation, killer tests should be performed when a stock is brought in from nature. Standard sensitives (e.g., cells of stock 152 of *P. triaurelia*) may be employed in such tests. Equal volumes of the culture to be tested and a culture of sensitive cells are mixed in a depression slide and observed with appropriate controls for prelethal effects. Mate killing can only be detected by mating sensitive cells with symbiont bearers. Any symbiont-free strain that will mate with an unknown strain is usually adequate. The mode of killing and the features of R bodies (if present), i.e., shape of the ends and the mode of unrolling of the different types of R body (*Fig. 18.6*), have been used for classification and determination of killer symbionts (for details, see Preer et al. 1974; Quackenbush 1988, and Pond et al. 1989). It has now become evident that R bodies and their genetic basis, plasmids or phages, are not adequate for classification of their bacterial hosts (Beier et al. 2002).

In contrast to the killer trait, other biological features may still be of great value for the classification and determination of certain symbiotic bacteria. Such features may be the high infectivity, unique life cycle, and also unique morphology of the infectious forms of the species of the genus *Holospora*; see "Biology of the Holosporaceae." These bacteria are host specific as well as nucleus specific, i.e., they infect only one type of nucleus, either the micronuclei or the macronuclei (Ossipov 1973; Ossipov et al. 1975; *Fig. 18.1*; see The Ciliate Cell as a Microcosm). *Holospora* bacteria establish short, reproductive forms that undergo binary fission as well as long, infective forms that leave the paramecium and infect others very efficiently (Ossipov and Podlipaev 1977; Wiemann and Görtz 1989; Wiemann 1989; Fujishima et al. 1990b; *Figs. 18.9*, *18.10*, and *18.11*). Namely, the infectious forms have a structure unique among bacteria, and their structure may well be used for identification.

## Orcein Staining of Intracellular Bacteria

A very useful staining technique that allows observations of intracellular bacteria in regular bright field and/or phase contrast is given by Beale and Jurand (1966): Paramecia are placed in a small drop on a slide, and as much of the fluid as possible is withdrawn by a micropipette or filter paper. The paramecia are lightly fixed by exposure to $OsO_4$ vapor for 6–10 s and immediately stained with a small drop of lacto-aceto-orcein (1 g of orcein dissolved in 25 ml of hot 45 % acetic acid, mixed with 25 ml of lactic acid, diluted with water 1:1, and then filtered). A coverslip is placed over the drop of stained paramecia and is lightly pressed down, flattening but not disrupting the cells. The preparations can then be observed with a 100X oil immersion objective. To remove lipids that sometimes obscure observation of endosymbionts, the paramecia can be treated with a drop of acetone or a 3:1 mixture of ethanol and acetic acid before staining. Orcein-stained preparations can be made permanent by either applying Vaseline around the edges of the coverslip or by the following procedure. The slide with the orcein preparation and coverslip is rapidly cooled in liquid nitrogen. The slide is immediately lifted off the coverslip with a razor blade. Cells will remain on the slide. The preparation is dehydrated by dipping it briefly into 50 %, 70 %, and absolute ethanol and adding a small drop of Euparal or other ethanol-soluble resins. The resin layer between slide and coverslip should be extremely thin for optimal optical conditions.

## The Taxa of Prokaryotic Symbionts of *Paramecium*

### Genus *Caedibacter*

Cells are straight rods or coccobacilli, 0.4–1.0 µm in diameter and 1.0–4.0 µm in length (Preer and Preer 1982). Usually less than 10 % but sometimes up to 50 % of the symbionts contain refractile (R) bodies (*Figs. 18.4*, *18.5*, and *18.6*). Cells containing R bodies are usually wider and longer than those that do not. In addition to R bodies, many spherical phage-like structures or covalently closed circular DNA plasmids are present in the central lumen of the cylinder formed by the structure. The symbionts are Gram negative and nonmotile.

The G + C content of the DNA is 40–44 mol%. Earlier work of this extensively studied genus has been reviewed by Preer et al. 1974; Preer and Preer 1984; and Pond et al. 1989 and recently Schrallhammer and Schweikert 2009.

An analysis of the 16S rDNA of *Caedibacter caryophilus*, a killer symbiont dwelling in the macronucleus of *Paramecium caudatum*, revealed an unusual insertion of 194 bp that was not present in mature 16S rRNA (Springer et al. 1993). It was shown that *C. caryophila* contained fragmented 16S rRNA. Insertion of more than 100 bp has been reported for bacterial 23S rDNAs (Roller et al. 1992). Comparable intervening sequences in 16S rRNAs have not been described in free-living or intracellular bacteria. Results obtained by Beier et al. (2002) give evidence that the genus *Caedibacter* is polyphyletic. Apparently, R bodies and their genetic basis, plasmids or phages, are not adequate for classification of their bacterial hosts. The following species have been described:

#### *Caedibacter taeniospiralis*

Cells are rods, 0.4–0.7 µm in diameter and 1.0–2.5 µm long (Preer and Preer 1982). The G + C content is 41 mol%. They are found in the cytoplasm of *P. tetraurelia* only. Their R bodies (*Fig. 18.5*) unroll from the inside and contain plasmids. Ingestion of R-body-containing symbionts by sensitive paramecia results in the development of clear small blisters on their surface in 2–3 h. Between 4 and 5 h, a bulge develops first in the posterior part of the oral side and later moves to the posterior aboral side. The position of the bulge gave rise to the designation "hump killer." Death takes place in 1–2 days, the corpses remaining intact for some time. *Caedibacter taeniospiralis* of *P. tetraurelia* stock 51K has been affiliated to γ-subgroup of Proteobacteria according to its ribosomal gene sequences (Beier et al. 2002). The type strain is found in ATCC strain 30632 (stock 51 of *P. tetraurelia*; see Preer et al. 1974).

#### *Caedibacter varicaedens*

The cells are rods, 0.4–1.9 µm in diameter and 2.0–4.0 µm long (Quackenbush 1982). The G + C content of the DNA is 40–41 mol%. They are found in the cytoplasm of *P. biaurelia*. Different strains cause vacuolization, paralysis, or rapid reverse rotation while swimming (spin-killing) of sensitive cells. After 4–6 h of swimming, vigorous rotation to the right becomes nearly uninterrupted and then becomes slower. R bodies (*Fig. 18.4*) unroll from the outside. The outer terminus of the unrolled R body is blunt. The R body is usually associated with bacteriophage capsids. The type strain is found in ATCC 30637 (stock 7 of *P. biaurelia*; see Preer et al. 1974).

#### *Caedibacter pseudomutans*

These are cigar-shaped rods, approximately 0.5 µm in diameter and 1.5 µm long (Quackenbush 1982). The G + C content is 44 mol%. They are found in the cytoplasm of *P. tetraurelia* and cause rapid reverse rotations of sensitive paramecia while swimming (spin-killers). The R bodies are of the *C. varicaedens* type. The type strain is found in ATCC strain 30633 (strain 51 ml of *P. tetraurelia*; see Preer et al. 1974).

#### *Caedibacter paraconjugatus*

Cells are small rods and contain phage-like structures (Quackenbush 1982). Less than 1 % of the symbionts contain R bodies of the *C. varicaedens* type. They are found in the cytoplasm of *P. biaurelia*. Ingestion of symbionts by sensitive strains does not produce any observable toxic effect. Cell-to-cell contact between host and sensitive paramecia is required for toxic effects to be observed in the sensitive paramecia (mate killers). The type strain is found in ATCC strain 30638 (stock 570 of *P. biaurelia*; see Preer et al. 1974).

#### *Caedibacter caryophilus*

Cells are rods, 0.4–0.7 µm wide and up to 2.5 µm long (Schmidt et al. 1987b; Euzeby 1997). Those with R bodies are larger than those without R bodies. The G + C content is 34.6 mol%. They are found in the macronucleus of *P. caudatum*. The R bodies unroll from the inside (with blunt outer terminus and sharp inner terminus) and are associated with phages. Width of R bodies after isolation is 0.8 µm. Sensitive strains are killed by paralysis. Coinfections with *Holospora obtusa*, *H. undulata*, and *H. elegans* may occur in natural populations (H.-D. Görtz, unpublished observations). According to its ribosomal gene sequences, *C. caryophilus* has been affiliated to the α-subgroup of Proteobacteria, the closest relative being *Holospora obtusa* (Springer et al. 1993). The type strain is found in ATCC strain 50168 (clone C221 of *P. caudatum*; see Schmidt et al. 1987b).

### Genus *Pseudocaedibacter*

Cells are rods, 0.25–0.7 µm in diameter and 0.5–4.0 µm long (Quackenbush 1982). They do not produce R-body-containing cells. The symbionts are Gram negative and nonmotile. The G + C content of the DNA is 35–39 mol%. The genus includes some species that confer a killer trait on their hosts, some that render them mate killers, and others that do not produce any killing ability. Four species have been described:

#### *Pseudocaedibacter conjugatus*

Formerly called mu particles, these cells are rods, 0.3–0.5 µm in diameter and 1.0–4.0 µm long (Quackenbush 1982). The G + C content of the DNA is 35–37 mol%. They are found in the cytoplasm of *P. primaurelia* and *P. octaurelia* where a mate-killer phenotype of the hosts is produced. The type strain is found in ATCC strain 30796 (stock 540 of *P. primaurelia*; see Preer et al. 1974).

#### *Pseudocaedibacter minutus*

Formerly known as "gamma particles," these cells are rods, often double but also single rods 0.25–0.35 µm in diameter and 0.5–1 µm long (Quackenbush 1982). The G + C content of the DNA is 38 mol%. They are found in the cytoplasm of *P. octaurelia*. In the host cell, the symbiont is surrounded by an extra set of membranes, apparently continuous with the endoplasmic reticulum of the host. The paramecia which bear these symbionts are very strong killers that cause sensitives to develop vacuoles, swell,

finally become spherical, and lyse. Death occurs after about 8 h. The type strain is found in ATCC strain 30699 (stock 214 of *P. octaurelia*; see Preer et al. 1974).

#### *Pseudocaedibacter falsus*

Formerly called "nu" and "pi particles," these cells are rods, 0.4–0.7 μm in diameter and 1.0–1.5 μm long. The G + C content of the DNA is 36 mol% (Quackenbush 1982). They have no known toxic actions and are found in the cytoplasm of *P. biaurelia*, *P. tetraurelia*, and *P. pentaurelia*. The type strain is found in ATCC strain 30640 (stock 1010 of *P. biaurelia*; see Preer et al. 1974).

#### *Pseudocaedibacter glomeratus*

These are small rods, about 0.3 μm wide and up to 1.2 μm long (Fokin and Ossipov 1986). They are found in the cytoplasm of *P. pentaurelia* and have no known toxic actions. Symbionts are individually enclosed in vacuoles which are tightly associated with the endoplasmic reticulum. The type strain is strain Bp 171 of *P. pentaurelia* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, St. Petersburg State University, Russia).

#### *Caedobacter chlorellopellens*

This organism, a symbiont living in the cytoplasm of *P. bursaria* and found to be antagonistic to symbiotic chlorellae, was named "*Caedobacter chlorellopellens*" by Skoblo et al. (1985), who did not know that the genus name *Caedobacter* had been changed to *Caedibacter*. *Caedibacter* was then restricted to symbionts that produce R bodies and confer killer traits upon their host paramecia (Preer and Preer 1982). The symbiont in *P. bursaria* is egg shaped, rarely rod shaped, 0.35 μm wide and up to 1.4 μm long. Since no R bodies were observed and the cells have no known toxic actions on sensitives, placement of the species in the genus *Pseudocaedibacter* seems more plausible. A renomination of the species seems necessary but should be done only after ribosomal sequences have been obtained.

## Genus *Lyticum*

Cells are large rods, 0.6–0.8 μm in diameter and 3.0–5.0 μm long (Preer and Preer 1982). They resemble bacilli in general appearance and bear numerous peritrichous flagella (◉ *Figs. 18.3* and ◉ *18.18*) but are not obviously motile. They are enclosed in vacuoles in the cytoplasm of their hosts. They do not contain R bodies. The G + C content is 27 mol% in one type and 45–49 mol% in another type. The symbionts are Gram negative. They produce toxins that kill sensitive paramecia by lysing them within 10–20 min (rapid-lysis killing). Two species have been described:

#### *Lyticum flagellatum*

Formerly known as "lambda particles," these cells are straight rods, 0.6–0.8 μm in diameter and 2.0–4.0 μm long (◉ *Figs. 18.3* and ◉ *18.18*) (Preer and Preer 1982). The G + C content of the DNA is 27 mol% in one strain and 49 mol% in another. They are found in the cytoplasm of *P. tetraurelia* and *P. octaurelia*. The type strain is found in ATTC strain 30700 (stock 299 of *P. octaurelia*; see Preer et al. 1974).

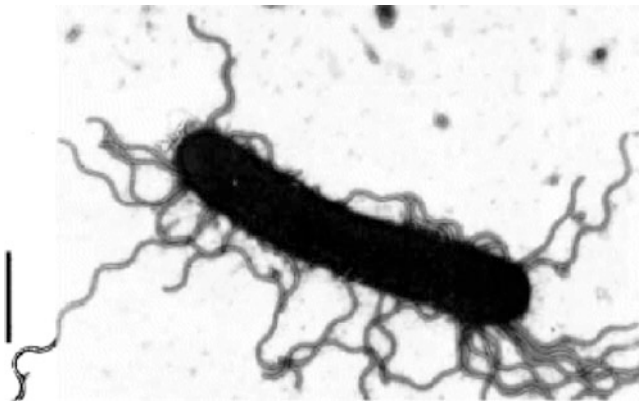


**Fig. 18.18**
**Electron micrograph of *Lyticum flagellatum* of *Paramecium octaurelia* stock 327. Negative staining with phosphotungstic acid. Bar = 1 μm (From Preer et al. 1974a)**

#### *Lyticum sinuosum*

Formerly, these were called "sigma particles" (Preer and Preer 1982). They are curved or spiral rods, 0.7–0.9 μm in diameter and 2.0–10 μm long, sometimes forming chains of 2–3 cells. The G + C content is 45 mol%. They are found in the cytoplasm of *P. biaurelia*. The type strain is found in ATCC strain 30696 (stock 114 of *P. biaurelia*; see Preer et al. 1974).

## Genus *Pseudolyticum*

These are large symbionts with numerous flagella (Boss et al. 1987). No motility has been observed. They may contain refractile bodies consisting of polyhydroxybutyric acid. No killer activity and no infectivity could be detected. Only one species is known:

#### *Pseudolyticum multiflagellatum*

Cells are straight rods, 1.0–2.0 μm in diameter and 3.5–14 μm long (Boss et al. 1987). They are found in the cytoplasm of *P. caudatum*. The symbionts are individually enclosed in vacuoles. The membrane of these vacuoles forms numerous projections that are continuous with the endoplasmic reticulum. No toxic actions are known.

## Genus *Tectibacter*

The species of this genus are distinguished from other symbionts by a layer of electron-dense material surrounding the outer of

the two membranes (Preer and Preer 1982). The symbionts have sparse peritrichous flagella and show slight motility on occasion. They appear not to be toxic. For a more detailed description, see Preer et al. (1974). Only one species has been described:

#### *Tectibacter vulgaris*

Formerly known as delta particles, these cells are straight rods, 0.4–0.7 µm in diameter and 1.0–2.0 µm long, and are Gram negative (Preer and Preer 1982). Hosts include the following: *P. primaurelia*, *P. biaurelia*, *P. tetraurelia*, *P. sexaurelia*, and *P. octaurelia*. The type strain is found in ATCC strain 30697 (stock 225 of *P. sexaurelia*; see Preer 1974a).

## Genus *Nonospora*

These rodlike symbionts live in the macronucleus (Fokin et al. 1987a). Flagella have not been observed. No toxic actions are known. The symbionts are retained in macronuclear fragments of exconjugants and enter macronuclear anlagen by fusion of old fragments with the anlagen (Fokin et al. 1987a). Only one species has been described:

#### *Nonospora macronucleata*

These symbionts are rodlike, 0.2–0.3 µm in diameter and mostly about 1.0 µm long, sometimes forming chains up to 10 µm long (Fokin et al. 1987a; Demar-Gervais 1976). The surface of the symbionts appears irregularly wavy in the electron microscope. They are found in the macronucleus of *P. caudatum*, often clustered in the center of the nucleus.

## Genus *Holospora*

Symbionts of this genus live in the micronucleus or the macronucleus (see The Ciliate Cell as a Microcosm) of *Paramecium* and exist in two forms: a short rod 1.0–3.0 µm long and 0.5–1.0 µm wide that can replicate; and a long form 5.0–20 µm long and 0.8–1 µm wide that cannot replicate (*Figs. 18.9–18.11*) (Gromov and Ossipov 1981; Hafkine 1890). The long form is released from the host and can infect other paramecia (*Fig. 18.8*). The infective form is differentiated into a refractile portion with a less electron-dense, pale tip, and a posterior part that contains typical bacterial cytoplasm, stains with DNA-specific dyes, and appears dark in phase contrast (*Fig. 18.9*). Nine species have been described (see *Table 18.1*): *Holospora undulata*, *H. obtusa*, *H. elegans*, *H. acuminata*, *H. caryophila*, *H. recta*, *H. curviuscula*, *H. bacillata*, and *H. curvata*.

The striking similarity of the biology and cytology of the different *Holospora* species led to their classification in one genus, although a comparison of the protein patterns of *H. obtusa* and *H. elegans* revealed great differences. Moreover, the hybridization of the total DNAs and a comparison of DNA banding patterns after digestion with restriction enzymes indicate that the two species may not be as closely related as originally assumed (Schmidt et al. 1987a). The differences at the molecular level may suggest that the genus *Holospora* has coevolved with ciliates for a long time.

*Holospora* were the first intracellular bacteria in *Paramecium* for which the phylogenetic position was determined (Amann et al. 1991). *Holospora obtusa*, *H. elegans*, and *H. undulata* belong to the α-group of Proteobacteria. The closest relative among other symbionts in ciliates was found to be *Caedibacter caryophilus*, and the closest relatives among other bacteria found up to now are *Rickettsia* and *Ehrlichia* (Amann et al. 1991; Springer et al. 1993). It is tempting to regard the striking biology (developmental cycle, host specificity for *Paramecium*, etc.) and the unique morphology of the infectious form as homologous features proving the close relationship and monophyletic origin of these bacteria. New observations, however, cast doubt on this possibility (Fokin et al. 1996).

The behavior of the infectious forms of certain *Holospora* species to assemble in the connecting piece of the dividing host nucleus is certainly highly advanced and must be regarded as an apomorphic feature. This behavior ensures that the infectious forms are specifically collected and released by the host cell. *Holospora caryophila*, *H. bacillata*, and *H. curvata* do not share this feature with the other holosporas (Fokin et al. 1996; Fokin and Sabaneyeva 1997), and it is not clear how the bacteria of these species leave their host nuclei. They either are more primitive than the other holosporas, as a quantitative separation of infectious forms and reproductive forms is not observed, or are phylogenetically not closely related. Two species groups may presently be distinguished in the genus *Holospora* (*Table 18.1*). It has been hypothesized that the unique behavior of the infectious form to deposit enormous amounts of periplasmic materials polarly could be encoded on a plasmid or phage genome. However, no plasmid or phage genome has been found (M. S. Rautian et al., unpublished results). It is consistent with this observation that the "more advanced" species tested (of which the infectious forms are collected in the separation spindle) were labeled by in situ hybridization using an oligonucleotide probe designed for *H. obtusa* Fokin et al. 1996).

An unnamed symbiont of this *Holospora*-type has repeatedly been observed in *Stentor multiformis* and in *S. polymorphus* (Görtz and Wiemann 1987); another one has been observed in the peritrich ciliate *Zoothamnium pelagicum* by Laval (1970). Similar morphology and life cycle were also observed in bacterial symbionts in the ciliates *Trithigmostoma cucullulus* (Görtz and Maier 1991) and *Spirostomum* sp. (Fokin et al. 2005). These observations indicate that symbionts of the genus *Holospora* are not restricted to *Paramecium*. Some strains of *Paramecium* were found to be harmed after infection with holosporas, whereas other strains apparently remained unaffected.

#### *Holospora undulata*

This organism lives in the micronucleus of *P. caudatum* (Hafkine 1890; Gromov and Ossipov 1981). Two forms are seen: a short, spindle-shaped, reproductive form, about 0.8 µm in diameter and 1.5–2.0 µm long, and a long, spiral-shaped infectious form

**Table 18.1**
**Descriptions of *Holospora* spp.**

| *Holospora*[a] | Morphology and size of infectious form | Host *Paramecium* | Nucleus | Species group[b] |
|---|---|---|---|---|
| "*H. acuminata*" | Straight, tapered ends, length 4–6 μm | *P. bursaria* | Micronucleus | I |
| "*H. bacillata*" | Straight, ends rounded, length 5–17 μm | *P. woodruffi* | Macronucleus | II |
| | | *P. calkinsi* | | |
| *H. caryophila* | Spiral, tapered ends, length 5–6 μm | *P. biaurelia* | Macronucleus | II |
| | | *P. novaurelia* | | |
| | | *P. caudatum* | | |
| "*H. curvata*" | Curved, length 12–20 μm | *P. calkinsi* | Macronucleus | II |
| "*H. curviuscula*" | Curved, tapered ends, length 6–10 μm | *P. bursaria* | Macronucleus | I |
| *H. elegans* | Straight, tapered ends, length 7–18 μm | *P. caudatum* | Micronucleus | I |
| *H. obtusa* | Straight, tapered ends, length 7–20 μm | *P. caudatum* | Macronucleus | I |
| "*H. recta*" | Straight, tapered ends, length 10–15 μm | *P. caudatum* | Micronucleus | I |
| *H. undulata* | Spiral, tapered ends, length 7–15 μm | *P. caudatum* | Micronucleus | I |

[a]Names in quotation marks are not validated
[b]Species of group I are characterized by an escape mechanism of the infectious form from the host nucleus that is regarded as higher evolved. The infectious forms are collected in the connecting piece of the dividing nucleus and later released by the host cell (Fig. 18.8). Bacteria of species of group I react with a "*Holospora*-specific" oligonucleotide probe in fluorescence in situ hybridization (Fokin et al. 1996). For species of group II, the exact mode by which infectious forms leave their host nuclei is not known. In this group, infectious forms are not collected in the connecting piece of a dividing nucleus (Fokin et al. 1996; Fokin and Sabaneyeva 1997). *Holospora* species of group II do not react with a "*Holospora*-specific" oligonucleotide probe in fluorescence in situ hybridization

that has tapered ends and is 7.0–15 μm long. The type strain is found in clone MI-48 of *P. caudatum* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, St. Petersburg State University, Russia).

#### *Holospora obtusa*

This organism lives in the macronucleus (Fig. 18.7; Hafkine 1890; Gromov and Ossipov 1981). Reproductive forms are short rods, 0.8 μm in diameter and 1.5–2.5 μm long. Infectious forms are long rods with rounded ends, 0.8–1.0 μm in diameter and 7.0–20 μm long. Reflecting the fine-structural organization, the infectious form shows a refractile part and a dark part in phase contrast light microscopy (Fig. 18.9). The type strain is found in clone M-115 of *P. caudatum* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, St. Petersburg State University, Russia).

#### *Holospora elegans*

This organism lives in the micronucleus (Hafkine 1890; Preer and Preer 1982). Reproductive forms are short rods, 0.8 μm in diameter and 1.5–2.0 μm long. Infectious forms are long rods with tapered ends, 0.6–0.8 μm in diameter and 7.0–18 μm long. The type strain is found in ATCC strain 50008 (stock C1O1 of *P. caudatum*; Görtz and Dieckmann 1980).

#### *Holospora acuminata*

This organism lives in the micronucleus of *P. bursaria* (Ossipov et al. 1980). Reproductive forms are fusiform rods, 0.6 μm in diameter and 2.0–2.5 μm long. Infectious forms are straight fusiform rods with tapered ends, 0.8–1.0 μm in diameter and 4.0–6.0 μm long. The type strain is found in stock AC61-10 of *P. bursaria* (deposited in the culture collection of the Laboratory of Invertebrate Zoology, Biological Research Institute, St. Petersburg State University, Russia).

#### *Holospora caryophila*

These organisms were formerly known as "alpha particles" (Preer 1969), live in the macronucleus of *P. biaurelia* (Fig. 18.2), and may also be found in *P. caudatum* (Görtz 1987; Preer and Preer 1982). Reproductive forms are thin, fusiform rods, 0.4 μm in diameter and 1.0–3.0 μm long. Infectious forms are spiral shaped with tapered ends, 0.5 μm in diameter and 5.0–6.0 μm long. The type strain is found in ATCC strain 30694 (stock 562 of *P. tetraurelia*; see Preer et al. 1974).

#### *Holospora recta*

This organism lives in the micronucleus of *P. caudatum* (Fokin 1991). Two forms are seen: a short, spindle-shaped, reproductive form, about 0.8 μm in diameter and 1.5–2.0 μm long and a long, straight infectious form (one end rounded, one end tapered) 10–15 μm long.

Whereas all other *Holospora* spp. listed here definitely appear to be good species, this status has been questioned for *H. recta* by Rautian and Ossipov (M. S. Rautian and D. V. Ossipov (1992), personal communication), who found that a strain of *H. elegans* had a number of individuals with *H. recta*-like features. The question may finally be resolved after molecular data have been obtained from analyses of the different strains.

#### *Holospora curviuscula*

Cells live in the macronucleus of *P. bursaria* (Borchsenius et al. 1983). They infect only certain strains of three syngens, and in other strains, the development into the infectious form is not completed. Occasionally, infection of both macro- and micronuclei was observed. Reproductive forms are fusiform rods, 0.8 µm in diameter and 1.5–2.0 µm long. Infectious forms are slightly curved rods with tapered ends, approximately 0.8 µm in diameter and 6.0–10 µm long.

#### *Holospora bacillata*

This organism lives in the macronucleus of *Paramecium calkinsi* (Fokin and Sabaneyeva 1993) and *Paramecium woodruffi* (Fokin et al. 1996; Fokin and Sabaneyeva 1997). Infectious forms are 5–17.0 µm long, 0.7–0.8 µm wide, straight, rounded at both ends.

#### *Holospora curvata*

Cells live in the macronucleus of *Paramecium calkinsi* (Fokin and Sabaneyeva 1993). Infectious forms are 12–20 µm long, 0.7–0.9 µm wide, curved, and rounded at both ends.

### Genus *Paraholospora*

Recently, a new bacterial genus *Paraholospora* with a single species described as "*Candidatus Paraholospora nucleivisitans*" was found (Eschbach et al. 2009). In phylogenetic trees of the SSU rDNA sequence, it branches at the basis of the *Holosporaceae* as a sister group. It differs from members of the genus *Holospora* in the intracellular cytoplasmic location and the missing life cycle.

#### *Candidatus* Paraholospora nucleivisitans

Cells live in the cytoplasm and occasionally in the macronucleus of *Paramecium sexaurelia* (Eschbach et al. 2009). Bacteria are bent or spiral shaped with a diameter of 0.5–0.8 µm and up to 25 µm in length. Cells are absent in the micronucleus. No spore formation or life cycle could be observed. Bacteria could not be cultivated outside their host.

## Symbionts of *Paramecium* Without Binomial Names

Jenkins (1970) described a Gram-negative bacterium living within bulbous distensions of the outer membrane of the nuclear envelopes of both the micro- and macronucleus of a strain of *P. multimicronucleatum*. This symbiont is a very short rod, sometimes appearing nearly coccoid, and approximately 0.35 µm in diameter with longer forms reaching 0.7 µm in length. It was named "epsilon."

Another symbiont, reported to occur in the perinuclear space, has been described by Fokin (1988). It was found inside the nuclear cisternae of the macronuclear envelope of *P. duboscqui*. The symbiont is 0.3 µm in diameter and 0.7–1.4 µm long, looks spindle shaped, and is Gram negative. No killing activity was observed when symbiont bearers were tested against nonsymbiont bearers.

A symbiont studied by Estève (1957) occurs in the macronucleus of *P. caudatum* and confers a killer trait on its host. When it was investigated cytologically, a greatly enlarged macronucleus was observed to contain numerous kappa-like bacteria, some of which contained R bodies. Electron micrographs of this bacterium showed spherical phages inside the R bodies. Schmidt and coauthors assumed that the symbiont is identical with *Caedibacter caryophilus* described by them (Schmidt et al. 1987b).

A so far unnamed symbiont found by Görtz and Freiburg (1984) living in the micronucleus of *P. bursaria* is a small rod, 0.5 µm in diameter and up to 2 µm long. Its ultrastructure suggests that it is a Gram-negative bacterium. No flagella were found, and no killing capacity of its host was observed. Another symbiont of the nonkiller type was discovered in the cytoplasm of *P. woodruffi*, a ciliate living in brackish water. The symbiont is a Gram-negative rod (0.2–0.8 µm in diameter and 0.6–2.5 µm long) that lacks flagella and contains hexagonal viroid particles (Fokin et al. 1987b).

In *P. sexaurelia* isolated from an aquarium with tropical fish, a bacterial endosymbiont was observed by Görtz (1981) to invade the macronucleus but not the micronucleus. Once the symbiont has entered the macronucleus, it tends to disappear from the cytoplasm. The bacterium is slightly curved with a diameter of 0.5–0.8 µm and up to 25 µm long. When present in the cytoplasm, the symbiont tends to be closely associated with food vacuoles. Once the symbiont has entered the macronucleus, it multiplies there without causing nuclear hypertrophy. After autogamy, the symbiont is found only in the cytoplasm and not in new macronuclear anlagen. It remains there until a new infection of the macronucleus occurs. The bacterium was observed again in a *P. sexaurelia* isolated by Fokin (Przybosz and Fokin 1997) from a pond in the Wilhelma (Zoological and Botanical Garden, Stuttgart).

*Table 18.2* summarizes the taxa of prokaryotic symbionts of *Paramecium* and provides a determinative key to their identification.

## Prokaryotic Symbionts of *Euplotes*

Endosymbiotic bacteria are also very common in *Euplotes*, a ciliate genus that comprises both freshwater and marine species (Heckmann 1983; Petroni et al. 2001). So far, only one of the symbionts has been given a binomial name. The others are still referred to by Greek letters, as was formerly customary for cytoplasmic elements. Several *Euplotes* symbionts remained unnamed or were provisionally given Latin letters as names when they were encountered. However, most of these symbionts are not well characterized.

**Table 18.2**

**Key to the prokaryotic symbionts of *Paramecium***

| |
|---|
| I. Host paramecia are killers or mate killers |
| A. Between 2 % and 50 % of the symbiont population contains R bodies |
| 1. Host paramecia are killers |
| a. Kill by producing aboral humps on sensitive paramecia; R bodies unroll from inside at low pH, reroll at high pH; found in *P. tetraurelia* |
| *Caedibacter taeniospiralis* |
| b. Kill in ways other than producing aboral humps |
| (1) Found in *P. tetraurelia*; R bodies unroll from the outside irreversibly when exposed to high temperature or certain detergents |
| *Caedibacter pseudomutans* |
| (2) Found in *P. biaurelia*; R bodies unroll from outside irreversibly when exposed to high temperature or certain detergents |
| *Caedibacter varicaedens* |
| (3) Found in *P. caudatum*, found in the macronucleus only; R bodies unroll from inside |
| *Caedibacter caryophilus* |
| 2. Host paramecia are mate killers |
| *Caedibacter paraconjugatus* |
| B. Symbiont population does not contain R bodies |
| 1. Host paramecia are killers |
| a. Rapid-lysis killer; symbionts are large flagellated cells |
| (1) Straight rods found in *P. tetraurelia*, *P. octaurelia* |
| *Lyticum flagellatum* |
| (2) Sinuous rods found in *P. biaurelia* |
| *Lyticum sinuosum* (Fig. 18.3) |
| b. Kill by vacuolization, symbionts are very small cells, often doublets |
| *Pseudocaedibacter minutus* |
| 2. Host paramecia are mate killers |
| *Pseudocaedibacter conjugatus* |
| II. Host paramecia are nonkillers |
| A. Symbionts are present only in the cytoplasm |
| 1. Symbionts lack flagella |
| a. Host: *P. bursaria*; egg shaped, rarely rod shaped, up to 1.4 μm long. Antagonistic to symbiotic algae (*Chlorella*) of the host |
| *Caedobacter chlorellopellens* |
| b. Hosts: *P. biaurelia*, *P. tetraurelia*, *P. pentaurelia*; rods, up to 1.2 μm long |
| *Pseudocaedibacter falsus* |
| c. Host: *P. pentaurelia*; rods, up to 1.2 μm long; the symbiont-containing vacuole is surrounded by endoplasmic reticulum |
| *Pseudocaedibacter glomeratus* |
| 2. Symbionts with flagella |
| a. Host: *P. caudatum*; rods; 3.5–14 μm long; refractile inclusions (polyhydroxy butyric acid); with numerous flagella but no motility observed |

**Table 18.2 (continued)**

| |
|---|
| *Pseudolyticum multiflagellatum* |
| b. Host: *P. primaurelia*; rods, up to 2 μm long, Gram negative with a thick surface layer visible in EM; with sparse flagella, occasionally slight motility; coexists with other symbionts such as *Caedibacter* and *Pseudocaedibacter* |
| *Tectibacter vulgaris* |
| B. Symbionts are present almost exclusively in the nuclei |
| 1. Symbionts exclusively in the micronucleus, show two morphologically distinct forms, and are highly infectious |
| a. Long form of symbiont (infectious form) spiral shaped, both ends tapered, 7–15 μm long; host: *P. caudatum* |
| *Holospora undulata* |
| b. Long form of symbiont (infectious form) straight rod with tapered ends |
| (1) Host: *P. caudatum*; long form 7–18 μm long |
| *Holospora elegans* |
| (2) Host: *P. bursaria*; long form 4–6 μm long |
| *Holospora acuminata* |
| c. Long form of symbiont (infectious form) straight rod, one end tapered, one end rounded, host: *P. caudatum* |
| *Holospora recta* |
| 2. Symbionts exclusively in the macronucleus |
| a. Symbionts are highly infectious, two morphologically distinct forms often observable |
| (1) Long form slightly spiral shaped, with tapered ends, 5–6 μm long; host: *P. biaurelia* (sometimes also observed in *P. caudatum*) |
| *Holospora caryophila* (Fig. 18.2) |
| (2) Long form straight rod, with rounded ends, 7–20 μm long; host: *P. caudatum* |
| (3) Long form straight rod, with rounded ends, 5–17 μm long; host: *P. calkinsi* or *P. woodruffi* |
| *Holospora bacillata* |
| (4) Long form slightly curved, with tapered ends, 6–10 μm long; host: *P. bursaria* |
| (5) Long form curved, both ends rounded, 12–20 μm long, host: *P. calkinsi* |
| *Holospora curvata* |
| b. Symbionts are weakly infectious, only one morphological form observable. Host: *P. caudatum*; rods small, about 1 μm long, sometimes forming ''chains'' that are up to 10 μm long; mostly aggregated in the center of the macronucleus; may coexist with *H. obtusa* or *H. undulata* |
| *Nonospora macronucleata* |
| C. Symbionts are present in the cytoplasm but occasionally invade the macronucleus |
| 1. Bacteria are bent or spiral shaped with a diameter of 0.5–0.8 μm and up to 25 μm in length. Host: *Paramecium sexaurelia* |
| *"Candidatus Paraholospora nucleivisitans"* Paraholospora nucleivisitans |

Based on the key proposed by Preer (1981) and on information from articles cited in this chapter

## Habitat of the *Euplotes* Symbionts

All except one of the bacterial symbionts of *Euplotes* are confined to the cytoplasm; see "The Ciliate Cell as a Microcosm." They can easily be observed with a phase contrast microscope. Fixation and staining of *Euplotes* with aceto-carmine or lacto-aceto-orcein can be helpful for the observation and identification of these endosymbionts; see "Orcein Staining of Intracellular Bacteria." When stained, *Polynucleobacter necessarius* (formerly omikron) and the closely related "omikron-like" bacteria reveal many nucleoids (Heckmann 1975; Heckmann et al. 1983; *Figs. 18.19* and *18.20*). It appears that most of the *Euplotes* symbionts cannot grow outside their hosts, although this has been investigated thoroughly only for *P. necessarius*. Like *Caedibacter* and other symbionts of the *P. aurelia* species complex, the *Euplotes* symbionts appear not to be infectious, at least under laboratory conditions, and some are killer symbionts similar to the killer symbionts of *Paramecium*; see "The Killer Trait in *Paramecium*." The symbionts found to date have not been observed to be harmful to their hosts, which, however, in some cases are converted by infection into killers or mate killers. *Polynucleobacter necessarius* and the closely related omikron-like bacteria are essential for their hosts (Heckmann 1983; Fujishima and Heckmann 1983).

## Isolation of the *Euplotes* Symbionts

*Euplotes minuta*, *E. crassus*, and *E. vannus* are marine species. They can be isolated from water samples containing algae or detritus collected at the seashore. All these species can be easily grown in the laboratory in seawater with *Dunaliella salina* as the food organism (for details, see Heckmann 1963). The other *Euplotes* species listed in *Table 18.3* are freshwater organisms that may be collected from ponds. They can be grown in a diluted soil medium (Ruthmann and Heckmann 1961) or in culture medium for *Euplotes* (CME; Kuhlmann and Heckmann 1989) and fed with *Chlorogonium elongatum* or *Chilomonas paramecium*. Most *Euplotes* species can also utilize bacteria as food.

Since the symbionts may differ in different strains of a host species, it is advisable to start *Euplotes* cultures from single cells so that the cultures maintained in the laboratory form clones. In most cases, this will ensure that one is dealing with homogeneous populations of endosymbionts. However, double and triple infections have also been observed.

*Polynucleobacter necessarius* is the only endosymbiont of *Euplotes* known so far that has been isolated. In this case, cells of strain 15 of *E. aediculatus* were homogenized mechanically, and the symbionts were then purified by applying the homogenate to an ECTEOLA column, followed by elution with phosphate buffer (Heckmann 1975; Schmidt 1982). The procedure basically followed the one developed for the isolation of kappa symbionts from *Paramecium* by Smith-Sonneborn and Van Wagtendonk (1964) and resulted in very clean preparations of symbionts.

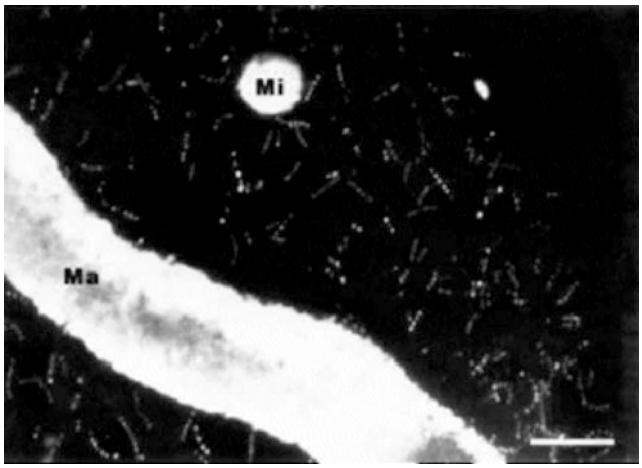



**Fig. 18.19**
**Fluorescence micrograph of *Polynucleobacter necessarius* in the cytoplasm of a slightly crushed *Euplotes aediculatus* cell after staining with N,N′-diethylpseudoisocyanin chloride. The symbionts are revealed by the DNA-specific yellow fluorescence of their nucleoids. *Ma* macronucleus and *Mi* micronucleus. Bar = 1 μm (From Heckmann 1975)**

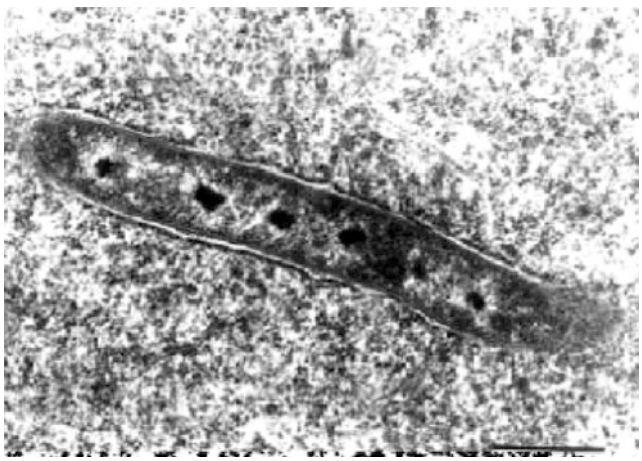


**Fig. 18.20**
**Electron micrograph of *Polynucleobacter necessarius*. *Longitudinal section*. The ultrastructure of this symbiont resembles that of Gram-negative bacteria. Bar = 0.5 μm (From Heckmann 1975)**

## Identification of the *Euplotes* Symbionts

The bacterial symbionts of *Euplotes* can be detected by direct observation with a phase contrast microscope, by observation of physiological effects produced on other *Euplotes* cells that lack symbionts, or by a combination of these methods. In the past, identification of symbionts of *Euplotes* has also been done using these methods. Now, at least for *Polynucleobacter necessarius*, formerly omikron particles, identification by FISH using appropriate oligonucleotide probes has become possible.

**Table 18.3**

**Characteristics of the prokaryotic endosymbionts of the genus *Euplotes***

| Symbiont designation | Euplotes species[a] | Site | Killing type | References |
|---|---|---|---|---|
| *Polynucleobacter necessarius* | *E. aediculatus (15)* | *Cy* | *NK* | Heckmann and Schmidt (1987) |
| *Polynucleobacter* sp. | *E. harpa* | *Cy* | *ND* | Vannini et al. (2005) |
| *Candidatus Francisella noatunensis subsp. endociliophora*[d] | *E. raikovi* | *Cy* | *NK* | Schrallhammer et al. (2010) |
| *Candidatus Devosia euplotes*[d] | *E. magnicirratus* | *Cy* | *ND* | Vannini et al. (2004) |
| *Candidatus Anadelfobacter veles*[d] | *E. harpa* | *Cy* | *ND* | Vannini et al. (2010) |
| *Candidatus Cyrtobacter comes*[d] | *E. harpa* | *Cy* | *ND* | Vannini et al. (2010) |
| *Omikron-like symbiont* | *E. eurystomus (25)* | *Cy* | *NK* | Heckmann et al. (1983) |
| *Omikron-like symbiont* | *E. plumipes (24)* | *Cy* | *NK* | Heckmann et al. (1983) |
| *Omikron-like symbiont* | *E. daidaleos (13)* | *Cy* | *NK* | Heckmann et al. (1983) |
| *Omikron-like symbiont* | *E. octocarinatus (11)* | *Cy* | *NK* | Heckmann et al. (1983) |
| *Omikron-like symbiont* | *E. patella (5)* | *Cy* | *NK* | Heckmann et al. (1983) |
| *Omikron-like symbiont* | *E. woodruffi (22)* | *Cy* | *NK* | Heckmann et al. (1983) |
| *Omikron-like symbiont* | *E. moebiusi* | *Cy* | *ND* | Foissner (1978) |
| *Omikron-like symbiont* | *E. harpa* | *Cy* | *ND* | Petroni et al. (2001) |
| *Epsilon* | *E. minuta* ($K_1$,$K_2$, $K_3$) | *Cy* | *K* | Heckmann et al. (1967) |
| *Epsilon-like* | *E. minuta* ($VF_{17}$) | *Cy* | *K + MK* | Heckmann et al. (1967) |
| *Eta* | *E. crassus* | *Cy* | *K* | Nobili et al. (1976) |
| *A* | *E. crassus* | *Cy* | *NK* | Rosati et al. (1976) |
| $B_1$ | *E. crassus* ($C_8$) | *Cy* | *MK* | Dini and Luporini (1976) |
| $B_3$ | *E. crassus* | *Cy* | *NK* | Rosati et al. (1976) |
| *C* | *E. crassus* | *Cy* | *NK* | Rosati et al. (1976) |
| *D* | *E. crassus* | *ND* | *ND* | Rosati et al. (1976) |
| *Unnamed* | *E. crassus* | *Ma* | *NK* | Rosati and Verni (1975) |
| *Unnamed* | *E. crassus* | *Cy* | *K + MK* | Demar-Gervais and Genermont (1975) |
| *Unnamed*[b] | *E. aediculatus* | *Cy* | *NK* | Heckmann et al. (1983) |
| *Unnamed*[c] | *E. octocarinatus* | *Cy* | *NK* | Heckmann et al. (1983)) |
| *Unnamed* | *E. harpa* | *Cy* | *ND* | Petroni et al. (2001) |

Abbreviations: *Cy* cytoplasm, *Ma* macronucleus, *NK* nonkiller, *K* killer, *MK* mate killer, and *ND* not determined

[a]The typical strain designation is given in parentheses

[b]Unnamed symbiont that is not omikron-like in *E. aediculatus* stocks 7 and 10

[c]Unnamed symbiont observed in addition to omikron-like symbionts in stock 11 of *E. octocarinatus*

[d]Scientific names given for uncultivated prokaryotes according to Stackebrandt et al. (2002)

## The Taxa of Prokaryotic Symbionts of *Euplotes*

### Genus *Polynucleobacter*

These are obligate endosymbiotic bacteria living in the cytoplasm of freshwater ciliates of the genus *Euplotes* (Heckmann and Schmidt 1987). Characterized by multiple nucleoids, these symbionts are essential for their host species and are nonmotile and Gram negative. The type species is *Polynucleobacter necessarius*.

Polynucleobacter was found to belong to the β-subclass of Proteobacteria and shows the closest relationship to members of the *Burkholderiaceae* (Hahn et al. 2009; Springer et al. 1996).

#### *Polynucleobacter necessarius*

This species was formerly called "omikron" (Heckmann and Schmidt 1987). Cells are slightly curved rods, about 0.3 µm in diameter and 2.5–7.5 µm long. If stained with DNA-specific dyes, usually 3–9, but in some cases, up to 12 intensely stained and regularly spaced dots become visible (*Fig. 18.19*). They are considered to be nucleoids. When examined with the electron microscope, these nucleoids differ from those of most free-living bacteria by exhibiting an electron-dense central core that resembles the chromatin of eukaryotes (*Fig. 18.20*). Whether this core is formed by proteins associated with DNA or some other material is not clear (Heckmann 1975). Recently, it was shown that free-living *Polynucleobacter necessarius* is distributed worldwide (Hahn 2003). *Polynucleobacter necessarius* strains revealed

the closest phylogenetic relationship between free-living and obligate endosymbiotic bacteria ever found (Vannini et al. 2007). As a result, the revision of *P. necessarius* led to the differentiation of free-living and endosymbiotic species at the subspecies level. The free-living subspecies is proposed to be named *Polynucleobacter necessarius subsp. asymbioticus*, while the endosymbiotic subspecies is named *P. necessarius subsp. necessarius* (Hahn et al. 2009). Both subspecies display a 99.1–99.4 % sequence similarity for the 16S rDNA gene. They differ significantly in genome size, visibility of nucleoids, cell length, and culturability (Hahn et al. 2009).

The symbionts are individually contained in vesicles, to which ribosomes are often attached. *Polynucleobacter necessarius subsp. necessarius* reproduces by transverse binary fission in a typical bacterial manner. However, the fission products have often been found to differ in size. Frequently, a 7 µm long rod containing eight to nine nucleoids was observed to bud off a 2.5 µm piece that contained three nucleoids (Heckmann 1975).

The G + C content of the DNA of endosymbiotic *P. necessarius* is 48 mol% by thermal denaturation. A value that was 2.8 mol% lower was found when the G + C content was calculated from the buoyant density, which was 1.7036 g/cm$^3$ (Schmidt 1982). The differences could be caused by rare bases; however, no DNA chemistry has been done for intracellular *P. necessarius*. The average DNA content of the symbiont was determined to be $5.8 \times 10^{-3}$ pg. Taking into account the average number of nucleoids, a DNA content of $0.5 \times 10^9$ Da per nucleoid was calculated. This value is in close agreement with the value of the kinetic complexity of the DNA, which was determined to be $0.57 \times 10^9$ Da when corrected for the G + C content (Schmidt 1982). Hahn et al. (2009) determined a genome size of 1.5–2.5Mbp for both subspecies of *P. necessarius* and estimated a G + C content of about 44–46 mol%. Equally, small genomes have been reported for *Lyticum flagellatum*, *Pseudocaedibacter conjugatus*, and *P. falsus*, endosymbionts of the *P. aurelia* species complex (Soldo and Godoy 1973a); for xenosomes of *Parauronema acutum* (Figueroa-de Soto and Soldo 1977); and for chlamydias, rickettsias, and mycoplasmas (Bak et al. 1969). The genomes of these organisms are the smallest known for cells. They code for only about 700–900 proteins.

Heckmann (1975) has shown that it is possible to remove *P. necessarius* from its host *E. aediculatus* by treating a rapidly growing culture with penicillin (100–500 units/ml) for 5–6 days. Aposymbiotic hosts may undergo one or two fissions but then stop multiplying and die about 15–20 days after the last fission. The same results were obtained with several other antibiotics or with sufficiently high doses of X-rays.

Reinfection and rescue of *E. aediculatus* have been achieved either by adding a cell homogenate from symbiont bearers or by injecting symbiont-containing cytoplasm (Heckmann 1975; Fujishima and Heckmann 1984a). The type strain is found in ATCC strain 30859 (clone 15 of *E. aediculatus*; see Heckmann and Schmidt 1987).

A phylogenetically and morphologically different endosymbiotic *Polynucleobacter* was described in *Euplotes harpa* from a brackish water of river estuaries of different geographical regions (Vannini et al. 2005). The bacteria were located in the cytoplasm of the ciliate cells embraced by a peribacterial membrane. Cells of *E. harpa* were found to be associated with a second endosymbiotic prokaryote affiliated with α-proteobacteria.

*Candidatus* Devosia euplotes Cells are about 0.5 µm wide and up to 2.5 µm long and located embraced by a peribacterial membrane in the cytoplasm of their host *Euplotes magnicirratus* (Vannini et al. 2004). Bacteria belong to α-proteobacteria.

*Candidatus* Francisella noatunensis subsp. endociliophora Bacterial Gram-negative endosymbionts are between 0.2–0.4 µm wide and 0.9–1.9 µm long and located dispersed in the cytoplasm of *Euplotes raikovi* without surrounding membranes (Schrallhammer et al. 2010).

*Candidatus* Anadelfobacter veles These are Gram-negative bacteria of the order Rickettsiales, inhabiting the cytoplasm of *Euplotes harpa* (Vannini et al. 2010). Cells are rod shaped and up to 2 µm wide and 6 µm long and surrounded by a host membrane within the cytoplasm of the ciliate. Found in an coinfection with bacteria of the genus Polynucleobacter (β-Proteobacteria).

*Candidatus* Cyrtobacter comes These are rod-shaped α-proteobacteria of the order Rickettsiales with an irregular surface, visible in ultrathin section for transmission electron microscopy (Vannini et al. 2010). Cells are up to 3 µm wide, up to 5 µm long and living without a surrounding peribacterial membrane in the cytoplasm of *Euplotes harpa*. Found in an coinfection with bacteria of the genus *Polynucleobacter* (β-Proteobacteria).

## The Omikron-Like Endosymbionts of *Euplotes*

These bacteria are very similar in appearance to *P. necessarius*. They were found in six freshwater *Euplotes* species (*E. eurystomus*, *E. plumipes*, *E. octocarinatus*, *E. patella*, and *E. woodruffi*) all having 9 type 1 fronto-ventral cirrus (FVC) pattern (9 FVC double dargyrome; Gates and Curds 1979) like *E. aediculatus*, the bearer of omikron, and are considered to be closely related. The symbionts may differ from *P. necessarius* in size, shape, and other features, but they always share with *P. necessarius* the characteristic of multiple nucleoids (Heckmann et al. 1983). Symbionts of this type, but about twice as large as *P. necessarius*, were first noticed by Fauré-Fremiet (1952) in stocks of *E. patella* and *E. eurystomus*. He also observed at that time that small doses of penicillin led to a loss of symbionts and to death of the host ciliates. He therefore suggested that the symbionts might be essential for survival of the ciliate. This was later confirmed when Heckmann et al. (1983) subjected a large number of stocks of *Euplotes* species with a 9 type 1 cirrus pattern to a penicillin treatment of the kind that had earlier been found to remove *P. necessarius*. They found that all the ciliates stopped dividing and eventually died when their symbionts were removed. Heckmann et al. (1986)

suggest that all *Euplotes* species with a 9 type 1 cirrus pattern suffer from a common deficiency that arose in a common ancestor of this group of organisms. This ancestor must have lived in symbiosis with a prokaryote that compensated for the acquired deficiency. *Polynucleobacter necessarius* and the omikron-like symbionts are considered to be progeny of this prokaryote.

## Symbionts of *Euplotes* without Binomial Names

As for the symbionts of *Paramecium*, many bacterial symbionts of *Euplotes* have not been given binomial names. For the time being, there is no information about their phylogenetic position. Nevertheless, they are included because they may be identified in further isolations by their host specificities and other distinct biological traits.

## The Epsilon Symbionts of *Euplotes*

The epsilon symbionts from killer strains $K_1$, $K_3$, and $K_7$ of *E. minuta*, collected from the Mediterranean Sea at Villefranche-sur-Mer (France), were described by Heckmann et al. (1967). They are small rods, about 0.4 µm in diameter and 0.8–2.5 µm long, when observed in freshly crushed cells with bright phase optics, and appear somewhat darker than free-living bacteria. When the killer strain $K_3$ was fixed in Schaudinn's fluid, hydrolyzed in 1 N HCl, and stained with basic fuchsin, as described by Dippell and Chao in Sonneborn (1950), several dozen darkly stained bodies were found scattered in the cytoplasm. Their staining properties, size, shape, and cellular distribution resembled that of *Caedibacter* species and other endosymbionts of *Paramecium*. Since epsilon particles were found only in killer strains, it is assumed that they are responsible for the killer phenotype of *E. minuta*.

Siegel and Heckmann (1966) noticed that certain strains of *E. minuta* killed certain other strains of this species when conjugation occurred. It was found that the sensitive cells formed vacuoles, ceased their normal swimming movements, settled to the bottom of the culture vessel, and finally lysed. These changes occurred within 1–4 days after the strains had been mixed. No evidence for killing was observed when cells of the three killer strains were mixed together.

A good example of the difficulties encountered in the identification of endosymbionts is provided by the killer particles of stock $VF_{17}$ of *E. minuta*. Heckmann et al. (1967) had noticed that this strain caused symptoms in sensitives slightly different from those observed in mixtures with the other *E. minuta* killers. Here, vacuole coalescence generally proceeded to the point where only one large vacuole was present in the posterior region of the sensitive, giving the whole affected organism a pear-shaped form. Nevertheless, no morphological differences between these symbionts and epsilon particles could be detected. Later, when stock $VF_{17}$ was employed for cross-breeding experiments in a study of the genetic control of cortical pattern in *E. minuta*, it was found that this stock not only killed sensitive strains via particles liberated into the medium but also acted as a mate killer. From a pair of conjugants, one generally developed into a healthy-looking exconjugant, whereas the other one failed to develop a large macronuclear anlage, became quiescent, and finally died. The surviving exconjugant clones had the same cortical pattern as stock $VF_{17}$ and were all found to act as killers. They were therefore cytoplasmic descendants of the mate-killer parent. That they were true hybrids, i.e., that they had received a gametic nucleus from the sensitive partner, was shown from presence of certain genetic markers followed in these crosses. Whether $VF_{17}$ was host to a mixed population of symbionts—with one type responsible for the killer trait and the other one for the mate-killer trait, both types being so similar that they could not be distinguished morphologically from each other—or whether a single class of particles present in $VF_{17}$ determined both traits cannot be decided. Both the killer trait and the mate-killer trait were lost concurrently with aging of the stock (Frankel 1973).

## The Eta Symbiont of *Euplotes*

The eta symbiont, which was observed only by electron microscopy, is round or oval shaped with an average diameter of 0.9 µm. It is bound by three juxtaposed membranes. The outermost membrane has ribosomes attached to it. The interior of the symbiont is granular with a few scattered fibrous strands (Rosati and Verni 1977). The symbiont was found in strains of *E. crassus* collected by Luporini (1974) from a site on the coast of Somalia on the Indian Ocean. It was named "eta" by Nobili et al. (1976), who investigated the killer trait conferred on its host by this symbiont. Sensitive testers, when exposed to the culture fluid of killer strains or to a homogenate prepared from cells of these lines, developed a large vacuole in the posterior part of the body within 6–24 h. Affected cells are gradually transformed into transparent spheres that eventually "explode."

## The $B_1$ Symbiont of *Euplotes*

A symbiont very similar in morphology to eta appears to be responsible for the mate-killer properties of strain $C_8$ of *E. crassus*; it was named "$B_8$." When cells of this strain were treated with penicillin, both the mate-killer trait and the symbiont disappeared. Dini and Luporini (1982) provided evidence for the existence of specific host genes required for maintenance of the $B_8$ particles.

## Inconspicuous Symbionts of *Euplotes*

The other symbionts of *Euplotes* listed in *Table 18.3* (A, $B_3$, C, D, and the unnamed ones) have been discovered by chance during electron microscopic investigations of certain strains. No special functions of these symbionts are known nor have any effects of the symbionts on their hosts been reported. Interestingly, in two cases, they have been observed in addition to omikron-like symbionts (Heckmann et al. 1983; Petroni et al. 2001).

## Ectosymbionts of *Euplotidium*

Peculiar ectosymbionts have been observed on the surface of the marine ciliate *Euplotidium itoi* (Fig. 18.21) and other species of the genus (Verni and Rosati 1990). Though they have been studied in detail, their phylogenetic position remained obscure over years until their relationship to *Verrucomicrobia* was elucidated (Petroni et al. 2000). It is the only ciliate symbiont belonging to *Verrucomicrobia*, while most symbionts of ciliates belong to the Proteobacteria. The authors found two morphologically different forms of the particles. At stage I of the life cycle, the organisms are spherical (1 μm in diameter; Fig. 18.22) and have a simple bacteria-like organization of their cell. These cells apparently divide by binary fission. Stage I epixenosomes may transform into stage II by gradually acquiring a more complex structure. Fully developed, stage II epixenosomes are larger (2.5 μm long) and egg shaped (Fig. 18.23). They contain a sophisticated extrusive structure of a ribbon coiled around a central core surrounded by a basket built of bundles of regularly arranged tubules. The tubules have a number of features in common with eukaryotic microtubules (Rosati et al. 1993a). On top of the extrusive structure in the distal part of the cell body, an electron-dense, dome-shaped structure lies underneath the cell membrane (Fig. 18.23). The dome-shaped structure contains DNA and basic proteins, and its ultrastructure resembles eukaryotic chromatin. The extrusive apparatus may be ejected upon appropriate stimuli (Rosati et al. 1993b).

### Habitat and Biology of Epixenosomes of *Euplotidium*

The ciliate hosts of epixenosomes, *Euplotidium itoi* and *E. arenarium*, were collected from tide pools along the rocky shore of the Ligurian Sea. As has been shown by Rosati, Verni, and collaborators, epixenosomes have defensive function for the host cell (Rosati et al. 1999). Predators such as the ciliate *Litonotus* easily ingested *Euplotidium* without epixenosomes, whereas they were not able to ingest *Euplotidium* with epixenosomes unless the ejecting capacity of the bacteria was inhibited.

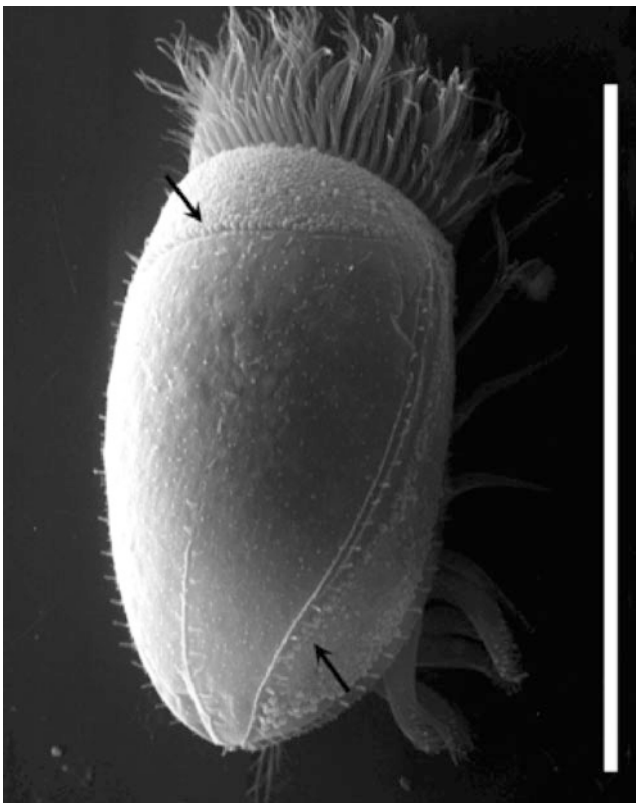


**Fig. 18.21**
**Scanning electron micrograph of *Euplotidium itoi*, *dorsal view*. Epixenosomes (*arrows*) are arranged in a band on the cortex of the ciliate. Bar = 100 μm (From Petroni et al. 2000. © 2000 National Academy of Sciences, USA)**

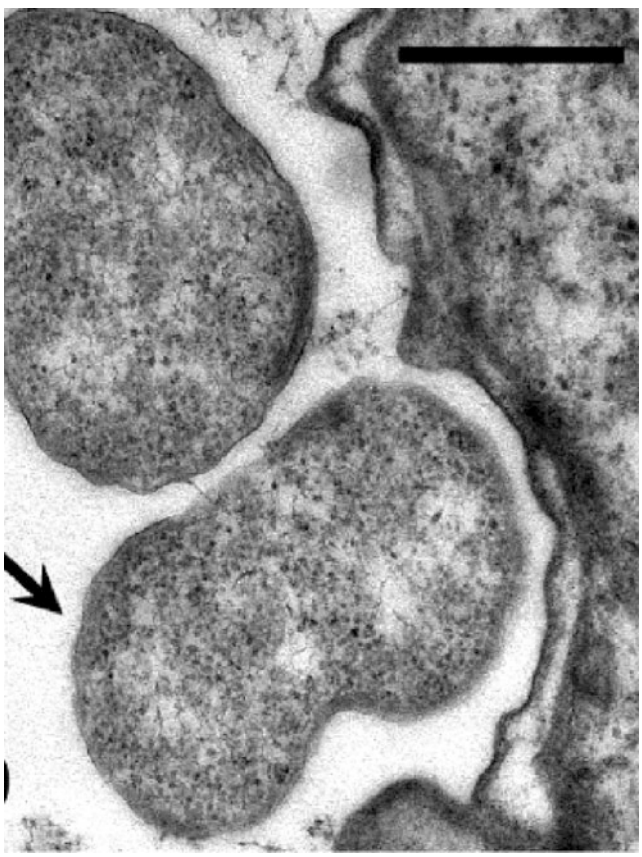


**Fig. 18.22**
**Epixenosomes (arrow) of stage I. Transmission electron micrograph of thin section. Bar = 1 μm (From Petroni et al. 2000. © 2000 National Academy of Sciences, USA)**

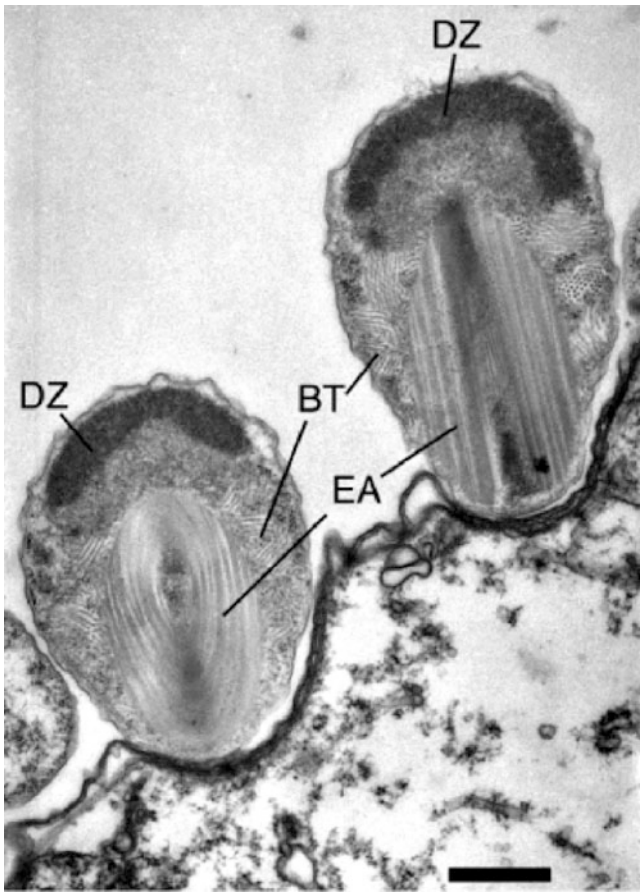



**Fig. 18.23**
**Epixenosomes of stage II. Sections of two bacteria at different levels. *DZ* apical (dome-shaped) zone, *EA* extrusive apparatus, and *BT* microtubule-like elements forming a basket around the extrusive apparatus. Bar = 1 μm (From Petroni et al. 2000. © 2000 National Academy of Sciences, USA)**

Epixenosomes are arranged in a broad cortical band on the surface of host cells (*Fig. 18.21*). Individual bacteria appear fixed in cup-shaped indentations of the ciliate cortex. At these sites, the cortex of host cells is different in symbiont-bearing ciliates compared to symbiont-free cells (Verni and Rosati 1990; Rosati 1999).

### Identification of Epixenosomes of *Euplotidium*

While it appears easy to identify epixenosomes of *Euplotidium* as such by their unique site specificity, arrangement on the host cell, and ultrastructure, their phylogenetic position long remained obscure. Earlier attempts of classification of these organisms did not give definite results. Only by using a Eukaryotes forward primer and a backward primer designed for Archaea did amplification of the small ribosomal subunit (SSU) rRNA gene become possible (Petroni et al. 2000). A probe designed for part of the sequence labeled epixenosomes on the host *E. arenarium*.

## The Xenosomes of *Parauronema acutum*

The term "xenosome" was coined by Soldo to denote the infectious particles that he found in the cytoplasm of the marine ciliate *Parauronema acutum* (Soldo et al. 1974). They were later shown to be small bacteria that possess multicopy genomes and resemble *Caedibacter taeniospiralis* (kappa) and other cytoplasmic symbionts of *Paramecium* in many respects; see "The Prokaryotic Symbionts of *Paramecium*." Two types of xenosomes were distinguished: killer xenosomes, which inhibit growth when taken up by susceptible ciliates, especially those of the genus *Uronema* (Soldo and Brickson 1978), and nonkiller xenosomes (Soldo et al. 1987). The xenosomes have not yet received binomial names.

### Habitat of the Xenosomes of *Parauronema acutum*

*Parauronema acutum* is a small hymenostome marine ciliate that can be maintained axenically. The culture medium is basically that developed for species of the *Paramecium aurelia* complex by Soldo and Van Wagtendonk (1969), distilled water being replaced by seawater (density 1.015–1.026 g/ml) and modified to contain asolectin (500 μg/ml) as the sole source of lipid (Soldo et al. 1974). The association with xenosomes is stable. Symbiont-bearing strains have now been maintained for more than 15 years.

Xenosomes released from host cells can infect symbiont-free strains of *P. acutum*. A single xenosome is capable of infecting a susceptible cell. However, a threshold of 100–200 xenosomes appears to be required before a single xenosome can infect a potential host (Soldo 1983). While phagocytosis is the usual way infectious bacteria enter host ciliates, Soldo and Brickson (1978) found that the symbionts in *Parauronema acutum*, termed "xenosomes" by those authors, penetrate the cell membrane at sites of the cortex other than the oral apparatus.

Soldo and coworkers measured the oxygen consumption of purified xenosome preparations and reported a rate of oxygen uptake of 1.3 nmoles $O_2$/min/mg of protein, which is higher than the oxygen uptake of *Bdellovibrio* and of the same order of magnitude as that of rickettsiae and *Caedibacter taeniospiralis*. Oxygen uptake is about 20 times lower than that of *E. coli*. The rate of oxygen consumption was found to be stimulated by various fatty acids, by intermediates of the glycolytic pathway, and by intermediates of the citrate cycle with the exception of citrate itself, which had no effect. Cyanide was found to be a potent inhibitor of oxygen consumption of xenosomes (Soldo 1983).

### Isolation of Xenosomes from *Parauronema acutum*

Xenosomes can be isolated from *Parauronema acutum* by the same procedures as those used for the isolation of *Paramecium*

symbionts (Soldo and Godoy 1974). It has been shown that killer xenosomes isolated in this way and purified with the help of Percoll gradients retain their ability to infect *P. acutum* and remain capable of killing sensitive *Uronema* strains (Soldo et al. 1986a). Results obtained from experiments in which isolated killer xenosomes lost their capacity to kill after they had been treated with various enzymes or had been coated with antibodies directed against the xenosomes indicate that the toxic principle of the xenosomes is a protein present at or near the surface (Soldo 1987).

### Identification of Xenosomes from *Parauronema acutum*

These symbionts are rod-shaped, Gram-negative bacteria, 0.3 µm in diameter and 0.8 µm long. The G + C content of the DNA is 33.9 mol%. The symbionts occur in the cytoplasm of the marine ciliate *Parauronema acutum* in numbers ranging from 50 to 300 per cell. Negative staining reveals the presence of flagella, which provide the symbionts with a spinning and darting motility when released from the host (Soldo 1987). The xenosomes were found to contain inclusions in the form of helical arrays. These structures (called "H bodies") are about 0.6 µm long and 0.026 µm wide. They occur singly and in multiples and extend almost the entire length of the symbiont. The H bodies were found in over 50 % of killer and nonkiller xenosomes (Soldo et al. 1987).

The genome size of xenosomes was found to be only 515 kbp, which is much smaller than that of free-living bacteria. Analytical measurements and data from sedimentation rate analyses led to the conclusion that the chromosomal DNA exists in the form of nine circularly permuted double-stranded DNA molecules, each about 515 kbp in length (Soldo et al. 1983). Both the small size and the multicopy nature of the genome are typical of bacterial symbionts rather than for free-living bacteria (Soldo and Godoy 1973a; Soldo 1987).

Killer and nonkiller xenosomes both contain plasmids (Soldo et al. 1986b; Soldo 1987). The nonkiller plasmids consist of two circular DNA duplexes, each 63 kbp in length. The killer plasmid consists of four circular 63-kbp DNA duplexes. When a ciliate, which previously harbored killer xenosomes but had been freed of them, is infected with nonkiller xenosomes, it becomes a killer. Soldo et al. (1986b) report that together with this transformation, the restriction pattern of the extrachromosomal DNA is altered. The mechanism by which this transformation takes place is unknown.

## Prokaryotic Symbionts of Ciliates from Anaerobic Environments

Ciliates living in an anaerobic habitat, such as the rumen or sewage sludge rich in hydrogen sulfide, show special adaptations. They usually lack mitochondria, bear hydrogenosomes, and are often associated with methanogenic bacteria (Finlay and Fenchel 1989; Stumm and Vogels 1989; Fenchel and Finlay 1991a; Narayanan et al. 2009). It has been shown that anaerobic ciliates generate their energy by converting carbohydrates to lactate, acetate, and butyrate and that they can remove reducing equivalents in the form of $H_2$ (Müller 1988). Production of $H_2$ by proton reduction involves the enzyme hydrogenase. However, this enzyme functions well only if the concentration of $H_2$ is kept low ($<10^{-5}$ atm). This requirement is apparently achieved by methanogenic bacteria, which consume $H_2$ and tend to be abundant in these habitats.

## Habitat of Prokaryotic Symbionts of Ciliates from Anaerobic Environments

An episymbiotic association with methanogenic bacteria was described for 11 species of rumen ciliates of the family Ophryoscolecidae (Vogels et al. 1980). The attached bacteria were rods 0.9–3.8 µm long and 0.6–0.7 µm wide that occurred as clusters or long chains. The symbionts were identified as methanogens (probably *Methanobrevibacter ruminantium*) on the basis of specific fluorescent coenzymes ($F_{350}$ and $F_{420}$; ❯ *Figs. 18.24* and ❯ *18.25*). Experiments with a fistulated sheep revealed a decrease of the association frequency of methanogenic bacteria with rumen ciliates when the hydrogen concentration in the rumen fluid increased and the reverse when hydrogen became scarce (Stumm et al. 1982). This is interpreted in favor of an interspecies transfer of hydrogen. The finding that rumen ciliates have hydrogenosomes, showing strong hydrogenase activity, supports this view (Yarlett et al. 1981). Stumm and Vogels (1989) propose that the attachment of hydrogen-consuming methanogens to rumen ciliates facilitates hydrogen removal, which is advantageous to both the ciliates and to their episymbionts. Fenchel and Finlay (1990; 1991a), however, have argued that the symbiosis is not of great advantage for the ciliates, since the external hydrogen tension is low and diffusion pathways are very short, the problem being greater for the bacteria to get hold of the $H_2$ that is produced than it is for the ciliate to get rid of it. Fenchel and Finlay (1991b) state that the presence of methanogens does not seem to be vital to the ciliates in any case that has been studied.

Episymbiotic bacteria have been known from sand-dwelling ciliates of anaerobic habitats for many years (Sauerbrey 1928; Kahl 1933, 1935; Fauré-Fremiet 1950b, 1951). They are firmly attached to the cell surface and appear to differ in size, shape, pigmentation, and wall structure from species to species (Fenchel et al. 1977; Fenchel and Finlay 1991a). Fauré-Fremiet (1950a) carried out a study of the symbionts of two species of the genus *Kentrophoros* (formerly *Centrophorella*). He investigated their symbionts and found them to be attached to the nonciliated dorsal side of these ribbon-shaped ciliates. The oblong symbionts were densely packed and protruded perpendicularly so that they appeared like bristles of a brush. They were Gram-negative, nonmotile rods and were observed to divide by longitudinal fission. Besides containing rather large quantities of polysaccharides (indicated by iodine treatment), they contained

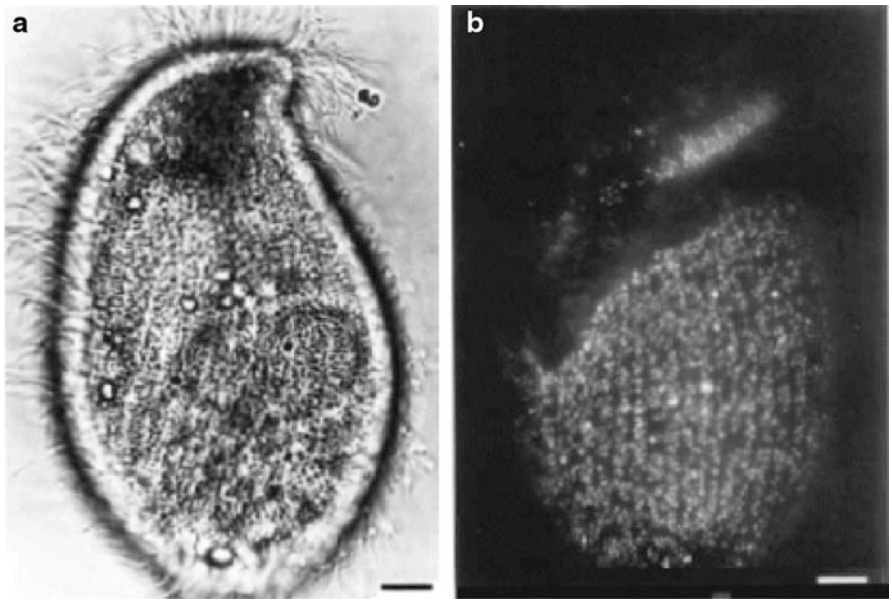



**Fig. 18.24**
***Metopus contortus*** **containing methanogenic endosymbionts: The methanogenic bacteria are located parallel to the kineties (*ciliary rows*) or the inner side of the cell. Cells are fixed with 1.2 % formaldehyde and 0.3 % glutaraldehyde. (a) Bright field micrograph. (b) Epifluorescence micrograph. Bars = 10 μm (From Van Bruggen et al. 1986)**

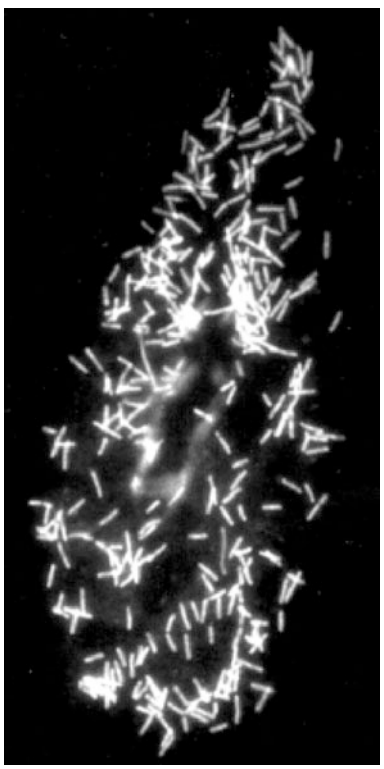


**Fig. 18.25**
**Autofluorescence of endosymbiotic methanogens in *Metopus palaeformis* (From Finlay and Fenchel 1992)**

dark refractive sulfur globules so that the host appeared black. The dark pigmentation is a strong indication that the symbionts are purple sulfur bacteria (Chromatiaceae). Fauré-Fremiet, who found the symbionts exclusively on the ciliates and neither on accompanying sand grains nor on glass slides that had been in the sand in which these ciliates lived for several days, considered the symbionts as obligately episymbiotic. Electron microscope studies by Raikov (1971, 1974) confirmed Fauré-Fremiet's observations and led to the discovery that the episymbiotic bacteria are phagocytized. He studied *Kentrophoros fistulosum* and *K. latum* and found that these ciliates take up the episymbiotic bacteria by pseudopodia-like cytoplasmic protrusions and enclose them in food vacuoles. This may occur at any place on the nonciliated body side of these ciliates, which have no special mouth structures. The unique form of phagocytosis discovered in these species has been named "random phagocytosis." Based on the content of the food vacuole of these ciliates, their epizoic bacteria appear to be their main nutrition source. Fourteen *Kentrophoros* species are now known. They all live in the interstices of sandy marine sediments.

Fenchel et al. (1977) examined marine sediment-dwelling ciliates for cytochrome oxidase activity and for fine-structural details. They found that many of the ciliates of this habitat lacked cytochrome oxidase activity and mitochondria but contained microbodies that were identified as hydrogenosomes (Van Bruggen et al. 1986). In addition, these ciliates harbored a species-specific flora of epi- and endosymbiotic bacteria. Large numbers of symbionts per cell were observed, ranging from about 1,000 bacteria per ciliate, corresponding to less

than 1 % of the host's biomass, to about 100,000 bacteria per ciliate, corresponding to about 20 % of the ciliate's biomass. Some of these anaerobic ciliates were found to contain both epi- and endosymbionts, and often more than one type of episymbiont was recognized. Fenchel et al. (1977) hypothesized that the bacteria and the ciliates interact metabolically.

Anaerobic conditions also exist in sediments that are rich in decaying plant material, such as those of freshwater ponds, lakes, ditches, swamps, and wastewater bioreactors. This habitat, also called "Faulschlamm" in German, was named "Sapropel" by Lauterborn (1901). Endosymbiotic bacteria in sapropelic ciliates were first described by Fauré- Fremiet (1909). Later, Liebmann (1937, 1938) noted that all the sapropelic ciliates that he investigated contained rodlike bacteria in their cytoplasm. In recent years, this habitat was thoroughly investigated (Stumm and Vogels 1989; Fenchel and Finlay 1991a; Hackstein and Stumm 1994), and it is now, next to the rumen, the best-studied anaerobic habitat. It is inhabited by large numbers of anaerobic protozoa and methanogenic bacteria. A survey of sapropelic ciliates by means of fluorescent microscopy revealed the presence of methanogenic bacteria inside the cells; they were spread throughout the cytoplasm in considerable quantities (Van Bruggen et al. 1983).

Electron microscopic investigations of sapropelic ciliates revealed the absence of mitochondria and the presence of microbodies. Because the bacteria were found to consume hydrogen, it has been hypothesized that the symbiont-associated microbodies are hydrogenosomes (Van Bruggen et al. 1986). In *Metopus striatus*, a Gram-positive rod-shaped bacterium was regularly found to be in close association with a hydrogenosome consisting of a granular matrix surrounded by a membrane (Van Bruggen et al. 1984). The bacterium was isolated and identified as *Methanobacterium formicicum*. A similar or even closer association of hydrogenosomes and methanogenic bacteria is reported for *Metopus contortus* (Van Bruggen et al. 1986; Finlay and Fenchel 1989; ❯ *Fig. 18.26*), *Plagiopyla nasuta* (Goosen et al. 1988), and *Plagiopyla frontata* (Embley and Finlay 1994). A direct proof was provided by Zwart et al. (1988), who demonstrated hydrogenase activity in microbodies of *Plagiopyla nasuta* and *Trimyema compressum*. Whereas methanogens are tightly packed with hydrogenosomes in ciliates such as *M. contortus* and *Plagiopyla frontata* (❯ *Fig. 18.27*) and *Trimyema* species (e.g., Finlay and Fenchel 1989, 1991a), the long methanogens found in *Metopus palaeformis* are not closely associated (Finlay and Fenchel 1991; ❯ *Fig. 18.28*). Symbionts in this ciliate appear to divide simultaneously, and growth rates of ciliates and methanogens are approximately equivalent (Fenchel and Finlay 1991b; Finlay and Fenchel 1992). A comparison of the phylogenetic diversity of endosymbiotic methanogens and anaerobic ciliates revealed that symbioses have formed repeatedly and independently (Finlay et al. 1993; Embley and Finlay 1994) with endosymbionts from the genus *Methanobacterium* and from the Methanomicrobiales. The authors found no congruence between the host and endosymbiont trees and thus no evidence of parallel speciation. Congeneric hosts in the genus *Plagiopyla* and *Metopus* contain endosymbionts which are more closely related to free-living methanogens than to each other (Embley and Finlay 1994).

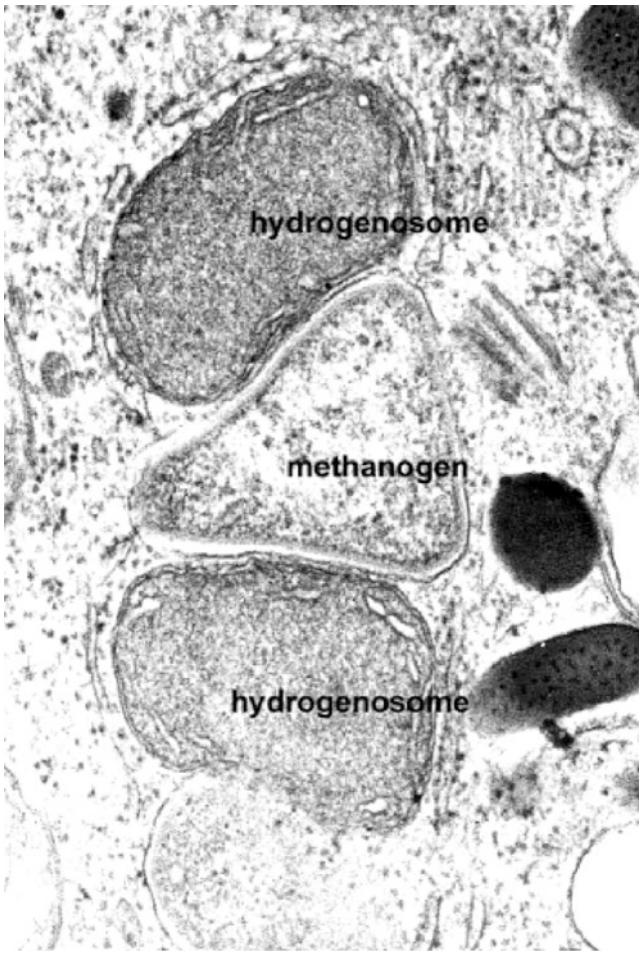



**Fig. 18.26**
**Symbiotic methanogen with hydrogenosomes in a transmission electron micrograph of the ciliate *Metopus contortus* (From Finlay and Fenchel 1989. Reprinted from Finlay and Fenchel 1989, © 1989, with permission from Elsevier Science)**

While many marine anaerobic ciliates have endosymbiotic methanogens, ectosymbiotic bacteria that are often found in addition to the endosymbionts are never methanogens. Fenchel and Finlay (1991a) assumed that the ectosymbionts are sulfate-reducing bacteria. Epibiotic bacteria have been reported also on the peritrichous ciliate *Zoothamnium niveum* collected in about 0.5–2.0 m depth on the vertical walls of a tidal channel cut into mangrove peat off the island of Twin Cays (Belize Barrier Reef, Carribean Sea; Bauer-Nebelsick et al. 1996a, b). The association appeared to be obligatory (the ciliate being unable to survive without its symbiont), and it was also shown that oxygen and sulfide were needed simultaneously. The white color of the bacteria is assumed to represent inclusions of elemental sulfur used as storage within the sulfide-oxidizing process. The bacteria were proven to be autotrophic. Epibiotic bacteria have been reported for a number of different species of *Zoothamnium* (cited in Bauer-Nebelsick et al. 1996a) but have not been further investigated.

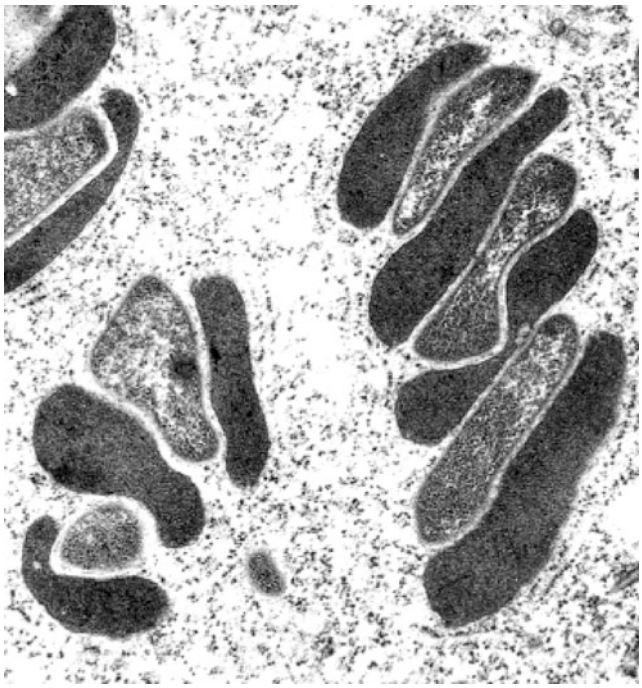


**Fig. 18.27**
**Stacks of alternating hydrogenosomes (*dark bodies*) and methanogens in a transmission electron micrograph of *Plagiopyla frontata* (From Embley and Finlay 1994)**

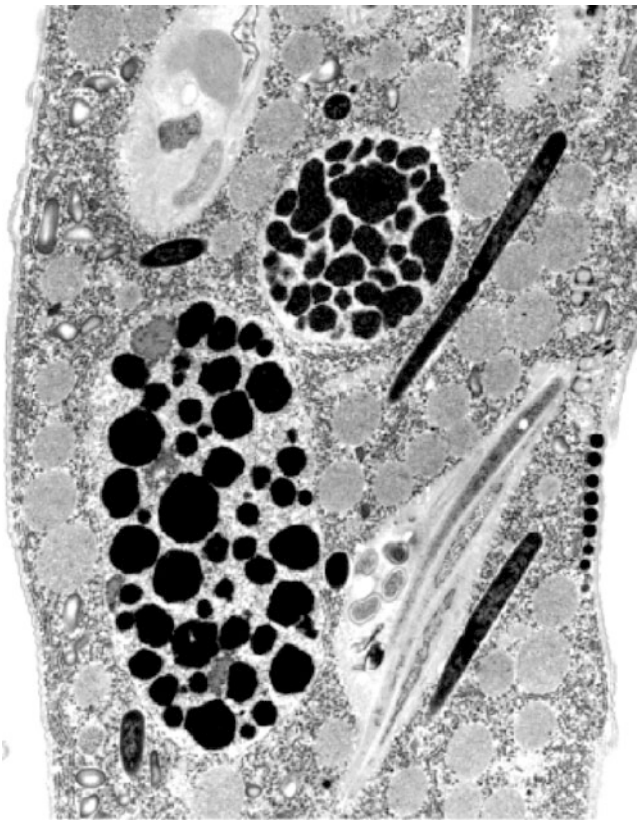


**Fig. 18.28**
**A transmission electron micrograph of *Metopus palaeformis* with *long rod-shaped* methanogens. *h* hydrogenosomes and *dv* digestive vacuole. Bar = 1 μm (From Finlay and Fenchel 1991)**

## Isolation of Prokaryotic Symbionts of Ciliates from Anaerobic Environments

For enrichment and culture of sapropelic ciliates, samples from anoxic natural sediments are introduced into mineral media under reducing conditions. Two media which have successfully been used for this purpose are given by Wagener and Pfennig (1987). They also reported the first monoxenic large-scale culture of an anaerobic ciliate. They enriched the ciliate *Trimyema compressum* from anoxic mud samples and then established a pure culture. The ciliate was fed with a bacterial strain isolated from the enrichment culture that proved capable of serving as food. During continued cultivation, *T. compressum* gradually lost its endosymbionts.

An isolation method for methanogenic bacteria from sapropelic ciliates has been described by Van Bruggen et al. (1986): A small number of ciliates is collected with a pipette and is washed free of potential contaminants in an isolation medium under a dissection microscope. The microscope is covered with a plastic hood flushed with nitrogen. The ciliates are then homogenized in an anaerobic glove box. The homogenate with the symbionts is plated on solid isolation medium containing penicillin G or lysozyme, which does not affect methanogenic bacteria. The homogenate is incubated at 22–37 °C under an atmosphere of $H_2/CO_2$ (80/20). In liquid media, growth can be followed by measuring the methane production.

## Identification of Prokaryotic Symbionts of Ciliates from Anaerobic Environments

The symbionts of several sapropelic ciliates have been identified as methanogenic bacteria. The endosymbionts of species of the ciliate *Metopus* (*Fig. 18.24*) were first recognized to be methanogens by their autofluorescence when irradiated with short-wavelength blue light (Van Bruggen et al. 1983, 1986). According to Doddema and Vogels (1978), epifluorescence microscopy can detect the presence of the deazaflavin coenzyme $F_{420}$ and the pterin compound $F_{342}$, both of which are specific for methanogens. The methanogenic character of the endosymbionts has subsequently been proven by studies of the isolated symbionts and measurements of their methane production in situ. The symbionts were finally identified as *Methanobacterium formicicum* (Van Bruggen et al. 1984). The methanogenic symbionts of *Plagiopyla nasuta* were also identified as *M. formicicum* (Goosen et al. 1988). This is a bacterium that often occurs in the sapropel; it is a slender, nonmotile rod, with a diameter of 0.4 μm and a length of 2–7 μm. It was identified by colony form, cell morphology, temperature and pH optimum, substrate specificity, DNA base composition, and type of coenzyme $F_{420}$. It appears likely that

*M. formicicum* is preadapted for endosymbiosis because symbiont-free lines of the ciliate *Trimyema compressum* could also successfully be infected with this bacterium (Wagener et al. 1990).

*Methanoplanus endosymbiosus* was isolated from the marine ciliate *Metopus contortus* (Van Bruggen et al. 1986). In liquid media, the isolated bacteria were irregular, nonmotile, nonsporeforming discs with a diameter of 1.6–3.4 µm. In a side view, they appeared as rods that were sometimes branched. The symbionts were osmotically fragile and lysed immediately when suspended in water. Growth and methanogenesis were observed with $H_2/CO_2$ or formate as substrates, with generation times of 7 or 12 h, respectively. The temperature range from growth was between 16 and 36 °C, with an optimum at 32 °C. The optimal pH range for growth was 6.8–7.3. Tungsten and NaCl at a concentration of 0.25 M were required for optimal growth. The G + C content of the DNA is 38.7 mol%.

*Methanosaeta* sp. found as endosymbionts in the ciliate *Metopus es* (Narayanan et al. 2009). Bacteria are belonging to archaeal methanogens. Archaea are rod shaped with an average length of about 3.4 µm which can be cultivated in a *Methanosaeta*-specific medium.

For detecting methanogenic endosymbionts, epifluorescence microscopy proved to be a powerful method. Even when symbionts are inside cells, their blue autofluorescence may be visible. Endosymbionts that fluoresce have been observed in the sapropelic ciliates *Brachonella spiralis*, *Caenomorpha medusula*, *C. universalis*, *Lacrymaria cucumis*, *Metopus es*, *M. laminarius* (Van Bruggen et al. 1983), *Parablepharisma pellitum*, *Metopus palaeformis*, and others (Fenchel and Finlay 1991b). They have not been found in ciliates from aerobic habitats.

## Electronic Supplementary Material

Video sequence showing killer trait. For the video, see the online version of *The Prokaryotes*.

Video sequence showing infection of *Paramecium* by *Holospora*. For the video, see the online version of *The Prokaryotes*.

Video sequence showing release of *Holospora*. For the video, see the online version of *The Prokaryotes*.

Video sequence showing the development of *Holospora*. For the video, see the online version of *The Prokaryotes*

## References


Abamo F, Dohra H, Fujishima M (2008) Fate of the 83-kDa periplamic protein of the infectious form of the endonuclear symbiotic bacterium *Holospora obtusa* during the infection process. FEMS Microbiol Lett 280:21–27

Allen SL, Nerad TA (1978) Method for the simultaneous establishment of many axenic cultures of *Paramecium*. J Protozool 25:134–139

Amann R, Springer N, Ludwig W, Görtz H-D, Schleifer K-H (1991) Identification in situ and phylogeny of uncultured bacterial endosymbionts. Nature 351:161–164

Amann RI, Ludwig W, Schleifer K-H (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Bak AL, Black FT, Christiansen C, Freundt EA (1969) Genome size of mycoplasmal DNA. Nature 224:1209–1210

Ball GH (1969) Organisms living on and in protozoa. In: Chen TT (ed) Research in protozoology. Pergamon, Oxford, pp 565–718

Bauer-Nebelsick M, Bardele CF, Ott J (1996a) A symbiosis between *Zoothamnium niveum* (Hemprich and Ehrenberg 1831) Ehrenberg 1838 (Oligohymenophorea, Peritrichida) and thiotrophic bacteria. I: redescription of the species based on light and scanning electron microscopic technique. Eur J Protistol 32:18–30

Bauer-Nebelsick M, Bardele CF, Ott J (1996b) Electron microscopic studies of *Zoothamnium niveum* (Hemprich & Ehrenberg 1831) Ehrenberg 1838 (Oligohymenophorea, Peritrichida), a ciliate with ectosymbiotic, chemmoautotrophic bacteria. Eur J Protistol 32:202–215

Beale GH, Jurand A (1966) Three different types of mate-killer (mu) particle in *Paramecium aurelia* (syngen 1). J Cell Sci 1:31–34

Beale GH, Jurand A, Preer JR Jr (1969) The classes of endosymbionts of *Paramecium aurelia*. J Cell Sci 5:65–91

Bedingfield G, Gibson I, Horne RW (1984) A comparative study of the structure of isolated refractile bodies (R-bodies) from *Paramecium* 1: the effect of treatment with EDTA and EGTA. Micron Microsc Acta 15:235–246

Beier CL, Horn M, Michel R, Schweikert M, Görtz H-D, Wagner M (2002) The genus *Caedibacter* comprises endosymbionts of *Paramecium* spp. related to the Rickettsiales (Alphaproteobacteria) and to *Francisella tularensis* (Gammaproteobacteria). Appl Environ Microbiol 68(12):6043–6050

Borchsenius ON, Skoblo II, Ossipov DV (1983) *Holospora curviuscula* – a new species of macronuclear symbiotic bacteria of *Paramecium bursaria*. Cytologia 25:91–97

Boss AO-L, Borchsenius ON, Ossipov DV (1987) *Pseudolyticum multiflagellatum* n. g, n. sp. – a new symbiotic bacterium in the cytoplasm of *Paramecium caudatum* (Ciliata, Protozoa). Cytologia 29:94–97

Bütschli O (1876) Studien über die ersten entwicklungsvorgänge der eizelle, die zelltheilung und die conjugation der infusorien. Abh Senckenb Naturforsch Ges 10:213–464

Bütschli O (1889) Protozoa. Abt. 111: infusoria und system der radiolaria. In: Bronn HG (ed) Klassen und ordnungen des thierreichs, vol 1. C.F. Winter, Leipzig, pp 1098–2035

Chen TT (1955) Paramecin 34, a killer substance produced by *Paramecium bursaria*. Proc Soc Exp Biol Med 88:541–543

Corliss JO (1985) Concept, definition, prevalence, and host-interactions of xenosomes (cytoplasmic and nuclear endosymbionts). J Protozool 32:373–376

Demar-Gervais C, Genermont J (1976) Recherches génétiques sur *Euplotes vannus*: les charactères "monster" et "killer". J Protozool 23 (Suppl) 8A

Dilts JA (1976) Covalently closed, circular DNA in kappa endosymbionts of *Paramecium*. Genet Res 627:161–170

Dilts JA (1986) The importance of the refractile body in expression of the killer trait in *Paramecium*. In: Wickner RB, Hinnebusch A, Lambowitz AM, Gunsalus IC, Hollaender A (eds) Extrachromosomal elements in lower eukaryotes. Plenum, New York

Dilts JA, Quackenbush RL (1986) A mutation in the R body-coding sequence destroys expression of the killer trait in *P tetraurelia*. Science 232:641–643

Dini F, Luporini P (1976) The mate-killer trait in a stock of *Euplotes crassus* (Dujardin) (Ciliata, Hypotrichida). Monitore Zool Ital (NS) 10:15–24

Dini F, Luporini P (1982) The inheritance of the mate-killer trait in *Euplotes crassus* (Hypotrichida, Ciliophora). Protistologica 18:179–184

Doddema HJ, Vogels GD (1978) Improved identification of methanogenic bacteria by fluorescence microscopy. Appl Environ Microbiol 36:752–754

Dohra H, Fujishima M (1999a) Cell structure of the infectious form of *Holospora*, an endonuclear symbiotic bacterium of the ciliate *Paramecium*. Zool Sci 16:93–98

Dohra H, Fujishima M (1999b) Effects of antibiotics on the early infection process of the macronuclear endosymbiotic bacterium *Holospora obtusa* of *Paramecium caudatum*. FEMS Microbiol Lett 179:473–477

Dohra H, Fujishima M, Hoshide K (1994) Monoclonal antibodies specific for periplasmic materials of the macronuclear specific bacterium *Holospora obtusa* of the ciliate *Paramecium caudatum*. Eur J Protistol 30:288–294

Dohra H, Yamamoto K, Fujishima M, Ishikawa H (1997) Cloning and sequencing of gene coding for a periplasmic 5.4 kDa peptide of the macronucleus-specific symbiont *Holospora obtusa* of the ciliate *Paramecium caudatum*. Zool Sci 14:69–75

Dohra H, Fujishima M, Ishikawa H (1998) Structure and expression of a GroE-homologous operon of a macronucleus-specific symbiont *Holospora obtusa* of the ciliate *Paramecium caudatum*. J Eukaryot Microbiol 4:71–79

Dorner RW (1957) Stability of paramecin 34 at different temperatures and pH values. Science 126:1243–1244

Ehrsam E, Görtz H-D (1999) Surface proteins of the gram-negative bacterium *Holospora obtusa* bind to macronuclear proteins of its host *Paramecium caudatum*. Eur J Protistol 35:304–308

Embley TM, Finlay BJ (1994) The use of small subunit rRNA sequences to unravel the relationships between anaerobic ciliates and their methanogen endosymbionts. Microbiology 140:225–235

Eschbach E, Pfannkuchen M, Schweikert M, Drutschmann D, Brümmer F, Fokin S, Ludwig W, Görtz HD (2009) "*Candidatus Paraholospora nucleivisitans*", an intracellular bacterium in *Paramecium sexaurelia* shuttles between the cytoplasm and the nucleus of its host. Sys Appl Microbiol 32:490–500

Estève J-C (1957) Une population de type "killer" chez *Paramecium caudatum* (Ehrenberg). Protistologica 14:201–207

Euzeby JP (1997) Revised nomenclature of specific or subspecific epithets that do not agree in gender with generic names that end in-bacter. Int J Syst Bacteriol 47(2):585–592

Fauré-Fremiet E (1909) Sur un cas de symbiose par un infusoir cilié. CR Soc Biol 67:113–114

Fauré-Fremiet E (1950a) Caulobactéries épizoiques associées aux Centrophorella (*Cilies holotriches*). Bull Soc Zool France 75:134–137

Fauré-Fremiet E (1950b) Ecologie des ciliés psammophiles litteraux. Bull Biol France-Belgique 84:35–75

Fauré-Fremiet E (1951) The marine sand dwelling ciliates of cape cod. Biol Bull 100:59–70

Fauré-Fremiet E (1952) Symbionts bacteriens des ciliés du genre *Euplotes*. CR Acad Sci 235:402–403

Favinger J, Stadtwald R, Gest H (1989) *Rhodospirillum centenum*, sp. nov., a thermotolerant cyst-forming anoxygenic photosynthetic bacterium. Ant V Leeuwenhoek 55:291–296

Fenchel T, Finlay BJ (1990) Anaerobic free-living protozoa: growth efficiencies and the structure of communities. FEMS Microbiol Ecol 74:269–276

Fenchel T, Finlay BJ (1991a) The biology of free-living anaerobic ciliates. Eur J Protistol 26:200–216

Fenchel T, Finlay BJ (1991b) Synchronous division of an endosymbiotic methanogenic bacterium in the anaerobic ciliate *Plagiopyla frontata* Kahl. J Protozool 38:22–28

Fenchel T, Perry T, Thane A (1977) Anaerobiosis and symbiosis with bacteria in free-living ciliates. J Protozool 24:154–163

Figueroa-de Soto VE, Soldo AT (1977) Characterization and base sequence homologies of the DNA of xenosomes and related particles. Int Congr Protozool 5:339

Finlay BL, Fenchel T (1989) Hydrogenosomes in some anaerobic protozoa resembling mitochondria. FEMS Microbiol Lett 65:187–190

Finlay BL, Fenchel T (1991) An anaerobic protozoon, with symbiotic methanogens, living in municipal landfill material. FEMS Microbiol Lett 65:169–180

Finlay BL, Fenchel T (1992) An anaerobic ciliate as a natural chemostat for the growth of endosymbiotic methanogens. Eur J Protistol 28:127–137

Finlay BJ, Embley TM, Fenchel T (1993) A new polymorphic methanogen, closely related to *Methanocorpusculum parvum*, living in stable symbiosis within the anaerobic ciliate *Trimyema sp.* J Gen Microbiol 139:371–378

Foissner W (1978) *Euplotes moebiusi f. quadricirratus* (Ciliata, Hypotrichida) II. The fine structure of some cytoplasmic organelles. Naturk Jahrb Stadt Linz 23:17–24

Fok A, Allen RD (1979) Axenic *Paramecium caudatum*. 1: mass culture and structure. J Protozool 6:463–470

Fok A, Allen RD (1988) The lysosome system. In: Görtz HD (ed) Paramecium. Springer, Berlin, pp 301–324

Fokin SI (1988) A bacterial symbiont of the macronucleus perinuclear space in the ciliate *Paramecium duboscqui*. Cytologia 30:632–635

Fokin SI (1991) *Holospora recta* sp. nov., a micronucleus-specific endobiont of the ciliate *Paramecium caudatum* (Russian with English summary). Cytologia 33:135–141

Fokin SI, Chivilev SM (1999) Brackish water Paramecium species and *Paramecium polycaryum*: morphometric analysis and some biological peculiarities. Acta Protozool 38:105–117

Fokin SI, Görtz HD (1993) *Caedibacter macronucleorum* sp. nov., a bacterium inhabiting the macronucleus of *Paramecium duboscqui*. Arch Protistenkd 143:319–324

Fokin SI, Görtz HD (2009) *Holospora bacteria* in *Paramecium* and their characterization. In: Fujishima M (ed) Endosymbionts in *Paramecium*. Microbiology Monpographs, vol 12. Springer, Berlin, Heidelberg, pp161–199

Fokin S, Karpov S (1995) Bacterial endocytobionts inhabiting the perinuclear space of Protista. Endocyt Cell Res 11:81–94

Fokin SI, Ossipov DV (1986) *Pseudocaedibacter glomeratus* n. sp. – A cytoplasmic symbiont of the ciliate *Paramecium pentaurelia*. Cytologia 28:1000–1004

Fokin SI, Sabaneyeva EV (1993) Bacterial endocytobionts of the ciliate *Paramecium calkinsi*. Eur J Protistol 29:390–395

Fokin SI, Sabaneyeva E (1997) Release of endonucleobiotic bacteria *Holospora bacillata* and *Holospora curvata* from the macronucleus of their host cells *Paramecium woodruffi* and *Paramecium calkinsi*. Endocyt Cell Res 12:49–55

Fokin SI, Skovorodkin IN (1991) *Holospora undulata* – endonucleobiont of the ciliate *Paramecium caudatum* in search for micronucleus. Cytology 33:64–75

Fokin SI, Skovorodkin IN (1997) Experimental analysis of the resistance of *Paramecium caudatum* (Ciliophora) against infection by bacterium *Holospora undulata*. Eur J Protisatol 33:214–218

Fokin SI, Ossipov DV, Skoblo II, Rautian MS, Sabaneyeva EV (1987a) Nonospora macronucleata g. n., sp. n–a vegetative nucleus symbiont of the ciliate *Paramecium caudatum*. Cytologia 29:963–970

Fokin SI, Boss AO-L, Ossipov DV (1987b) Virus-containing cytoplasmic symbiont of the ciliate Paramecium woodruffi. Cytologia 29:1303–1306

Fokin SI, Brigge T, Brenner J, Görtz H-D (1996) *Holospora* species infecting the nuclei of *Paramecium* appear to belong into two groups of bacteria. Eur J Protistol 32(suppl 1):19–24

Fokin SI, Schweikert M, Brümmer F, Gőrtz H-D (2005a) *Spirostomum* spp. (Ciliophora, Protista) – a new suitable system for endocytobiosis research. Protoplasma 255:93–102

Fokin SI, Schweikert M, Fujishima M (2005b) Recovery of the ciliate *Paramecium multimicronucleatum* following bacterial infection with *Holospora obtusa*. Eur J Protistol 41:129–138

Frankel J (1973) Dimensions of control of cortical patterns in *Euplotes*: the role of preexisting structure, the clonal life cycle, and the genotype. J Exp Zool 183:71–94

Freiburg M (1985) Isolation and characterization of macronuclei of *Paramecium caudatum* infected with the macronucleus-specific bacterium *Holospora obtusa*. J Cell Sci 73:389–398

Fujishima M (1993) Control of morphological changes of the endonuclear symbiont *Holospora* of the ciliate *Paramecium*. In: Sato S, Ishida M, Ishikawa H (eds) Endocytobiology, vol V. Tübingen University Press, Tübingen, pp 505–508

Fujishima M (2009) Infection and maintenance of *Holospora* species in *Paramecium caudatum*. In: Endosymbionts in *Paramecium*. Microbiology Monpographs Vol 12, Fujishima, M. (Ed.). Springer Berlin, Heidelberg, pp. 201–225

Fujishima J, Fujita M (1985) Infection and maintenance of *Holospora obtusa*, a macronuclear-specific bacterium of the ciliate *Paramecium caudatum*. J Cell Sci 76:179–187

Fujishima M, Heckmann K (1984a) Intra-and interspecific transfer of endosymbionts in *Euplotes*. J Exp Zool 230:339–345

Fujishima M, Nagahara K (1984b) Isolation of endonuclear symbiont *Holospora obtusa* from mass cultures of *Paramecium caudatum*. In: Proceedings of the annual meeting of the zoological society of Japan, vol 55. Toryokosku Press, Morioka, Japan, pp 36–40

Fujishima J, Nagahara K, Kojima K (1990a) Changes in morphology, buoyant density and protein composition in differentiation from the reproductive short form to the infectious long form of *Holospora obtusa*, a macronucleus-specific symbiont of the ciliate *Paramecium caudatum*. Zool Sci 7:849–860

Fujishima M, Sawabe H, Iwatsuki K (1990b) Scanning electron microscopic observation of differentiation from the reproductive short form to the infectious long form of *Holospora obtusa*. J Protozool 37:123–128

Fujishima M, Dohra H, Kawai M (1997) Quantitative changes in periplasmic proteins of the macronucleus-specific bacterium *Holospora obtusa* in the infection process of the ciliate *Paramecium caudatum*. J Euk Microbiol 44:636–642

Fujishima M, Kawai M, Yamamoto M (2005) *Paramecium caudatum* acquires heat-shock resistance in ciliary movement by infection with the endonuclear symbiotic bacterium *Holospora*. Zool Sci 23:1161

Gates MA, Curds CR (1979) The dargyroome of the genus *Euplotes*. Bull Br Mus Nat Hist (Zool) 35:127–200

Gellissen G, Michaelis G (1987) Gene transfer: mitochondria to nucleus. In: Lee JJ, Fredrick R (eds) Endocytobiology, vol III. Academy of Science, New York, pp 391–401

Gibson I (1974) The endosymbionts of *Paramecium*. Crit Rev Microbiol 3:243–273

Gibson I (1984) A comparison of the refractile bodies (R-bodies) of certain bacteria. III. Nucleotide sequence homologies and R-body function. Micron Microsc Acta 15:253–273

Gibson I, Beale GH (1961) Genic basis of the mate-killer trait in *Paramecium aurelia* stock 540. Genet Res 2:82–91

Goosen NK, Hormenans AMC, Hillebrand SJW, Stumm CK, Vogels GD (1988) Cultivation of the sapropelic ciliate *Plagiopyla nasuta* Stein and isolation of the endosymbiont *Methanobacterium formicicum*. Arch Microbiol 150:165–170

Görtz H-D (1981) Ein neues symbiontisches Bakterium in *Paramecium sexaurelia*. Verh Dtsch Zool Ges 74:227

Görtz H-D (1983) Endonuclear symbionts in ciliates. In: Jeon KW (ed) Intracellular symbiosis. International review of cytology, supplement 14. Academic, New York, pp 145–176

Görtz H-D (1987) Different endocytobionts simultaneously colonizing ciliate cells. Ann NY Acad Sci 503:261–268

Görtz H-D (1992) Bactéries endosymbiotiques de Paramecium. Ann Biolog 73:41–49

Görtz H-D (2001) Intracellular bacteria in ciliates. Int Microbiol 4(3):143–150

Görtz H-D, Dieckmann J (1980) Life cycle and infectivity of *Holospora elegans* Hafkine, a micronucleus-specific symbiont of *Paramecium caudatum* (Ehrenberg). Protistologica 16:591–603

Görtz H-D, Fokin SI (2009) Diversity of endosymbiotic bacteria in *Paramecium*. In: Fujishima M (ed) Endosymbionts in *Paramecium*. Microbiology Monpographs, vol 12. Springer, Berlin/Heidelberg, pp 131–160

Görtz H-D, Freiburg M (1984) Bacterial symbionts in the micronucleus of *Paramecium bursaria*. Endocyt Cell Res 1:37–46

Görtz H-D, Fujishima M (1983) Conjugation and meiosis of *Paramecium caudatum* infected with the micronucleus-specific bacterium *Holospora elegans*. Eur J Cell Biol 32:86–91

Görtz H-D, Maier G (1991) A bacterial infection in a ciliate from sewage sludge. Endocyt Cell Res 8:45–52

Görtz H-D, Wiemann M (1987) Colonization of the ciliate *Stentor multiformis* by three different endocytobionts. Endocyt Cell Res 4:177–184

Görtz H-D, Wiemann M (1989) Route of infection of the bacteria *Holospora elegans* and *Holospora obtusa* into the nuclei of *Paramecium caudatum*. Eur J Protistol 24:101–1090

Görtz H-D, Freiburg M, Wiemann M (1988) Polypeptide differences between infectious and reproductive forms of *Holospora obtusa*, an endonucleobiotic bacterium in *Paramecium caudatum*. Endocyt Cell Res 5:233–244

Görtz H-D, Ahlers N, Robenek H (1989) Ultrastructure of the infectious and reproductive forms of *Holospora obtusa*, a bacterium infecting the macronucleus of *Paramecium caudatum*. J Gen Microbiol 135:3079–3085

Görtz H-D, Lellig S, Miosga O, Wiemann M (1990) Changes in fine structure and polypeptide pattern during development of *Holospora obtusa*, a bacterium infecting the macronucleus of *Paramecium caudatum*. J Bacteriol 172:5664–5669

Görtz H-D, Benting J, Ansorge I, Freiburg M (1992) Cell surface proteins of the infectious forms of the symbiotic bacterium *Holospora obtusa*. Symbiosis 14:391–397

Grimes GW, Preer JR Jr (1971) Further observations on the correlation between kappa and phage-like particles in *Paramecium*. Genet Res 18:115–116

Gromov BV, Ossipov DV (1981) *Holospora* (ex Hafkine 1890) nom. rev., a genus of bacteria inhabiting the nuclei of paramecia. Int J Syst Bacteriol 31:348–352

Hackstein JHP, Stumm CK (1994) Methane production in terrestrial arthropods. Proc Natl Acad Sci 91:5441–5445

Hafkine M-W (1890) Maladies infectieuses des paramécies. Ann Inst Pasteur (Paris) 4:148–162

Hahn MW (2003) Isolation of strains belonging to the cosmopolitan *Polynucleobacter necessarius* cluster from freshwater habitats located in three climatic zones. Appl Environ Microbiol 69:5248–5254

Hahn MW, Lang E, Brandt U, Wu QL, Scheuerl T (2009) Emended description of the genus *Polynucleobacter* and the species *P necessarius* and proposal of two subspecies *P. necessarius* subspecies necessarius subsp. nov. and *P. necessarius* subsp. A symbioticus subsp nov. Int J Syst Evol Microbiol 59(8):2002–2009

Hausmann K, Bradbury PC (eds) (1996) Ciliates—cells as organisms. Gustav Fischer-Verlag, Stuttgart

Heatherington S (1934) The sterilization of protozoa. Biol Bull 67:315–321

Heckmann K (1963) Paarungstypsystem und genabhängige Paarungstypdifferenzierung bei dem hypotrichen Ciliaten *Euplotes vannus*. O F Müller Arch Protistenk 106:393–421

Heckmann K (1975) Omikron, ein essentieller Endosymbiont von *Euplotes aediculatus*. J Protozool 22:97–104

Heckmann K (1983) Endosymbionts of *Euplotes*. In: Jeon KW (ed) Intracellular symbiosis International review of cytology. Academic, New York, pp 111–144

Heckmann K, Frankel J (1968) Genic control of cortical pattern in *Euplotes*. J Exp Zool 168:11–38

Heckmann K, Görtz HD (1991) Prokaryotic symbionts of ciliates. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KD (eds) The prokaryotes, 2nd edn. Springer, New York, pp 3865–3890

Heckmann K, Schmidt HJ (1987) *Polynucleobacter necessarius* gen. nov., spe nov., an obligately endosymbiotic bacterium living in the cytoplasm of *Euplotes aediculatus*. Int J Syst Bacteriol 37:456–457

Heckmann K, Preer JR Jr, Straetling WH (1967) Cytoplasmic particles in the killers of *Euplotes minuta* and their relationship to the killer substance. J Protozool 1:360–363

Heckmann K, ten Hagen R, Görtz H-D (1983) Fresh-water *Euplotes* species with a 9 type 1 cirrus pattern depend upon endosymbionts. J Protozool 30:284–289

Heckmann K, Schmidt HJ, Fujishima M (1986) Omikron and omikron-like endosymbionts of *Euplotes*. In: Wickner RB, Hinnebusch A, Lambowitz AM, Gunsalus IC, Hollaender A (eds) Extrachromosomal elements of lower eukaryotes. Plenum, New York, pp 327–335

Henry SM (1966) Symbiosis: vol I. Associations of microorganisms, plants, and marine organisms. Academic, New York/London

Hernández-Romero D, Lucas-Elío P, López-Serrano D, Solano F, Sanchez-Amat A (2003) Marinomonas mediterranea is a lysogenic bacterium that synthesizes R-bodies. Microbiology 149:2679–2686

Heruth DP, Pond FR, Dilts JA, Quackenbush RL (1994) Characterization of genetic determinants for R body synthesis and assembly in *Caedibacter taeniospiralis* 47 and 116. J Bacteriol 176:3559–3567

Holtzman HE (1959) A kappa-like particle in a non-killer stock of *Paramecium aurelia*, syngen 5. J Protozool 6(Suppl):26

Hori M, Fujishima M (2003) The endosymbionts bacterium *Holospora obtusa* enhances heat-shock gene expression of the host *Paramecium caudatum*. J Eukaryot Microbiol 50:293–298

Hori M, Fujii K, Fujishima M (2008) Micronucleus-specific bacterium *Holospora elegans* irreversibly enhances stress gene expression of the host *Paramecium caudatum*. J Eukaryot Microbiol 55:515–521

Iwatani K, Dohra H, Lang FB, Burger G, Hori M, Fujishima M (2005) Translocation of an 89-kDa periplasmic protein is associated with *Holospora* infection. Biochem Biophys Res Comm 337:1198–2015

Jenkins RA (1970) The fine structure of a nuclear envelope associated endosymbiont of *Paramecium*. J Gen Microbiol 61:355–359

Jeon KW, Jeon MS (1976) Scanning electron microscope observations of *Amoeba proteus* during phagocytosis. J Protozool 23:83–86

Kahl A (1933) Ciliata libera et ectocommensalia. In: Grimpe G, Wagler E (eds) Die Tierwelt der Nord-und Ostsee, Teil II Akademische Verlagsgesellschaft Becker & Erler, Leipzig, Germany, pp 29–146

Kahl A (1935) Urtiere oder protozoa I: Wimpertiere oder ciliata (infusoria). 4: peritricha und chonotricha nachtrag. In: Dahl F (ed) Die tierwelt Deutschlands. Gustav Fischer, Jena, pp 806–842

Kanabrocki JA, Lalucat J, Cox BJ, Quackenbush RL (1986) Comparative study of refractile (R) bodies and their genetic determinants: relationship of type 51 R bodies to R bodies produced by *Pseudomonas taeniospiralis*. J Bacteriol 168:1019–1022

Kawai M, Fujishima M (2000) Invasion of the macronucleus of *Paramecium caudatum* by the bacterium *Holospora obtusa*: fates of the bacteria and timing of invasion steps. Eur J Protistol 36:46–52

Kirby H Jr (1941) Organisms living on and in protozoa. In: Calkins GN, Summers FM (eds) Protozoa in biological research. Columbia University Press, New York, pp 1009–1113

Kuhlmann H-W, Heckmann K (1989) Adolescence in *Euplotes octocarinatus*. J Exp Zool 251:316–328

Kusch J, Stremmel M, Schweikert M, Adams V, Schmidt HJ (2000) The toxic symbiont *Caedibacter caryophila* in the cytoplasm of *Paramecium novaurelia*. Microb Ecol 40:330–335

Kusch J, Czubatinski L, Wegmann S, Hübner M, Alter M, Albrecht P (2001) Competitive advantages of Caedibacter-infected paramecia. Protist 153:47–58

Lalucat J, Meyer O, Mayer F, Pares R, Schlegel HG (1979) R bodies in newly isolated free-living hydrogen-oxidizing bacteria. Arch Microbiol 121:9–15

Lalucat J, Wells B, Gibson I (1986) Relationships between R bodies of certain bacteria. Micron Microsc Acta 17:243–245

Landis WG (1981) The ecology, role of the killer trait, and interactions of five species of the *Paramecium aurelia* complex inhabiting the littoral zone. Can J Zool 59:1734–1743

Landis WG (1987) Factors determining the frequency of the killer trait within populations of the *Paramecium aurelia* complex. Genetics 115:197–206

Lauterborn R (1901) Die "sapropelische" Lebewelt. Zool Anz 24:50–55

Laval M (1970) Présence de bactéries intranucléaires chez Zoothamnium pelagicum. In: 7th Internernational Congress on Electron Microscoscopy, Grenoble, pp 403–404

Liebmann H (1937) Bakteriensymbiose bei Faulschlammziliaten. Biol Zbl 57:442–445

Liebmann H (1938) Biologie und Chemismus der Bleilochsperre. Arch Hydrobiol 33:1–81

Luporini P (1974) Killer and autogamous strains of *Euplotes crassus* Dujardin from the Somalien coast. Monit Zool Ital (NS) 5(Suppl):129–132

Margulis L, Fester R (1991) Symbiosis as the source of evolutionary innovation. MIT Press, Cambridge, MA

Meenaghan H, Reilly K, Wells B, Gibson I (1984) A comparison of the refractile bodies (R-bodies) of certain bacteria. II: effects of pH on the structure. Micron Microsci Acta 15:247–252

Mueller JA (1963) Separation of kappa particles with infective activity from those with killing activity and identification of the infective particles in *Paramecium aurelia*. Exp Cell Res 30:492–508

Müller J (1856) Beobachtungen an Infusorien. Monatsber Königl Preuβ Akad Wissensch, Berlin, Juli, pp 389–393

Müller M (1988) Energy metabolism of protozoa without mitochondria. Ann Rev Microbiol 42:465–488

Nakamura Y, Aki M, Aikawa T, Hori M, Fujishima M (2004) Differences in gene expression of the ciliate *Paramecium caudatum* caused by endonuclear symbiosis with *Holospora obtusa*, revealed using differential display reverse transcribed PCR. FEMS Microbiol Lett 240:209–213

Narayanan N, Krishnakumar B, Anupama VN, Manilal VB (2009) *Methanosaeta sp.*, the major endosymbionts of *Metopus es*. Res Microbiol 160:600–607

Nobili R, Rosati G, Verni F (1976) The killer-trait in Euplotes crassus. Boll Zool 43:251–258

Ossipov DV (1973) Specific infectious specificity of the omega-particles, micronuclear symbiotic bacteria of *Paramecium caudatum*. Cytologia 15:211–217

Ossipov DV (1981) Problems of nuclear heteromorphism in the unicellular organisms. Nauka, Leningrad

Ossipov DV, Podlipaev SA (1977) Electron microscope examinations of early stages of infection of *Paramecium caudatum* by bacterial symbionts of the macronucleus (Iota-bacteria). Acta Protozool 16:289–308

Ossipov DV, Skoblo II, Rautian MS (1975) Iota-particles, macronuclear symbiotic bacteria of the ciliate *Paramecium caudatum* clone M 115. Acta Protozool 14:263–280

Ossipov DV, Skoblo II, Borchsenius ON, Rautian MS, Podlipaev SA (1980) *Holospora acuminata* – a new species of symbiotic bacterium from the micronucleus of the cilíate *Paramecium bursaria*. Focke Cytolog 22:922–929

Ossipov DV, Skoblo II, Borchsenius ON, Lebedeva NA (1993) Interactions between *Paramecium bursaria* (Protozoa, Ciliophora, Hymenostomatida) and their nuclear symbionts. I: phenomenon of symbiogenic lysis of the bacterium *Holospora acuminata*. Eur J Protistol 29:61–71

Paulin JJ (1996) Morphology and cytology of ciliate. In: Hausmann K, Bradbury PC (eds) Ciliates: cells as organisms. Gustav Fischer-Verlag, Stuttgart, pp 1–40

Petroni G, Spring S, Schleifer KH, Verni F, Rosati G (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

Petroni G, Dini F, Verni F, Rosati G (2001) A molecular approach to the tangled intragenic relationships underlying phylogeny in *Euplotes* (Ciliophora, Spirotricha). Molec Phylogenet Evol 22(1):118–130

Pond FR, Gibson I, Lalucat J, Quackenbush RL (1989) R-body producing bacteria. Microbiol Rev 53:25–67

Preer JR Jr (1948a) A study of some properties of the cytoplasmic factor "kappa" in *Paramecium aurelia*, variety 2. Genetics 33:349–404

Preer JR Jr (1948b) The killer cytoplasmic factor kappa: Its rate of reproduction, the number of particles per cell, and its size. Am Natural 82:35–42

Preer JR Jr (1950) Microscopically visible bodies in the cytoplasm of the "killer" strains of *Paramecium aurelia*. Genetics 35:344–362

Preer LB (1969) Alpha, an infectious macronuclear symbiont of *Paramecium aurelia*. J Protozool 16:570–578

Preer JR Jr (1977) The killer system in Paramecium-kappa and its viruses. Microbiology 1977:576–578

Preer LB (1981) Prokaryotic symbionts of *Paramecium*. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds) The prokaryotes. Springer, New York, pp 2127–2136

Preer JR Jr, Jurand A (1968) The relationship between virus-like particles and R bodies of *Paramecium aurelia*. Genet Res 12:331–340

Preer LB, Preer JR Jr (1964) Killing activity from lysed particles of *Paramecium*. Genet Res 5:230–239

Preer JR Jr, Preer LB (1967) Virus-like bodies in killer-paramecia. Proc Natl Acad Sci USA 58:1774–1781

Preer JR Jr, Preer LB (1982) Revival of names of protozoan endosymbionts and proposal of *Holospora caryophila* nom. nov. Int J Syst Bacteriol 32:140–141

Preer JR Jr, Preer LB (1984) Endosymbionts of protozoa. In: Krieg NR (ed) Bergey's manual of systematic bacteriology, vol 1. Williams and Wilkins, Baltimore, pp 795–811

Preer JR Jr, Stark P (1953) Cytological observations on the cytoplasmic factor "kappa" in *Paramecium aurelia*. Exp Cell Res 5:478–491

Preer JR Jr, Hufnagel LA, Preer LB (1966) Structure and behavior of "R" bodies from killer paramecia. J Ultrastr Res 15:131–143

Preer JR Jr, Preer LB, Rudman B, Jurand A (1971) Isolation and composition of bacteriophage-like particles from kappa of killer paramecia. Mole Gen Genet 111:202–208

Preer LB, Jurand A, Preer JR Jr, Rudman B (1972) The classes of kappa in *Paramecium aurelia*. J Cell Sci 11:581–600

Preer JR Jr, Preer LB, Jurand A (1974a) Kappa and other endosymbionts of *Paramecium aurelia*. Bacteriol Rev 38:113–163

Preer LB, Rudman B, Preer JR Jr (1974b) Induction of R bodies by ultraviolet light in killer paramecia. J Gen Microbiol 80:209–215

Przybosz E, Fokin SI (1997) Species of the *Paramecium aurelia* complex Sonneborn in Germany. Arch Protistenkd 148:167–172

Quackenbush RL (1978) Genetic relationships among bacterial endosymbionts of *Paramecium aurelia*: polynucleotide sequence relationship among members of *Caedobacter*. J Gen Microbiol 108:181–187

Quackenbush RL (1982) Validation of the publication of new names and new combinations previously published outside the IJSB. List No. 8. Int J Syst Bacteriol 32:266–268

Quackenbush RL (1983) Plasmids from bacterial endosymbionts of hump-killer paramecia. Plasmid 9:298–306

Quackenbush RL (1988) Endosymbionts of killer paramecia. In: Görtz HD (ed) Paramecium. Springer, Berlin, pp 406–418

Quackenbush RL, Burbach JA (1983) Cloning and expression of DNA sequences associated with the killer trait of *Paramecium tetraurelia* stock 47. Proc Natl Acad Sci USA 80:250–254

Quackenbush RL, Cox BJ, Kanabrocki JA (1986) Extrachromosomal elements of extrachromosomal elements of *Paramecium* and their extrachromosomal elements. In: Wickner RB, Hinnebusch A, Lambowitz AM, Gunsalus IC, Hollaender A (eds) Extrachromosomal elements in lower eukaryotes. Plenum, New York, pp 265–278

Raikov IB (1971) Bactéries épizoiques et mode de nutrition du cilié psammophile Kentrophorus fistulosum Fauré-Fremiet (étude au microscope electronique). Protistologica 7:365–378

Raikov IB (1974) Etude ultrastructurale des bactéries épizoiques et éndozoiques de Kentrophorus latum Raikov, cilié holotriche mésopsammique. Cahiers de Biologie Marine 15:379–393

Read CP (1970) Parasitism and symbiology. Ronald Press, New York

Roller C, Ludwig W, Schleifer K-H (1992) Gram-positive bacteria with a high DNA G-C content are characterized by a common insertion with their 23S rRNA genes. J Gen Microbiol 138:1167–1175

Rosati G (1999) Epixenosomes: symbionts of the hypotrich ciliate *Euplotidium itoi*. Symbiosis 26:1–24

Rosati G, Verni F (1975) Macronuclear symbionts in *Euplotes crassus* (Ciliata, Hypotrichida). Bull Zool 42:231–232

Rosati G, Verni F (1977) Bacteria-like endosymbionts in *Euplotes crassus*. In: Hutner SH (ed) 5th Iternational Congress of Protozoology, Abstract Society of Protozoology, New York, 443 pp

Rosati G, Verni F, Luporini P (1976) Cytoplasmic bacteria-like endosymbionts in *Euplotes crassus* (Dujardin) (Ciliata, Hypotrichida). Monitore Zool Ital (NS) 10:449–460

Rosati G, Lenzi P, Verni F (1993a) "Epixenosomes": peculiar epibionts of the protozoon ciliate *Euplotidium itoi*. Do their cytoplasmic tubules consist of tubulin? Micron 24:465–471

Rosati G, Verni F, Lenzi P (1993b) "Epixenosomes": peculiar epibionts of the ciliate *Euplotidium itoi*. The formation of the extrusive apparatus and the ejecting mechanism. Eur J Protistol 29:238–245

Rosati G, Petroni G, Quochi S, Modeo L, Verni F (1999) Epixenosomes: peculiar epibionts of the hyprotrich ciliate *Euplotidium itoi* defend their host against predators. J Eukaryot Microbiol 46:278–282

Ruthmann A, Heckmann K (1961) Formwechsel und struktur des makronukleus von *Bursaria truncatella*. Arch Protistenkd 105:313–340

Sabaneyeva EV, Derkacheva ME, Benken KA, Fokin SI, Vainio S, Skovorodkin IN (2009) Actin-based mechanism of holospora obtusa trafficking in *Paramecium caudatum*. Protist 160:205–219

Sauerbrey E (1928) Beobachtungen über einige neue oder wenig bekannte marine Ciliaten. Arch Protistenkd 61:355–407

Schmidt HJ (1982) Isolation of omikron-endosymbionts from mass cultures of *Euplotes aediculatus* and characterization of their DNA. Exp Cell Res 140:417–425

Schmidt HJ (1984) Studies on protein synthesis in kappa particles. J Gen Microbiol 130:1517–1523

Schmidt HJ, Freiburg M, Görtz HD (1987a) Comparison of the infectious forms of two bacterial endonucleobionts, *Holospora elegans* and *H. obtusa*, from the ciliate *Paramecium caudatum*. Microbios 49:189–197

Schmidt HJ, Görtz HD, Quackenbush RL (1987b) *Caedibacter caryophila* sp. nov., a killer symbiont inhabiting the macronucleus of *Paramecium caudatum*. Int J Syst Bacteriol 37:459–462

Schmidt HJ, Pond FR, Görtz HD (1987c) Refractile bodies (R bodies) from the macronuclear killer particle *Caedibacter caryophila*. J Cell Sci 88:177–184

Schmidt HJ, Görtz HD, Pond FR, Quackenbush RL (1988) Characterization of *Caedibacter* endonucleobionts from the macronucleus of *Paramecium caudatum* and the identification of a mutant with blocked R-body synthesis. Exp Cell Res 174:49–57

Schneller MV (1958) A new type of killing action in a stock of *Paramecium aurelia* from Panama. Proc Ind Acad Sci 67:302

Schneller MV, Sonneborn TM, Mueller JA (1959) The genetic control of kappa-like particles in *Paramecium aurelia*. Genetics 44:533–534

Schönefeld U, Alfermann AW, Schultz JE (1986) Economic mass cultivation of *Paramecium tetraurelia* on a 200-liter scale. J Protozool 33:222–225

Schrallhammer M, Schweikert M (2009) The killer effect of *Paramecium* and its causative agents. In: Fujishima M (ed) Endosymbionts in *Paramecium*. Microbiology Monpographs, vol 12. Springer, Dortrecht/Heidelberg/London/New York, pp 228–246

Schrallhammer M, Schweikert M, Vallesi A, Verni F, Petroni G (2010) Detection of a novel subspecies of *Francisella noatunensis* as endosymbiont of *Euplotes raikovi*. Microb Ecol 61:455–464

Siegel RW (1953) A genetic analysis of the mate-killer trait in *Paramecium aurelia*, variety 8. Genetics 38:550–560

Siegel RW, Heckmann K (1966) Inheritance of autogamy and the killer trait in *Euplotes minuta*. J Protozool 13:34–48

Simon EM, Schneller MV (1973) The preservation of ciliated protozoa at low temperature. Cryobiology 10:421–426

Sitte P (1993) Symbiogenetic evolution of complex cells and complex plastids. Eur J Protistol 29:131–143

Skoblo II, Lebedeva NA (1986) Infection of the nuclear apparatus of *Paramecium bursaria* (Ciliata) by the symbiotic bacterium *Holospora curviuscula*. Cytologia 28:367–372

Skoblo II, Borchsenius ON, Lebedeva NA, Ossipov DV (1985) A new species of symbiotic bacteria of *Paramecium bursaria*, Ciliophora, Protozoa. Cytologia 27:1292–1297

Skoblo II, Borchsenius ON, Lebedeva NA, Ossipov DV (1990) Symbiogenic lysis of bacteria *Holospora acuminata* in the generative nucleus of the ciliate Paramecium bursaria. Cytologia 32:515–519

Smith IE (1961) Purification of kappa particles of *Paramecium aurelia*, stock 51. Am Zool 1:390

Smith-Sonneborn JE, Van Wagtendonk WJ (1964) Purification and chemical characterization of kappa of stock 51, *Paramecium aurelia*. Exp Cell Res 33:50–59

Smurov AO, Fokin SI (1999) Resistence of *Paramecium* species (Ciliophor, Peniculia) to salinity of environment. Protistology 1:43–53

Soldo AT (1963) Axenic culture of Paramecium: some observations on the growth behavior and nutritional requirements of a particle-bearing strain of *Paramecium aurelia* 299 lambda. Ann NY Acad Sci 108:380–388

Soldo AT (1974) Intracellular particles in *Paramecium*. In: Van Wagtendonk WJ (ed) Paramecium: a current survey. Elsevier, Amsterdam, pp 377–442

Soldo AT (1983) The biology of the xenosome, an intracellular symbiont. In: Jeon KW (ed) Intracellular symbiosis. Int Rev Cytol, Suppl 14. Academic Press, New York, pp 79–109

Soldo AT (1987) *Parauronema acutum* and its xenosomes, a model system. J Protozool 34:447–451

Soldo AT, Brickson SA (1978) Observation on the ultrastructure, mode of infectivity and host range of xenosomes. Tissue Cell 10:609–618

Soldo AT, Godoy GA (1973a) Molecular complexity of *Paramecium* symbiont lambda deoxyribonucleic acid: evidence for the presence of a multicopy genome. J Mol Biol 73:93–108

Soldo AT, Godoy GA (1973b) Observations on the production of folic acid by symbiont lambda particles of *Paramecium aurelia* stock 299. J Protozool 20:502

Soldo AT, Godoy GA (1974) The molecular complexity of mu and pi symbiont DNA of *Paramecium aurelia*. Nucl Acid Res 1:387–396

Soldo AT, Van Wagtendonk WJ (1969) The nutrition of *Paramecium aurelia*, stock 299. J Protozool 16:500–506

Soldo AT, Godoy GA, Van Wagtendonk WJ (1966) Growth of particle-bearing and particle-free *Paramecium aurelia* in axenic culture. J Protozool 13:492–497

Soldo AT, Van Wagtendonk WJ, Godoy GA (1970) Nucleic acid and protein content of purified endosymbiote particles of *Paramecium aurelia*. Biochim Biophys Acta 204:325–333

Soldo AT, Godoy GA, Brickson SA (1974) Infectious particles in a marine ciliate. Nature 249:284–286

Soldo AT, Brickson SA, Larin F (1983) The size and structure of the DNA genome of symbiont xenosome particles in the ciliate *Parauronema acutum*. J Gen Microbiol 129:1317–1325

Soldo AT, Brickson SA, Castiglione GA, Freytag AF (1986a) Association of transformation of xenosomes from nonkiller to killer with extrachromosomal DNA. J Bacteriol 169:96–102

Soldo AT, Brickson SA, Freytag A (1986b) Structure and characterization of extrachromosomal DNA of killer xenosomes, intracellular symbionts of a marine protozoan. In: Wickner RB, Hinnebusch A, Lam AM, Gunsalus IC, Hollaender A (eds) Extrachromosomal elements in lower eukaryotes. Plenum, New York, pp 291–302

Soldo AT, Brickson SA, Castiglione GA (1987) Inclusion bodies in xenosomes purified on percoll gradients as revealed by electron microscopy. J Protozool 34:6–9

Sonneborn TM (1938) Mating types, toxic interactions and heredity in *Paramecium aurelia*. Science 88:503

Sonneborn TM (1943) Gene and cytoplasm. I: the determination and inheritance of the killer character in variety 4 of *P aurelia*. II: the bearing of determination and inheritance of characters in *P aurelia* on problems of cytoplasmic inheritance, pneumococcus transformation, mutations and development. Proc Natl Acad Sci USA 29:329–343

Sonneborn TM (1950) Methods in the general biology and genetics of *Paramecium aurelia*. J Exp Zool 113:87–148

Sonneborn TM (1959) Kappa and related particles in *Paramecium*. Adv Virus Res 6:229–356

Sonneborn TM (1970) Methods in *Paramecium* research. In: Prescott M (ed) Methods in cell physiology, vol 4. Academic, New York, pp 241–339

Sonneborn TM (1975) The *Paramecium aurelia* complex of fourteen sibling species. Trans Am Microsci Soc 94:155–178

Sonneborn TM, Mueller JA, Schneller MV (1959) The classes of kappa-like particles in *Paramecium aurelia*. Anat Rec 134:642

Springer N, Ludwig W, Amann R, Schmidt HJ, Görtz HD, Schleifer KH (1993) Occurrence of fragmented 16S rRNA in an obligate bacterial endosymbiont of *Paramecium caudatum*. Proc Natl Acad Sci USA 90:9892–9895

Springer N, Amann R, Ludwig W, Schleifer K-H, Schmidt HJ (1996) *Polynucleobacter necessarius*, an obligate bacterial endosymbiont of the hypotrichous ciliate *Euplotes aediculatus*, is a member of the beta-subclass of Proteobacteria. FEMS Microbiol Lett 135:333–336

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PA, Kampfe P, Maiden MC, Nesme X, Rossello-Mora R, Swings J, Truper HG, Vauterin L, Ward AC, Whitma WB (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. IJSEM 52:1043–1047

Stumm CK, Vogels GD (1989) Relations of autotrophic bacteria to protozoa. In: Schlegel HG, Bwien B (eds) Autotrophic bacteria. Springer, Berlin, pp 177–191

Stumm CK, Gijzen HJ, Vogels GD (1982) Association of methanogenic bacteria with bovine rumen ciliates. Br J Nutr 47:95–99

Takayanagi T, Hayashi S (1964) Cytological and cytogenetical studies on *Paramecium polycarium* V Lethal interactions in certain stocks. J Protozool 11:128–132

Thiele JO, Honer-Schmid J, Wahl GK, Schultz JE (1980) A new method for axenic mass cultivation of *Paramecium tetraurelia*. J Protozool 27:118–121

Van Bruggen JJA, Stumm CK, Vogels GD (1983) Symbiosis of methanogenic bacteria and sapropelic protozoa. Arch Microbiol 136:89–95

Van Bruggen JJA, Zwart KB, Van Assema RM, Stumm CK, Vogels GD (1984) *Methanobacterium formicicum*, an endosymbiont of the anaerobic ciliate *Methopus striatus*. McMurrich Arch Microbiol 139:1–7

Van Bruggen JJA, Zwart KB, Hermans JGF, Van Hove EM, Stumm CK, Vogels GD (1986) Isolation and characterization of *Methanoplanus endosymbiosus* sp nov., an endosymbiont of the marine sapropelic ciliate *Methopus contortus*. Quennerstedt Arch Microbiol 144:367–374

Van Wagtendonk WJ, Soldo AT (1970) Methods used in the axenic cultivation of *Paramecium aureli*. In: Prescott M (ed) Methods in cell physiology, vol 4. Academic, New York, pp 117–130

Vannini C, Rosati G, Verni F, Petroni G (2004) Identification of the bacterial endosymbionts of the marine ciliate *Euplotes magnicirratus* (Ciliophora, Hypotrichia) and proposal of "*Candidatus Devosia euplotis*". Int J Sys Evol Microbiol 54:1151–1156

Vannini C, Petroni G, Verni F, Rosati G (2005) *Polynucleobacter* bacteria in the brackish-water species *Euplotes harpa* (Ciliata, Hypotrichia). J Euk Microbol 52:116–122

Vannini C, Ferrantini F, Schleifer KH, Ludwig W, Verni F, Petroni G (2010) "*Candidatus Anadelfobacter veles*" and "*Candidatus Cyrtobacter comes*" two new Rickettsiales species hosted by the protist ciliate *Euplotes harpa* (Ciliophora, Spirotrichea). Appl Env Microbiol 76:4047–4054

Vannini C, Pöckl M, Petroni G, Wu QL, Lang E, Stackebrandt E, Schrallhammer M, Richardson PM, Hahn MW (2007) Endosymbiosis in statu nascendi: close phylogenetic relationship between obligatory endosymbiotic and obligately free-living *Polynucleobacter* strains (Betaproteobacteria). Environ Microbiol 9(2):347–359.

Verni F, Rosati G (1990) Peculiar epibionts in *Euplotidium itoi* (Ciliata, Hypotrichida). J Protozool 37:337–343

Vogels GD, Hoppe WF, Stumm CK (1980) Association of methanogenic bacteria with rumen ciliates. Appl Environ Microbiol 40:608–612

Wagener S, Pfennig N (1987) Monoxenic culture of the anaerobic ciliate *Trimyema compressum* Lackey. Arch Microbiol 149:4–11

Wagener S, Bardele CF, Pfennig N (1990) Functional integration of *Methanobacterium formicicum* into the anaerobic ciliate Trimyema compressum. Arch Microbiol 153:496–501

Wells B, Horne RW (1983) The ultrastructure of *Pseudomonas avena*. II: intracellular refractile (R body) structure. Micron Microsc Acta 14:329–344

Wichterman R (1953) The biology of Paramecium. Plenum Press, New York

Wiemann M (1989) The release of *Holospora obtusa* from *Paramecium caudatum* observed with a new device for extended in vivo microscopy. J Protozool 36:176–179

Wiemann J, Görtz H-D (1989) Release of the endonucleobiotic bacterium *Holospora elegans* from its host cell *Paramecium caudatum*. Eur J Protistol 25:100–108

Wiemann M, Görtz H-D (1991) Identification and localization of major stage-specific polypeptides of infectious *Holospora obtusa* with monoclonal antibodies. J Bacteriol 173:4842–4850

Yarlett N, Hann AC, Lloyd D, Williams A (1981) Hydrogenosomes in the rumen protozoon *Dasytricha ruminantium* Schuberg. Biochem J 200:365–372

Zwart KB, Goosen NK, Van Schijndel MW, Broers CAM, Stumme CK, Vogels GD (1988) Cytochemical localization of hydrogenase activity in the anaerobic protozoa *Trichomonas vaginalis*, *Plagiopyla nasuta* and *Trimyema compressum*. J Gen Microbiol 134:2165–2170

# 19 Bacteriocyte-Associated Endosymbionts of Insects

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## Introduction

Intracellular associations between bacteria and insects are widespread in nature (Baumann and Moran 1997; Buchner 1965; Dasch et al. 1984; Douglas 1989; Houk and Griffiths 1980). Extensive studies of the natural history of such associations have led to the conclusion that they are commonly found in insects that utilize diets containing an excess of one class of compounds but a deficiency of some essential nutrients (Buchner 1965; Dadd 1985). It was thought that the function of the endosymbionts was to rectify this imbalance by the synthesis of these essential nutrients for the host. Extensive compilations of the occurrence of endosymbionts in different groups of insects are found in Buchner (1965) and Dasch et al. (1984). Because most of the prokaryotes involved in such associations are not cultivable on common laboratory media, their characterization had to await the development of recombinant DNA methodology. The past 10 years have witnessed the initiation of studies on the intracellular association of prokaryotes with a variety of insect hosts. In this chapter, we will provide an overview of the evolution and, where possible, genetics and physiology of such recently studied associations. A summary of some of their features is presented in ❯ *Table 19.1*, and the phylogeny of the endosymbionts based on 16S rDNA is presented in ❯ *Fig. 19.1*.

The diversity of symbiotic associations and problems of definitions have been previously discussed and will not be considered here (Smith and Douglas 1987; Werren and O'Neill 1997). Some of the phylogenetic studies have included few host taxa and are thus not entirely conclusive; nevertheless, current results suggest that most of the associations considered in this chapter have common features and represent a relatively well-defined type. To aid presentation, we will describe these common features, which are established from recent, largely molecular, studies as well as from older investigations based on morphological analyses. References to the earlier studies are found in Buchner (1965), who arrived at similar conclusions. References to recent studies are given as each association is considered.

The associations listed in ❯ *Table 19.1* and ❯ *Fig. 19.1* are the results of infections of various insect lineages with different prokaryotes. These associations became stable, resulting in the emergence of a new composite (of host and endosymbiont)

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**Table 19.1**
**General properties of the considered endosymbiotic associations**[a]

| Host category[b] | Principal host food source | Symbiont designation | 16S rRNA group or other taxonomic designation |
|---|---|---|---|
| Order: Homoptera | | | |
| Suborder: Sternorrhyncha | | | |
| Superfamily: Aphidoidea | | | |
| *Aphids* | Phloem sap | *Buchnera aphidicola* | γ-*Proteobacteria* |
| | | S-endosymbiont | *Enterobacteriaceae*[c] |
| Superfamily: Psylloidea | | | |
| *Psyllids* | Phloem sap | P-endosymbiont | γ-*Proteobacteria* |
| | | S-endosymbiont | γ-*Proteobacteria* |
| Superfamily: Aleyrodoidea | | | |
| *Whiteflies* | Phloem sap | P-endosymbiont | γ-*Proteobacteria* |
| | | S-endosymbiont | *Enterobacteriaceae* |
| Superfamily: Coccoidea | | | |
| Family: Pseudococcidae | | | |
| *Mealybugs* | Phloem sap | P-endosymbiont | β-*Proteobacteria* |
| Order: Diptera | | | |
| Family: Muscidae | | | |
| Genus: *Glossina* | | | |
| *Tsetse flies* | Vertebrate blood | *Wigglesworthia glossinidia* (P-endosymbiont) | γ-*Proteobacteria* |
| | | *Sodalis glossinidius* (S-endosymbiont) | *Enterobacteriaceae* |
| Order: Coleoptera | | | |
| Family: Curculionidae | | | |
| Genus: *Sitophilus* | | | |
| *Weevils* | Stored grain | P-endosymbiont | *Enterobacteriaceae* |
| Order: Hymenoptera | | | |
| Family: Formicidae | | | |
| Genus: *Camponotus* | | | |
| *Carpenter ants* | Plant nectar, honeydew, detritus, and other sources | P-endosymbiont | γ-*Proteobacteria* |
| Order: Orthoptera | | | |
| Superfamily: Blattoidea | | | |
| *Cockroaches* | Universalists | *Blattabacterium cuenoti* | Flavobacterium-Bacteroides group |
| Order: Isoptera | | | |
| Family: Mastotermitidae | | | |
| Genus: *Mastotermes* | | | |
| *Termites* | Dead wood | *Blattabacterium cuenoti* | Flavobacterium-Bacteroides group |

[a]See text for references
[b]Taxonomy of the host according to Borror et al. (1989)
[c]As defined by Brenner (1984)

organism. The endosymbiont became heritable through the acquisition of mechanisms ensuring vertical, maternal transmission to progeny. The association also became obligate, or beneficial, for host growth. Because the host depended on the association, and because horizontal or infectious transmission between hosts did not occur, the phylogeny of the endosymbionts is congruent with the phylogeny of the hosts. With some exceptions, heritable associations tend to become mutualistic (Lipsitch et al. 1995; Werren and O'Neill 1997). In most cases, the host cannot survive without the endosymbiont, or the elimination of the endosymbiont has a deleterious effect. Although the advantage for the host is in most cases apparent,

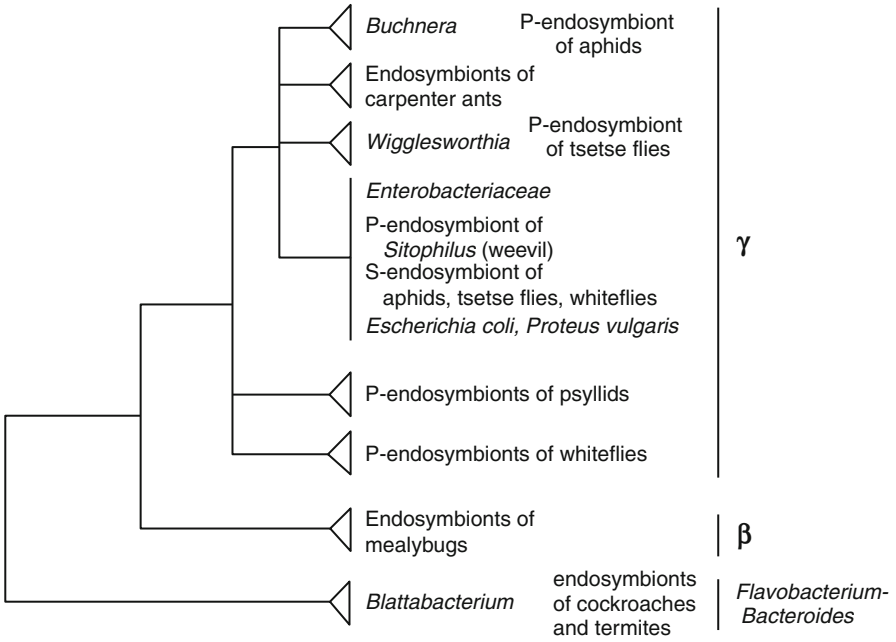


**Fig. 19.1**
**Phylogenetic tree resulting from parsimony analysis of insect endosymbionts based on 16S rDNA sequence analysis. P-, primary endosymbiont; S-, secondary endosymbiont; Greek letters, subdivisions of the Proteobacteria. References given in text**

the advantage for the endosymbiont is not always clear. Perhaps it is more correct to think that the host domesticates the endosymbiont for its own welfare, utilizing functions that are present in the prokaryote but lacking in the host (Douglas and Smith 1989; Maynard Smith and Szathmáry 1995). In this chapter, the organism which is present in all the species of an insect group and which appears to be of essential value to the host is designated by either its scientific name or, if one is lacking, by the term primary (P-) endosymbiont.

Superimposed on this fundamental association may be associations with additional endosymbionts. Although these are heritable, they appear to be the result of multiple independent infections, horizontal transmission, or both. Since these endosymbionts may be absent in some hosts, their contribution to the welfare of the organism may not be major or essential. These organisms are designated as secondary (S-) endosymbionts. In this connection, it is relevant that some bacterial strains may exist within the body cavity of insects for long periods without obvious deleterious effects, thus serving as potential endosymbiont precursors of endosymbiotic associations (Boman and Hultmark 1987; Faye 1978).

These conclusions are tentative and, with the possible exception of the *Sitophilus*-endosymbiont association, are probably applicable to most of the endosymbiotic associations considered in this chapter. One well-studied, contrasting association is between many arthropods and the intracellular prokaryote, *Wolbachia* (O'Neill et al. 1997). Although this organism is typically heritable, being transmitted maternally, results of phylogenetic studies imply some incidence of horizontal exchange between very different lineages. *Wolbachia* causes a number of different reproductive alterations favoring the spread of infected host lineages and has properties characteristic of a parasite (O'Neill et al. 1997). Recent work may necessitate a modification of this conclusion because newly discovered *Wolbachia* in filarial worms appear to be essential for host survival and may show phylogenetic congruence with their hosts, indicating vertical evolution (Bandi et al. 1998, 1999).

## Symbionts of Insects Which Utilize Plant Sap as Food

Aphids, psyllids, whiteflies, and mealybugs share a number of common structural and nutritional properties (Borror et al. 1989) and constitute four separate lineages within the suborder Sternorrhyncha (order Homoptera; Campbell et al. 1994; von Dohlen and Moran 1995). All of these groups feed predominantly or exclusively on plant phloem sap. This mode of life necessitates the penetration of plant tissue by flexible tubular mouthparts (stylets) and the ingestion of plant phloem sap. This diet is unbalanced, as it is rich in carbohydrates but deficient in amino acids and other nitrogenous compounds (Dadd 1985; Sandström and Pettersson 1994). Because of the low concentrations of nitrogenous compounds, phloem-feeding insects ingest a large amount of plant sap and then excrete the excess sugar as honeydew. This mode of feeding is conducive to the transmission of plant viruses, and members of the Homoptera are important vectors of viral plant pathogens (Blackman and Eastop 1984; Gray and Banerjee 1999; Sylvester 1985). In addition, these insect populations may reach enormous numbers on plants, causing nutrient deprivation, leaf curling, and gall formation (Borror et al. 1989).

In spite of these common properties, aphids, whiteflies, psyllids, and mealybugs have different prokaryotic P-endosymbionts (Table 19.1, Fig. 19.1). These insects, like other animals, require ten essential amino acids, and endosymbionts are thought to upgrade the diet by synthesizing these essential amino acids and providing them to the host (Baumann et al. 1995, 1997a, b; Douglas 1989; Moran and Telang 1998). Of these four symbiotic associations, the most extensively studied is that between *Buchnera* (the P-endosymbiont) and aphids. This association will be considered in some detail and followed by a brief discussion of three other associations.

## Aphid Endosymbionts

### *Buchnera*: The Primary Endosymbiont of Aphids

#### Phylogeny

The initial characterization of the 16S rDNA gene of *Buchnera* involved the use of an *Escherichia coli* 16S rDNA hybridization probe to perform a restriction enzyme and Southern blot analysis on total *Acyrthosiphon pisum* DNA, which established that this gene was present as a single copy (Unterman et al. 1989). Subsequently, three overlapping DNA fragments were cloned and the 16S rDNA gene was sequenced. In addition, bacteriomes were dissected from the aphid and the DNA purified. Restriction enzyme and Southern blot analysis gave the same results with whole aphid DNA and DNA obtained from dissected bacteriomes, indicating that the bacteriomes were the source of endosymbiont DNA. In all subsequent studies, the 16S rDNA was obtained by PCR amplification using whole aphid DNA preparations cloned into plasmid vectors and then sequenced (Munson et al. 1991b).

Based on 16S rDNA analysis, *Buchnera* is a distinct lineage within the γ-3 subgroup of the Proteobacteria (Fig. 19.1; Moran et al. 1993; Munson et al. 1991b; Unterman et al. 1989; van Ham et al. 1997). The closest known organisms are the endosymbionts of carpenter ants, endosymbionts of tsetse flies (*Wigglesworthia*), and members of the Enterobacteriaceae as defined by Brenner (Brenner 1984; Aksoy 1995a, b; Schröder et al. 1996). Phylogenetic analyses based on 16S rDNA indicate that these organisms are four separate lineages but do not permit firm conclusions regarding their relationships to one another. *Buchnera* contains a single copy of rRNA genes which are arranged as two transcription units, 16S rRNA and tRNAGlu-23S rRNA-5S rRNA (Munson et al. 1993; Rouhbakhsh and Baumann 1995). This organization of the rRNA genes into two transcription units is somewhat rare but also has been found in *Wolbachia* (Bensaadi-Merchermek et al. 1995) and *Rickettsia* (Andersson et al. 1998), organisms which are in the α-subdivision of the Proteobacteria and also associated with insects. In the endosymbionts of carpenter ants, the rRNA genes are also split into two transcription units (C. Sauer and R. Gross, personal communication), whereas in *Wigglesworthia* (Aksoy 1995b) and the Enterobacteriaceae, the order is 16S–23S (Berlyn 1998). The organization of the rRNA genes into two transcription units suggests a possible relationship between *Buchnera* and the endosymbionts of carpenter ants.

The results of phylogenetic analyses involving all of the currently available *Buchnera* sequence information are presented in Fig. 19.2. Most of the characterized endosymbionts are from the family Aphididae. Based on 16S rDNA, *Buchnera* forms one clade within which two well-supported subclades are apparent. These are the aphids of the Aphididae and the Sc and Mr from the tribe Fordini in the family Pemphigidae. Additional studies using a portion of *trpB* (Fig. 19.2b) confirmed some of these relationships and provided further resolution within the genus *Uroleucon*. These relationships are in broad agreement with the results of evolutionary studies of plasmid-associated *trpE*, *leuBCD*, and *repA1* (Fig. 19.2c, d, e; Baumann et al. 1997b, 1999b; Bracho et al. 1995; Rouhbakhsh et al. 1996, 1997; Silva et al. 1998; van Ham et al. 1997, 1999). Within the genus *Uroleucon*, the phylogeny based on *trpB* is in good agreement with the more extensive analysis of host phylogeny based on mitochondrial and nuclear genes (Clark et al. 2000; Moran et al. 1999).

The congruence of phylogenies derived from *Buchnera* chromosomal and plasmid genes, as well as host mitochondrial and nuclear genes, is strong evidence for a vertical mode of evolution with no exchange of either bacteria or plasmids among host lineages (Moran and Baumann 1994). An implication of the congruence between the phylogenies of *Buchnera* and corresponding aphid hosts is that dates for branch points inferred from fossil aphids can be extended to ancestral *Buchnera* (Moran et al. 1993). A further implication is that modern *Buchnera* descend from an infection of a common ancestor of all modern aphids. From the aphid fossil record, we can infer that this infection by a free-living bacterium must have occurred at least 150–250 million years ago. The divergence in 16S rDNA of modern *Buchnera* is consistent with this hypothesis of an ancient infection followed by cospeciation of *Buchnera* and hosts.

*Buchnera* shows a rate of base substitution in its 16S rDNA that is about twice as great as that in related free-living bacteria based both on relative rate comparisons with free-living taxa and on comparisons of rates calibrated with respect to absolute time (Clark et al. 1999b; Moran 1996). The elevated substitution rate of *Buchnera* relative to that of related free-living bacteria extends to genes encoding proteins (Brynnel et al. 1998; Clark et al. 1999b; Moran 1996; Wernegreen and Moran 1999). Based on calibrated rates for protein-coding genes, synonymous sites evolve about twice as fast and nonsynonymous sites about six times as fast in *Buchnera* as in *E. coli*/*Salmonella typhimurium*, based on an absolute time scale (Table 19.3). The rate differences are considerably greater on a scale based on generations, since *Buchnera* appears to have fewer generations per year than do natural populations of enteric bacteria (Clark et al. 1999b).

The most plausible explanation for the faster rate of substitution in *Buchnera* is that the population structure of *Buchnera*, involving strictly vertical transmission of a small inoculum between hosts, results in greater levels of genetic drift, which

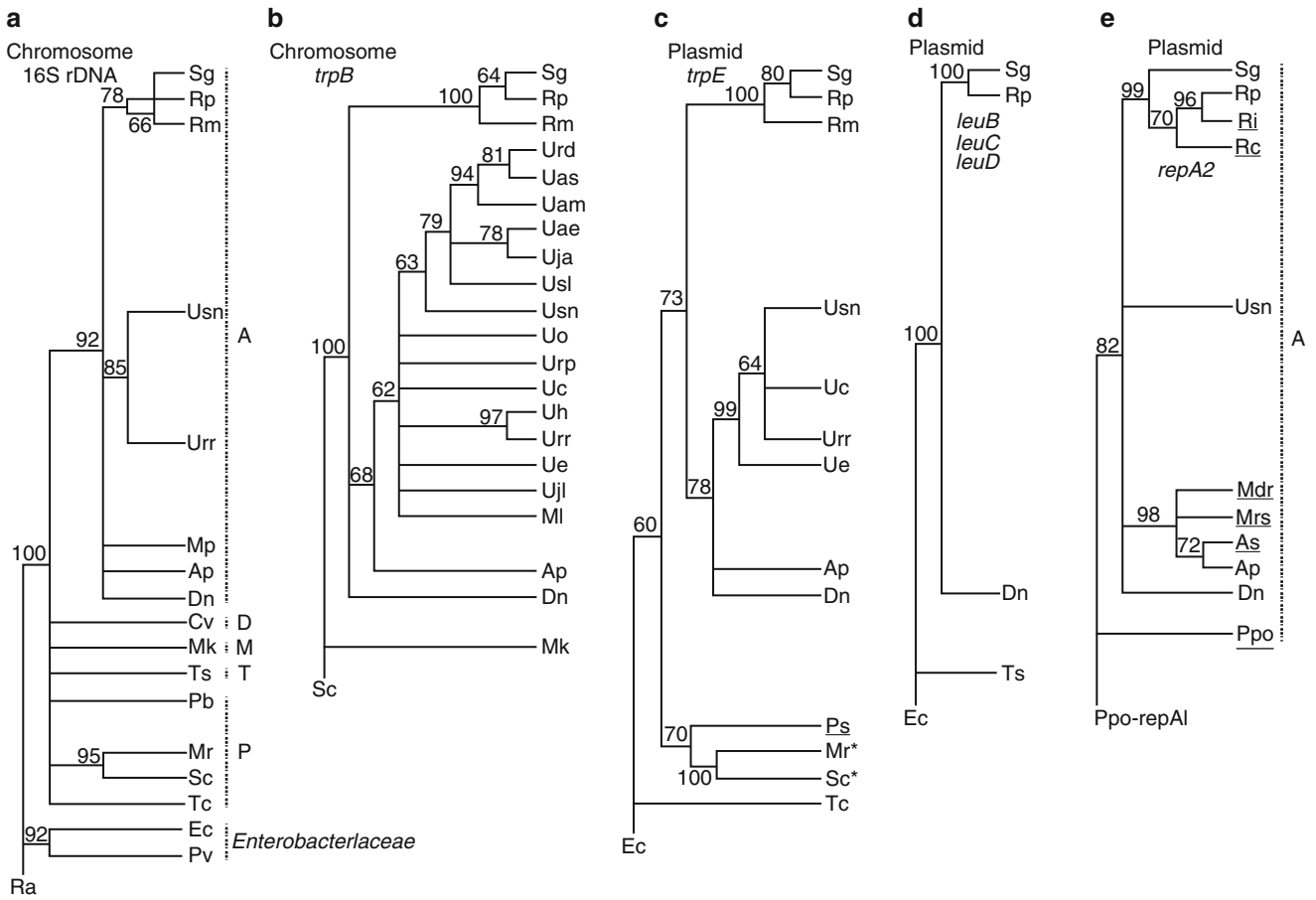


**Fig. 19.2**

**Phylogenetic trees resulting from parsimony analyses using Buchnera (a) 16S rDNA, (b) trpB, (c) trpE, (d) leuB, leuC, and leuD, and (e) repA1. Numbers at nodes are bootstrap percentages from parsimony searches (1,000 replicates). Abbreviations designating the insect hosts are given in *Table 19.2*. In (a), Enterobacteriaceae: Ec(*E. coli*), Pv(*P. vulgaris*); Ra(*Ruminobacter amylophilus*). *Dashed lines* in (a) designate aphid species within one family: *A* Aphididae, *D* Drepanosiphidae, *M* Mindaridae, *T* Thelaxidae, *P* Pemphigidae. *Dashed line* in (e) designates aphids within one family. *Underlined abbreviations* in (c) and (e) refer to aphid species not included in the other analyses. * in (c) designates chromosomal genes. For references see text**

can increase the fixation rate of mildly deleterious mutations. Several observations support this explanation. First, the rate increase is found at all genes and is concentrated at sites, such as nonsynonymous sites, that are expected to be under selective constraint (Moran 1996; Wernegreen and Moran 1999). Second, polypeptide compositions are consistently biased toward amino acids that allow more adenine and thymine in the DNA sequence, indicating that mutational bias has affected protein evolution. Third, faster substitution rates in 16S rDNA are observed in other insect endosymbionts that share a similar transmission mode, suggesting that the endosymbiotic lifestyle has repeatedly produced the same changes in patterns of molecular evolution. Finally, the 16S rRNA secondary structure of *Buchnera* and other endosymbionts has lower thermal stability than that of related free-living bacteria, as expected if the DNA base substitutions are mildly deleterious (Lambert and Moran 1998).

The 16S rDNA substitution rate of *Buchnera* is about 35 times greater than that of homologous regions of 18S rDNA of hosts, based on comparisons of pairwise divergences of corresponding aphid and *Buchnera* taxa (Moran et al. 1995). Thus, the hypothesis of a universal rate of substitution in rDNA is not even approximately true.

## Taxonomy

The genus *Buchnera* contains one species, *Buchnera aphidicola*, and the type strain is the endosymbiont of the aphid *Schizaphis graminum* (Munson et al. 1991a). Currently this species name designates the lineage consisting of the P-endosymbionts of aphids. There are over 4,000 species of aphids (Blackman and Eastop 1984; Remaudière and Remaudière 1997) of which only 35 have been characterized by molecular methods. Consequently our conclusions are based on a very small sample of aphid species. Although 16S rDNA has been useful for showing the monophyletic origin of aphid endosymbiosis and the establishment of

**Table 19.2**
**Abbreviations of aphid species used in this chapter**

| Abbreviation | Aphid species | Family | Tribe |
|---|---|---|---|
| Rc | *Rhopalosiphum cerasifoliae* | Aphididae | Aphidini |
| Ri | *Rhopalosiphum insertum* | Aphididae | Aphidini |
| Rm | *Rhopalosiphum maidis* | Aphididae | Aphidini |
| Rp | *Rhopalosiphum padi* | Aphididae | Aphidini |
| Sg | *Schizaphis graminum* | Aphididae | Aphidini |
| Ap | *Acyrthosiphon pisum* | Aphididae | Macrosiphini |
| As | *Aulacorthum solani* | Aphididae | Macrosiphini |
| Dn | *Diuraphis noxia* | Aphididae | Macrosiphini |
| Ml | *Macrosiphoniella ludovicianae* | Aphididae | Macrosiphini |
| Mp | *Myzus persicae* | Aphididae | Macrosiphini |
| Mdr | *Metopolophium dirhodum* | Aphididae | Macrosiphini |
| Mrs | *Macrosiphum rosae* | Aphididae | Macrosiphini |
| Uae | *Uroleucon aeneum* | Aphididae | Macrosiphini |
| Uam | *Uroleucon ambrosiae* | Aphididae | Macrosiphini |
| Uas | *Uroleucon astronomus* | Aphididae | Macrosiphini |
| Uc | *Uroleucon caligatum* | Aphididae | Macrosiphini |
| Ue | *Uroleucon erigeronense* | Aphididae | Macrosiphini |
| Uh | *Uroleucon helianthicola* | Aphididae | Macrosiphini |
| Uja | *Uroleucon jaceae* | Aphididae | Macrosiphini |
| Ujl | *Uroleucon jaceicola* | Aphididae | Macrosiphini |
| Uo | *Uroleucon obscurum* | Aphididae | Macrosiphini |
| Urd | *Uroleucon rudbeckiae* | Aphididae | Macrosiphini |
| Urp | *Uroleucon rapunculoidis* | Aphididae | Macrosiphini |
| Urr | *Uroleucon rurale* | Aphididae | Macrosiphini |
| Usl | *Uroleucon solidaginis* | Aphididae | Macrosiphini |
| Usn | *Uroleucon sonchi* | Aphididae | Macrosiphini |
| Ppo | *Pterocomma populeum* | Aphididae | Pterocommatinae |
| Cv | *Chaitophorus viminalis* | Drepanosiphidae | Chaitophorini |
| Mk | *Mindarus kinseyi* | Mindaridae | Mindarini |
| Mr | *Melaphis rhois* | Pemphigidae | Fordini |
| Sc | *Schlechtendalia chinensis* | Pemphigidae | Fordini |
| Pb | *Pemphigus betae* | Pemphigidae | Pemphigini |
| Ps | *Pemphigus spyrothecae* | Pemphigidae | Pemphigini |
| Tc | *Tetraneura caerulescens* | Pemphigidae | Eriosomatini |
| Ts | *Thelaxes suberi* | Thelaxidae | |

major aphid subgroups, it is far too conserved to be useful for defining relationships among endosymbionts of closely related aphids. Some success has been obtained by the use of other, less conserved, molecules (Fig. 19.2). The 16S rDNA sequence difference of *Buchnera* in Sg and Sc (the most distantly related aphids) is about the same as that between *E. coli* and *Proteus vulgaris*. Thus, subsequent studies using less conserved molecules will probably indicate that *Buchnera* should be subdivided into new species. So far, no studies have addressed the range of variation within endosymbionts of a single aphid species.

## Habitat

### Location and Ultrastructure

During their reproductive phase, aphids contain within their body cavity a bilobed structure called a bacteriome consisting of 60–90 uninucleate, polyploid cells called bacteriocytes (Douglas and Dixon 1987). These cells are filled with host-derived vesicles containing *Buchnera* (Fig. 19.3a). This organism is spherical or oval in shape, 2–4 m in diameter, with a cell wall consisting of two-unit membranes, as is characteristic of Gram-negative

**Table 19.3**
**Substitution rates in *Buchnera* and enteric bacteria[a]**

| Species pair | Estimated time of divergence | Synonymous rate | | Nonsynonymous rate | |
|---|---|---|---|---|---|
| | | Absolute[b] | Generation[c] | Absolute[b] | Generation[c] |
| *Buchnera* (Sg/Dn) | 50–70 MY | 6.8–9.5 | 0.14–0.19 | 1.0–1.4 | 0.02–0.03 |
| *Buchnera* (Sc/Mr) | 50–70 MY | 5.1–7.2 | 0.17–0.24 | 1.1–1.6 | 0.04–0.05 |
| *E. coli/S. typhimurium* | 100–150 MY | 2.9–4.4 | 0.03–0.04 | 0.1–0.2 | 0.001–0.002 |

[a]Based on comparisons of over 5,100 codons (Clark et al. 1999b)
[b]Substitutions/site/$10^{\circ}$ years
[c]Substitutions/site/$10^{\circ}$ generations

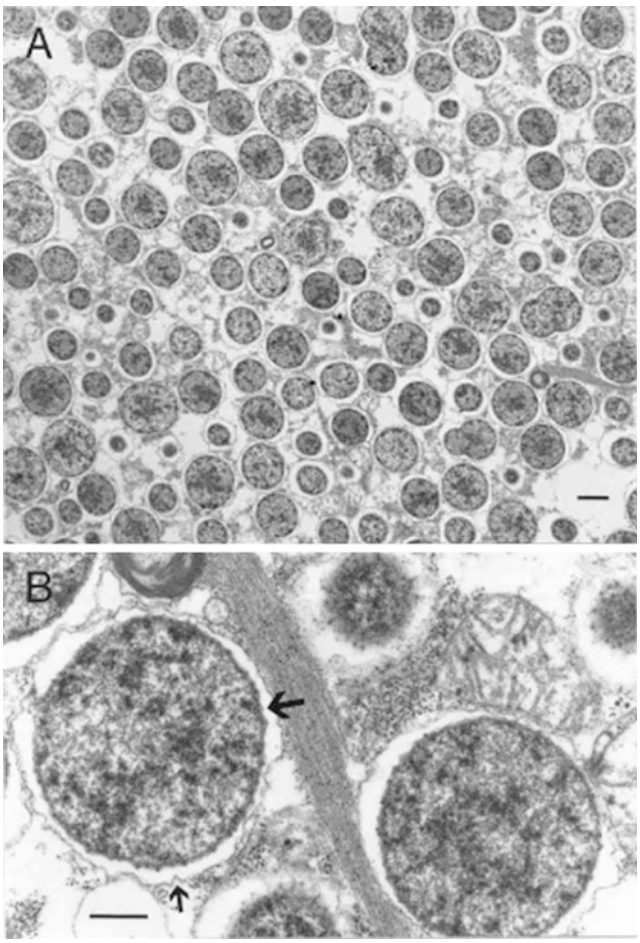


**Fig. 19.3**
**Electron micrographs of *Buchnera*, the P-endosymbiont of aphids. (a) Endosymbionts within a bacteriocyte, bar = 1 m. (b) Higher magnification showing the Gram-negative cell wall (*large arrow*) and the vesicle membrane (*small arrow*), bar = 0.5 m (Photos courtesy of Marv Kinsey and Don McLean)**

bacteria (*Fig. 19.3b*; Akhtar and van Emden 1994; Griffiths and Beck 1973; Hinde 1971b; McLean and Houk 1973). A thin layer corresponding to peptidoglycan has been detected (Houk et al. 1977). The presence of peptidoglycan also is indicated by penicillin-induced alterations of the cell wall as well as by chemical analysis (Griffiths and Beck 1974; Houk et al. 1977).

### Number of Endosymbionts

*Buchnera* contains one copy of the 16S rRNA gene (Munson et al. 1991b, 1993; Unterman et al. 1989) and one copy of *groEL* (Ohtaka et al. 1992; Hassan et al. 1996). The number of copies of *Buchnera* 16S rRNA genes in the aphid Sg was studied by quantitative PCR (Baumann and Baumann 1994) and was found to be 0.5–1.2 $\times$ $10^7$ $mg^{-1}$ aphid wet weight. Using quantitative hybridization of a *Buchnera groEL* probe, the number of genome copies in Ap was estimated at 1–2 $\times$ $10^7$ $mg^{-1}$ aphid wet weight (Humphreys and Douglas 1997). In both of these studies, the number of *Buchnera* cells was assumed to be identical to the number of genome copies. However, a recent study has demonstrated that *Buchnera* (Ap) is polyploid, containing an average of about 120 genomes per cell (Komaki and Ishikawa 1999). If the average number of genomes per endosymbiont is relatively constant, then the number of endosymbionts is about 100-fold less than estimated previously, or about 105 $mg^{-1}$ aphid wet weight. This value is considerably lower than the estimates for Ap of 1.6–1.8 $\times$ $10^6$ endosymbionts $mg^{-1}$ aphid wet weight, based on microscopic enumeration of the endosymbionts (Harrison et al. 1989).

### Growth and Reproduction

In their most active stage, aphids are wingless females, which reproduce by parthenogenesis, giving birth to live young. There is telescoping of generations in that the mother aphid contains embryos that, in turn, may contain other embryos (Dixon 1973, 1992). Studies on the growth of Sg (Baumann and Baumann 1994) have indicated that newly born aphids weigh 24 µg and contain 2 $\times$ $10^5$ copies of the *Buchnera* genome. The increase in the number of endosymbiont genomes parallels the increase in the weight of the aphid. The maximum weight is reached in 9–10 days at which time the aphid weighs 540 µg and contains 5.6 $\times$ $10^6$ *Buchnera* genomes. The endosymbionts are partitioned between maternal and embryonic bacteriocytes. In a mature aphid, most of the *Buchnera* genomes are found in the embryos (Humphreys and Douglas 1997). The first young are born in 8–9 days; each aphid can produce 60–80 live young during its lifetime. Douglas and Dixon (1987) showed that, during the period of growth, there is a concomitant increase in the maternal bacteriocyte volume as well as a small drop in bacteriocyte number. In the adult aphid, the number of maternal bacteriocytes in the bacteriome undergoes a sharp decrease

probably due, in part, to their dispersion within the abdomen, their degradation, as well as the degradation of *Buchnera* (Brough and Dixon 1990; Douglas and Dixon 1987; Griffiths and Beck 1973; Hinde 1971a).

Aphids may also produce sexual forms with the females depositing eggs that overwinter and hatch in the spring. *Buchnera* is maternally transmitted (transovarial transmission) to both developing embryos and eggs. Maternal bacteriocytes adjacent to an embryo near the blastoderm stage form a small opening through which the endosymbionts pass. *Buchnera* then moves through the intervening hemolymph and enters a nearby opening on the oocyte surface. During early embryonic development, the presumptive bacteriocytes form, and the endosymbionts migrate into these cells (Buchner 1965; Blackman 1987; Hinde 1971a). Symbiont invasion of eggs also occurs from the dispersed bacteriocytes, and they can be observed as an aggregate at the posterior pole of the mature egg (Buchner 1965; Brough and Dixon 1990). *Buchnera* and bacteriomes appear to be nearly universal in aphids (Buchner 1965). However, some species of the tribe Cerataphidini lack both and instead contain yeastlike extracellular symbionts within their body cavity (Buchner 1965; Fukatsu and Ishikawa 1992a, 1996). Some species of aphids may produce dwarf males and/or sterile female soldiers that may also lack endosymbionts (Buchner 1965; Fukatsu and Ishikawa 1992b; Fukatsu et al. 1994).

## Physiology

### Nutrition and Metabolism

Plant sap, the diet of aphids, has an excess of carbohydrate relative to amino acids and other nitrogenous compounds (Dadd 1985; Douglas 1998; Sandström and Pettersson 1994; Sandström and Moran 1999). Aphids, like other insects, are thought to require 10 preformed amino acids, and these essential amino acids are present in low amounts in plant sap. Some species of aphids can grow on synthetic diets even in the absence of essential amino acids. Adding antibiotics to such diets results in the elimination of endosymbionts and the failure of the aphids to reproduce. There is some sparing effect when the essential amino acids are included in the antibiotic-containing diet. These experiments have generally been interpreted as indicating that one of the functions of *Buchnera* is the synthesis of essential amino acids for the aphid host (reviewed by Baumann et al. 1995; Douglas 1998). A major problem is that compared with growth on plants, growth on artificial diets is poor and generally limited to a few generations. In addition, aphid growth on complete synthetic diets in the presence of antibiotics is even worse, indicating that *Buchnera* provides nutrients or functions that cannot be provided by the artificial diets. The effects of antibiotics on a number of aphid properties have been recently reviewed (Wilkinson 1998). There is a further complication with some of the nutritional studies, in which physiological effects have been attributed to the removal of *Buchnera*. The aphid strain used may also contain S-endosymbionts, as is true of the strain of the aphid Ap used in the studies of A. E. Douglas and her collaborators (Douglas and Prosser 1992; Wilkinson 1998). The S-endosymbiont probably does not perform any essential functions for the host (see section on "Secondary Endosymbionts of Aphids" in this chapter); nevertheless, the use of antibiotics eliminates both endosymbionts, and consequently the observed effects of this loss may not be attributable solely to the loss of *Buchnera*.

Currently one of the more complete studies involves the essential amino acid tryptophan. Using a strain of Ap containing an S-endosymbiont, Douglas and Prosser (1992) have shown a sparing effect of tryptophan in chlortetracycline-containing synthetic diets on aphid growth. In addition, they detected low levels of tryptophan synthase in *Buchnera* and found that activity was absent in chlortetracycline-treated and thus endosymbiont-free aphids. The assays used (Smith and Yanofsky 1962) crude extracts of whole aphids as well as preparations enriched in endosymbionts and measured the disappearance of the substrate indole and not the appearance of the product tryptophan. Indole or indole derivatives may be substrates for a variety of reactions catalyzed by enzymes found in crude extracts of insects. No information was provided about the linearity of increasing enzyme activity with increasing crude extract concentration. In spite of possible difficulties with this assay, the dependence of the reaction on "the substrate [sic] pyridoxal phosphate" (Douglas and Prosser 1992) is consistent with it being a measure of tryptophan synthase activity.

Using synthetic diets containing radiolabeled sulfate, it was shown that *Buchnera* can reduce this compound to the level of hydrogen sulfide and incorporate it into methionine and cysteine and that these amino acids are found in aphid tissue (Douglas 1988). Using $^{14}$C-radiolabeled amino acids, it was found that the synthesis of the essential amino acids arginine, threonine, isoleucine, and lysine was reduced or eliminated by the inclusion of rifampicin in the diet (Liadouze et al. 1996). Sasaki and Ishikawa (1995) also showed that treatment of aphids with rifampicin eliminated the incorporation of dietary $^{15}$N-glutamine into the essential amino acids arginine, histidine, isoleucine and/or leucine, phenylalanine, threonine, and valine.

Glutamine is the predominant amino acid in phloem and also in aphid hemolymph (Sandström and Pettersson 1994; Sasaki et al. 1990). Isolated bacteriocytes were found to take up glutamine and convert it to glutamate, which subsequently was taken up by *Buchnera* (Sasaki and Ishikawa 1995). Isolated endosymbionts incorporated the nitrogen of glutamine into the essential amino acids isoleucine, leucine, valine, and phenylalanine as well as a number of other amino acids, and these amino acids were excreted into the suspending medium. Whitehead and Douglas, however, could not find any evidence for excretion of amino acids by *Buchnera* (cited in Douglas 1997).

Using synthetic diets, Nakabachi and Ishikawa (1999) demonstrated a requirement for riboflavin by rifampicin-treated aphids. These results indicate that *Buchnera* is required for the synthesis of at least one vitamin for the aphid host.

Whitehead and Douglas (1993) isolated vesicles containing *Buchnera* and showed that they readily took up acetic, glutamic, and aspartic acid as well as tricarboxylic acid cycle intermediates

and oxidized them to $CO_2$. Oxygen consumption was also detected and was greatly reduced by KCN. These results suggest the presence of a tricarboxylic acid cycle in the endosymbionts and indicate a respiratory metabolism. The latter conclusion is consistent with the presence of a gene for a subunit of NADH dehydrogenase I, an enzyme involved in the generation of a proton motive force during respiration, and of all the genes for ATP synthase, a membrane-associated enzyme which utilizes the proton motive force for the synthesis of ATP (Table 19.4).

### Gene Expression

*Buchnera* messenger RNA (mRNA) has been detected for a variety of genes encoding proteins involved in amino acid biosynthesis (Table 19.4). This includes genes for amino acids of the glutamate (*argA*) and aspartate (*thrB*) families (Nakabachi and Ishikawa 1997), the shikimate pathway (*aroH*), as well as the biosynthetic pathway for tryptophan (*trpE*, *trpD*, *trpA*), branched-chain amino acids (*ilvI*, *ilvD*, *leuA*), and histidine (*hisG*; Baumann et al. 1999a). *Buchnera* mRNA has been detected also for a gene involved in the biosynthesis of riboflavin (*ribE*; Nakabachi and Ishikawa 1999) as well as for genes involved in the heat shock response (*groEL*, *groES*, *dnaK*, *dnaJ*; Table 19.4; Sato and Ishikawa 1997a, b). Numerous *Buchnera* proteins have also been detected by immunological methods. These include *GroEL*, *GroES*, and *DnaK* (Kakeda and Ishikawa 1991; Sato and Ishikawa 1997b) as well as ribosomal protein S1 (the product of *rpsA*; Clark et al. 1996) and the protein involved in septum formation during cell division (the product of *ftsZ*; Table 19.4; Baumann and Baumann 1998).

In bacteria, rRNA genes are transcribed from strong promoters. Comparisons of the regions upstream of rRNA genes from six species of *Buchnera* indicated conservation of sequences resembling the −35 and −10 regions of $\sigma^{70}$ promoters as well as boxA and boxC (Munson et al. 1993; Rouhbakhsh 1995). Similar putative −35 and −10 regions were found in *Buchnera* plasmids containing genes for tryptophan and leucine biosynthesis (Baumann et al. 1999b; Rouhbakhsh et al. 1996; Silva et al. 1998).

### GroEL Overproduction and Its Significance

In *Buchnera*, the chaperonin, GroEL, constitutes a major fraction of the total protein (Sato and Ishikawa 1997a). In addition, GroEL is present in aphid hemolymph (van den Heuvel et al. 1994). Overproduction of GroEL is a characteristic of some endosymbionts and pathogens in the intracellular environment (Hogenhout et al. 1998). This protein mediates the folding of peptides into their functional forms as well as the repair of damaged proteins (Gross 1996). *Buchnera* GroEL is able to complement *E. coli* mutants (Ohtaka et al. 1992). GroEL has been localized in maternal and embryonic *Buchnera* by immunohistochemistry (Fukatsu and Ishikawa 1992c). Electron micrographs indicate that the purified *Buchnera* GroEL has the characteristic double-ring appearance observed with the *E. coli* protein (Filichkin et al. 1997; Hara and Ishikawa 1990). The endosymbiont protein has ATPase activity, and in the presence of *E. coli*, GroES could reconstitute denatured *Rhodospirillum*

**Table 19.4**
**Genes of *Buchnera* from the aphid *S. graminum*[a]**

| Gene symbol | Gene product description | Linkage group[b] |
|---|---|---|
| I. *Small-Molecule Metabolism* | | |
| B. *Energy metabolism* | | |
| 1. *Glycolysis* | | |
| *gap A* | Glyceraldehyde-3-phosphate dehydrogenase | 13 |
| *tpiA* | Triosephosphate isomerase | 3 |
| 5. *Pentose phosphate pathway* | | |
| (a) *Oxidative branch* | | |
| *gnd* | Gluconate-6-phosphate dehydrogenase | 2 |
| 7. *Respiration* | | |
| (a) *Aerobic* | | |
| *nuoC(D)*[c] | NADH dehydrogenase I, subunits cd | |
| (c) *Electron transport* | | |
| *fdx* | Ferredoxin | 1 |
| *fpr* | Ferredoxin-NADP reductase | 12 |
| 9. *ATP proton motive force* | | |
| *atp A* | ATP synthase, α-subunit | 1 |
| *atp B* | ATP synthase, subunit a | 1 |
| *atp C* | ATP synthase, ϵ-subunit | 1 |
| *atp D* | ATP synthase, β-subunit | 1 |
| *atp E* | ATP synthase, subunit c | 1 |
| *atp F* | ATP synthase, subunit b | 1 |
| *atp G* | ATP synthase, γ-subunit | 1 |
| *atp H* | ATP synthase, -subunit | 1 |
| D. *Amino acid biosynthesis* | | |
| 1. *Glutamate family* | | |
| *argA*[d] | N-acetylglutamate synthase | |
| *argH* | Argininosuccinate lyase | 7 |
| 2. *Aspartate family* | | |
| *dapD* | Succinyldiaminopimelate aminotransferase | 4 |
| *thrA* | Aspartokinase I | |
| *thrB*[d] | Homoserine kinase | |
| 3. *Serine family* | | |
| *cysE* | Serine acetyltransferase | 7 |
| *serC* | Phosphoserine aminotransferase | 3 |
| 4. *Aromatic amino acid family* | | |
| *aroA* | 5-Enolpyruvylshikimate-3-phosphate synthase | 3 |
| *aroC* | Chorismate synthase | 2 |
| *aroE* | Dehydroshikimate reductase | 8 |
| *aroH* | 3-Deoxy-D-*arabino*-heptolosonate-7-phosphate synthetase (DAHP synthetase) | 10 |
| *trpA* | Tryptophan synthase, A protein | 5 |
| *trpB* | Tryptophan synthase, B protein | 5 |

**Table 19.4 (continued)**

| Gene symbol | Gene product description | Linkage group[b] |
|---|---|---|
| *trpC(F)* | Indole-3-glycerol-phosphate synthetase and *N*-(5-phosphoribosyl)anthranilate isomerase | 5 |
| *trpD* | Phosphoribosylanthranilate transferase | 5 |
| *trpE* (p)[e] | Anthranilate synthase, A subunit | 16 |
| *trpG* (p)[e] | Anthranilate synthase, B subunit (glutamine amidotransferase) | 16 |
| 5. *Histidine* | | |
| *hisA* | *N*-(5′-phospho-L-ribosyl-formimino)-5-amino-1-(5′-phosphoribosyl)-4-imidazolecarboxamide isomerase | 2 |
| *hisB* | Imidazoleglycerol-phosphate dehydratase and histidinol-phosphate phosphatase | 2 |
| *hisC* | Histidinol-phosphate aminotransferase | 2 |
| *hisD* | Histidinol dehydrogenase | 2 |
| *hisF* | Imidazoleglycerol-phosphate synthase subunit (with HisH) | 2 |
| *hisG* | ATP phosphoribosyltransferase | 2 |
| *hisH* | Glutamine amidotransferase subunit (with HisF) | 2 |
| *hisI* | Phosphoribosyl-AMP cyclohydrolase and phosphoribosyl-ATP pyrophosphatase | 2 |
| 7. *Branched-chain family* | | |
| *ilvC* | Acetohydroxy acid isomeroreductase | 1 |
| *ilvD* | Dihydroxyacid dehydratase | 1 |
| *ilvH* | Acetohydroxyacid synthase, small subunit | 4 |
| *ilvI* | Acetohydroxyacid synthase, large subunit | 4 |
| *leuA* (p)[e] | 2-Isopropylmalate synthase | 15 |
| *leuB* (p)[e] | 3-Isopropylmalate dehydrogenase | 15 |
| *leuC* (p)[e] | Isopropylmalate isomerase subunit | 15 |
| *leuD* (p)[e] | Isopropylmalate isomerase subunit | 15 |
| F. *Purines, pyrimidines, nucleosides, and nucleotides* | | |
| 3. *2′-Deoxyribonucleotide metabolism* | | |
| *dcd* | 2′-Deoxycytidine 5′-triosephosphate deaminase | 2 |
| *trxB* | Thioredoxin reductase | 3 |
| G. *Biosynthesis of cofactors, prosthetic groups, and carriers* | | |
| 9. *Riboflavin* | | |
| *ribEd* | Riboflavin synthase, β-chain | |
| 10. *Thioredoxin, glutaredoxin, and glutathione* | | |
| *trxA* | Thioredoxin | 1 |
| II. *Broad Regulatory Functions* | | |
| *rpoD* | RNA polymerase, $\sigma^{70}$ subunit | 7 |
| *rpoHd* | RNA polymerase, $\sigma^{32}$ subunit, regulation of proteins induced at high temperature | |
| III. *Macromolecule Metabolism* | | |
| A. *Synthesis and modification of macromolecules* | | |
| 1. *rRNA and "stable" RNAs* | | |
| *rrf* | 5S rRNA | 8 |
| *rrl* | 23S rRNA | 8 |
| *rrs* | 16S rRNA | 11 |
| 2. *Ribosomal protein synthesis and modification* | | |
| *rplL* | 50S ribosomal protein L7/L 12 | 9 |
| *rplT* | 50S ribosomal protein L20 | 10 |
| *rpmH* | 50S ribosomal protein L34 | 1 |
| *rpmI* | 50S ribosomal protein L35 | 10 |
| *rpsA* | 30S ribosomal protein S1 | 3 |
| *rpsD* | 30S ribosomal protein S4 | 14 |
| *rpsK* | 30S ribosomal protein S11 | 14 |
| 4. *tRNAs* | | |
| *tRNAGlu* | Glutamate-tRNA | 8 |
| *tRNAPhe* | Phenylalanine-tRNA | 1 |
| *tRNATrp* | Tryptophan-tRNA | 1 |
| 5. *Aminoacyl tRNA synthetases and their modification* | | |
| *argS* | Arginine tRNA synthetase | 11 |
| *aspS* | Aspartic tRNA synthetase | 3 |
| *cysS* | Cysteine tRNA synthetase | 8 |
| *metG* | Methionine tRNA synthetase | 2 |
| *serS* | Serine tRNA synthetase | 3 |
| *thrS* | Threonine tRNA synthetase | 10 |
| *trmE* | tRNA methyltransferase | 1 |
| 7. *DNA replication, restriction/modification, and recombination* | | |
| *dnaA* | DNA biosynthesis, initiation of chromosome replication, global transcription regulator | 1 |
| *dnaG* | DNA biosynthesis, DNA primase | 7 |
| *dnaN* | DNA polymerase III holoenzyme, β-subunit | 1 |
| *dnaQ* | DNA polymerase III holoenzyme, ε-subunit | 11 |
| *gidA* | Chromosome replication? | 1 |
| *gyrB* | DNA gyrase subunit B | 1 |
| *himD* | Integration host factor, β-subunit | 3 |
| *rep* | Rep helicase, ssDNA-dependent ATPase | 1 |
| 8. *Protein translation and modification* | | |
| *efp*[f] | Elongation factor EF-P | |
| *infC* | Initiation factor IF-3 | 10 |
| *tuf*[g] | Elongation factor EF-Tu | |
| 9. *RNA synthesis, RNA modification, and DNA transcription* | | |
| *rho* | Transcription termination factor Rho | 1 |
| *rpoA* | RNA polymerase, α-subunit | 14 |
| *rpoB* | RNA polymerase, β-subunit | 9 |
| *rpoC* | RNA polymerase, β′-subunit | 9 |
| 11. *Phospholipids* | | |
| *clh*[h] | Cardiolipin synthase | |
| B. *Degradation of macromolecules* | | |
| 1. *RNA* | | |
| *rnh* | RNase H | 11 |
| *rnpA* | RNase P | 1 |

**Table 19.4 (continued)**

| Gene symbol | Gene product description | Linkage group[b] |
|---|---|---|
| 3. *Proteins, peptides, and glycopeptides* | | |
| *hslU* | Heat shock protein, protease? | 12 |
| *htrA* | Periplasmic serine protease and heat shock protein | 4 |
| *soh*[i] | Periplasmic protease | |
| C. *Cell envelope* | | |
| 2. *Surface polysaccharides, lipopolysaccharides, and antigens* | | |
| *kdtB* | Putative enzyme of lipopolysaccharide synthesis | 12 |
| 4. *Murein sacculus and peptidoglycan* | | |
| *ddlB* | D-alanine-D-alanine ligase | 6 |
| *murC* | L-alanine-adding enzyme, UDP-*N*-acetylmuramate-alanine ligase | 6 |
| *murE* | *meso*-Diaminopimelate-adding enzyme | 4 |
| IV. *Cell Processes* | | |
| B. *Chaperones* | | |
| *dnaJ*[d] | Heat shock protein | |
| *dnaKd* | Heat shock protein (Hsp 70), DNA biosynthesis | |
| *groEL* | Heat shock protein (Hsp 60) | 1 |
| *groES* | Heat shock protein (Hsp 10) | 1 |
| *hscA* | Cold shock protein (Hsp 70) | 1 |
| *hscB* | Cold shock protein | 1 |
| C. *Cell division* | | |
| *ftsA* | Cell division protein, complexes with FtsZ | 6 |
| *ftsI* | Septum formation; penicillin binding protein 3; peptidoglycan synthase | 4 |
| *ftsL* | Cell division protein; ingrowth of wall at septum | 4 |
| *ftsZ* | Cell division, forms circumferential ring | 6 |
| E. *Protein and peptide secretion* | | |
| *secB* | Protein export, molecular chaperone | 7 |
| V. *Other* | | |
| F. *Adaptations and atypical conditions* | | |
| *ibp* | Heat shock protein, HSP20 family | 12 |
| *Additional ORFs* | | |
| *bcp* | *E. coli homolog* Bacterioferritin comigratory protein 1788825 | 2 |
| *nifS* | 1788879 | 1 |
| *γibN* | 1790040 | 7 |
| *γjEA* | Hypothetical lysine tRNA synthase homolog 1790599 | 12 |
| 10 kDa | YIDD_ECOLI | 1 |
| 39 kDa | 1790589 | 1 |
| 60 kDa | 1790140 | 1 |
| *ORF113* | 1786351 | 6 |
| *ORF128* | 1788878 | 1 |
| *ORF177* | 1788671 | 2 |

**Table 19.4 (continued)**

| Gene symbol | Gene product description | Linkage group[b] |
|---|---|---|
| *ORF194* | 1788860 | 1 |
| *ORF217* | 1787362 | 13 |
| *ORF235* | 1786354 | 6 |
| *ORF312* | 1786270 | 4 |
| *ORF340* | 1788543 | 4 |
| *ORF453* | 1788858 | 1 |
| *ORFI* | 1786406 | 11 |
| *ORFV* | 1787508 | 5 |
| *ORFVI* | 1787507 | 5 |
| *P14* | 1787506 | 5 |
| *ORFA* | 1788269 Transmembrane protein? | 5 |
| *ORFB* | 1787524 | 5 |
| *ORFC* | 1787361 ABC transporter protein? ATP-binding site? | 13 |
| *ORFD* | 1789158 | 13 |
| pLeu | | |
| *repA1* (p)[e] | Related to RepA protein of IncFII plasmids | 15 |
| *repA2* (p)[e] | Related to RepA protein of IncFII plasmids | 15 |
| ORF1 (p)[e] | Related to *E. coli* 1789376 | 15 |

[a]Gene list arranged according to the classification of gene products of Riley and Labedan (1996). Genes from *Buchnera* from other species of aphids are indicated
[b]See *Table 19.5*
[c]From *Buchnera* (Usn)
[d]From *Buchnera* (Ap) (Nakabachi and Ishikawa 1997, 1999; Sato and Ishikawa 1997a, b)
[e]p, plasmid-associated gene
[f]From *Buchnera* (Mp) (Hogenhout et al. 1998)
[g]From Brynnel et al. (1998)
[h]From *Buchnera* (Dn, Usn)
[i]From *Buchnera* (Sc) (Lai et al. 1995)

*rubrum* ribulose-1,5-bisphosphate carboxylase (Kakeda and Ishikawa 1991).

The *Buchnera groESL* operon organization resembles that of *E. coli* (Hassan et al. 1996; Hogenhout et al. 1998; Ohtaka et al. 1992). Upstream of *groES* are nucleotide sequences characteristic of the −35 and −10 regions of $\sigma^{32}$ promoters. A message of 2.1 kb (containing both *groES* and *groEL*) is made by the endosymbiont using only this promoter (Sato and Ishikawa 1997a). It is not understood why GroES is low in the endosymbiont, in contrast to the high quantities of GroEL (Kakeda and Ishikawa 1991). The genes for $\sigma^{32}$ (*rpoH*) as well as *dnaKJ* have been cloned and sequenced (Sato and Ishikawa 1997a, b). The latter also are transcribed solely from a $\sigma^{32}$ promoter. In *E. coli* as well as other organisms, transcription of the *groESL* operon and the *dnaKJ* operon is part of the $\sigma^{32}$ regulon, and their synthesis is increased by heat shock (Gross 1996). It would appear that this mode of regulation is modified in *Buchnera*

**Table 19.5**
**Order of genes on DNA fragments of *Buchnera* from the aphid *S. graminum*[a]**

| | Chromosomal genes |
|---|---|
| 1. | (34.7 kb, AF008210) *39 kDa-groEL-groES-tRNAPhe-trmE-60 kDa-rnpA-rpmH-dnaA-dnaN-gyrB-atpCDGAHFEB-gidA-ORF194-0RF453-fdx-hscA-hscB-ORF128-nifS-tRNATrp-ilvD-ilvC-rep-trxA-rho* |
| 2. | (12.8 kb, AF067228) *bcp-aroC-ORF177-hisG-hisD-hisC-hisB-hisH-hisA-hisF-hisI-gnd-dcd-metG* |
| 3. | (11.5 kb, L43549) *aspS-trxB-serS-serC-aroA-rpsA-himD-tpiA* |
| 4. | (9.7 kb, AF060492) *dapD-htrA-ORF340-IlvI-ilvH-ORF312-ftsL-ftsI-murE* |
| 5. | (8.4 kb, Z19055) *ORFB-ORFA-trpD-trpC(F)-trpB-trpA-ORFV-ORFVI-P14* |
| 6. | (6.8 kb, AF012886) *murC-ddlB-ftsA-ftsZ-ORF113-ORF235* |
| 7. | (6.5 kb, M90644) *dnaG-rpoD-cysE-secB-yibN-argH* |
| 8. | (6.1 kb, U09230) *aroE-tRNAglu-rrl-rrf-cysS* |
| 9. | (5.0 kb, Z11913) *rplL-rpoB-rpoC* |
| 10. | (4.5 kb, U11066) *aroH-thrS-infC-rpmI-rplT* |
| 11. | (4.4 kb, L18927) *argS-rrs-ORF1-rnh-dnaQ* |
| 12. | (4.1 kb, AF108665) *hslU-ibp-fpr-yjeA-kdtB* |
| 13. | (3.9 kb, U11045) *ORFC-ORF217-gapA-ORFD* |
| 14. | (0.9 kb, M74510) *rpsK-rpsD-rpoA* |
| | Plasmid-associated genes |
| 15. | (8.0 kb, AF041836) *leuA-leuB-leuC-leuD-repA1-ORF1-repA2* |
| 16. | (3.6 kb, Z21938) *trpEG* |

[a]Numbers followed by parenthesis indicate linkage groups, numbers within parentheses indicate size of fragment and GenBank number. See *Table 19.4* for description of gene products

(Sato and Ishikawa 1997a, b). Synthesis of *groESL* and *dnaKJ* mRNA is constitutive and is not increased by heat shock. This conclusion is supported by the observation that there is no increase in the level of total GroEL in aphids shifted from 23 °C to 33 °C for one day (Baumann et al. 1996).

Baumann et al. (1996) arrived at an estimate of the amount of GroEL per *Buchnera* cell, based on the premise that the endosymbiont contained only one genome copy. Recently it has been shown that *Buchnera* is polyploid, containing an average of 120 genome copies per cell (Komaki and Ishikawa 1999). If this result is incorporated into the calculation, an impossible excess of GroEL would be present in each endosymbiont cell. Since protein extracts of whole aphids were used in the estimation of GroEL content, this result could be explained by the finding that GroEL is also present in the hemolymph (van den Heuvel et al. 1994).

Perhaps the major economic effect of aphids on agriculture is their ability to transmit plant viruses (Blackman and Eastop 1984; Gray and Banerjee 1999; Sylvester 1985). *Buchnera*-derived GroEL has been implicated in the survival of luteoviruses in the hemolymph (Filichkin et al. 1997; van den Heuvel et al. 1994; Hogenhout et al. 1998). These viruses replicate in the plant and are ingested by aphids when they feed on phloem sap. Subsequently they are transported from the digestive tract into the hemolymph and from there into the salivary gland for transmission to plants via salivary secretions. The viruses are retained in an infective form (without replication) in the hemolymph throughout the life span of the aphid. There is evidence that the GroEL that is found in the hemolymph coats the virus particles and protects them from host defenses. A region in *Buchnera* GroEL has been identified which is essential for binding to the virus (Hogenhout et al. 1998), and similarly a portion of a viral capsid protein has been identified as the region to which the endosymbiont GroEL binds (van den Heuvel et al. 1997). Transmission of plant viruses may be advantageous to the aphid because infected plants have higher levels of nutrients in their sap (Blua et al. 1994).

An additional reason for the constitutive synthesis of high amounts of GroEL by *Buchnera* may be to compensate for the accumulated amino acid substitutions which have occurred at a high rate in this endosymbiont (Moran 1996; *Table 19.3*). These slightly deleterious changes may result in proteins of decreased stability, and the high levels of GroEL may compensate for these changes, allowing proper folding and retention of function. A similar role for chaperones in masking deleterious mutations has been recently suggested on the basis of work on *Drosophila* heat shock protein (Pennisi 1998; Rutherford and Lindquist 1998).

## Genetics

### Genome Analysis

The guanine + cytosine (G + C) content of *Buchnera* is about 28 mol% (Clark et al. 1998a; Ishikawa 1987). The genome size of the endosymbiont from the aphid Ap has been found to be 0.657 Mb (Charles and Ishikawa 1999). This is considerably below such free-living organisms as *E. coli* (4.6 Mb; Blattner et al. 1997) and *Haemophilus influenzae* (1.8 Mb; Fleischmann et al. 1995) and the intracellular pathogens, *Chlamydia trachomatis* (1.0 Mb; Stephens et al. 1998) and *Rickettsia prowazekii* (1.1 Mb; Andersson et al. 1998). The *Buchnera* genome is somewhat larger than that of the pathogen *Mycoplasma genitalium* (0.58 Mb; Fraser et al. 1995). An unusual property of the *Buchnera* genome is that it appears to be present as about 120 copies per cell (Komaki and Ishikawa 1999).

Approximately 130 kb of DNA have been sequenced from *Buchnera* (from Sg) (Baumann and Baumann 1998; Baumann et al. 1995; Clark et al. 1998a, b; Thao and Baumann 1998). The choice of this aphid was predicated on the fact that it contains only one endosymbiont as indicated by morphological examinations as well as extensive restriction enzyme and Southern blot analysis of whole aphid DNA, using probes for many different genes. The latter results indicated that, with the exception of plasmid-amplified DNA, only one copy of the targeted genes was present in the endosymbiont genome. In many cases, the DNA that was used for cloning was also endosymbiont-enriched.

There were many independently cloned DNA fragments with identical overlapping sequences, indicating that the aphids did not contain several closely related endosymbionts. At least 20 kb of DNA also have been sequenced from *Buchnera* of each of the aphids Dn, Sc, and Mr (Baumann et al. 1998a; Clark et al. 1999b; Lai et al. 1995, 1996). *Table 19.4* lists, under different functional categories, the genes found in *Buchnera*, primarily in (Sg). The order of these genes in the DNA fragments is presented in *Table 19.5*. A total of 126 open reading frames were detected, of which 101 corresponded to *E. coli* genes with known function. The remaining 25 open reading frames all had homologs of no known function in the *E. coli* chromosome (Blattner et al. 1997). *Table 19.6* presents the codon usage of the *Buchnera* structural genes. As expected from the G + C content, there is a strong bias for A and T, especially in the third codon position. This bias also affects the composition of proteins, favoring amino acids for which codons contain more A and T (Moran 1996; Clark et al. 1999b).

*Buchnera* was found to contain *dnaA*, encoding a protein which initiates bidirectional chromosome replication, and *ftsZ*, encoding a protein involved in septum formation during cell division (Baumann and Baumann 1998; Lai et al. 1992a). Among other genes that were found are those encoding proteins for peptidoglycan synthesis, cell division, DNA replication, DNA transcription, ribosomal proteins, amino acid tRNA synthases, ATP synthase, electron transport, protein secretion, and glycolysis. In addition, genes for three tRNAs were detected. Genes encoding homologs of proteins involved in the *E. coli* heat shock response (*groEL*, *groES*, *htrA*, *dnaK*, *dnaJ*) and the cold shock response (*hscA*, *hscB*) were also detected (Clark et al. 1998a; Hassan et al. 1996; Ohtaka et al. 1992; Sato and Ishikawa 1997b). Nakabachi and Ishikawa (1999) detected a gene (*ribE*) encoding a protein involved in riboflavin biosynthesis. In addition, some of the genes encoding enzymes for the biosynthesis of aromatic amino acids (shikimate pathway, tryptophan branch), branched-chain amino acids (isoleucine, valine, leucine), lysine, cysteine, and serine as well as genes for the complete pathway of histidine biosynthesis were found (Clark et al. 1998a, b; Thao and Baumann 1998). The presence of genes for enzymes of amino acid biosynthesis is in marked contrast to the obligate intracellular pathogens *Rickettsia prowazekii* and *Chlamydia trachomatis*, as well as such fastidious organisms as *Mycoplasma genitalium* and *Borrelia burgdorferi*, all of which lack genes encoding enzymes of amino acid biosynthesis (Andersson et al. 1998; Fraser et al. 1995, 1997; Stephens et al. 1998). Retention of amino acid biosynthetic genes by *Buchnera* probably reflects the role of these pathways in the mutualistic association with the host aphids. Overall, these results on gene content indicate that *Buchnera* has many of the properties of free-living bacteria and would appear to be, in many respects, a self-contained, physiologically autonomous unit enclosed within bacteriocyte-derived vesicles.

Currently the most interesting comparison of the *Buchnera* genome is with the recently sequenced obligate intracellular pathogen *R. prowazekii*, an organism which is a member of the α-subdivision of the Proteobacteria (Andersson et al. 1998).

**Table 19.6**
**Codon usage of *Buchnera* from the aphid *S. graminum*[a]**

| AA | Codon | Fraction | AA | Codon | Fraction |
|---|---|---|---|---|---|
| Phe | UUU - | 0.933 | Ala | GCA - | 0.470 |
| Phe | UUC - | 0.067 | Ala | GCG - | 0.052 |
| Leu | UUA - | 0.663 | Tyr | UAU - | 0.854 |
| Leu | UUG - | 0.091 | Tyr | UAC - | 0.146 |
| Leu | CUU - | 0.132 | His | CAU - | 0.865 |
| Leu | CUC - | 0.011 | His | CAC - | 0.135 |
| Leu | CUA - | 0.087 | Gln | CAA - | 0.887 |
| Leu | CUG - | 0.016 | Gln | CAG - | 0.113 |
| Ile | AUU - | 0.576 | Asn | AAU - | 0.863 |
| Ile | AUC - | 0.075 | Asn | AAC - | 0.137 |
| Ile | AUA - | 0.349 | Lys | AAA - | 0.918 |
| Met | AUG - | 1.000 | Lys | AAG - | 0.082 |
| Val | GUU - | 0.474 | Asp | GAU - | 0.875 |
| Val | GUC - | 0.054 | Asp | GAC - | 0.125 |
| Val | GUA - | 0.407 | Glu | GAA - | 0.913 |
| Val | GUG - | 0.065 | Glu | GAG - | 0.087 |
| Ser | UCU - | 0.448 | Cys | UGU - | 0.826 |
| Ser | UCC - | 0.039 | Cys | UGC - | 0.174 |
| Ser | UCA - | 0.273 | Trp | UGG - | 1.000 |
| Ser | UCG - | 0.034 | Arg | CGU - | 0.348 |
| Ser | AGU - | 0.177 | Arg | CGC - | 0.045 |
| Ser | AGC - | 0.029 | Arg | CGA - | 0.148 |
| Pro | CCU - | 0.456 | Arg | CGG - | 0.013 |
| Pro | CCC - | 0.069 | Arg | AGA - | 0.416 |
| Pro | CCA - | 0.420 | Arg | AGG - | 0.030 |
| Pro | CCG - | 0.055 | Gly | GGU - | 0.464 |
| Thr | ACU - | 0.474 | Gly | GGC - | 0.042 |
| Thr | ACC - | 0.041 | Gly | GGA - | 0.446 |
| Thr | ACA - | 0.433 | Gly | GGG - | 0.048 |
| Thr | ACG - | 0.052 | Ter | UAA - | 0.887 |
| Ala | GCU - | 0.426 | Ter | UAG - | 0.094 |
| Ala | GCC - | 0.052 | Ter | UGA - | 0.019 |

[a]Based on 19,037 codons (Clark et al. 1998a)

Compared to the sequenced bacterial genomes, this organism is unusual in that 24 % of its DNA is noncoding. In addition it has a number of pseudogenes. These findings are interpreted as a stage in the adaptation of *R. prowazekii* to an intracellular lifestyle, involving the loss of genes encoding metabolic pathways for products that are provided by the host. Currently about 20 % of the *Buchnera* genome has been sequenced, and the organization of the genes in these genome fragments is highly compact and similar to that of other bacteria (Clark et al. 1998a). These findings indicate that if, as seems probable, *Buchnera* originated from an organism with a larger genome (Charles and Ishikawa 1999), then the reduction in the genome size has

**Table 19.7**
**Designations and characteristics of *Buchnera* plasmids[a]**

| | |
|---|---|
| 1. | *pTrpEG.* Two or more DnaA boxes in a putative origin of replication (Fig. 19.5a–h). Arrangement of DnaA boxes varies, one conserved pattern is recognized and designated at *ori*-3.6. (Fig. 19.5a–c). The plasmids usually consist of tandem repeats of identical or similar units containing gene(s) for a putative anthranilate synthase (TrpEG), the first enzyme of the tryptophan biosynthetic pathway |
| 2. | *pTrpEG-R.* Plasmid contains genes for putative replication initiation proteins (*repAC1, repAC2*) which are related to replication initiation proteins of broad host range plasmids of the IncA/C group (Fig. 19.5i, 19.7c). Within the DNA encoding the C-terminal end of the replication initiation proteins and/or downstream of this gene are 19 nt-long repeats which correspond to putative interons (Fig. 19.5i). Plasmid consists of tandem repeats of similar units and contains genes for a putative anthranilate synthase (TrpEG), the first enzyme of the tryptophan biosynthetic pathway |
| 3. | *pLeu.* Plasmid contains genes for putative replication initiation proteins (*repA1, repA2*) which are related to replication initiation proteins of plasmids of the IncFII group (Fig. 19.9a–c). Putative origin of replication downstream of *repA1*. Plasmids contain one copy of genes encoding for enzymes of leucine biosynthesis (*leuA, leuB, leuC, leuD*) |

[a]For references see text

already been accomplished and what has been retained is the essential gene complement required for the endosymbiotic association.

### Plasmid-Associated Amplification of Biosynthetic Genes

Some species of aphids have *Buchnera* in which genes for enzymes of amino acid biosynthesis are amplified on plasmids (Bracho et al. 1995; Lai et al. 1994; van Ham et al. 1999). This plasmid-associated gene amplification has been interpreted as an adaptation of *Buchnera* to an endosymbiotic association in which one of its functions is the overproduction of amino acids. This interpretation is based on analogies with other prokaryotic systems in which gene amplification is viewed as an attribute of prokaryotic genome plasticity allowing the organism to adapt to new environments (for recent reviews see Romero and Palacios 1997; Roth et al. 1996). Currently *Buchnera* has been found to contain three different types of plasmids. The properties of these plasmids are summarized in Table 19.7. The plasmid-amplified genes encode the first enzyme of the tryptophan biosynthetic pathway (TrpEG) and four enzymes (LeuA, LeuB, LeuC, LeuD) of the leucine portion of the branched-chain amino acid biosynthetic pathway. For purposes of grouping of plasmid types and ease of presentation, we have used the plasmid designations given in Table 19.7 followed by the abbreviation corresponding to the aphid species (Table 19.2). When first used, the designation is followed by the original plasmid name given in parentheses (if applicable). There are a number of other examples of plasmid amplification of biosynthetic genes. Amplification has been interpreted as a means of increasing the rate of synthesis of end products. These include genes of purine biosynthesis in *Borrelia* (Margolis et al. 1994), cysteine biosynthesis in *Synechococcus* (Nicholson et al. 1995), and histamine biosynthesis in *Vibrio* (Barancin et al. 1998).

#### The trpEG-Containing Plasmids and Gene Silencing

Figure 19.4 is an outline of the aromatic amino acid biosynthetic pathway. It consists of a common portion leading to chorismate (shikimate pathway) and branches to (1) phenylalanine and tyrosine as well as (2) tryptophan. In the shikimate pathway, arrows that have designations correspond to genes detected in *Buchnera* (Sg). The activity of the tryptophan biosynthetic pathway is regulated by anthranilate synthase (TrpEG) which is feedback inhibited by tryptophan (Crawford 1989). In *Buchnera* from 11 species of aphids, *trpEG* has been found to be plasmid-associated (Fig. 19.5; Baumann et al. 1997b; Lai et al. 1994, 1996; Rouhbakhsh et al. 1996, 1997; van Ham et al. 1999). The remaining genes of the pathway [*trpDC(F)BA*] have been found to have a chromosomal location in all cases examined [*Buchnera* (Sg, Dn, Sc, Mr)] (Baumann et al. 1998a; Clark et al. 1999b; Lai et al. 1995). In contrast to the situation in Aphididae, in *Buchnera* (Sc, Mr), *trpEG* is not plasmid-associated but is present as one copy on the endosymbiont chromosome (Fig. 19.4; Clark et al. 1999b; Lai et al. 1995).

The structure of plasmids of the pTrpEG type usually consists of tandem repeats of a nearly identical unit (Fig. 19.5a–h). In *Buchnera* (Sg, Rp), the plasmids contain four tandem repeats of a 3.6 kb unit; in *Buchnera* (Rm), the plasmid consists of one 3.6 kb unit; while in *Buchnera* (Ap) plasmids containing 5, 6, or 10 tandem repeats are found. *Buchnera* (Sg) contains about 4 plasmids per endosymbiont chromosome resulting in a 16-fold *trpEG* amplification. In *Buchnera* (Sc, Mr), in which *trpEG* is chromosomal, these two genes are found between *fpr* and *hslU* (Fig. 19.6). In *Buchnera* (Sg), in which *trpEG* is plasmid-associated, *trpEG* is absent from this chromosomal location, consistent with its transfer to the plasmid (Fig. 19.6). Instead *ibp* is present at this position, suggesting a concomitant or a subsequent acquisition of this gene (Clark et al. 1999a).

The plasmids in Fig. 19.5a–h all share in common the presence of 2–5 DnaA boxes which are 9 nt-long sequences to which the DnaA protein binds, thereby initiating chromosomal replication (Messer and Weigl 1996). There is also considerable variation in the length of the repeated units (2.6–3.6 kb). Within this plasmid group, a readily recognized subset contains a unique arrangement of three DnaA boxes and a conserved region upstream of *trpEG* which has been designated as *ori-3.6* (Fig. 19.5a–c; Lai et al. 1996; Rouhbakhsh et al. 1996). *Buchnera* in aphids of the genus *Uroleucon* (which is derived from *Buchnera* within the cluster that has *ori-3.6*-containing plasmids, Fig. 19.2b, c) have *trpEG* units which show a considerable size range and substantial differences in the arrangement of the DnaA boxes (Fig. 19.5d–g; Baumann et al. 1997b; Rouhbakhsh et al. 1997). All of these *Buchnera* are from aphid species of the family Aphididae (Fig. 19.2a). Plasmid

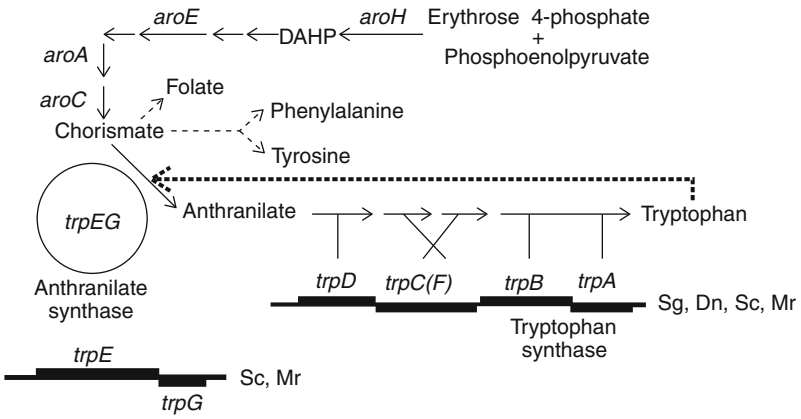


**Fig. 19.4**

**Outline of the pathway for the biosynthesis of aromatic amino acids. *Arrows*, single enzymatic reactions; *dashed arrows*, several enzymatic reactions; *striped arrow*, feedback inhibition; genetic designations above *striped line*, genes detected in *Buchnera* (Sg); other genes, detected in *Buchnera* from the designated host aphids; *circle*, plasmid containing one or multiple copies of trpEG; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate. For a description of genes, see *Table 19.4*; for references see text**

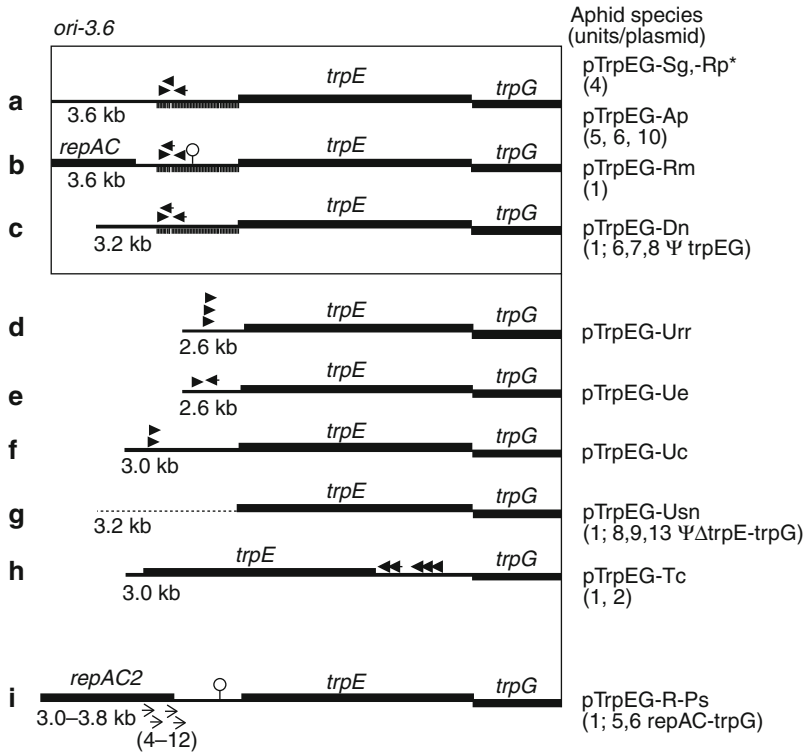


**Fig. 19.5**

**Genetic maps of the repeated units which constitute trpEG-containing plasmids. *Filled arrowheads*, position and direction of DnaA boxes which are components of a putative origin of replication; *circle* on stem in (b) and (i), position of a 19 nt sequence similar to the interon of the broad host range plasmid RA1; ori-3.6, putative origin of replication found primarily in plasmids consisting of 3.6 kb repeat units (*boxed*); *striped line*, conserved sequence; *dashed line*, DNA that has not been sequenced; *arrow* in (i), 19 nt repeated sequence corresponding to a putative interon; Rp*, TrpEG-Rp also contains a remnant of repAC. For references see text**

pTrpEG-Tc (pBTc2; *Fig. 19.5h*) is from an aphid within the family Pemphigidae (van Ham et al. 1999). The predominant form of this plasmid consists of one 3.0-kb unit; a minor form consists of two units. A distinctive feature is the presence of DnaA boxes between *trpE* and *trpG* (*Fig. 19.5h*) and not upstream of *trpEG* as is the case of the other plasmids (*Fig. 19.5a–g*). However, since there is considerable rearrangement of the DnaA boxes within plasmids in *Buchnera*

of *Uroleucon* (Fig. 19.5d–g), it is plausible that the arrangement of DnaA boxes within pTrpEG-Tc is not a fundamental difference but a variation on the arrangement observed in the other *trpEG*-containing plasmids (Fig. 19.5a–g).

A totally different *trpEG*-containing plasmid is pTrpEG-R-Ps (pBPs2; Fig. 19.5i), which does not contain DnaA boxes but instead has putative replication initiation proteins (RepAC) which are related to those of plasmids of the broad host range group IncA/C (van Ham et al. 1999). Within the DNA encoding the C-terminal portion of *repAC* and/or downstream of it are 4–12 repeats of a 19 nt-long sequence corresponding to a putative interon. In addition, there is a single copy of a 19 nt-long sequence similar to the interon sequence of IncAC plasmid RA1 (Llanes et al. 1996). Curiously pTrpEG-Rm (Fig. 19.5b) contains in its DNA a gene for a putative RepAC protein and the 19 nt-long sequence similar to that found in plasmid RA1, while pTrpEG-Rp from a closely related aphid has a remnant of *repAC* (van Ham et al. 1999).

In several pTrpEG plasmids, the expression of most of the *trpEG* copies appears to be silenced (Baumann et al. 1997b; Lai et al. 1996; van Ham et al. 1999). In *Buchnera* (Dn), there are about two copies of pTrpEG-Dn for each endosymbiont genome (Lai et al. 1996). Plasmid pTrpEG-Dn consists of a single 3.2-kb unit containing an open reading frame corresponding to the putative protein TrpEG (Fig. 19.7a). This is followed by a 2.6-kb unit containing *trpEG* pseudogenes and 5–7 repeats of a 3.2-kb unit also containing *trpEG* pseudogenes. (By pseudogenes, we mean segments of DNA which are clearly recognizable as *trpEG* but which contain numerous frameshifts and stop codons preventing the synthesis of an intact protein.) A comparison of the sequences between the 3.2 kb fragments with and without pseudogenes indicated 244 differences of which 93 % were localized in an approximately 900-bp DNA segment which included the putative promoter and the N-terminus of *trpE* (Fig. 19.7a). These changes should result in the reduction or elimination of mRNA synthesis; if messenger is made, it would be translated into short peptides because of the numerous frame shifts and stop codons in the region of the N-terminus of *trpE*.

Another instance of *trpEG* silencing is illustrated by pTrpEG-Usn (Fig. 19.7b). This plasmid consists of a 3.2-kb unit of *trpEG* followed by 10–14 2.1 kb units consisting of DNA with a deletion of about 56 % of the N-terminal region of *trpE* and an intact trpG. A more remarkable example of gene silencing is found in pTrpEG-R-Ps (Fig. 19.7c), which consists of a 3.6–3.8-kb unit containing *repAC2-trpEG* followed by 1.8 kb units consisting of *repAC1*, a deletion of *trpE*, and an intact *trpG* (van Ham et al. 1999). Preceding the latter is a short DNA

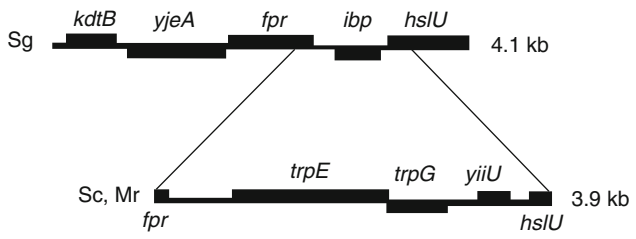


**Fig. 19.6**

**Genetic map of similar chromosomal DNA fragments from *Buchnera* (Sg) as well as (Sc, Mr). *Buchnera* (Sg) lacks trpEG due to its presence on a plasmid, while in *Buchnera* (Sc, Mr), trpEG is chromosomal. For a description of genes, see Table 19.4; for references, see text**

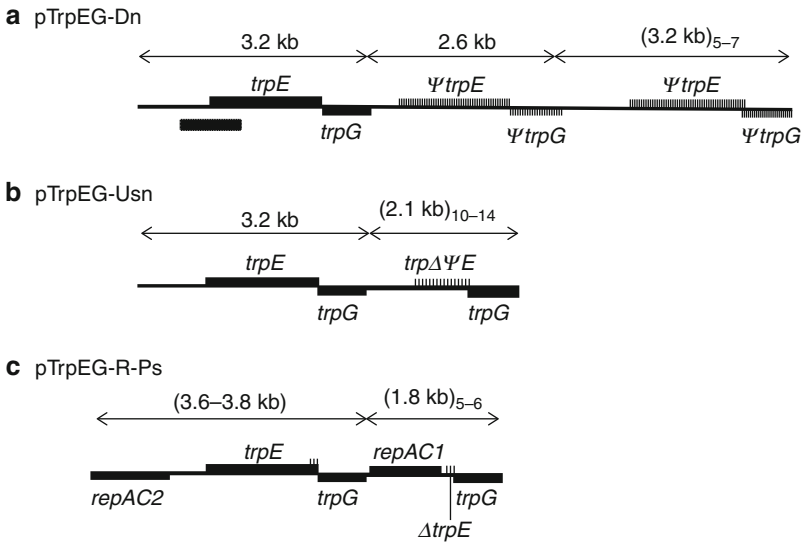


**Fig. 19.7**

**Genetic map of plasmids which contain silenced trpEG. *Stippled line* in (a), region which in the pseudogene containing fragments has most of the changes; *striped line* in (a) and (b), pseudogenes (Ψ); δ in (b), deletion of the N-terminal portion of trpE; *striped line* in (c), sequence homologous to the end of trpE. For references see text**

segment which appears to be a remnant of DNA encoding the C-terminus of *trpE*. The structure of this plasmid suggests that, in *Buchnera* (Ps) of the family Pemphigidae, *trpEG* amplification had an origin independent of that of the remaining plasmids shown in ❯ *Fig. 19.5*. The initial plasmid probably contained tandem repeats of *repAC-trpEG*. Subsequently there was selection pressure for gene silencing resulting in a plasmid with one intact copy of *repAC-trpEG* and tandem repeats of *repAC-trpG* which contain a deletion of the putative promoter region and most of *trpE*.

**Speculation Concerning trpEG Amplification** The following is a summary of the results obtained from studies of *trpEG* in *Buchnera*. (1) *trpEG* amplification is widespread in *Buchnera* within the family Aphididae and is also present in at least two members of the family Pemphigidae. In *Buchnera* from two aphid species of the latter family, *trpEG* is chromosomal and is found in the same location. In most cases, *trpEG* amplification is affected by plasmids consisting of tandem repeats of the same or similar unit. (2) Evolution of plasmid-associated *trpEG* is vertical, that is, *Buchnera* from different aphid species do not exchange plasmids. (3) The *trpEG*-containing plasmids constitute at least two replicon types, one of which is based on the presence of DnaA boxes, while the other is based on the presence of interons and replication initiation proteins related to plasmids of the IncA/C group. *Buchnera* from the related aphids Rm and Rp which belong to the first replicon type also have a gene and/or the remnant of a gene for a replication initiation protein related to plasmids of the IncA/C group. (4) In *trpEG* plasmids of both replicon types, gene silencing of some of the *trpEG* tandem repeats is observed. (5) In *Buchnera* (Sg), the sequence of two of the 3.6-kb units is virtually identical, as is the sequence of the 2.6- and 3.2-kb *trpEG* pseudogene-containing units of *Buchnera* (Dn). While results above are derived from genetic analyses, the interpretations that follow are speculative and based in large part on analogies with other prokaryotic systems.

Gene amplification is currently viewed as a reversible aspect of genome plasticity which occurs at a frequency considerably higher than that of mutation in structural genes (Romero and Palacios 1997; Roth et al. 1996). Gene amplification is frequently used by an organism as a means of increasing the amount of a growth-limiting enzyme to levels beyond that achieved by gene regulation of expression. One of the functions of *Buchnera* is the synthesis of essential amino acids (including tryptophan) for the aphid host. In almost all prokaryotes, the limiting enzyme TrpEG (which is feedback inhibited by tryptophan) regulates the activity of the tryptophan biosynthetic pathway. The *Buchnera* enzyme is probably also feedback inhibited by tryptophan since *trpE* has the conserved amino acid residues that are involved in feedback inhibition (Lai et al. 1994). To overproduce tryptophan, the activity of TrpEG must be increased, the potential effect of tryptophan accumulation on activity must be overcome, or both. Since even in high concentrations of tryptophan the activity of TrpEG is not fully inhibited, an increase in enzyme protein will result in increased tryptophan production. This has been the case in experiments on overproduction of tryptophan for industrial purposes; an increase in allosterically inhibitable TrpEG is the primary means of achieving excretion of high amounts of tryptophan into the medium (Katsumata and Ikeda 1993).

In free-living bacteria, gene amplification is frequently transient. Its persistence depends on a constant selective pressure, the absence of which leads to a rapid decrease in number of repeats primarily by means of RecA-mediated homologous recombination (Roth et al. 1996). There may be differences or fluctuations in the levels of tryptophan in aphid diets; furthermore, the high level of TrpEG protein may impose an energy burden on the endosymbiont. Both of these conditions could provide short-term selective pressure for the elimination of plasmids from *Buchnera* (Baumann et al. 1997a). Consequently some mechanism of stabilization may be necessary for the maintenance of *trpEG*-containing plasmids. The gene *recA* has been cloned and sequenced from many bacterial taxa, and adequate oligonucleotide primers are available for its amplification by PCR (Eisen 1995). We have made extensive unsuccessful attempts to detect this gene by PCR, suggesting that it may be absent from *Buchnera* or is greatly modified. Once plasmid stabilization occurs, the aphid may encounter conditions in which *trpEG* amplification is no longer necessary due to availability of tryptophan in the diet. If the usual mechanisms (homologous recombination?) which effect a decrease in *trpEG* amplification are absent, then one way of reducing the potentially wasteful synthesis of TrpEG is gene silencing.

In the past, we have speculated that *trpEG* amplification is a property of rapidly growing aphids and that gene silencing may occur when following plasmid stabilization, the diet of aphids is nutritionally enriched (Baumann et al. 1997a; Lai et al. 1994, 1995, 1996). Recent studies have indicated that these speculations are overly simplistic. It has been suggested that the lack of *trpEG* amplification in *Buchnera* (Sc, Mr) which are in the Pemphigidae is due to the slow growth rate of these aphids compared to that of aphids within the Aphididae (Lai et al. 1995). There are, however, few studies on the growth rate of aphids, and the finding of amplification in *Buchnera* (Ps, Tc) which are in the family Pemphigidae and presumably also have a slow growth rate makes this explanation questionable. Dn causes major tissue histolysis of plants, and it has been suggested that the presence of higher amounts of tryptophan in the diet may be the explanation for gene silencing (Lai et al. 1996). Recent studies on amino acid composition of ingested phloem sap are not strongly supportive of this hypothesis as a sole explanation for the presence of pseudogenes (Sandström and Moran 1999; Sandström et al. 2000; Telang et al. 1999). *Buchnera* (Rp) and *Buchnera* (Sg) are similar in that both have pTrpEG consisting of four tandem repeats of a 3.6-kb unit (❯ *Fig. 19.5a*). In spite of this similarity, Rp causes essentially no modification of the amino acid composition of plant phloem, while Sg causes substantial increases (Sandström et al. 2000). Dn contains a plasmid with *trpEG* pseudogenes (❯ *Fig. 19.7a*). The ingested diet of Dn has approximately doubled concentrations of tryptophan, yet the changes it causes in the amino acid composition of phloem are less than those caused by Sg. This suggests that

pseudogene formation is not solely the result of increased dietary tryptophan (Sandström et al. 2000; Telang et al. 1999). However, in the case of Usn, the presence of *trpEG* pseudogenes is consistent with the finding of unusually high levels of essential amino acids in the phloem diet (Sandström and Moran 2000). Thus, the availability of nutrients in plant sap may be a partial explanation for *trpEG* amplification and pseudogene formation, but other factors must also be involved.

In this connection, it should be mentioned that the past speculations attempt to correlate adaptations of *Buchnera* with properties of the aphid host (growth rate, modification of nutrient content of plant sap). The environment of the endosymbiont is the bacteriocyte vesicle, which harbors the endosymbiont. This environment is a reflection of the activities of the aphid host and is a function of its ability to obtain nutrients from the plant as well as its demands on the biosynthetic attributes of the endosymbiont. Therefore, host properties, such as the efficiency of nutrient uptake from the plant and their transformation and delivery, may determine the nutritional parameters within the bacteriocyte vesicles and impose the selective pressure resulting in *Buchnera* adaptation to the endosymbiotic association.

Some of the phenomena encountered in pTrpEG from *Buchnera* also have been found in other systems. Promoter inactivation by multiple sequence changes is the mechanism used for silencing the expression of the *Bordetella pertussis* toxin gene (Gross and Rappuoli 1988) and the expression of the *Bordetella* urease gene cluster (McMillan et al. 1998). The changes resemble those observed in gene silencing of *trpEG* in *Buchnera* (Dn; ❯ *Fig. 19.7a*). Multiple copies of nearly identical chromosomal enzyme-encoding genes have been found in *Thiobacillus ferrooxidans* (Kusano et al. 1991) and *Nitrosospira* sp. (Norton et al. 1996). This situation is similar to that found with the repeats of *trpEG* and *trpEG* pseudogenes and has led to the postulation of mechanisms for the preservation of sequence identity of the repeated units (Klotz and Norton 1998).

The phylogenetic trees constructed on the basis of plasmid-associated genes are congruent with the phylogenetic trees based on *Buchnera* chromosomal genes (❯ *Fig. 19.2a–c*). One exception, the basal position of *Buchnera* (Tc; ❯ *Fig. 19.2c*), is probably an artifact arising from the more rapid change of *trpE* in this lineage (van Ham et al. 1999). These results strongly suggest that there is no exchange of *trpEG*-containing plasmids between endosymbionts of different aphids. Plasmids of the pTrpEG type (❯ *Fig. 19.5a–h*) could have an endogenous origin. DnaA boxes are found in other locations of the *Buchnera* genome (Clark et al. 1998c), and their assembly with *trpEG* could generate a separate replicon. In contrast to these plasmids, the *repAC* genes of pTrpEG-R are related to replication initiation proteins of IncA/C plasmids. Thus, this plasmid may be the result of an invasion of *Buchnera* by an exogenous plasmid that recombined with endosymbiont genes, resulting in their amplification. It has been established that some bacteria may persist for a long time in insects; conceivably, such organisms transferred their plasmids to *Buchnera* during the infection of embryos or eggs at a stage at which the endosymbionts are not sequestered within bacteriocytes. It is, however, difficult to explain the presence of *repAC* and its remnant in *Buchnera* (Rm, Rp). One possibility is that it is the result of another invasion by a similar plasmid. However, organization of *ori-3.6* in *Buchnera* (Rm, Rp) closely resembles that of *Buchnera* of related aphids, suggesting a common plasmid origin for this group (❯ *Fig. 19.5a–c*). Alternatively, van Ham et al. (1999) suggested that a plasmid of the pTrpEG-R type is the ancestor of all *trpEG* amplification plasmids. This hypothesis requires the subsequent occurrence of multiple losses of *repAC* and the interons in pTrpEG-R type plasmids and the acquisition of DnaA boxes in *Buchnera* of Aphididae.

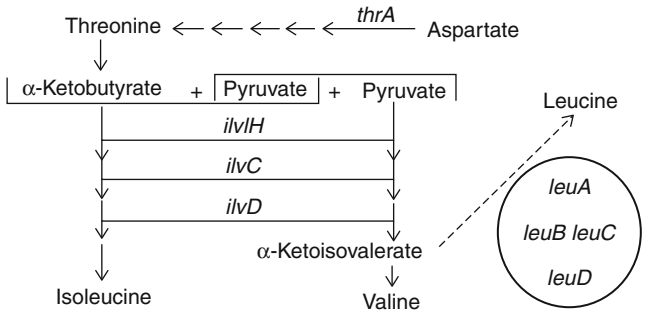


**Fig. 19.8**
**Outline of the pathway for branched-chain amino acid biosynthesis. *Arrows*, single enzymatic reactions; *horizontal lines*, enzymatic activities functional in both isoleucine and valine biosynthesis; *dashed arrow*, four enzymatic reactions; *circle*, plasmid containing genes for leucine biosynthesis. thrA was detected in *Buchnera* (Sg); ilvIH, ilvC, and ilvD were detected in *Buchnera* (Sg, Dn, Sc, Rm); leuACBD were detected in *Buchnera* (Sg, Dn, Rm). For a description of genes, see ❯ *Table 19.4*; for references, see text**

**pLeu Plasmids** ❯ *Figure 19.8* is an outline of the pathway of branched-chain amino acid biosynthesis. The gene for aspartokinase (*thrA*) has been found in *Buchnera* (Sg). The genes *ilvIH*, *ilvC*, and *ilvD* have been found in *Buchnera* (Sg, Dn, Sc, Mr) and encode three enzymes which function in both the isoleucine and valine biosynthetic pathways (Clark et al. 1998c, 1999b; Thao and Baumann 1998). The pathway of leucine biosynthesis is a branch off the valine pathway (❯ *Fig. 19.8*). Bracho et al. (1995) found that in *Buchnera* (Rp), the genes for leucine biosynthesis (*leuABCD*) were present on a plasmid (❯ *Fig. 19.9a*). This plasmid represents a third type (❯ *Table 19.7*), designated pLeu, which is characterized by the presence of genes (*repA1*, *repA2*) encoding putative replication initiation proteins related to those of plasmids of the IncFII incompatibility group. Besides these genes, pLeu also contains *ORF1* encoding a putative membrane-associated protein. The closely related *Buchnera* (Sg, Rp, Dn) all contain very similar plasmids of 7.8–8.0 kb in which the genes are arranged in the same order (❯ *Fig. 19.9a*; Baumann et al. 1999a; Bracho et al. 1995). In *Buchnera* from the more distantly related pLeu-Ppo (❯ *Fig. 19.2e*), there are rearrangements of the *repA* genes and

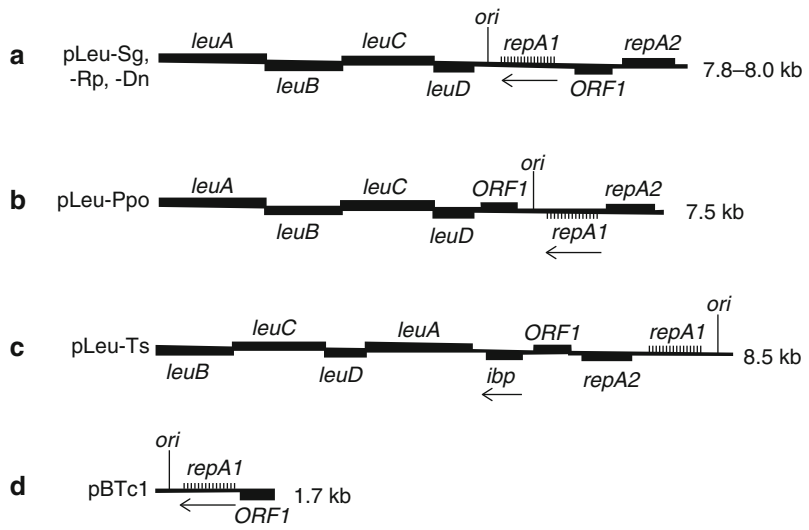


**Fig. 19.9**

**Genetic maps of pLeu plasmids. Unless indicated by an arrow, transcription is left to right. Ori, putative origin of replication; *striped line*, repA1 downstream of which is ori. For a description of genes, see *Table 19.4*; for references, see text**

*ORF1* (*Fig. 19.9b*; Silva et al. 1998). All of these aphids are within the family Aphididae. In pLeu-Ts (pBTs1; *Fig. 19.9c*), which is from an aphid of the family Thelaxidae, there is also a rearrangement of the *leu* genes as well as an acquisition of *ibp* which encodes a heat shock protein (van Ham et al. 1997). All of these plasmids have a conserved region, downstream of *repA1*, which is probably an origin of replication (*ori*) (Baumann et al. 1999; Bracho et al. 1995; van Ham et al. 1997). Remarkably pBTc1 (*Fig. 19.9d*), a 1.7-kb plasmid from *Buchnera* (Tc), which is found in an aphid belonging to the family Pemphigidae, contains only *ori*, *repA1*, and *ORF1* and probably constitutes a minimal replicon.

In *Buchnera* (Sg), there are about 24 copies of pLeu per endosymbiont genome, while in *Buchnera* (Dn), there are only two copies (Thao et al. 1998). This difference in functional gene copy number parallels that observed with pTrpEG in the endosymbionts of these two aphid species. In the case of pLeu, in which only one copy of the genes is present, the reduction in amplification in *Buchnera* (Dn) is achieved by means of a reduction of copy number. In pTrpEG, which contains tandem repeats of the same unit, the reduction in amplification is accomplished by means of pseudogene formation (*Fig. 19.7a*).

The similarities of pLeu plasmids suggest a single origin with pBTc1 (*Fig. 19.9d*) being the ancestral state (Baumann et al. 1999). In the lineage common to the Aphididae and the Thelaxidae, there was probably a duplication of *repA1* and the acquisition of *leu* genes. This was followed by a rearrangement of the genes and in one lineage the acquisition of *ibp* [for another interpretation, see van Ham et al. (1997)]. Silva et al. (1998) sequenced *repA2* from *Buchnera* of six additional aphids. A phylogeny based on this gene (*Fig. 19.2e*) as well as a more limited analysis based on *leu* genes (*Fig. 19.2d*) is congruent with trees established on the basis of other *Buchnera* chromosomal and plasmid genes. These results indicate that the pLeu plasmids are not exchanged among endosymbionts from different aphid species and that their evolution is vertical, as is the case with the *trpEG*-containing plasmids.

**Unanswered Questions, Other Possible Adaptations** One unanswered question is why only the *trp* and *leu* genes are amplified. The endosymbiont produces other essential amino acids for the aphid host, and their overproduction would in principle also be enhanced by plasmid amplification. It has been speculated that aphids make indole acetic acid which is involved in gall formation (Forrest 1987). In many plant pathogens that cause gall formation, tryptophan is the precursor of indole acetic acid (Patten and Glick 1996). Amplification of *trpEG* is, however, found in aphids that do not produce galls (Sg, Dn, Rp, Usn) and is absent in some that do (Sc, Mr). Consequently this does not appear to be a probable explanation for *trpEG* amplification. Leucine, lysine, valine, arginine, and threonine are the most common amino acid in aphids (Sandström and Moran 1999), yet only genes for leucine biosynthesis have been detected on plasmids. It is possible that, in other cases where amino acids are overproduced for the host, an increase in enzyme activity is obtained by increasing the expression of the gene(s) by promoter modification. Alternatively changes of the allosteric properties of regulated enzymes may allow retention of activity in the presence of end products.

A possible example of the latter is *cysE* of *Buchnera* (Sg; Lai and Baumann 1992b). This gene encodes an enzyme of the biosynthetic pathway of cysteine, and its activity is regulated by cysteine feedback inhibition (Kreditch 1996). It has been established that the amino acids at the C-terminus of the *E. coli* enzyme are involved in cysteine feedback inhibition (Denk and Böck 1987). The *Buchnera* (Sg) enzyme lacks these C-terminal amino acids and consequently is probably not

subject to feedback inhibition by the end product; this change would result in cysteine overproduction (Lai et al. 1992b).

## Secondary Endosymbionts of Aphids

Besides *Buchnera*, many aphids have additional endosymbionts usually called secondary (S-) endosymbionts (Buchner 1965; Houk and Griffiths 1980; Moran and Baumann 1994). In many cases, these endosymbionts are spheres or rod-shaped with different width and length; they were initially recognized by differences in size and shape from the round or oval *Buchnera*. The S-endosymbionts are also maternally inherited. They have not been extensively studied, and most of the available information is for the S-endosymbionts of the aphid Ap. Electron microscopic studies have shown that the rod-shaped S-endosymbionts are located within vesicles found in the flattened, syncytial, sheath cells which surround the bacteriome (Griffiths and Beck 1973). Using a probe derived from *E. coli* 16S rDNA and restriction enzyme and Southern blot analysis of total aphid DNA, it was found that the S-endosymbiont from Ap contained a single copy of the 16S rDNA gene (Unterman et al. 1989). DNA obtained from dissected bacteriocytes gave the same restriction pattern, indicating that the S-endosymbiont was located in the bacteriome. Two DNA fragments of 2.3 kb each were cloned, and the 16S rDNA sequence determined. A phylogenetic analysis indicated that the S-endosymbiont was a member of the Enterobacteriaceae (*Fig. 19.1*). As in other members of this family, the 16S rDNA gene of the S-endosymbiont was directly upstream of 23S rDNA (Unterman and Baumann 1990).

Chen and Purcell (1997) found that 88 % of the strains of Ap had the S-endosymbiont. In addition, the S-endosymbionts of Ap and *Macrosiphum rosae* were identical, suggesting recent infection or horizontal transmission. Interestingly, it was also found that the hemolymph of 48 % of Ap strains contained a rod-shaped organism which had a 16S rDNA sequence nearly identical to that of *Rickettsia bellii*, an organism found in ticks (Chen et al. 1996). As a result of these studies, strains of Ap became available which had (1) only the S-endosymbiont, (2) only the *Rickettsia* sp., as well as (3) neither of these two organisms. Chen et al. (1996) injected one or both of these organisms into Ap, which originally lacked both, and observed their effect on fecundity, longevity, and the length of the reproductive period. The results were complex in that they were affected by the plant on which the aphids grew and by the temperature of growth. At 20 °C, both the S-endosymbiont and the *Rickettsia* sp. reduced the fecundity, longevity, and reproductive period of Ap on clover but had no significant effects on Ap grown on alfalfa or sweet pea (Chen et al. 1996). In some cases, both of these organisms appeared to cause an increase in the fitness of Ap when grown at 25 °C. These results suggest that both the S-endosymbiont and the *Rickettsia* sp. can have either a deleterious or a beneficial effect on the host, depending on the environmental conditions (Chen et al. 1996). The S-endosymbiont, upon injection into the closely related species, *Acyrthosiphon kondoi*, was found to be pathogenic. Although the rate of maternal transmission of both the S-endosymbiont and the *Rickettsia* sp. was high, one instance of S-endosymbiont loss was observed (Chen and Purcell 1997).

Studies based on light and electron microscopy have suggested that some S-endosymbionts in some aphid species may inhabit syncytial cells or, possibly, bacteriocytes that appear similar to those containing *Buchnera* (Buchner 1965; Hinde 1971b; Iaccarino and Tremblay 1973). Fukatsu and Ishikawa (1993) surveyed 61 aphids for the presence of S-endosymbionts. Previously it was found that *Buchnera* (Ap) overproduced GroEL (Kakeda and Ishikawa 1991). Using anti-*E. coli* GroES for immunoprecipitation of *Buchnera* (Ap) GroES, it was concluded that *Buchnera* produced low levels of this protein (Kakeda and Ishikawa 1991). This antiserum as well as anti-*E. coli* GroEL was used to detect GroEL and GroES in immunoblots of whole aphid extracts as well as for histochemical detection in thin sections of aphids. From these experiments, it was concluded that the synthesis of substantial amounts of GroES by S-endosymbionts distinguishes them from *Buchnera* and that this property can be used for the identification of S-endosymbionts (Fukatsu and Ishikawa 1993). These studies have a number of problems that limit their general applicability to the survey of S-endosymbionts. The principal one is the use of antisera against *E. coli* GroES for the detection of cross-reactivity of GroES from organisms that have an unknown relationship to *E. coli*. Since it is probable that many of the S-endosymbionts are members of the Enterobacteriaceae, a stronger cross-reaction would be expected with their proteins than with the proteins from *Buchnera*; consequently, an increased reactivity need not indicate a major difference in the amount of the protein. Conversely, in those cases where the S-endosymbiont is not a member of the Enterobacteriaceae, the distant relationship may preclude a strong cross-reaction (Eremeeva et al. 1998). The cross-reactivity of the *Buchnera* and S-endosymbiont proteins with the anti-*E. coli* protein antisera has not been compared. Finally, the relative production of GroES may vary among S-endosymbionts of different types.

The studies of Fukatsu and Ishikawa (1993, 1998), in which thin sections of aphids were stained by immunohistochemistry and examined by light microscopy, do suggest that in many aphids the S-endosymbionts occupy bacteriocytes distinct from those containing *Buchnera*. The authors also state that the S-endosymbionts have a variety of different shapes. The methods used and the photographs presented do not, however, allow adequate visualization of cell shape and the resolution of bacteriocyte structure. In addition, the designation of some of the endosymbionts as *Buchnera* or S-endosymbionts appears to be arbitrary.

Fukatsu et al. (1998) used group-specific oligonucleotide probes for in situ detection of aphid P- and S-endosymbionts. A universal eubacterial 16S rRNA probe was used as well as probes specific for the 23S rRNA γ- and β-subdivision Proteobacteria. The sequences of the latter two probes differ by only one nucleotide. *Buchnera* and S-endosymbionts all

hybridized with the 16S eubacterial probe, although the intensities of the signal differed considerably. Curiously the putative P-endosymbionts of two out of seven aphids did not hybridize with the 23S γ-subdivision probe. Using the total DNA preparation from these aphids, the 16S rDNA was amplified, cloned, and sequenced. Two sequences were detected in each aphid DNA preparation, and one of these was related to *Buchnera* 16S rDNA. Based on this result, it was concluded that these aphids contained *Buchnera* but that their 23S rDNA gene was changed to such an extent that hybridization with the γ-subdivision probe no longer occurred. This conclusion is questionable since the 23S rDNA of *Buchnera* (Sg, Dn, Sc, Mr), which span the diversity of aphid hosts (*Fig. 19.2*), contains the exact sequence complementary to the probe used (Clark et al. 1999b; Rouhbakhsh and Baumann 1995). The S-endosymbiont of *Tetraneura radicicola* hybridized with the probe to the β-subdivision but not to the γ-subdivision, suggesting that this endosymbiont is a member of the former group. Since there is only a single nucleotide difference between these two probes, a confirmation of this conclusion by sequencing the rDNA from this organism seems desirable. The S-endosymbiont from two other aphid species did not hybridize with either the γ- or β-subdivision probe. In view of the technical difficulties encountered with some of the specimens, these conclusions cannot be interpreted as indicating that the S-endosymbionts of these aphids belong to different bacterial groups.

### Absence of a Stable Bacterial Flora in Aphid Guts

Aphids maintained under clean conditions do not appear to have a bacterial gut flora (Douglas 1990; Grenier et al. 1994; Harada and Ishikawa 1993). Older aphids as well as aphids reared under crowded conditions may acquire a gut flora which appears to consist of members of the Enterobacteriaceae (*Serratia*, *Erwinia*), *Pseudomonas*, *Staphylococcus*, and *Bacillus* (Grenier et al. 1994; Harada and Ishikawa 1993). These organisms are frequently associated with plant surfaces. None of these organisms has a close relationship to *Buchnera*, precluding a recent common ancestor from which they and *Buchnera* are descended. The presence of this bacterial flora has a deleterious effect on aphid performance (Grenier et al. 1994). The actual bacterial numbers have not been established. Harada et al. (1996) have isolated 38 bacterial strains from the guts of 20 aphids. This hardly suggests the presence of an indigenous bacterial flora and the numbers are insignificant compared to the numbers of *Buchnera* or the S-endosymbionts. These studies indicate that the guts of aphids are generally sterile, but under certain conditions, a transient bacterial gut flora may be present.

## Endosymbionts of Other Plant Sap-Utilizing Insects

Psyllids, whiteflies and mealybugs are three separate lineages of the suborder Sternorrhyncha and contain P-endosymbionts from three distinct bacterial groups (*Table 19.1*, *Fig. 19.1*). These insects have obligatory sexual reproduction with the young hatching from eggs (Borror et al. 1989). The endosymbionts are housed within bacteriocytes, and at least during some stage of the insect's life cycle, the bacteriocytes are associated with the ovarioles, resulting in the transmission of endosymbionts to the eggs (Buchner 1965).

### Psyllid Endosymbionts

Fukatsu and Nikoh (1998) sequenced the 16S rDNA of endosymbionts from *Anomoneura mori*, while Spaulding and von Dohlen (1998) performed a similar study of the endosymbionts of *Blastopsylla occidentalis*, *Pachypsylla venusta*, and *Trioza magnoliae*. These authors came to the same conclusion, namely, that the P-endosymbionts of psyllids constitute a distinct lineage within the γ-subdivision of the Proteobacteria (*Fig. 19.1*). These endosymbionts have an unusual property, namely, the lowest known G + C content of any 16S rDNA (36.4 mol%). In addition, this lineage appeared to have a substantial acceleration of the rate of evolutionary change within the 16S sequence. *A. mori*, *B. occidentalis*, and *T. magnoliae* all had different S-endosymbionts, which belong in the γ-subdivision. Only the P-endosymbiont was found in *P. venusta* (Spaulding and von Dohlen 1998). The studies were limited to few taxa but were consistent with cospeciation of the P-endosymbiont and the psyllid host and multiple acquisitions of S-endosymbionts (Fukatsu and Nikoh 1998; Spaulding and von Dohlen 1998).

Psyllids contain bilobed bacteriomes made up of round uninucleate bacteriocytes and a multinucleate syncytial region (Buchner 1965). Many psyllids have endosymbionts in both the bacteriocytes and the syncytium; some have endosymbionts only within the bacteriocytes (Buchner 1965). Using electron microscopy, Chang and Musgrave (1969) and Waku and Endo (1987) found that two psyllid species have endosymbionts in the bacteriocytes which are distinguishable from those found in the syncytium and that both endosymbiont types have a Gram-negative cell wall. In general, the bacteriocyte-associated endosymbionts are more numerous than the syncytium-associated endosymbionts (Buchner 1965). Fukatsu and Nikoh (1998), using an oligonucleotide probe specific for the P- or S-endosymbiont, showed by means of in situ hybridization that the former was localized in the bacteriocytes while the latter was in the syncytium (*Fig. 19.10*). There is currently no information on the requirement of the endosymbiont(s) by the psyllid host or on their function. The similarity in diet between psyllids and aphids raises the possibility that psyllid endosymbionts may provide nutrients as do *Buchnera*.

### Whitefly Endosymbionts

16S rDNA sequences have been obtained for endosymbionts of *Bemisia tabaci*, *B. argentifolii* (previously *B. tabaci* B biotype), *Siphoninus phillyreae*, and *Trialeurodes vaporariorum*

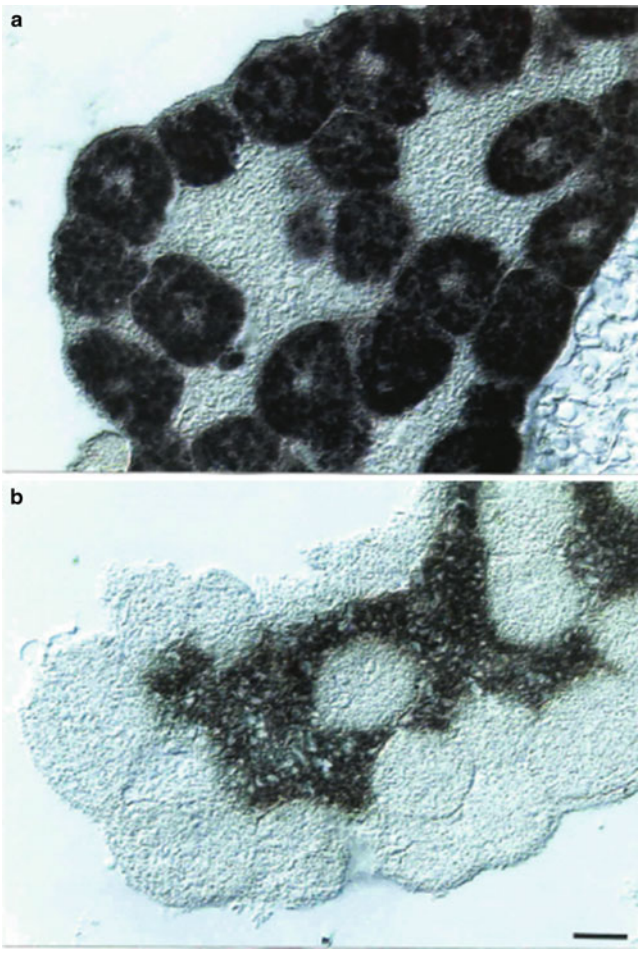


**Fig. 19.10**
**Light micrographs of a bacteriome of the psyllid *Anomoneura mori*. (a) In situ hybridization using an oligonucleotide probe specific for the P-endosymbiont 16S rRNA, which reacts with bacteriocytes containing these endosymbionts. (b) In situ hybridization using an oligonucleotide probe specific for the 16S rRNA, of the S-endosymbiont of this psyllid, which reacts with the endosymbiont located in the syncytium. Bar = 20 μm (From Fukatsu and Nikoh (1998) with permission from the authors and ASM Press)**

(Clark et al. 1992). The P-endosymbionts of these insects are a lineage within the γ-subdivision of the Proteobacteria (Fig. 19.1). *B. tabaci* and *B. argentifolii* have an S-endosymbiont which is a member of the Enterobacteriaceae. The P-endosymbionts and the S-endosymbionts from these two species have identical 16S rDNA sequences, consistent with their close relationship (Brown et al. 1995; Clark et al. 1992).

The ultrastructure of the endosymbionts of *B. tabaci*, *B. argentifolii*, and *T. vaporariorum* has been studied by electron microcopy (Costa et al. 1993b, 1995). There is evidence for at least two morphological types. Whiteflies are unusual in that they transmit an entire bacteriocyte containing endosymbionts to the egg (Buchner 1965; Costa et al. 1996). Some prokaryote-specific antibiotics affect the growth and development of whiteflies, indicating a requirement for the endosymbiont(s) by the host (Costa et al. 1993a, 1997).

## Mealybug Endosymbionts

16S rDNA sequences have been obtained for endosymbionts of *Pseudococcus longispinus*, *P. maritimus*, and *Dysmicoccus neobrevipes* (Munson et al. 1992), and these organisms were found to be a distinct lineage within the β-subdivision of the Proteobacteria (Fig. 19.1). The morphology of endosymbionts from several mealybug species has been studied by means of electron microscopy (Tremblay 1989). Within the bacteriocytes the endosymbionts appear to be embedded in mucous spherules of unknown composition. There is no information on the function or the requirement for the endosymbionts by the host.

## Tsetse Fly Endosymbionts

Tsetse flies (genus *Glossina*) are important vectors of trypanosomes, which are causative agents of African sleeping sickness and various diseases of animals (Harwood and James 1979). They have a somewhat unusual reproductive cycle in that the female gives birth to fully grown larvae. Only one larva is carried at a time within the uterus. During this stage the larva is fed nutritive fluids from special glands, commonly known as "milk glands." The female requires several blood meals to complete the development period of each larva, and it is these blood meals which result in the transmission of trypanosomes (Harwood and James 1979).

Tsetse may be associated with three prokaryotes: (1) *Wigglesworthia* (P-endosymbionts), (2) *Sodalis* (S-endosymbionts), and (3) *Wolbachia*. The last are parasites found in reproductive tissue and causing reproductive disorders (O'Neill et al. 1997) and will not be considered here.

## *Wigglesworthia*: The Primary Endosymbiont of Tsetse Flies

### Phylogeny

Based on 16S rDNA, *Wigglesworthia* was found to constitute a distinct lineage within the γ-3 subgroup of the Proteobacteria (Fig. 19.1; Aksoy et al. 1995; Chen et al. 1999). These organisms are related to but distinct from *Buchnera* of aphids and the P-endosymbionts of carpenter ants (Fig. 19.1). Using the host rDNA transcribed spacer-2, it was found that the phylogeny of the host was the same as that of *Wigglesworthia*, indicating cospeciation of the host and the endosymbiont (Chen et al. 1999). These results suggest a single infection of a tsetse ancestor with a bacterium followed by long-term vertical transmission of the endosymbiont, that is, a lack of exchange of *Wigglesworthia* between different tsetse fly species. The age of this association has been estimated to be at least 50 million years

**Table 19.8**
**Species of tsetse (*Glossina*) for which the 16S rDNA sequence of *Wigglesworthia* has been determined**

| |
|---|
| *G. austeni* |
| *G. brevipalpis* |
| *G. fuscipes* |
| *G. morsitans centralis* |
| *G. m. morsitans* |
| *G. palpalis gambiensis* |
| *G. p. palpalis* |
| *G. tachinoides* |

Chen et al. (1999)

(Aksoy et al. 1997). A list of the species of tsetse flies for which the 16S rDNA of *Wigglesworthia* has been sequenced is presented in *Table 19.8*.

#### Taxonomy

The genus *Wigglesworthia* contains one species, *W. glossinidia*, which designates the lineage consisting of the P-endosymbionts of tsetse flies (Aksoy 1995b). The type strain of this species is the P-endosymbiont of *G. morsitans morsitans*.

#### Habitat

Tsetse flies contain a U-shaped bacteriome located in the anterior region of the gut, which is made up of bacteriocytes containing *Wigglesworthia* (Aksoy 1995b; Aksoy et al. 1995). These endosymbionts have a Gram-negative cell wall and are somewhat pleomorphic, occurring mostly as 4–5-m-long rods. They are found free (not enclosed within host-derived vesicles) in the bacteriocyte cytoplasm. *Wigglesworthia* is maternally transmitted. Since neither the milk gland nor the developing eggs contain *Wigglesworthia*, the mechanism of their transmission is not known (Aksoy et al. 1997).

#### Physiology

The feeding of tsetse flies on animals immunized with *Wigglesworthia* results in elimination of the P-endosymbiont and sterility of the flies (Nogge 1976). A similar effect is observed upon treatment of tsetse with prokaryote-specific antibiotics (Aksoy et al. 1995; Nogge 1976, 1982). These results indicate that the P-endosymbiont is essential for reproduction. There is evidence that one of the functions of the *Wigglesworthia* is the production of B-complex vitamins (Nogge 1982). *Wigglesworthia* produces a high level of GroEL (Aksoy 1995a). In this respect, it is similar to a number of other endosymbionts as well as other intracellular organisms (Hogenhout et al. 1998).

#### Genetics

The *Wigglesworthia* genome has one copy of the 16S rRNA gene (Aksoy 1995a). In this organism, 16S rRNA gene is directly upstream of 23S rRNA gene, suggesting that, as in the case of many other bacteria, these genes are a part of a single transcription unit. The presence of one copy of the rRNA operon is characteristic of slow-growing bacteria and also is found in several other endosymbionts (Baumann et al. 1995).

### Sodalis: The Secondary Endosymbiont of Tsetse Flies

Tsetse flies may also contain S-endosymbionts. These are primarily found within midgut cells but also have been detected in hemolymph and in a variety of other tissues excluding ovaries (Aksoy et al. 1997; Beard et al. 1993b; Cheng and Aksoy 1999). Their numbers are age-dependent, being higher in older insects (Cheng and Aksoy 1999). The 16S rDNA has been sequenced from the S-endosymbionts of five different tsetse fly species, and it was found that they are members of the Enterobacteriaceae (Aksoy et al. 1997; Beard et al. 1993b). The sequences were found to be virtually identical, indicating either multiple recent infections with the same organism or horizontal transmission of the S-endosymbiont. The S-endosymbiont is maternally transmitted via the "milk gland" secretions to developing larvae (Aksoy et al. 1997).

The S-endosymbionts have been cultivated in cell-free liquid media (Beard et al. 1993b) and recently on solid media (Dale and Maudlin 1999). The latter allowed a phenotypic characterization of this organism and led to its assignment into a new genus and species, *Sodalis glossinidius* (Dale and Maudlin 1999). This species consists of Gram-negative rods 1–1.5 m in diameter and 2–12 m in length. It is microaerophilic, lacking catalase, and has a relatively limited capacity for carbohydrate utilization.

The S-endosymbiont has seven copies of 16S rDNA, a number which is similar to that found in rapidly growing free-living organisms (Aksoy 1995a). Plasmids of 80 kb and about 130 kb have been detected in these organisms (Beard et al. 1993b). The S-endosymbiont has been transformed with pSUP204, and plasmid-encoded resistance to ampicillin, tetracycline, and chloramphenicol was expressed (Beard et al. 1993b). Similarly the S-endosymbiont has been transformed with a pSUP204 derivative, which expressed the green fluorescent protein, allowing ready visualization of this organism in insect tissues (Cheng and Aksoy 1999).

In one case, the S-endosymbiont from one tsetse fly species when microinjected into another species became pathogenic, killing the flies within 48 h (Cheng and Aksoy 1999). This result is similar to the observations made with the S-endosymbiont of aphids (Chen and Purcell 1997). The variation in the number of S-endosymbionts and their possible absence from some insects suggest that they do not perform a function essential for the survival of tsetse flies.

## Sitophilus (Weevils) Endosymbionts

Weevils of the genus *Sitophilus* are major pests of stored grain (Borror et al. 1989). The female bores a hole in kernels and deposits an egg. The larva develops inside the grain from

which the young adults emerge. Three related species have been studied with respect to their endosymbionts, *S. oryzae*, *S. granarius*, and *S. zeamais* (Dasch et al. 1984). Of these three species, the most extensive studies deal with *S. oryzae* (Nardon and Grenier 1988). In addition, weevils may harbor the pathogen, *Wolbachia* (O'Neill et al. 1997).

### Phylogeny

Early studies of *Sitophilus*-endosymbiont morphology and the G + C content of its DNA suggested that weevils have different endosymbionts (Dasch 1975; Dasch et al. 1984; Grinyer and Musgrave 1966; Musgrave and Grinyer 1968). *S. oryzae* has one endosymbiont with a G + C content of about 54 mol%, *S. granarius* has one with a G + C content of 50 mol%, while *S. zeamais* appears to have both endosymbionts (Dasch et al. 1984; Heddi et al. 1998). One type of endosymbiont 16S rDNA sequence was detected in *S. oryzae*, and two types were detected in *S. zeamais* (Campbell et al. 1992; Heddi et al. 1998). Phylogenetic analysis indicated that all of these endosymbionts are members of the family Enterobacteriaceae (*Fig. 19.1*).

### Habitat

In larvae, endosymbionts are present in bacteriocytes which make up a bacteriome located at the junction of the foregut and the midgut as well as in the rudimentary ovaries (Charles et al. 1995; Nardon and Grenier 1988). The endosymbionts are transmitted via the eggs. *S. oryzae* endosymbionts are rod-shaped, 5–15 m long, and free (not within host-derived vesicles) in the cytoplasm (Dasch et al. 1984; Nardon and Grenier 1988).

### Physiology

*S. oryzae* may be cured of endosymbionts by treatment with heat or antibiotics (Baker and Lum 1973; Nardon and Grenier 1988). Such aposymbiotic weevils are softer and paler, have an increased development time, and the fertility of their eggs is reduced (Nardon and Grenier 1988). On some nutrient-rich grains, they can grow indefinitely. Aposymbiotic weevils lack bacteriomes, indicating that the endosymbiont triggers their development. Naturally occurring aposymbiotic weevils also may be found. There is evidence that one of the functions of the endosymbiont is the synthesis of vitamins as well as possibly phenylalanine or tyrosine (Baker 1975, 1979; Wicker and Nardon 1982). An additional function is the conversion of excess methionine in the diet to methionine sulfoxide (Gasnier-Fauchet and Nardon 1986). Aposymbiotic weevils also have mitochondria with reduced levels of enzymes involved in respiration (Heddi et al. 1991). Isolated endosymbionts of *S. oryzae* do not consume oxygen and lack a number of enzymes of respiratory metabolism (Heddi et al. 1991, 1993). These results suggest that they have an anaerobic metabolism. As in the case of *Buchnera* and *Wigglesworthia*, the endosymbionts of *S. oryzae* overproduce GroEL (Charles et al. 1997b). Unlike *Buchnera*, the endosymbionts of *S. oryzae* have a heat shock response, as is indicated by an increase of *groEL* mRNA (Charles et al. 1997b).

### Genetics

The genome of the endosymbiont of *S. oryzae* is 3.0 Mb and has two copies of the rRNA operon (Charles et al. 1997a). In addition, the endosymbiont contains a plasmid of about 138 kb. This genome size puts the endosymbiont within the range of many free-living bacteria (Heddi et al. 1998).

## Comparisons with Other Associations

Although the information is somewhat limited, comparison of the *Sitophilus*-endosymbiont association with other insect-endosymbiont associations suggests that the former has some unique features. In several insect endosymbiotic associations, the S-endosymbionts are members of the Enterobacteriaceae, suggesting that organisms within this lineage have an enhanced capacity to enter into such associations. An interesting feature of the *Sitophilus* association is that these organisms are the sole endosymbionts. The morphological diversity of the endosymbionts, the large endosymbiont genome size, and the fact that aposymbiotic weevils are viable suggest that the associations arose through multiple recent infections and that major adaptations resulting in obligatory mutual interdependence of both partners have not evolved as yet. This association may consequently be an example of an endosymbiosis at an early stage of its development.

## Carpenter Ant Endosymbionts

Ants feed on complex diets, and the presence of endosymbionts has been reported in only two groups. These two groups are the genus *Formica* and the genus *Camponotus*, commonly known as the carpenter ants (Borror et al. 1989; Buchner 1965; Dasch et al. 1984). Both groups can use a broad range of food types but typically utilize plant nectar and honeydew (the liquid feces of sap-feeding Homoptera) as major components of their diet. Only in the carpenter ants have symbionts been studied using modern methods, and we focus on these. The contribution of ant endosymbionts to host nutrition is not clear (Dasch et al. 1984).

### Phylogeny

The G + C content of the DNA of carpenter ants is 30–32 mol% (Dasch 1975; Dasch et al. 1984). The sequence of the 16S rDNA has been determined for endosymbionts of the species *Camponotus floridanus*, *C. rufipes*, *C. ligniperdus*, and

*C. herculeanus* (Schröder et al. 1996). A phylogenetic analysis indicated that the endosymbionts constitute a distinct, monophyletic group related to, but different from, the endosymbionts of aphids, tsetse flies, and the members of the Enterobacteriaceae (*Fig. 19.1*). The order of branching reflects the relationships between the carpenter ant species and is consistent with a single infection and subsequent vertical evolution of the endosymbionts. The age of the association is estimated at over 100 million years (Schröder et al. 1996). In endosymbionts of carpenter ants, the 16S rRNA gene is not directly upstream of the 23S rRNA gene, suggesting that these genes are organized in two transcription units, as is the case in *Buchnera* (C. Sauer and R. Gross, personal communication).

### Habitat

In both workers and queens of *Camponotus*, the endosymbionts are located in bacteriocytes, which are intercalated between epithelial cells of the midgut (*Fig. 19.11a*; Buchner 1965; Schröder et al. 1996). The endosymbionts are rods of 1 µm in width to 5–15 µm in length (*Fig. 19.11b*). They have a Gram-negative type cell wall and are free (not enclosed in host-derived vesicles) in the cytoplasm (Schröder et al. 1996). Transmission is via infection of the ovaries and incorporation into the eggs.

## *Blattabacterium*-Endosymbionts of Cockroaches and Termites

Cockroaches (order Blattaria) utilize a complex diet and harbor prokaryotic endosymbionts (Dasch et al. 1984). It has been hypothesized that cockroaches and termites (order Isoptera) are phylogenetically related (Kambhampati 1995). Common ancestry is suggested from the fact that the wood-eating cockroach, *Cryptocercus punctulatus*, has a cellulose-digesting protozoal gut flora which is similar to that of termites (reviewed in Bandi and Sacchi 1999). In addition, *Mastotermes darwiniensis*, a primitive termite, lays eggs in rows resembling those made by cockroaches (Borror et al. 1989; Sacchi et al. 1998b).

### Phylogeny

The G + C content of the DNA of *Blattabacterium* is 26–28 mol% (Dasch 1975; Dasch et al. 1984). Phylogenetic analysis of the 16S rDNA from *Blattabacterium* of cockroaches and the termite *M. darwiniensis* indicates that the endosymbionts form a distinct lineage within the *Flavobacterium-Bacteroides* group of bacteria (Bandi et al. 1994, 1995; *Fig. 19.1*). The phylogenetic tree obtained on the basis of endosymbiont 16S rDNA is the same as that derived from host taxonomy. This result is consistent with a single infection in an ancestor of cockroaches and termites and vertical evolution of the endosymbionts, that is, a lack of endosymbiont exchange among different species. *M. darwiniensis* is the only termite known to harbor

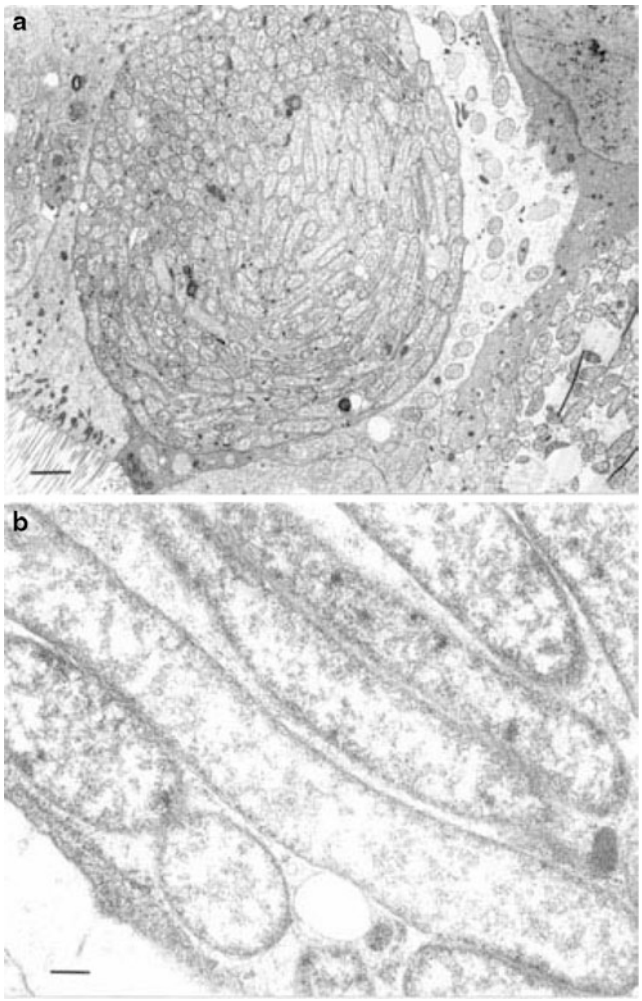


**Fig. 19.11**
**Electron micrographs of the endosymbionts of the carpenter ant *Camponotus floridanus*. (a) Bacteriocyte containing the endosymbionts, bar = 3.0 µm; (b) ultrastructure of the endosymbionts showing the Gram-negative cell wall and the absence of a vesicular membrane, bar = 0.3 µm (Photos courtesy of C. Sauer and R. Gross)**

*Blattabacterium*, suggesting that, with the exception of this termite, the endosymbionts were eliminated in the lineage leading to the present termite species (Bandi and Sacchi 1999; Bandi et al. 1997). The 16S rDNA sequence has been determined for endosymbionts of the cockroach species *Periplaneta australasiae*, *P. americana*, *Blatella germanica*, *Pycnoscelus surinamensis*, *Nauphoeta cinerea*, and *C. punctulatus*, as well as the termite species *M. darwiniensis* (Bandi et al. 1994, 1995). The association between *Blattabacterium* and termites is estimated to be 135–300 million years old (Bandi et al. 1995).

### Taxonomy

The genus *Blattabacterium* contains one species, *B. cuenoti*, and currently designates the lineage consisting of the

bacteriocyte-associated endosymbionts of cockroaches and one termite (Bandi et al. 1995; Dasch et al. 1984). The type strain is the endosymbiont of *Blatta orientalis* (Dasch et al. 1984).

## Habitat

*Blattabacterium* is found in bacteriocytes, polyploid cells which are found within the abdominal fat bodies of cockroaches and the termite *M. darwiniensis* (Fig. 19.12a, Bigliardi et al. 1995; Sacchi et al. 1996, 1998a, b). The endosymbionts are rods of 1 μm in width and 1.6–9 μm in length (Fig. 19.12b; Dasch et al. 1984). They have a Gram-negative cell wall and are located within vesicles derived from the host cell. The bacteriocytes originate from plasmatocytes, which are phagocytic cells present in the hemolymph. There are extensive electron microscopic studies that document the infection and differentiation of plasmatocytes into bacteriocytes and the transmission of the endosymbionts to oocytes and eggs (Bigliardi et al. 1995; Sacchi et al. 1996, 1998a, b).

## Physiology

Aposymbiotic cockroaches can be obtained by rearing the insects on antibiotic-containing foods (Bandi and Sacchi 1999; Dasch et al. 1984). The resulting insects can be propagated on enriched diets. The aposymbiotic insects are smaller in size, light in color, and have a reduced fecundity and an increased development time. They also have an increased level of uric acid in fat bodies, suggesting that one function of *Blattabacterium* is nitrogen recycling (Cochoran 1985). The proximity of bacteriocytes and urate cells (which store uric acid) in the fat body and the presence of adhesion sites between their plasma membranes suggest direct metabolic interactions between these cells (Sacchi et al. 1998a). There is also evidence that the bacteria provide essential amino acids for the host (Henry 1962).

## Isolation

P-endosymbionts have been isolated from aphids and also from *Sitophilus*. In the case of aphids, the starting material is usually whole insects; in the case of *Sitophilus*, the starting material is dissected bacteriomes. Since the two methods are similar, only the method for the isolation of *Buchnera* from aphids will be considered. Details of the isolation of the endosymbionts from *Sitophilus* are described by Heddi et al. (1991).

The methods for the isolation of *Buchnera* have been developed by Ishikawa (1982), Sasaki and Ishikawa (1995), and Charles and Ishikawa (1999). The resulting preparations are suitable for isolation of high molecular weight DNA for genome analysis as well as for physiological studies. The best criteria of purity have involved examination of the preparations by electron microscopy, which also allows determination of whether the endosymbionts are still within host-derived vesicles. Both the aphids and the endosymbionts have a similar mol% G + C in their DNA (Ishikawa 1987; Unterman and Baumann 1990). Consequently, endosymbiont DNA cannot be separated from host DNA by CsCl density gradient centrifugation.

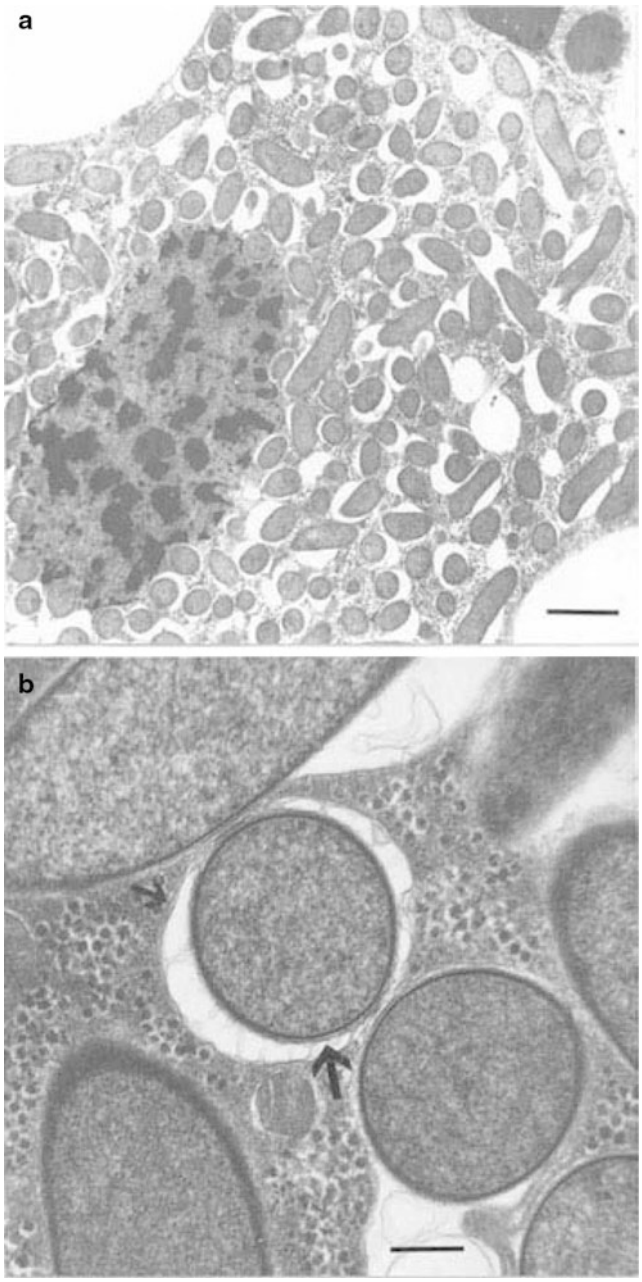


**Fig. 19.12**
**Electron micrographs of *Blattabacterium cuenoti*, the endosymbiont of cockroaches and termites. (a) Endosymbiont within bacteriocytes of the cockroach *Periplaneta americana*, bar = 3.0 μm (Photo courtesy of L. Sacchi); (b) ultrastructure of the endosymbiont of *Cryptocercus punctulatus* showing the Gram-negative cell wall (*large arrow*) and the vesicular membrane (*small arrow*), bar = 0.3 μm (From Sacchi et al. (1998) with permission of the authors and Balaban Publishers)**

As starting material, it is best to use an aphid strain that has only *Buchnera* and lacks S-endosymbionts. All of the reagents and equipment are kept on ice, and the procedures are performed as rapidly as possible. Approximately 2–3 g (wet weight) aphids are transferred to a 1.5-cm-diameter tissue grinder. Ten milliliters of buffer A of Ishikawa (1982) is added, and the aphids are ground with a loose fitting plunger for 5 min. [Buffer A contains 0.25 M sucrose, 35 mM Tris–HCl (pH 7.6), 25 mM KCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol.] The preparation is then passed through a double layer of a nylon mesh to remove large particulate material. The filtrate is brought to a volume of about 100 mL with buffer A and then quickly passed through a 100-µm nylon filter followed by filtration through 20- and 10-µm nylon filters (in some cases filtration through 5- and 3-µm filters is also performed). Only slight vacuum pressure is applied during the last two filtration steps. The suspension is centrifuged in a swinging bucket rotor for 6–10 min at 1,500 g. The pellets are gently resuspended in 1–2 mL buffer A and centrifuged through a Percoll gradient (12,000 g, 15 min). The gradient consists of 27–70 % Percoll in buffer A, 5 % PEG 6000, 1 % Ficoll, and 1 % bovine serum albumin (Pharmacia Biotech, Uppsala, Sweden; Charles and Ishikawa 1999). Mitochondria are in the upper phase, host nuclei are in the pellet, and *Buchnera* appears as a green band.

## Identification

Currently none of the P-endosymbionts has been cultured, and consequently identification is based primarily on sequence analysis of their 16S rDNAs. Since these studies are just beginning, we will briefly discuss the methods used for both their characterization and identification. The success of the approach used in most of the studies has been dependent on the use of fresh or frozen insect samples which meet the following criteria: (1) the predominant bacterial flora of the insect consists of one or a few endosymbiont types, (2) there is no significant gut flora, and (3) the samples are relatively clean.

An ideal study would utilize the full-cycle rRNA analysis formulated by Amann et al. (1995). In this approach, the 16S rDNA is amplified by PCR and sequenced. Based on comparisons with 16S rDNA(s) in databases, the organism(s) is identified. Specific oligonucleotide probes are designed and used in in situ hybridization to identify the endosymbiont associated with the sequence. This approach is extremely useful when more than one endosymbiont is present and, in the case of insects, has been applied to the identification of two types of psyllid endosymbionts (Fukatsu and Nikoh 1998).

In studies in which the primary goal was a phylogenetic characterization of the P-endosymbiont, one approach used is an initial thorough study of one or more representative insects that can be cultivated or are readily available. Then characterization is extended to taxa obtained as field collections and preserved in dry ice or absolute ethanol. For example, in the initial study of aphid endosymbionts, the insect specimen chosen (Ap) was known from electron microscopy to harbor two morphologically distinct endosymbionts (Unterman et al. 1989). Using total aphid DNA, a probe to *E. coli* 16S rDNA, and restriction enzyme and Southern blot analysis, only two restriction maps could be constructed corresponding to the two endosymbionts. There were differences in the intensities of the bands, indicating that one of the endosymbionts was present in larger numbers than the other. In addition, the results were consistent with the presence of only one copy of the 16S rRNA gene per endosymbiont genome. Based on the restriction maps, DNA fragments containing 16S rRNA genes of both endosymbionts were cloned and sequenced. In addition, DNA was isolated from dissected bacteriomes, and restriction enzyme and Southern blot analysis indicated the association of both endosymbionts with the bacteriome. Subsequently, restriction enzyme and Southern blot analysis using DNA from other aphid species indicated the presence of only one copy of the 16S rRNA gene, a finding consistent with only one or a single predominant endosymbiont (Munson et al. 1991b). Upon amplification of the 16S rDNA by PCR, the sequences detected were all related to *Buchnera*. This approach is suitable for the study of insects in which there is one predominant P-endosymbiont and does not exclude the possibility of not detecting S-endosymbionts that are present in lower numbers.

Instead of initial studies involving restriction enzyme and Southern blot analysis, it is much more convenient to use oligonucleotides complementary to the front and back of 16S rDNA and PCR to amplify DNA fragments for cloning, sequencing, or both. Localization of the endosymbiont may be performed by dissection of different tissues, purification of the DNA, and amplification of the 16S rDNA by PCR (Aksoy et al. 1995; Bandi et al. 1994, 1995; Schröder et al. 1996). Differences in the types of rDNA amplified may be established by restriction fragment polymorphism (Clark et al. 1992; Fukatsu and Nikoh 1998). Once a pattern of relationship is established, this method may be applied using whole insect DNA. It should be noted that there are potential problems associated with PCR such as selective amplification of some sequences and hybrid formation, which are reviewed by von Wintzingerode et al. (1997).

One additional approach to the detection of different kinds of organisms in the DNA samples is to use oligonucleotide primers that are complementary to the front of the 16S rDNA and the middle of the 23S rDNA. In bacteria in which the order of genes is 16S–23S (most species), there is a spacer region between the genes the length of which is labile and usually differs among species. If, after PCR, several bands are observed, they probably correspond to different bacteria. This approach has been applied to the cloning of P-endosymbionts of psyllids and to the putative S-endosymbionts which differ in the size of their 16S–23S spacer (Thao, M. L. and Baumann, P., unpublished observations).

Specific oligonucleotide primers also can be made complementary to unique sequences of the endosymbiont 16S and 23S rDNA. This has been done for *Buchnera* 16S rDNA (Rouhbakhsh et al. 1994). Another approach is to take advantage of unique linkage relationships. *Buchnera*-specific probes have been made which span the region *argS-16SrDNA* and

*aroE-23SrDNA* (Munson et al. 1993; Rouhbakhsh and Baumann 1995; Rouhbakhsh et al. 1994). The latter is especially useful, since most bacteria have 16S rDNA directly upstream of 23S rDNA.

## Application

Aphids, psyllids, whiteflies, and mealybugs, which utilize plant sap as food, are of major economic importance in that they may cause plant debilitation and the transmission of a variety of plant pathogens (Gray and Banerjee 1999; Sylvester 1985). Tsetse flies, which suck blood, are important in the transmission of human and animal disease, especially in tropical regions of Africa (Harwood and James 1979). Since these organisms are dependent on their P-endosymbionts for survival, an understanding of the genetics and physiology of the endosymbionts may be of use in devising strategies, which are based on interference with their endosymbionts, for the control of these insect pests. An additional approach, which is currently being developed, is the potential use of endosymbionts to produce compounds that would result in modification of the vector competence of an insect. Such an insect would be unable to harbor and/or transmit the disease agent. This approach has been discussed by Beard et al. (1993a) and Durvasula et al. (1997).

## References


Akhtar S, van Emden HF (1994) Ultrastructure of the symbionts and mycetocytes of bird cherry aphid (*Rhopalosiphum padi*). Tissue Cell 26:513–522

Aksoy S (1995a) Molecular analysis of the endosymbionts of tsetse flies: 16S rDNA locus and over-expression of a chaperonin. Insect Mol Biol 4:23–29

Aksoy S (1995b) *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidia* sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse flies. Int J Syst Bacteriol 45:848–851

Aksoy S, Pourhosseini AA, Chow A (1995) Mycetome endosymbionts of tsetse flies constitute a distinct lineage related to the Enterobacteriaceae. Insect Mol Biol 4:15–22

Aksoy S, Chen X, Hypsa V (1997) Phylogeny and potential transmission routes of midgut-associated endosymbionts of tsetse (Diptera: Glossinidae). Insect Mol Biol 6:183–190

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Andersson SGE, Zomorodipour A, Andersson JO, Sicheritz-Ponten T, Alsmark UCM, Podowski RM, Naslund AK, Eriksson AS, Winkler HH, Kurland CG (1998) The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. Nature 396:133–140

Baker JE (1975) Vitamin requirements of larvae of *Sitophilus oryzae*. J Insect Physiol 21:1337–1342

Baker JE (1979) Requirements for the essential dietary amino acids of larvae of the rice weevil. Environ Entomol 8:451–453

Baker JE, Lum PTM (1973) Development of aposymbiosis in larvae of *Sitophilus oryzae* by dietary treatment with antibiotics. J Stored Prod Res 9:241–245

Bandi C, Damiani G, Magrassi L, Grigolo A, Fani R, Sacchi L (1994) Flavobacteria as intracellular symbionts in cockroaches. Proc R Soc Lond B Biol Sci 257:43–48

Bandi C, Sironi M, Damiani G, Magrassi L, Nalepa CA, Laudani U, Sacchi L (1995) The establishment of intracellular symbiosis in an ancestor of cockroaches and termites. Proc R Soc Lond B Biol Sci 259:293–299

Bandi C, Sironi M, Nalepa CA, Corona S, Sacchi L (1997) Phylogenetically distant intracellular symbionts in termites. Parassitologia 39:71–75

Bandi C, Anderson TJC, Genchi C, Blaxter ML (1998) Phylogeny of *Wolbachia* in filarial nematodes. Proc R Soc Lond B Biol Sci 265:2407–2414

Bandi C, McCall JW, Genchi C, Corona S, Venco L, Sacchi L (1999) Effects of tetracycline on the filarial worms *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia*. Int J Parasitol 29:357–364

Bandi C, Sacchi L. Intracellular symbiosis. In: Abe T, Higashi M, Bignel D (eds) Termites: their symbiosis, behavior, and global diversification. Kluwer, Dordrecht (in press)

Barancin CE, Smoot JC, Findlay RH, Actis LA (1998) Plasmid-mediated histamine biosynthesis in the bacterial fish pathogen *Vibrio anguillarum*. Plasmid 39:235–244

Baumann L, Baumann P (1994) Growth kinetics of the endosymbiont *Buchnera aphidicola* in the aphid *Schizaphis graminum*. Appl Environ Microbiol 60:3440–3443

Baumann L, Baumann P (1998) Characterization of ftsZ, the cell division gene of *Buchnera aphidicola* (endosymbiont of aphids) and detection of the product. Curr Microbiol 36:85–89

Baumann P, Moran NA (1997) Non-cultivable microorganisms from symbiotic associations of insects and other hosts. Antonie Van Leeuwenhoek 72:39–48

Baumann P, Baumann L, Lai CY, Roubakhsh D, Moran NA, Clark MA (1995) Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. Annu Rev Microbiol 49:55–94

Baumann P, Baumann L, Clark MA (1996) Levels of *Buchnera aphidicola* chaperonin GroEL during growth of the aphid *Schizaphis graminum*. Curr Microbiol 32:279–285

Baumann P, Moran NA, Baumann L (1997a) The evolution and genetics of aphid endosymbionts. Bioscience 47:12–20

Baumann L, Clark MA, Rouhbakhsh D, Baumann P, Moran NA, Voegtlin DJ (1997b) Endosymbionts (Buchnera) of the aphid *Uroleucon sonchi* contain plasmids with trpEG and remnants of trpE pseudogenes. Curr Microbiol 35:18–21

Baumann L, Baumann P, Moran NA (1998a) The endosymbiont (Buchnera) of the aphid *Diuraphis noxia* contains all the genes of the tryptophan biosynthetic pathway. Curr Microbiol 37:58–59

Baumann P, Baumann L, Clark MA, Thao ML (1998b) *Buchnera aphidicola*: the endosymbiont of aphids. ASM News 64:203–209

Baumann L, Baumann P, Thao ML (1999a) Detection of messenger RNA transcribed from genes encoding enzymes of amino acid biosynthesis in *Buchnera aphidicola* (endosymbiont of aphids). Curr Microbiol 38:135–136

Baumann L, Baumann P, Moran MA, Sandström J, Thao ML (1999b) Genetic characterization of plasmids containing genes encoding enzymes of leucine biosynthesis in endosymbionts (Buchnera) of aphids. J Mol Evol 48:77–85

Beard CB, O'Neill SL, Tesh RB, Richards FF, Aksoy S (1993a) Modification of arthropod vector competence via symbiotic bacteria. Parasitol Today 9:179–183

Beard CB, O'Neill SK, Mason P, Mandelco L, Woese CR, Tesh RB, Richards FF, Aksoy S (1993b) Genetic transformation and phylogeny of bacterial symbionts from tsetse. Insect Mol Biol 1:123–131

Bensaadi-Merchermek N, Salvado JC, Cagnon C, Karama S, Mouches C (1995) Characterization of the unlinked 16S rDNA and 23S-5S rRNA operon of Wolbachia pipientis, a prokaryotic parasite of insect gonads. Gene 165:81–86

Berlyn MKB (1998) Linkage map of Escherichia coli K-12, edition 10: the traditional map. Microbiol Mol Biol Rev 62:814–984

Bigliardi E, Selmi MG, Corona S, Bandi CA, Sacchi L (1995) Membrane systems in endocytobiosis. III. Ultrastructural features of symbionts and vacuolar membrane in bacteriocytes of the wood-eating cockroach Cryptocercus punctulatus (Dictyoptera, Cryptocercidae). Boll Zool 62:235–238

Blackman RL (1984) Reproduction, cytogenetics and development. In: Minks AK, Harrewijn P (eds) Aphids: their biology, natural enemies and control, vol 2A. Elsevier Biomedical Press, Amsterdam, pp 163–195

Blackman RL, Eastop VF (1984) Aphids on the world's crops. Wiley, Chichester, UK

Blattner FR, Plunkett G, Bloch CA, Perna NT, Burland V, Riley M, ColladoVides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997) The complete genome sequence of Escherichia coli K-12. Science 277:1453–1462

Blua MJ, Perring TM, Madore MA (1994) Plant virus-induced changes in aphid population development and temporal fluctuations in plant nutrients. J Chem Ecol 20:691–707

Boman HG, Hultmark D (1987) Cell-free immunity in insects. Annu Rev Microbiol 41:103–126

Borror DJ, Triplehorn CA, Johnson NF (1989) An introduction to the study of insects. Harcourt Brace College, Fort Worth

Bracho AM, Martínez-Torres D, Moya A, Latorre A (1995) Discovery and molecular characterization of a plasmid localized in Buchnera sp., bacterial endosymbiont of the aphid Rhopalosiphum padi. J Mol Evol 41:67–73

Brenner DJ (1984) Enterobacteriaceae. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 1. Williams and Wilkins, Baltimore, pp 408–506

Brough CN, Dixon AFG (1990) Ultrastructural features of egg development in oviparae of vetch aphid, Megoura viciae. Tissue Cell 22:51–63

Brown JK, Frohlich DR, Rosell RC (1995) The sweetpotato or silverleaf whiteflies—biotypes of Bemisia tabaci or a species complex. Annu Rev Entomol 40:511–534

Brynnel EU, Kurland CG, Moran NA, Andersson SGE (1998) Evolutionary rates for tuf genes in endosymbionts of aphids. Mol Biol Evol 15:574–582

Buchner P (1965) Endosymbiosis of animals with plant microorganisms. Interscience, New York

Campbell BC, Bragg TS, Turner CE (1992) Phylogeny of symbiotic bacteria of four weevil species (Coleoptera, Curculionidae) based on analysis of 16S ribosomal DNA. Insect Biochem Mol Biol 22:415–421

Campbell BC, Stefen-Campbell JD, Gill RJ (1994) Evolutionary origin of whiteflies (Hemiptera: Sternorrhyncha: Aleyrodidae) inferred from 18S rDNA sequences. Insect Mol Biol 3:73–88

Chang KP, Musgrave AJ (1969) Histochemistry and ultrastructure of the mycetome and its "symbiotes" in the pear psylla, Psylla pyricola Foerster (Homoptera). Tissue Cell 1:597–606

Charles H, Ishikawa H (1999) Physical and genetic map of the genome of Buchnera, the primary endosymbiont of the pea aphid Acyrthosiphon pisum. J Mol Evol 48:142–150

Charles H, Ishikawa H, Nardon P (1995) Presence of a protein specific of endocytobiosis (symbionin) in the weevil Sitophilus. C R Acad Sci Paris Ser III 318:35–41

Charles H, Condemine G, Nardon C, Nardon P (1997a) Genome size characterization of the principal endocellular symbiotic bacteria of the weevil Sitophilus oryzae, using pulsed field gel electrophoresis. Insect Biochem Mol Biol 27:345–350

Charles H, Heddi A, Guillaud J, Nardon C, Nardon P (1997b) A molecular aspect of symbiotic interactions between the weevil Sitophilus oryzae and its endosymbiotic bacteria: over-expression of a chaperonin. Biochem Biophys Res Commun 239:769–774

Chen D-Q, Purcell AH (1997) Occurrence and transmission of facultative endosymbionts in aphids. Curr Microbiol 34:220–225

Chen DQ, Campbell BC, Purcell AH (1996) A new rickettsia from a herbivorous insect, the pea aphid Acyrthosiphon pisum (Harris). Curr Microbiol 33:123–128

Chen XA, Li S, Aksoy S (1999) Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus Glossina and its bacteriome-associated endosymbiont, Wigglesworthia glossinidia. J Mol Evol 48:49–58

Cheng Q, Aksoy S (1999) Tissue tropism, transmission and expression of foreign genes in vivo in midgut symbionts of tsetse flies. Insect Mol Biol 8:125–132

Clark MA, Baumann L, Munson MA, Baumann P, Campbell BC, Duffus JE, Osborne LS, Moran NA (1992) The eubacterial endosymbionts of whiteflies (Homoptera, Aleyrodoidea) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. Curr Microbiol 25:119–123

Clark MA, Baumann L, Baumann P, Rouhbakhsh D (1996) Ribosomal protein S1(RpsA) of *Buchnera aphidicola*, the endosymbiont of aphids: characterization of the gene and detection of the product. Curr Microbiol 32:89–94

Clark MA, Baumann L, Baumann P (1998a) Sequence analysis of a 34.7-kb DNA segment from the genome of Buchnera aphidicola (endosymbiont of aphids) containing groEL, dnaA, the atp operon, gidA, and rho. Curr Microbiol 36:158–163

Clark MA, Baumann L, Baumann P (1998b) Buchnera aphidicola (aphid endosymbiont) contains genes encoding enzymes of histidine biosynthesis. Curr Microbiol 37:356–358

Clark MA, Baumann P, Moran MA (1999a) Buchnera plasmid-associated trpEG probably originated from a chromosomal location between hslU and fpr. Curr Microbiol 38:309–311

Clark MA, Moran NA, Baumann P (1999b) Sequence evolution in bacterial endosymbionts having extreme base compositions. Mol Biol Evol 16:1586–1598

Clark MA, Moran NA, Baumann P (2000) Cospeciation between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (Uroleucon) and pitfalls of testing for phylogenetic congruence. Evolution 54(2):517–525

Cochoran DG (1985) Nitrogen excretion in cockroaches. Annu Rev Entomol 30:29–49

Costa HS, Ullman DE, Johnson MW, Tabashnik BE (1993a) Antibiotic oxytetracycline interferes with *Bemisia tabaci* (Homoptera, Aleyrodidae) oviposition, development, and ability to induce squash silverleaf. Ann Entomol Soc Am 86:740–748

Costa HS, Westcot DM, Ullman DE, Johnson MW (1993b) Ultrastructure of the endosymbionts of the whitefly, *Bemisia tabaci* and *Trialeurodes vaporariorum*. Protoplasma 176:106–115

Costa HS, Westcot DM, Ullman DE, Rosell R, Brown JK, Johnson MW (1995) Morphological variation in *Bemisia* endosymbionts. Protoplasma 189:194–202

Costa HS, Toscano NC, Henneberry TJ (1996) Mycetocyte inclusion in the oocytes of *Bemisia argentifolii* (Homoptera, Aleyrodidae). Ann Entomol Soc Am 89:694–699

Costa HS, Henneberry TJ, Toscano NC (1997) Effects of antibacterial materials on *Bemisia argentifolii* (Homoptera: Aleyrodidae) oviposition, growth, survival, and sex ratio. J Econ Entomol 90:333–339

Crawford IP (1989) Evolution of a biosynthetic pathway—the tryptophan paradigm. Annu Rev Microbiol 43:567–600

Dadd RH (1985) Nutrition: organisms. In: Kerkut GA, Gilbert LI (eds) Comprehensive insect physiology, biochemistry, and pharmacology, vol 4. Pergamon, Elmsford, pp 315–319

Dale C, Maudlin I (1999) *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans* morsitans. Int J Syst Bacteriol 49:267–275

Dasch GA (1975) Morphological and molecular studies on intracellular bacterial symbiotes of insects. Yale University, New Haven

Dasch GA, Weiss E, Chang K-P (1984) Endosymbionts of insects. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 1. Williams and Wilkins, Baltimore, pp 811–833

Denk D, Böck A (1987) L-Cysteine biosynthesis in *Escherichia coli*: nucleotide sequence and expression of the serine acetyltransferase (cysE) gene from wild-type and a cysteine-excreting mutant. J Gen Microbiol 133:515–525

Dixon AFG (1973) Biology of aphids. Edward Arnold, London

Dixon AFG (1992) Constraints on the rate of parthenogenetic reproduction and pest status of aphid. Invertebr Reprod Dev 22:159–163

Douglas AE (1988) Sulfate utilization in an aphid symbiosis. Insect Biochem 18:159–163

Douglas AE (1989) Mycetocyte symbiosis in insects. Biol Rev Camb Philos Soc 64:409–434

Douglas AE (1990) Nutritional interactions between *Myzus persicae* and its symbionts. In: Campbell RK, Eikenbary RD (eds) Aphid-plant genotype interactions. Elsevier Biomedical Press, Amsterdam, pp 319–327

Douglas AE (1997) Parallels and contrasts between symbiotic bacteria and bacterial-derived organelles: evidence from *Buchnera*, the bacterial symbiont of aphids. FEMS Microbiol Ecol 24:1–9

Douglas AE (1998) Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu Rev Entomol 43:17–37

Douglas AE, Dixon AFG (1987) The mycetocyte symbiosis of aphids: variation with age and morph in virginoparae of *Megoura viciae* and *Acyrthosiphon pisum*. J Insect Physiol 33:109–113

Douglas AE, Prosser WA (1992) Synthesis of the essential amino acid tryptophan in the pea aphid (*Acyrthosiphon pisum*) symbiosis. J Insect Physiol 38:565–568

Douglas AE, Smith DC (1989) Are endosymbioses mutualistic? Trends Ecol Evol 4:350–352

Durvasula RV, Gumbs A, Panackal A, Kruglov O, Aksoy S, Merrifield RB, Richards FF, Beard CB (1997) Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. Proc Natl Acad Sci USA 94:3274–3278

Eisen JA (1995) The RecA Protein as a model molecule for molecular systematic studies of bacteria—comparison of trees of RecAs and 16S rRNAs from the same species. J Mol Evol 41:1105–1123

Eremeeva ME, Ching WM, Wu YL, Silverman DJ, Dasch GA (1998) Western blotting analysis of heat shock proteins of Rickettsiales and other eubacteria. FEMS Microbiol Lett 167:229–237

Faye I (1978) Insect immunity: early fate of bacteria injected in a *Saturniid* pupae. J Invertebr Pathol 31:19–26

Filichkin SA, Brumfield S, Filichkin TP, Young MJ (1997) In vitro interactions of the aphid endosymbiotic SymL chaperonin with barley yellow dwarf virus. J Virol 71:569–577

Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM, McKenney K, Sutton G, Fitzhugh W, Fields C, Gocayne JD, Scott J, Shirley R, Liu LI, Glodek A, Kelley JM, Weidman JF, Phillips CA, Spriggs T, Hedblom E, Cotton MD, Utterback TR, Hanna MC, Nguyen DT, Saudek DM, Brandon RC, Fine LD, Fritchman JL, Fuhrmann JL, Geoghagen NSM, Gnehm CL, McDonald LA, Small KV, Fraser CM, Smith HO, Venter JC (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 269:469–512

Forrest JMS (1987) Galling aphids. In: Minks AK, Harrewijn P (eds) Aphids: their biology, natural enemies and control, vol 2A. Elsevier, Amsterdam, pp 341–353

Fraser CM, Gocayne JD, White O, Adams MD, Clayton RA, Fleischmann RD, Bult CJ, Kerlavage AR, Sutton G, Kelley JM, Fritchman JL, Weidman JF, Small KV, Sandusky M, Fuhrmann J, Nguyen D, Utterback TR, Saudek DM, Phillips CA, Merrick JM, Tomb JF, Dougherty BA, Bott KF, Hu PC, Lucier TS, Peterson SN, Smith HO, Hutchison CA, Venter JC (1995) The minimal gene complement of *Mycoplasma genitalium*. Science 270:397–403

Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, White O, Ketchum KA, Dodson R, Hickey EK, Gwinn M, Dougherty B, Tomb JF, Fleischmann RD, Richardson D, Peterson J, Kerlavage AR, Quackenbush J, Salzberg S, Hanson M, vanVugt R, Palmer N, Adams MD, Gocayne J, Weidman J, Utterback T, Watthey L, McDonald L, Artiach P, Bowman C, Garland S, Fujii C, Cotton MD, Horst K, Roberts K, Hatch B, Smith HO, Venter JC (1997) Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature 390:580–586

Fukatsu T, Ishikawa H (1992a) A novel eukaryotic extracellular symbiont in an aphid, *Astegopteryx styraci* (Homoptera, Aphididae, Hormaphidinae). J Insect Physiol 38:765–773

Fukatsu T, Ishikawa H (1992b) Soldier and male of an eusocial aphid *Colophina arma* lack endosymbiont: implications for physiological and evolutionary interaction between host and symbiont. J Insect Physiol 38:1033–1042

Fukatsu T, Ishikawa H (1992c) Synthesis and localization of symbionin, an aphid endosymbiont protein. Insect Biochem Mol Biol 22:167–174

Fukatsu T, Ishikawa H (1993) Occurrence of chaperonin-60 and chaperonin-10 in primary and secondary bacterial symbionts of aphids—implications for the evolution of an endosymbiotic system in aphids. J Mol Evol 36:568–577

Fukatsu T, Ishikawa H (1996) Phylogenetic position of yeast-like symbiont of *Hamiltonaphis styraci* (Homoptera, Aphididae) based on 18S rDNA sequence. Insect Biochem Mol Biol 26:383–388

Fukatsu T, Ishikawa H (1998) Differential immunohistochemical visualization of the primary and secondary intracellular symbiotic bacteria of aphids. Appl Entomol Zool 33:321–326

Fukatsu T, Nikoh N (1998) Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). Appl Environ Microbiol 64:3599–3606

Fukatsu T, Aoki S, Kurosu U, Ishikawa H (1994) Phylogeny of *Cerataphidini aphids* revealed by their symbiotic microorganisms and basic structure of their galls: implications for host-symbiont coevolution and evolution of sterile soldier castes. Zool Sci 11:613–623

Fukatsu T, Watanabe K, Sekiguchi Y (1998) Specific detection of intracellular symbiotic bacteria of aphids by oligonucleotide-probed in situ hybridization. Appl Entomol Zool 33:461–472

Gasnier-Fauchet F, Nardon P (1986) Comparison of methionine metabolism in symbiotic and aposymbiotic larvae of *Sitophilus oryzae* L. (Coleoptera: curculionidae). II. Involvement of the symbiotic bacteria in the oxidation of methionine. Comp Biochem Physiol 85B:251–254

Gasnier-Fauchet F, Gharib A, Nardon P (1986) Comparison of methionine metabolism in symbiotic and aposymbiotic larvae of *Sitophilus oryzae* L. (Coleoptera: Curculionidae) I. Evidence for a glycine *N*-methyltransferase-like activity in the aposymbiotic larvae. Comp Biochem Physiol 85B:245–250

Gray SM, Banerjee N (1999) Mechanisms of arthropod transmission of plant and animal viruses. Microbiol Mol Biol Rev 63:128–148

Grenier AM, Nardon C, Rahbe Y (1994) Observations on the micro-organisms occurring in the gut of the pea aphid *Acyrthosiphon pisum*. Entomol Exp Appl 70:91–96

Griffiths GW, Beck SD (1973) Intracellular symbiotes of the pea aphid, *Acyrthosiphon pisum*. J Insect Physiol 19:75–84

Griffiths GW, Beck SD (1974) Effect of antibiotics on intracellular symbiotes in the pea aphid, *Acyrthosiphon pisum*. Cell Tissue Res 148:287–300

Grinyer I, Musgrave AJ (1966) Ultrastructure and peripheral membranes of the mycetomal microorganism of *Sitophilus granarius* (L. coleoptera). J Cell Sci 1:181–186

Gross CA (1996) Function and regulation of heat shock proteins. In: Neidhard FC (ed) *Escherichia coli* and *Salmonella*, vol 1. ASM Press, Washington, DC, pp 1382–1399

Gross R, Rappuoli R (1988) Positive regulation of pertussis toxin expression. Proc Natl Acad Sci USA 85:3913–3917

Hara E, Ishikawa H (1990) Purification and partial characterization of symbionin, an aphid endosymbiont-specific protein. Insect Biochem 20:421–427

Harada H, Ishikawa H (1993) Gut microbe of aphid closely related to its intracellular symbiont. Biosystems 31:185–191

Harada H, Oyaizu H, Ishikawa H (1996) A consideration about the origin of aphid intracellular symbiont in connection with gut bacterial flora. J Gen Appl Microbiol 42:17–26

Harrison CP, Douglas AE, Dixon AFG (1989) A rapid method to isolate symbiotic bacteria from aphids. J Invertebr Pathol 53:427–428

Harwood RF, James MT (1979) Entomology in human and animal health. Macmillan, New York

Hassan AKM, Moriya S, Baumann P, Yoshikawa H, Ogasawara N (1996) Structure of the dnaA region of the endosymbiont, *Buchnera aphidicola*, of the aphid *Schizaphis graminum*. DNA Res 3:415–419

Heddi A, Lefebvre F, Nardon P (1991) The influence of symbiosis on the respiratory control ratio (RCR) and the ADP/O Ratio in the adult weevil-*Sitophilus oryzae* (Coleoptera, Curculionidae). Endocytobiosis Cell Res 8:61–73

Heddi A, Lefebvre F, Nardon P (1993) Effect of endocytobiotic bacteria on mitochondrial enzymatic activities in the weevil *Sitophilus oryzae* (Coleoptera, Curculionidae). Insect Biochem Mol Biol 23:403–411

Heddi A, Charles H, Khatchadourian C, Bonnot G, Nardon P (1998) Molecular characterization of the principal symbiotic bacteria of the weevil *Sitophilus oryzae*: a peculiar G–C content of an endocytobiotic DNA. J Mol Evol 47:52–61

Henry SM (1962) The significance of microorganisms in the nutrition of insects. Trans N Y Acad Sci 24:676–683

Hinde R (1971a) The control of mycetome symbiotes of the aphids *Brevicoryne brassicae*, *Myzus persicae*, and *Macrosiphon rosae*. J Insect Physiol 17:1971–1800

Hinde R (1971b) The fine structure of mycetome symbiotes of the aphids *Brevicoryne brassicae*, *Myzus persicae*, and *Macrosiphum rosae*. J Insect Physiol 17:2035–2050

Hogenhout SA, van derWilk F, Verbeek M, Goldbach RW, van den Heuvel JFJM (1998) Potato leafroll virus binds to the equatorial domain of the aphid endosymbiotic GroEL homolog. J Virol 72:358–365

Houk EJ, Griffiths GW (1980) Intracellular symbiotes of the *Homoptera*. Annu Rev Entomol 25:161–187

Houk EJ, Griffiths GW, Hadjokas NE, Beck SD (1977) Peptidoglycan in the cell wall of the primary intracellular symbiote of the pea aphid. Science 198:401–403

Humphreys NJ, Douglas AE (1997) Partitioning of symbiotic bacteria between generations of insect: a quantitative study of a *Buchnera* sp. in the pea aphid (*Acyrthosiphon pisum*) reared at different temperatures. Appl Environ Microbiol 63:3294–3296

Iaccarino FM, Tremblay E (1973) Comparazione ultrastrutturale della disimbiosi di *Macrosiphum rosae* (L.) e *Dactynotus jaceae* (L.) (Homoptera, Aphididae). Boll Lab Entomol Agrar Filipo Silvestri 30:319–335

Ishikawa H (1982) Isolation of the intracellular symbionts and partial characterizations of their RNA species of the elder aphid, *Acyrthosiphon magnoliae*. Comp Biochem Physiol 72B:239–247

Ishikawa H (1987) Nucleotide composition and kinetic complexity of the genomic DNA of an intracellular symbiont in the pea aphid *Acyrthosiphon pisum*. J Mol Evol 24:205–211

Kakeda K, Ishikawa H (1991) Molecular chaperon produced by an intracellular symbiont. J Biochem 110:583–587

Kambhampati S (1995) A phylogeny of cockroaches and related insects based on DNA sequence of mitochondrial ribosomal RNA genes. Proc Natl Acad Sci USA 92:2017–2020

Katsumata R, Ikeda M (1993) Hyperproduction of tryptophan in Corynebacterium glutamicum by pathway engineering. Biotechnology 11:921–925

Klotz MG, Norton JM (1998) Multiple copies of ammonia monooxygenase (amo) operons have evolved under biased AT/GC mutational pressure in ammonia-oxidizing autotrophic bacteria. FEMS Microbiol Lett 168:303–311

Komaki K, Ishikawa H (1999) Intracellular symbionts of aphids are bacteria with numerous genomic copies. J Mol Evol 48:717–722

Kreditch NM (1996) Biosynthesis of cysteine. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*, vol 1. ASM Press, Washington, DC, pp 514–527

Kusano T, Takeshima T, Inoue C, Sugawara K (1991) Evidence for two sets of structural genes coding for ribulose bisphosphate carboxylase in *Thiobacillus ferrooxidans*. J Bacteriol 173:7313–7323

Lai CY, Baumann P (1992a) Genetic analysis of an aphid endosymbiont DNA fragment homologous to the rnpA-rpmH-dnaA-dnaN-gyrB region of eubacteria. Gene 113:175–181

Lai CY, Baumann P (1992b) Sequence analysis of a DNA fragment from *Buchnera aphidicola* (an endosymbiont of aphids) containing genes homologous to dnaG, rpoD, cysE andsecB. Gene 119:113–118

Lai CY, Baumann L, Baumann P (1994) Amplification of trpEG: adaptation of *Buchnera aphidicola* to an endosymbiotic association with aphids. Proc Natl Acad Sci USA 91:3819–3823

Lai C-Y, Baumann P, Moran NA (1995) Genetics of the tryptophan biosynthetic pathway of the prokaryotic endosymbiont (*Buchnera*) of the aphid *Schlechtendalia chinensis*. Insect Mol Biol 4:47–59

Lai CY, Baumann P, Moran N (1996) The endosymbiont (*Buchnera* sp.) of the aphid *Diuraphis noxia* contains plasmids consisting of trpEG and tandem repeats of trpEG pseudogenes. Appl Environ Microbiol 62:332–339

Lambert JD, Moran NA (1998) Deleterious mutations destabilize ribosomal RNA in endosymbiotic bacteria. Proc Natl Acad Sci USA 95:4458–4462

Liadouze I, Febvay G, Guillaud J, Bonnot G (1996) Metabolic fate of energetic amino acids in the aposymbiotic pea aphid *Acyrthosiphon pisum* (Harris) (Homoptera, Aphididae). Symbiosis 21:115–127

Lipsitch M, Nowak MA, Ebert D, May RM (1995) The population dynamics of vertically and horizontally transmitted parasites. Proc R Soc Lond B Biol Sci 260:321–327

Llanes C, Gabant P, Couturier M, Bayer L, Plesiat P (1996) Molecular analysis of the replication elements of the broad-host-range RepA/C replicon. Plasmid 36:26–35

Margolis N, Hogan D, Tilly K, Rosa PA (1994) Plasmid location of *Borrelia purine* biosynthesis gene homologs. J Bacteriol 176:6427–6432

Maynard Smith J, Szathmáry E (1995) The major transitions in evolution. W. H. Freeman Spektrum, Oxford/New York

McLean DL, Houk EJ (1973) Phase contrast and electron microscopy pf the mycetocytes and symbiotes of the pea aphid, *Acyrthosiphon pisum*. J Insect Physiol 19:625–633

McMillan DJ, Mau M, Walker MJ (1998) Characterisation of the urease gene cluster in *Bordetella bronchiseptica*. Gene 208:243–251

Messer W, Weigl C (1996) Initiation of chromosome replication. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*, vol 2. ASM Press, Washington, DC, pp 1579–1601

Moran NA (1996) Accelerated evolution and Muller's ratchet in endosymbiotic bacteria. Proc Natl Acad Sci USA 93:2873–2878

Moran N, Baumann P (1994) Phylogenetics of cytoplasmically inherited microorganisms of arthropods. Trends Ecol Evol 9:15–20

Moran NA, Telang A (1998) Bacteriocyte-associated symbionts of insects: a variety of insect groups harbor ancient prokaryotic endosymbionts. Bioscience 48:295–304

Moran NA, Munson MA, Baumann P, Ishikawa H (1993) A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. Proc R Soc Lond B Biol Sci 253:167–171

Moran NA, von Dohlen CD, Baumann P (1995) Faster evolutionary rates in endosymbiotic bacteria than in cospeciating insect hosts. J Mol Evol 41:727–731

Moran NA, Kaplan ME, Gelsey MJ, Murphy TG, Scholes EA (1999) Phylogeny and evolution of the aphid genus Uroleucon based on nuclear and mitochondrial DNA sequences. Syst Entomol 24:85–93

Munson MA, Baumann P, Kinsey MG (1991a) *Buchnera* gen. nov. and *Buchnera aphidicola* sp. nov., a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. Int J Syst Bacteriol 41:566–568

Munson MA, Baumann P, Clark MA, Baumann L, Moran NA, Voegtlin DJ, Campbell BC (1991b) Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. J Bacteriol 173:6321–6324

Munson MA, Baumannn P, Moran MA (1992) Phylogenetic relationships of the endosymbionts of mealybugs (Homoptera: Pseudococcidae) based on 16S rDNA sequences. Mol Phylogenet Evol 1:26–30

Munson MA, Baumann L, Baumann P (1993) Buchnera aphidicola (a prokaryotic endosymbiont of aphids) contains a putative 16S rRNA operon unlinked to the 23S rRNA-encoding gene: sequence determination, and promoter and terminator analysis. Gene 137:171–178

Musgrave AJ, Grinyer I (1968) Membranes associated with the disintegration of mycetomal micro-organisms in *Sitophilus zeamais* (Mots. Coleoptera). J Cell Sci 3:65–70

Nakabachi A, Ishikawa H (1997) Differential display of mRNAs related to amino acid metabolism in the endosymbiotic system of aphids. Insect Biochem Mol Biol 27:1057–1062

Nakabachi A, Ishikawa H (1999) Provision of riboflavin to the host aphid, *Acyrthosiphon pisum*, by endosymbiotic bacteria, *Buchnera*. J Insect Physiol 45:1–6

Nardon P, Grenier AM (1988) Genetical and biochemical interactions between the host and its endocytobiotes in the weevil *Sitophilus* (Coleoptera, Curculionidae) and other related species. In: Scannerini S (ed) Cell to cell signals in plant, animal and microbial symbiosis. Springer, Heidelberg, pp 255–270

Nicholson ML, Gaasenbeek M, Laudenbach DE (1995) Two enzymes together capable of cysteine biosynthesis are encoded on a cyanobacterial plasmid. Mol Gen Genet 247:623–632

Nogge G (1976) Aposymbiotic tsetse flies. Glossina morsitans morsitans obtained by feeding adults on rabbits immunized specifically with symbionts. J Insect Physiol 24:299–304

Nogge G (1982) Significance of symbionts for the maintenance of an optimal nutritional state of successful reproduction in hematophagous arthropods. Parasitology 82:299–304

Norton JM, Low JM, Martin G (1996) The gene encoding ammonia monooxygenase subunit A exists in three nearly identical copies in *Nitrosospira* sp. NpAV. FEMS Microbiol Lett 139:181–188

O'Neill A, Hoffman A, Werren JH (1997) Influential passengers; inherited microorganisms and arthropod reproduction. Oxford University Press, Oxford

Ohtaka C, Nakamura H, Ishikawa H (1992) Structures of chaperonins from an intracellular symbiont and their functional expression in *Escherichia coli* groE mutants. J Bacteriol 174:1869–1874

Patten CL, Glick BR (1996) Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol 42:207–220

Pennisi E (1998) Evolution—heat shock protein mutes genetic changes. Science 282:1796

Remaudière G, Remaudière M (1997) Catalogue des aphididae du monde. Institut National de la Recherche Agronomique, Paris

Riley M, Labedan B (1996) *Escherichia coli gene* products: physiological functions and common ancestries. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*, vol 2. ASM Press, Washington, DC, pp 2118–2202

Romero D, Palacios R (1997) Gene amplification and genomic plasticity in prokaryotes. Annu Rev Genet 31:91–111

Roth JR, Benson N, Galitski T, Haack K, Lawrence JG, Miesel L (1996) Rearrangements of the bacterial chromosome: formation and applications. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*, vol 2. ASM Press, Washington, DC, pp 2256–2276

Rouhbakhsh D, Baumann P (1995) Characterization of a putative 23S-5S rRNA operon of *Buchnera aphidicola* (endosymbiont of aphids) unlinked to the 16S rRNA-encoding gene. Gene 155:107–112

Rouhbakhsh D, Moran NA, Baumann L, Voegtlin DJ, Baumann P (1994) Detection of *Buchnera*, the primary prokaryotic endosymbiont of aphids, using the polymerase chain reaction. Insect Mol Biol 3:213–217

Rouhbakhsh D, Lai CY, von Dohlen CD, Clark MA, Baumann L, Baumann P, Moran NA, Voegtlin DJ (1996) The tryptophan biosynthetic pathway of aphid endosymbionts (*Buchnera*): genetics and evolution of plasmid-associated anthranilate synthase (trpEG) within the Aphididae. J Mol Evol 42:414–421

Rouhbakhsh D, Clark MA, Baumann L, Moran NA, Baumann P (1997) Evolution of the tryptophan biosynthetic pathway in *Buchnera* (aphid endosymbionts): studies of plasmid-associated trpEG within the genus *Uroleucon*. Mol Phylogenet Evol 8:167–176

Rutherford SL, Lindquist S (1998) Hsp90 as a capacitor for morphological evolution. Nature 396:336–342

Sacchi L, Corona S, Grigolo A, Laudani U, Selmi MG, Bigliardi E (1996) The fate of the endocytobionts of *Blattella germanica* (Blattaria, Blattellidae) and *Periplaneta americana* (Blattaria, Blattidae) during embryo development. Ital J Zool 63:1–11

Sacchi L, Nalepa CA, Bigliardi E, Corona S, Grigolo A, Laudani U, Bandi C (1998a) Ultrastructural studies of the fat body and bacterial endosymbionts of *Cryptocercus punctulatus* Scudder (Blattaria: Cryptocercidae). Symbiosis 25:251–269

Sacchi L, Nalepa CA, Bigliardi E, Lenz M, Bandi C, Corona S, Grigolo A, Lambiase S, Laudani U (1998b) Some aspects of intracellular symbiosis during embryo development of *Mastotermes darwiniensis* (Isoptera: Mastotermitidae). Parassitologia 40:309–316

Sandström J, Moran N (1999) How nutritionally imbalanced is phloem sap for aphids? Entomol Exp Appl 91:203–210

Sandström J, Pettersson J (1994) Amino acid composition of phloem sap and the relation to intraspecific variation in pea aphid (*Acyrthosiphon pisum*) performance. J Insect Physiol 40:947–955

Sandström J, Telang A, Moran NA (2000) Nutritional enhancement of host plants by aphids-a comparison of three aphid species on grasses. J Insect Physiol 46:33–40

Sasaki T, Ishikawa H (1995) Production of essential amino acids from glutamate by mycetocyte symbionts of the pea aphid, *Acyrthosiphon pisum*. J Insect Physiol 41:41–46

Sasaki T, Aoki T, Hayashi H, Ishikawa H (1990) Amino acid composition of the honeydew of symbiotic and aposymbiotic pea aphids *Acyrthosiphon pisum*. J Insect Physiol 36:35–40

Sato S, Ishikawa H (1997a) Expression and control of an operon from an intracellular symbiont which is homologous to the groE operon. J Bacteriol 179:2300–2304

Sato S, Ishikawa H (1997b) Structure and expression of the dnaKJ operon of Buchnera, an intracellular symbiotic bacteria of aphid. J Biochem 122:41–48

Schröder D, Deppisch H, Obermayer M, Krohne G, Stackebrandt E, Holldobler B, Goebel W, Gross R (1996) Intracellular endosymbiotic bacteria of *Camponotus* species (carpenter ants): systematics, evolution and ultrastructural characterization. Mol Microbiol 21:479–489

Silva FJ, van Ham RCHJ, Sabater B, Latorre A (1998) Structure and evolution of the leucine plasmids carried by the endosymbiont (*Buchnera aphidicola*) from aphids of the family Aphididae. FEMS Microbiol Lett 168:43–49

Smith DC, Douglas AE (1987) The biology of symbiosis. Edward Arnold, London

Smith OH, Yanofsky C (1962) Enzymes involved in the biosynthesis of tryptophan. Methods Enzymol 5:794–806

Spaulding AW, von Dohlen CD (1998) Phylogenetic characterization and molecular evolution of bacterial endosymbionts in psyllids (Hemiptera: Sternorrhyncha). Mol Biol Evol 15:1506–1513

Stephens RS, Kalman S, Lammel C, Fan J, Marathe R, Aravind L, Mitchell W, Olinger L, Tatusov RL, Zhao QX, Koonin EV, Davis RW (1998) Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. Science 282:754–759

Sylvester ES (1985) Multiple acquisition of viruses and vector-dependent prokaryotes: consequences on transmission. Annu Rev Entomol 30:71–88

Telang A, Sandström J, Dyreson E, Moran NA (1999) Feeding damage by *Diuraphis noxia* results in nutritionally enhanced phloem diet. Entomol Exp Appl 91:403–412

Thao ML, Baumann P (1998) Sequence analysis of a DNA fragment from *Buchnera aphidicola* (aphid endosymbiont) containing the genes dapD-htrA-ilvI-ilvH-ftsL-ftsI-murE. Curr Microbiol 37:214–216

Thao ML, Baumann L, Baumann P, Moran NA (1998) Endosymbionts (*Buchnera*) from the aphids *Schizaphis graminum* and *Diuraphis noxia* have different copy numbers of the plasmid containing the leucine biosynthetic genes. Curr Microbiol 36:238–240

Tremblay E (1989) Coccoidea endosymbiosis. In: Schwemmler W, Gassner G (eds) Insect endocytobiosis: morphology, physiology, genetics, evolution. CRC Press, Boca Raton, pp 145–173

Unterman BM, Baumann P (1990) Partial characterization of ribosomal RNA operons of the pea-aphid endosymbionts: evolutionary and physiological implications. In: Campbell RK, Eikenbary RD (eds) Aphid-plant genotype interactions. Elsevier Biomedical Press, Amsterdam, pp 329–350

Unterman BM, Baumann P, McLean DL (1989) Pea aphid symbiont relationships established by analysis of 16S rRNAs. J Bacteriol 171:2970–2974

van den Heuvel JFJM, Verbeek M, van der Wilk F (1994) Endosymbiotic bacteria associated with circulative transmission of potato leafroll virus by *Myzus persicae*. J Gen Virol 75:2559–2565

van den Heuvel JFJM, Bruyere A, Hogenhout A, ZieglerGraff V, Brault V, Verbeek M, van derWilk F, Richards K (1997) The N-terminal region of the luteovirus readthrough domain determines virus binding to *Buchnera* GroEL and is essential for virus persistence in the aphid. J Virol 71:7258–7265

van Ham RCHJ, Moya A, Latorre A (1997) Putative evolutionary origin of plasmids carrying the genes involved in leucine biosynthesis in *Buchnera aphidicola* (endosymbiont of aphids). J Bacteriol 179:4768–4777

van Ham RCHJ, Martínez-Torres D, Moya A, Latorre A (1999) Plasmid-encoded anthranilate synthase (TrpEG) in Buchnera aphidicola from the family Pemphigidae. Appl Environ Microbiol 65:117–125

von Dohlen CD, Moran NA (1995) Molecular phylogeny of the Homoptera—a paraphyletic taxon. J Mol Evol 41:211–223

von Wintzingerode F, Gobel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21:213–229

Waku Y, Endo Y (1987) Ultrastructure and life cycle of the symbionts in a Homopteran insect, *Anomoneura mori* Schwartz (Psyllidae). Appl Entomol Zool 22:630–637

Wernegreen JJ, Moran NA (1999) Evidence for genetic drift in endosymbionts (*Buchnera*): analyses of protein-coding genes. Mol Biol Evol 16:83–97

Werren JH, O'Neill SL (1997) The evolution of heritable symbionts. In: O'Neill SL, Hoffman AA, Werren JH (eds) Influential passengers; inherited microorganisms and arthropod reproduction. Oxford University Press, Oxford, pp 1–41

Whitehead LF, Douglas AE (1993) A metabolic study of *Buchnera*, the intracellular bacterial symbionts of the pea aphid *Acyrthosiphon pisum*. J Gen Microbiol 139:821–826

Wicker C, Nardon P (1982) Development responses of symbiotic and aposymbiotic weevils *Sitophilus oryzae* L. (Coleoptera, Curculionidae) to a diet supplemented with aromatic amino-acids. J Insect Physiol 28:1021–1024

Wilkinson TL (1998) The elimination of intracellular microorganisms from insects: an analysis of antibiotic-treatment in the pea aphid (*Acyrthosiphon pisum*). Comp Biochem Physiol A Comp Physiol 119:871–881

# 20 *Vibrio fisheri*: Squid Symbiosis

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## Introduction

### Overview

The marine bioluminescent bacterium *Vibrio fischeri* forms a highly specific mutualistic symbiosis with the Hawaiian bobtail squid *Euprymna scolopes*. The study of this symbiosis over the past 20 years has been aided by the nature of the interaction itself: the squid hatch without *V. fischeri* but rapidly acquire them from the seawater, and thus experimentally, wild-type or mutant bacteria can be added to the seawater and the process of colonization examined. Once the bacteria colonize, they bioluminesce, and this natural light production provides a noninvasive means of monitoring colonization. Furthermore, tools such as green fluorescent protein (GFP) have been engineered to permit visualization of bacteria at all stages of colonization in the transparent symbiotic tissue of juvenile squid. The bacterium can be readily manipulated genetically, and the genome sequences of multiple strains are known, making it feasible to test specific genes for their roles in bacteria-host interactions. Finally, investigations into the biology of the squid and its symbiotic organ, the light organ, provide a framework for developing hypotheses to be tested. The result is a robust model that is continually yielding novel insights.

In this chapter, we describe in detail the biology of *V. fischeri* as it relates to the ability of this microbe to form a specific symbiosis with *E. scolopes*. We begin with an introduction to the ecology of *V. fischeri* and its squid host. Then, to provide a basis for understanding the symbiosis, we describe the structure of the symbiotic light organ and give an overview of what is known about the dynamics of colonization and specificity in the symbiosis. We then discuss host development and the known roles of the bacteria and bacterial signals in developmental processes. With this foundation, we then describe a number of bacterial genes and phenotypes whose roles in symbiosis have been investigated, including the processes of bacterial bioluminescence, biofilm formation, motility, and iron uptake. Finally, we conclude with a brief discussion of evolution and our perspectives on the field and its future.

### Ecology of *V. fischeri* and Its Squid Host *E. scolopes*

It is not possible to appreciate the biology of *V. fischeri* fully without first understanding the environments that it experiences during its life cycle. As a marine bacterium, *V. fischeri* primarily resides in seawater, which contains dissolved salts at a concentration of 3.5%. The dissolved salts include sodium, chloride, magnesium, sulfate, calcium, and potassium. In this environment, *V. fischeri* can be found free-living in seawater and also associated with sediment (Lee and Ruby 1992, 1994b). It also can be found colonizing animal hosts.

The best known of these animal associations are exquisitely evolved light-organ symbioses in which *V. fischeri* colonizes the light-emitting organs of certain fishes and squids, generating bioluminescence used by the host in exchange for nutrients and a privileged niche. For example, *V. fischeri* colonizes light organs in monocentrid "pinecone" fishes of the genera *Cleidopus*

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or *Monocentris*, both found in the Pacific Ocean (Fitzgerald 1977; Ruby and Nealson 1976), and in sepiolid "bobtail" squids of the genera *Sepiola* or *Euprymna*, which are found in the Mediterranean Sea and Pacific Ocean, respectively (Fidopiastis et al. 1998; Jones et al. 2006; Nishiguchi 2002; Nishiguchi et al. 1998; Wei and Young 1989). Although its role as a bioluminescent symbiont is well studied, the association of *V. fischeri* with hosts is not restricted to monospecific light-organ symbioses. It has also been isolated from multi-species gut consortia of fish (Ramesh and Venugopalan 1989; Ruby and Morin 1979; Sugita and Ito 2006) and from chitinous structures on the invertebrate hydrozoans *Aglaophenia tubiformis* and *Halopteris diaphana* (Stabili et al. 2008).

Although it is a close associate of marine animals, the genetic and physiological capacity of *V. fischeri* is unlike that of obligate symbionts (Ochman and Moran 2001; Ruby et al. 2005). Both its demonstrated metabolic flexibility and its genomic content indicate that *V. fischeri* is able to grow under a range of conditions using any of several substrates, unlike many obligate symbionts that evolve reduced genomes adapted to a relatively simple and constant host environment. The metabolic diversity and genomic content of *V. fischeri* suggest that an important component of this bacterium's life history occurs outside of specific symbioses, consistent with the observation that *V. fischeri* has been found free-living in different marine environments, both aerobic and anaerobic, in the water column and in sediments (Garcia-Amado et al. 2011; Jones et al. 2007; Lee and Ruby 1994b; Orndorff and Colwell 1980; Ruby et al. 1980). While it is possible that *V. fischeri* isolated from sediments and the water column had recently cycled through a host, it also has been isolated in regions far from any known light-organ symbioses, such as the Sargasso Sea and coastal waters off the northeastern United States.

Despite its frequent association with hosts and its phylogenetic relationship to known pathogens such as *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Tantillo et al. 2004), to our knowledge, *V. fischeri* has never been documented as a pathogen. Its inability to grow at 37 °C certainly restricts it from causing human infections, and it has not been observed to cause disease in marine organisms even at permissive temperatures. The apparent nonpathogenicity of *V. fischeri* contrasts with its relative *Vibrio salmonicida*, which causes cold-water vibriosis in salmonids, and with another common bioluminescent marine bacterium, *Vibrio harveyi*, which apparently is responsible for some die-off events in aquacultured shrimp. *V. fischeri* has been isolated from aquaculture tanks in fish-rearing facilities (Alcaide 2003; Montes et al. 2006), and in one instance, it was isolated from organs of diseased aquacultured fish (Lamas et al. 1990); however, it was not shown to be causal to morbidity or mortality. It seems plausible that in this isolated incident, *V. fischeri* may have been a secondary opportunist flourishing in a host already compromised by another microbe. Overall, although the *V. fischeri* genome encodes homologs of virulence factors found in other members of the *Vibrionaceae* (Ruby et al. 2005), this species appears to enter benign or beneficial associations with hosts.

Among its specialized light-organ symbioses, the best studied is that between *V. fischeri* and *E. scolopes*, the Hawaiian bobtail squid (Fig. 20.1a). *E. scolopes* is a nocturnal predator that feeds on polychaetes and shrimp. The hatchlings (Fig. 20.2a) are typically only ~3–4 mm long but can grow to be ten times that length (Moynihan 1983; Shears 1988). The adults likely live less than a year in the wild (Hanlon et al. 1997; Singley 1983) and are found near the Hawaiian coast in shallow, sandy reef areas, in a few meters or even as little as a few centimeters of water. It is unclear the extent to which this habitat reflects where they live versus where researchers typically search for them. There are reports of *E. scolopes* being found well outside the reefs and even at depths of 200 m (Berry 1912), but for convenience, researchers typically have stayed closer to shore. These animals appear to be solitary except when they are mating, which has been observed both in shallow water and in captivity (Fig. 20.1b). The ability to maintain and mate *E. scolopes* in laboratory aquaria has underpinned its development as a model experimental system.

After mating, *E. scolopes* females lay clutches of eggs that lack *V. fischeri* symbionts. Upon hatching, each new generation must acquire *V. fischeri* from the surrounding seawater (Wei and Young 1989), a phenomenon that allows researchers in controlled laboratory environments to compare animals infected with different strains or with no *V. fischeri* symbionts at all. If *V. fischeri* is present, infection occurs within hours, and it is so efficient that no uninfected *E. scolopes* has ever been found in the wild. The animal maintains a monospecific culture of *V. fischeri* in its light organ. As discussed below, this specific infection with *V. fischeri* triggers a developmental program in the light-organ tissue. Although the light organ undergoes large morphological changes, the animals maintain a monospecific culture of *V. fischeri* throughout their life, allowing the squid to exploit the symbionts' bioluminescence.

Whether to hide from predators or prey, *E. scolopes* makes extensive use of camouflaging (Anderson and Mather 1996; Shears 1988), a general strategy that appears to include their use of *V. fischeri* bioluminescence. The animals cover themselves with sand and use chromatophores to change colors among a natural-looking palette (Fig. 20.1c). They can even be observed swimming with a sand coat, which they can discard quickly (Shears 1988). Similarly, the *V. fischeri* symbionts are apparently used in a strategy referred to as "counterillumination," where ventrally directed bioluminescence is used to obscure the squid's silhouette from organisms beneath it in the water column. The strongest evidence that this is the function of symbiotic bioluminescence includes the architecture of the organ (McFall-Ngai and Montgomery 1990) and the observation that the light emitted is controlled to directly correlate with the ambient downwelling light (Jones and Nishiguchi 2004). Although a nutritional or other benefit of the symbionts cannot be ruled out, *E. scolopes* raised through a complete life cycle without *V. fischeri* did not appear compromised (Claes and Dunlap 2000), further reinforcing the idea that symbiont bioluminescence is the main advantage to the host.

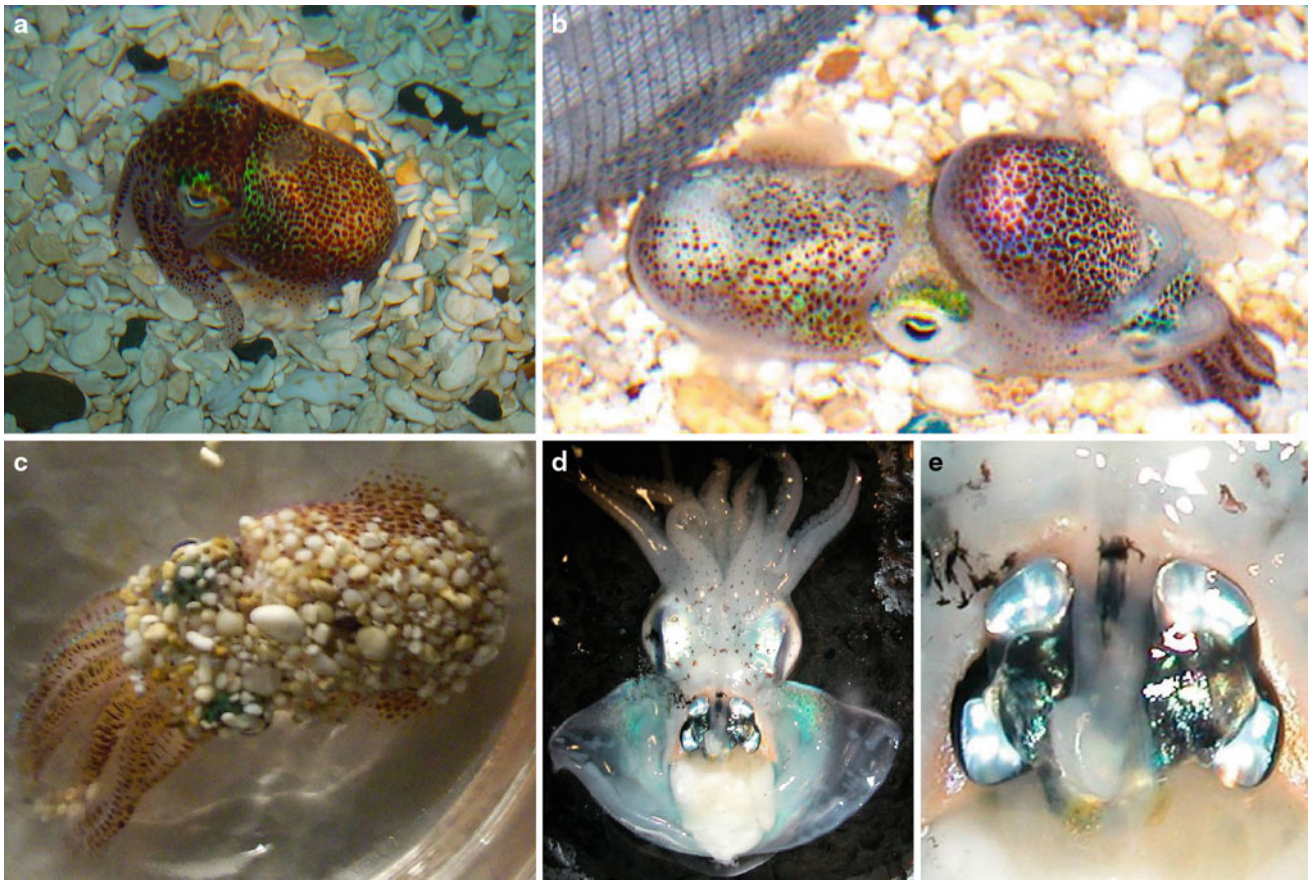


**Fig. 20.1**
**Adult *E. scolopes*. (a) Adult *E. scolopes* sitting on coral sand; (b) a mating pair of *E. scolopes*; (c) sand-covered *E. scolopes*; (d) ventrally dissected *E. scolopes*; (e) a close-up view of the adult light organ (Image from panel a was taken from Stabb and Millikan (2009), while images from b and c are courtesy of Kati Geszvain)**

The counterillumination model above and other possible explanations for the use of bioluminescent symbionts by the squid were reviewed recently (Stabb and Millikan 2009).

The advantage of the symbiosis for *V. fischeri* appears clearer. *V. fischeri* is provided nutrients in the *E. scolopes* light organ, and this supports its rapid growth (Ruby and Asato 1993). Furthermore, *V. fischeri* cells also appear to benefit from the host immune system, which maintains an exclusive relationship with the bacteria, protecting them from predation or competition by other microbes. Each morning, the squid expel most of the *V. fischeri* cells in their light organ out into the environment and then support regrowth of the remaining symbionts throughout the day (Boettcher et al. 1996) (*Fig. 20.3*). Given this daily venting and re-culturing, one would expect to find relatively high populations of *V. fischeri* in habitats occupied by *E. scolopes*, which indeed has been observed (Lee and Ruby 1994b). Other ecological studies (Lee and Ruby 1994a) support the idea that in shallow, sandy Hawaiian reefs occupied by *E. scolopes*, the ability to colonize this squid is advantageous for *V. fischeri*. Taken together, the evidence suggests that this symbiosis is a mutualism that benefits both partners.

Given the evidence for mutualism, it is not surprising that *V. fischeri* isolates from *E. scolopes* appear to have coevolved with this host. There is considerable evidence that certain strains of *V. fischeri* have adapted to be especially proficient colonizers of *E. scolopes* (Lee and Ruby 1994a; Mandel et al. 2009; Nishiguchi 2002; Nishiguchi et al. 1998; Schuster et al. 2010), and repeated passage of strains through *E. scolopes* in the laboratory has shown that less proficient colonizers can evolve into more effective symbionts (Schuster et al. 2010). Interestingly, the gene encoding the regulatory sensor RscS, discussed further below, appears to be a key genetic acquisition in the evolution of *V. fischeri*, leading to more proficient colonization of *Euprymna* hosts in the Pacific (Mandel et al. 2009).

As a coevolved mutualism, the *V. fischeri-E. scolopes* symbiosis resembles many specific bacterium-host interactions found in nature. Given several features that make it experimentally tractable, it serves as a powerful natural model for such associations. Although *V. fischeri* may not require *E. scolopes* or other light-organ symbioses to survive and grow in the environment, in locales where hosts are available, light-organ colonization appears to be very important in its ecology. Thus, studies of

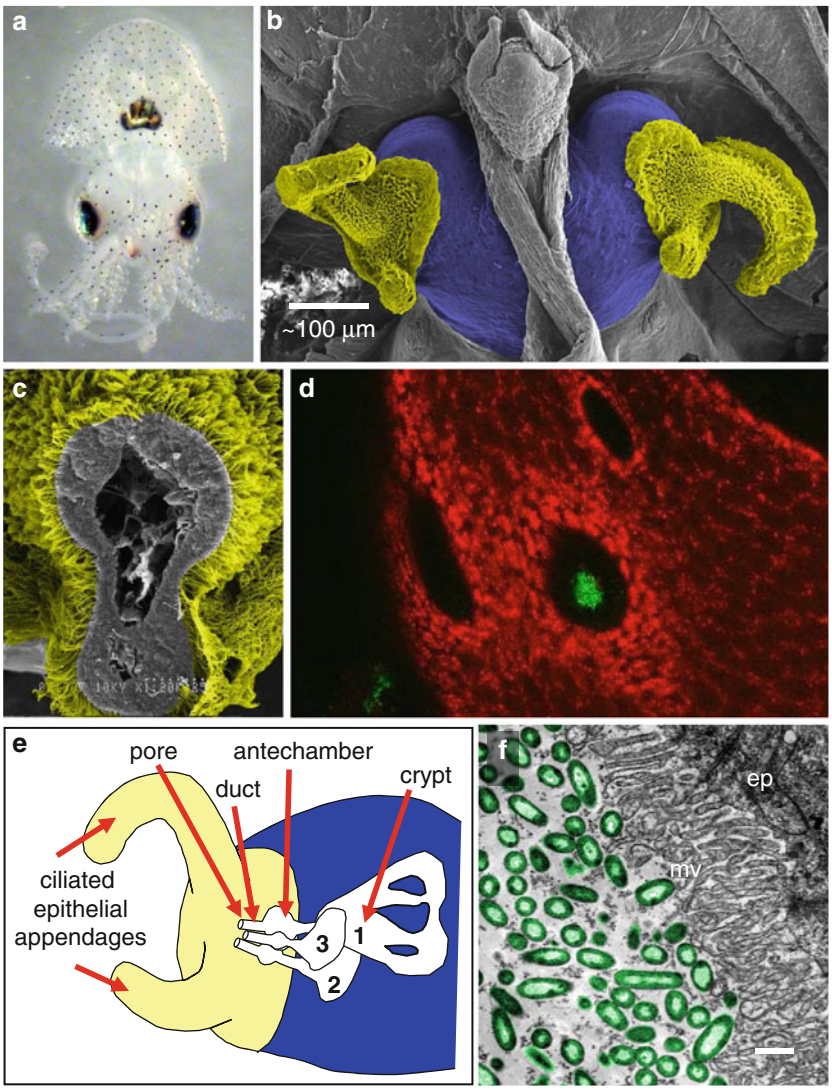


**Fig. 20.2**
**Juvenile E. scolopes. (a) Juvenile *E. scolopes*, with the light organ prominent as a *black* shape in the *center* of the mantle; (b) an image, generated with scanning electron microscopy, in which the ciliated epithelial appendages are seen extending from the surface and the underlying light-organ tissues are apparent; (c) a cross section of one of the appendages revealing a blood sinus; (d) the surface of part of the light organ depicting three pores, in/near one of which is an aggregate of *V. fischeri* cells; (e) cartoon depicting the structure of one-half of the juvenile light organ with three crypts labeled (*1*, *2*, and *3*); (f) cross section of a region of a colonized crypt. *mv* microvilli, *ep* epithelium (The image from panel a was previously published (Dunn and Stabb 2008a), and the image in panel d is cropped from a version that was previously published (Yip et al. 2006))**

*V. fischeri* infecting *E. scolopes* address issues directly significant to the ecology of the bacterium in nature, and they can elucidate our understanding of bacterium-host symbioses in general.

## Structure of Light Organ, Dynamics of Colonization, and Development

### Structure of Light Organ

In adult *E. scolopes*, *V. fischeri* cells reside within a complex, bilobed organ at a level in excess of $10^9$ bacteria or approximately $10^{11}$ cells per ml of light-organ fluid (Boettcher and Ruby 1990; Nyholm and McFall-Ngai 1998) (Fig. 20.1d). It is at these high cell densities that the bacterial contribution to the symbiosis, bioluminescence, is produced. The adult organ contains several tissues, including lens and reflector tissues that direct and modulate the light (Fig. 20.1e). Of note, the light organ occupies a significant portion of the space within the squid's body cavity (mantle), a feature that suggests the relative importance of this organ and the symbiosis to the life cycle of the animal.

Juvenile *E. scolopes*, which hatch without symbionts (aposymbiotic), are first exposed to *V. fischeri* cells when the animal ventilates seawater into its mantle cavity. Derived from

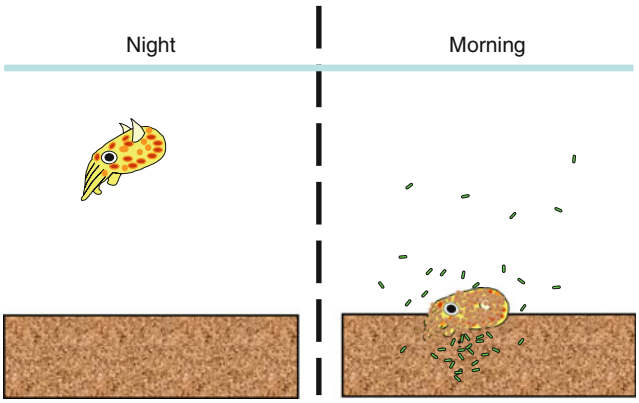


**Fig. 20.3**
**Daily behavior of *E. scolopes*. The cartoon depicts the behavior of *E. scolopes*, which forages for food in the water column at night, and in the morning, expels 90 % of its bacteria and buries in the sand for the day**

an outgrowth of the digestive tract (Montgomery and McFall-Ngai 1993), the juvenile light organ (*Fig. 20.2b* & *e*) features two sets of ciliated surface appendages that project into the mantle cavity (McFall-Ngai and Ruby 1991). The cilia on these appendages, along with the cilia decorating ridges on either side of the organ, entrain the bacteria-containing ambient seawater toward pores that serve as the entrances to the light organ (McFall-Ngai and Ruby 1998) (*Fig. 20.2d* & *e*). In addition to the cilia, the surface of the light organ is coated with mucus that is secreted from the epithelial cells that line the appendages (Nyholm et al. 2000, 2002). The directed movement of the cilia and surface-secreted mucus are thought to promote attachment of *V. fischeri* carried into the squid with the ventilated seawater. Thus, at a very early stage in colonization, *V. fischeri* experiences a mucus-coated surface, an environment that is vastly different from seawater.

A small aggregate of *V. fischeri* cells accumulates on the surface of the symbiotic light organ, then ultimately individual cells track into pores to enter the organ (*Fig. 20.2d*) (Nyholm et al. 2000). A total of six pores exist, three on each side of the organ. They range in size from 5 to 15 μm in diameter (Montgomery and McFall-Ngai 1993). Thus, *V. fischeri* cells, which are approximately 1–2 μm in length (Millikan and Ruby 2003; Ruby and Asato 1993), are substantially smaller than the pores through which they enter. Bacterial motility appears to be important for entry, as nonmotile bacteria appear to aggregate but do not migrate to the pores (Nyholm et al. 2000).

The six pores open into six ducts or tube-like extensions from the surface (*Fig. 20.2e*) (Montgomery and McFall-Ngai 1993). Passage through the ducts appears to be a challenge: although heterologous species such as *V. parahaemolyticus* appear competent to reach the duct, they fail to colonize (Nyholm et al. 2000). The duct contains mucus, cilia that appear to beat outward toward the pores, and antimicrobial molecules (Davidson et al. 2004; McFall-Ngai and Ruby 1998; McFall-Ngai 1999; Small and McFall-Ngai 1999). To progress to colonization, *V. fischeri* must be able to overcome these challenges and others, as described in greater detail below.

Each of the six ducts leads to an antechamber (Sycuro et al. 2006), a small chamber outside of the larger deep crypt where most colonizing cells eventually reside (*Fig. 20.2e*). Each set of antechambers has an average size and complexity that corresponds to that of the deep crypt with which it is associated. For example, the antechamber of crypt 1 has an average cross-sectional area of 1,380 $\mu m^2$, while the antechamber of crypt 3 has its largest cross-sectional dimension in the range of 510 $\mu m^2$ (Sycuro et al. 2006) (*Fig. 20.2e*). The antechambers are not permissive to persistent colonization, likely because this is a region of the light organ with extensive antimicrobial activities, such as nitric oxide (NO) production (Davidson et al. 2004). Little else is known about the antechambers. To reach their respective deep crypts, the bacteria must exit the antechamber through a bottleneck region that has small dimensions (between 5 and 9 μM in width) that limit passage.

Like the antechambers, the three sets of deep crypts have different characteristics with respect to size and complexity. In the newly hatched juvenile, the largest and most developed is known as deep crypt 1 (or simply crypt 1), while the smallest and least developed is crypt 3. Crypt 2 is intermediate between the other two. Because of the complexity of the deep crypt tissues, no estimate has been made of the area or volume of these spaces. Each of the deep crypts in the uncolonized juvenile is lined with columnar epithelial cells. The microvilli on the surfaces of these cells in colonized animals provide points of direct contact with the bacterial symbionts (*Fig. 20.2f*). It is within these confined deep crypt spaces that multiplication to high cell density ensues, resulting in colonization of the host by *V. fischeri*. Sycuro et al. (2006) reported that there is no particular order to which the six deep crypts become colonized. Similarly, Dunn et al. (2006) noted that, while bacterial gene expression was altered in crypt 3 relative to the other crypts, the timing of colonization did not appear different. Thus, there appear to be differences in crypt structure and maturity in hatchlings, but the differences do not interfere with colonization. Rapid growth of the bacteria is supported by host-provided nutrients, including amino acids presented in the form of peptides (Graf and Ruby 1998), and likely oxygen. When a sufficiently high cell density is achieved, the production of bioluminescence is induced (Ruby and Asato 1993).

It is readily apparent from this brief description that *V. fischeri* experiences a variety of environments during its passage into the deep crypts where multiplication and colonization occurs. *V. fischeri* must transit from the nutrient-limited seawater to the mucus-lined surfaces of the light organ, through the apparently challenging environments of the ducts and antechambers, to reach the nutrient-rich, hospitable environment of the deep crypts where rapid growth is possible and a generation time as low as 30 min is estimated (Ruby and Asato 1993). Each stage likely requires the expression of a distinct set of traits that permit *V. fischeri*—and no other bacteria—to successfully navigate these challenges.

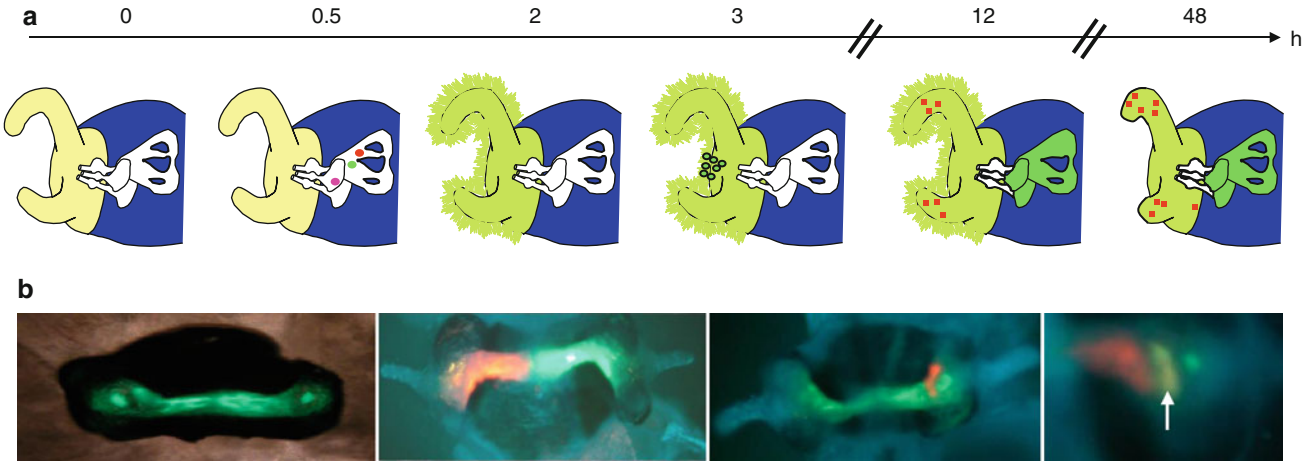


**Fig. 20.4**

**Initiation of colonization. (a) An approximate time line of some of the known events in colonization during the first 48 h is depicted. Following hatching (0 h), the light organ is transiently permissive to entry by bacteria (0.5 h). Mucus shedding (represented by *fuzzy shading* on the surface of the organ) is induced (2 h), promoting surface aggregation by *V. fischeri* (*ovals*) (3 h). Colonization occurs (*darker shading*) (12 h) and triggers apoptosis (represented by *dots*), regression of the appendages, and cessation of mucus secretion (48 h). (b) Images of light organs clonally or dually colonized by GFP- or RFP-expressing *V. fischeri* cells. The segregation of initiating cells is apparent by the separation of colors; in the last panel, mixing of cells can be seen (*white arrow*) (Images from panel b were previously published Dunn et al. (2006))**

## Dynamics of Symbiosis

One of the most interesting facets of the *V. fischeri*-squid symbiosis is its dynamic nature. The squid are nocturnal animals, and many of their behaviors are cued to the day/night cycle. For example, the animals forage for food at night, but bury in the sand during the day (*Fig. 20.3*) (Moynihan 1983). In addition, juveniles hatch from eggs at dusk; thus, these newly hatched squid become colonized at night. That event begins a cycle of colonization, expulsion, and regrowth: every dawn, colonized squid expel 90% of their bacterial symbionts by means of a muscle-induced contraction (*Fig. 20.3*) (Boettcher et al. 1996; Graf and Ruby 1998; Lee and Ruby 1994b; Nyholm and McFall-Ngai 1998; Ruby and Asato 1993). In the adult, the result is the release of a toothpaste-like gel of acellular matrix along with bacteria and host cells (Nyholm and McFall-Ngai 1998). The remaining 5–10% of the bacterial population repopulates the light organ. The consequence of this phenomenon is that both the bacteria and their host experience a changing environment daily. In this section, we describe some of the known molecular details that correspond to the rhythm of the squid's biology, including the early events specifying colonization and subsequent daily events that influence the interaction between the partners.

Detailed studies of the initiation of colonization revealed a surprising fact: within the first hour following hatching (in fact, as early as 20 min), the light organ is permissive to entry by both *V. fischeri* and non-*V. fischeri* bacteria, as well as other similarly sized particles (*Fig. 20.4a*) (Nyholm et al. 2002). Both Gram-negative and Gram-positive bacteria with sizes of 1 μm in diameter could be observed in the crypt spaces in the first hour. Fluorescent beads with a 1-μm diameter could also be observed in the deep crypts, but not beads with larger diameters (2 μm or 10 μm) or cells (*Bacillus cereus*) with a diameter of 5 μm. This phenomenon was labeled the permissive period, since even nonsymbiotic bacteria can gain entry. However, no viable bacteria could be recovered from light organs at this time using plating techniques, and furthermore, no particles (bacteria or beads) could be detected 2 h after inoculation (Nyholm et al. 2002). Thus, following entry, the bacteria and beads appear to be removed, likely by host defense cells (hemocytes) (Nyholm et al. 2009), and the time between 1 and 2 h after hatching represents a nonpermissive period.

Within 1–2 h following hatching, mucus secretion occurs from cells within the ciliated epithelial appendages in animals exposed to bacteria, but not in response to beads (*Fig. 20.4a*) (Nyholm et al. 2002). Sialomucin is the predominant mucin type found on the surface of the epithelial fields, but neutral mucins can also be detected. Although sialomucin can be found on the surfaces of the appendages of squid maintained in filter-sterilized seawater, no shedding of mucus occurs. Mucus shedding could be induced by the addition of peptidoglycan (PG) but not lipopolysaccharide (LPS) (Nyholm et al. 2002). Mucus secretion also could be induced by PG-coated beads too large to enter the light organ, indicating that entry is not necessary and that a receptor for PG must be present on the surface of the light organ. These data indicate that the early permissive period is not essential for induction of mucus secretion, if PG is present in the seawater. In contrast to several developmental events discussed below that require *V. fischeri* and also involve PG, this induction of mucus secretion occurs even if *V. fischeri* is absent from the seawater, indicating that the squid are simply sensing the presence of bacteria.

Because the onset of mucus secretion coincides with the beginning of the nonpermissive period, experiments were undertaken to determine if mucus secretion causes the block to particle entry. Newly hatched juvenile squid were exposed to PG for 3 h to induce mucus secretion. These animals were then exposed to GFP-labeled nonsymbiont *V. parahaemolyticus*, and the number of animals with these bacteria in their crypts determined. The results indicated that reduced numbers of animals with bacteria could be detected relative to animals that had not been exposed to PG and thus not shedding mucus. The authors of this study concluded that mucus secretion contributes to, but is not wholly responsible for, the block to the permissive period (Nyholm et al. 2002).

The production of mucus promotes the ability of *V. fischeri* to aggregate on the surface of the light organ and subsequently to enter and colonize (Fig. 20.4a). Once *V. fischeri* has colonized, however, mucus shedding is downregulated: the amount of mucus secreted from 72-h aposymbiotic animals was significantly greater than that of 48-h colonized animals (Nyholm et al. 2002). Furthermore, *V. fischeri* could aggregate on the light organs of aposymbiotic animals upon exposure at any point during the first 4 days, but could not aggregate on the light organs of 48-h symbiotic animals, which shed relatively little mucus (Fig. 20.4a). Presumably, once colonization is achieved, there is no longer a need for mucus to promote bacterial aggregation, and thus it is downregulated; this decrease in mucus production likely also restricts colonization by undesired species. In further support of the relationship between colonization and mucus shedding, symbiotic animals that were cured of their bacteria through antibiotic treatment exhibited an increase in mucus shedding, which once again promoted *V. fischeri* aggregation. It should be noted that cured animals, while able to shed more mucus and permit aggregation, exhibited reduced levels of aggregation, likely due to the reduced numbers of mucus-secreting epithelial cells resulting from apoptosis and regression of the ciliated appendages (Fig. 20.4a), developmental events that occur in the same time frame (described below) (Nyholm et al. 2002). Finally, the deep crypt spaces of colonized animals contained increased mucus compared to uncolonized animals (Nyholm et al. 2002). This effect was opposite to the downregulation that occurs on the light-organ surface, indicating that mucus secretion in the crypts may promote symbiosis.

*V. fischeri* also downregulates the production by the host of nitric oxide (NO), which is relatively high in uncolonized hatchlings (Davidson et al. 2004). NO synthesis carries both a cost to produce and a risk (of oxidative damage) to the host. By shutting down mucus and regressing the appendages, the squid has greatly reduced the opportunity for further colonization and, potentially, can now relax its defenses. Sustained activation might ultimately jeopardize maintenance of symbiont colonization, and thus, this change in NO may reflect an accommodation for *V. fischeri*. Alternatively, it might reflect a signaling function for NO (see below). Regardless, it is clear that the result of colonization is to decrease subsequent attachment and superinfection. These phenotypes demonstrate the influence of *V. fischeri* on the biology of its host.

This tight control over initiation of symbiosis begs the questions, how many cells are necessary to initiate colonization and how many different *V. fischeri* cells can successfully colonize the light organ of a single squid? These questions were experimentally addressed in a series of studies. Early studies indicated that squid could contain more than one strain. For example, bacteria with different plasmid profiles could be isolated from the light organ of a single field-caught adult animal (Boettcher and Ruby 1994). In addition, when a mixture of two strains was used to inoculate juvenile squid, some colonized animals contained both strains, indicating that multiple strains could colonize (Lee and Ruby 1994a). Furthermore, a marked strain could be introduced into a colonized animal, albeit at a very low frequency (Lee and Ruby 1994a); likely the poor efficiency of superinfection resulted from decreased mucus shedding and loss of the ciliated surface appendages.

A subsequent study evaluated how different numbers of bacteria impacted colonization proficiency. Generally, at different inoculation dosages, the percentage of squid that had associated bacteria at an early time point (3 h) was similar to the percentage of squid that ultimately became colonized (McCann et al. 2003). Inoculation with as few as 250 *V. fischeri* cells in 4 ml during a short (3-h) period of time was sufficient to promote colonization of 50% of the animals, indicating that colonization by *V. fischeri* is an efficient process (McCann et al. 2003). At inoculation levels above 1,000 bacteria, colonization occurred 100% of the time. With increasing doses of bacteria, the efficiency of colonization increased, as determined by the decreasing time to onset of luminescence, a trait that is governed by cell density. However, beyond a certain point, no further increase in efficiency was obtained with increasing numbers, suggesting that the process rather than the number of bacteria becomes limiting.

The same study asked whether three strains of *V. fischeri* could simultaneously colonize a single squid (McCann et al. 2003). The strains differed only by distinct antibiotic markers that did not substantially impact colonization proficiency of a single strain. At a low inoculation dose (500 cells), all colonized squid contained a single strain. When the dosage was increased to 5,000 cells, most squid contained two strains but not three. Finally, at high doses (16,000 and 27,000), a significant percentage of squid contained all three strains. Thus, although multiple strains can co-colonize, it appears that, at levels similar to those found in nature (Lee and Ruby 1992), only one or two bacteria generally colonize a single animal.

A subsequent study evaluated co-colonization using two strains that differed by a fluorescent tag (red or green fluorescent protein) and visual examination of crypt colonization using epifluorescence microscopy (Dunn et al. 2006). When squid were inoculated with moderate doses of the two strains (for a total of 2,000–7,000 CFU/ml), most animals were colonized with both strains. Interestingly, however, most animals with mixed infections contained pockets of either red or green fluorescence, and only rarely did light organs contain a region with a mixture of red and green cells (Fig. 20.4b). These data further support the idea that a few cells initiate colonization, and even when multiple strains colonize, they often become

segregated within the light organ, presumably because it is often only a single cell that initially reaches each deep crypt and becomes the dominant colonist there.

These two experimental studies, which suggest that low numbers of *V. fischeri* cells initiate colonization, are consistent with a subsequent analysis examining the population structure of *V. fischeri*. Wollenberg and Ruby (2009) used an extensive PCR-based analysis along with phenotypic analyses and modeling to evaluate the number of different strains of *V. fischeri* in light organs of field-caught animals. These analyses support the prediction that one or two bacteria colonize each crypt, resulting in the mixed population structure found within the adult light organ (Wollenberg and Ruby 2009).

As mentioned earlier, *E. scolopes* expel ~90% of their bacterial symbionts every dawn (Boettcher et al. 1996; Graf and Ruby 1998; Lee and Ruby 1994b; Ruby and Asato 1993). Intriguingly, for juveniles, different crypts are emptied with different efficiencies: bacteria are expelled from deep crypt 1 to a greater extent than crypt 2, with crypt 3 being least effectively emptied (Sycuro et al. 2006). Sycuro et al. (2006) suggest two possibilities for these results: the expulsion efficiency (1) reflects the relative developmental maturity of the crypts or (2) is due to position effects (the muscle contraction is less effective in the area where crypt 3 resides). These features of the juvenile could impact the identity and number of specific symbiont strains over time. Indeed, one study determined that a specific mutant strain of *V. fischeri* was preferentially expelled from the light organ in mixed colonization experiments (Millikan and Ruby 2004). Understanding the dynamics of crypt expulsion and how it relates to colonization competitiveness and persistence is an important area of future investigation.

Linked to but separate from the expulsion event is another rhythm established in *E. scolopes*, the variation of bacterial bioluminescence over the day/night cycle. Luminescence increases and is highest in the hours preceding darkness; light production is as much as 100-fold lower at other times (Boettcher et al. 1996). This rhythm of increasing and decreasing light emission is disrupted if animals are kept in either constant light or constant darkness, indicating that it is a diel rhythm rather than a circadian rhythm (Boettcher et al. 1996). Furthermore, the amount of specific bioluminescence, or the amount of light produced on a per-cell basis, varies over the day/night cycle: when symbiotic luminescence levels are high prior to the onset of darkness, the amount of symbiotic light per cell matches that produced by newly released bacteria (Boettcher et al. 1996). However, in periods of low symbiotic luminescence, the specific luminescence is lower in symbiotic cells relative to newly released cells. These data indicate that the squid controls (inhibits) light production or emission. The daily expulsion of bacteria at dawn contributes to the change in light emission over the daily cycle: a peak of luminescence can be observed at the transition from dark to light that is not observed for animals kept in constant light or dark conditions. The peak of light corresponds to the release of bacteria into the seawater; following this expulsion event, the levels of luminescence by the squid are clearly decreased. However, although expulsion clearly contributes to a drop in light intensity, it is insufficient to fully account for the observed patterns, as light emission decreases steadily over a number of hours prior to the expulsion event.

Another mechanism by which the squid controls light emission is by physically blocking it. It is clear that adult animals can direct, control, and conceal the light produced by their bacterial partner using a muscle-controlled ink sac, along with lens and reflector tissues (McFall-Ngai and Montgomery 1990). These tissues direct the light downward to match the downwelling moonlight and modulate light emission under a variety of natural environmental conditions (e.g., full moon and new moon), thus permitting the squid to use counterillumination as a defense mechanism (Jones and Nishiguchi 2004). However, the light organs of newly hatched juvenile squid do not seem to have sufficiently developed accessory tissues to account for the observed diel rhythm (Boettcher et al. 1996; McFall-Ngai and Montgomery 1990; Montgomery and McFall-Ngai 1993). Thus, it was speculated that another mechanism must be in place to account for the daily modulation of bioluminescence.

The currently favored model is that the level of specific luminescence varies over the daily cycle due to changes in oxygen levels provided to the symbiont by the host (Boettcher et al. 1996). Luminescence is an oxygen-dependent reaction, and thus decreased oxygen availability could lead to decreased luminescence. The finding that newly released bacteria exhibit a spike in their levels of light production is consistent with the idea that oxygen is more readily available in seawater and, as a limiting reagent in the light organ, can rapidly change the specific luminescence level. Regardless of the cause, changes in luminescence are part of the daily rhythm of the symbiosis.

A series of studies has investigated the influence of *V. fischeri* and the daily rhythm on host transcription and protein production. One of these studies generated a set of 11 expressed tag sequence (EST) cDNA libraries from juvenile animals at different times and either with or without *V. fischeri* cells (colonized or uncolonized) (Chun et al. 2006). A large number (46%) of the non-redundant set of sequences appeared to represent unique sequences, as they had no matches in the database at the time. Perhaps the most significant finding from the initial description of this tool was that the biggest set of unique transcript fragments was obtained from animals that had been colonized for 2 days (48 h). This finding agrees with previous proteomic work showing that 48 h was the earliest time point at which differences in protein profiles between colonized and uncolonized animals could be detected (Doino Lemus and McFall-Ngai 2000). At this point in colonization, numerous developmental changes are occurring or are being induced by the bacteria, including apoptosis and regression, and crypt cell swelling (Nyholm and McFall-Ngai 2004). The proteomic study also found that some proteins that appeared specifically in 48-h symbiotic animals but not in uncolonized animals were not symbiosis-specific, as the same proteins could be detected in uncolonized animals at a later time point (96 h) (Doino Lemus and McFall-Ngai 2000). These data indicate that the bacteria accelerate certain host developmental events in addition to inducing symbiosis-specific gene expression.

The EST database permitted the construction of a microarray, which was used to probe changes in host gene expression in response to the symbiosis. Specifically, a subsequent study investigated how host transcription was affected by colonization, luminescence, and the LuxI-produced autoinducer pheromone that induces luminescence (as described in greater detail below) (Chun et al. 2008). Perhaps not surprisingly, the biggest influence on the response of the host was the presence of the bacteria, with hundreds of transcripts altered in response to colonization. The host also responded to light production and, to a much lesser extent, the presence of autoinducer. Further investigation of the transcriptional responses and the impact of those changes on host activities will provide important insights into host colonization.

A second microarray study analyzed gene expression of both *V. fischeri* and its host at 6-h intervals to capture the changes that drive or derive from the squid's daily rhythm (Wier et al. 2010). For the host, about 10% of genes present in the array (a ~14,000 gene EST library) exhibited changes in expression. The 6-h intervals on either side of dawn showed the greatest differences in host gene expression, notably including changes in transcripts encoding proteins with cytoskeletal functions. In the interval prior to dawn, there was a substantial upregulation in expression of this suite of genes, while in the period after dawn, they were substantially downregulated, suggesting cytoskeletal remodeling was induced then decreased in the intervals bracketing dawn. An examination of the crypt epithelium revealed that structural changes indeed occurred right around dawn (Wier et al. 2010). Throughout most of the 24-h period, the host epithelial cells appear "normal": the cells are highly polarized and have microvilli on the surfaces that interact with the symbionts. However, around dawn, the cell surfaces appear effaced, and portions appear to be released into the crypts as vesicles (Nyholm and McFall-Ngai 1998; Wier et al. 2010). Within a few hours, however, the normal appearance is restored. Thus, there is a daily cycle of structural change in the host epithelium coincident with symbiont expulsion.

The same microarray experiment confirmed that the symbionts also changed their gene expression: 17% of genes changed during one of the four intervals, with the greatest period of regulatory change occurring in the interval after dawn (Wier et al. 2010). A deeper analysis indicated that genes involved in the catabolism of chitin were upregulated in the period prior to dawn and downregulated after dawn and throughout the day. In contrast, genes involved in glycerol metabolism were upregulated after dawn. Wier et al. (2010) proposed that host vesicles, which become abundant around dawn, would be a rich source of glycerophospholipids, from which glycerol could be released (Wier et al. 2010). In support of the idea that the symbionts could incorporate fatty acids from host vesicles into their membranes, symbiont cells contained dramatically different lipid profiles compared to cultured *V. fischeri* (Wier et al. 2010). Together, these data suggest that the metabolism of *V. fischeri* undergoes dynamic changes over the course of each day, depending on the availability of nutrients.

In addition to light production and, likely, metabolism, *V. fischeri* undergoes developmental changes that are reflected in the daily rhythm. Motility, which is necessary for *V. fischeri* to enter and reach the deep crypts, appears unnecessary upon colonization as deep-crypt-localized *V. fischeri* have no flagella (Ruby and Asato 1993). In addition, *V. fischeri* cells undergo a change in morphology: within 24 h of symbiosis, the cells have become smaller and rounder than culture-grown cells or symbionts within the first 12 h of symbiosis (Ruby and Asato 1993). The signals and genes responsible for inducing these changes remain unknown.

In summary, the light-organ symbiosis is dynamic. The bacteria and their host influence each other's gene expression and induce developmental events, promoting, among other things, an exquisite specificity in partner selection and reducing competition by others, including late-arriving *V. fischeri*. For the host, some changes, once initiated, are irreversible, while others require the continuous presence of the bacteria. Numerous events occur on a daily cycle driven by a number of factors, including bacterial expulsion, available nutrients and (probably) available oxygen, and bacterial growth. This dynamic nature must be considered when assessing the requirements for specific bacterial traits, which may be important at one stage or one temporal period, but not another. This is one example where the laboratory "batch culture" may actually somewhat reflect a natural process in the wild.

## Specificity and Host Defenses

The discrete localization of the *V. fischeri* light-organ infection and the inability of other bacteria to colonize this tissue demonstrate an exquisite level of control by the host. As further evidence that host tolerance of the symbiont is regulated, we have observed that when juvenile squid that are colonized by *V. fischeri* become nutritionally stressed, the animals are able to entirely clear the *V. fischeri* infection (Stabb, unpublished data). Thus, while the light organ is receptive to infection and able to support the rapid growth of *V. fischeri*, the squid are able both to prevent other bacteria from colonizing and to keep the *V. fischeri* symbionts themselves in check. The mechanisms of specificity and control of the infection maintained by the host remain intriguing and somewhat mysterious, although a great deal is now known. It appears that specificity is achieved through multiple layers of enrichment, eventually "winnowing" *V. fischeri* symbionts away from unwanted interlopers (Nyholm and McFall-Ngai 2004). The underlying mechanisms involve physical barriers, physiological constraints, and immune functions that include both broad-spectrum antimicrobial compounds and potentially a more microbe-specific population of macrophage-like hemocyte cells (*Fig. 20.5*).

Cilia and mucus in the ducts may constitute an impediment to infection (Nyholm and McFall-Ngai 1998). A sticky mucus also facilitates adherence to the outside of the light organ by planktonic cells, but such adherence could constitute a barrier to the movement required to traverse the ducts. Once the

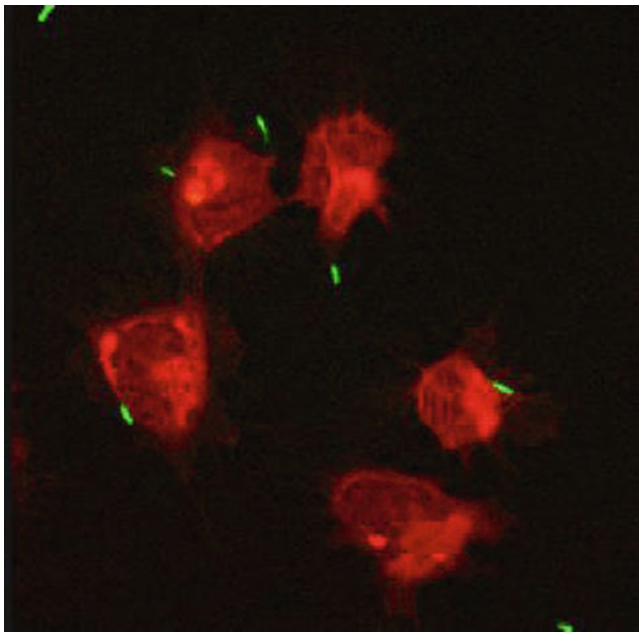

**Fig. 20.5**
**Host defense by *E. scolopes*. Hemocytes, part of the innate defense of *E. scolopes*, are shown binding to *V. fischeri* bacteria (This image was taken by Andrew Collins and generously provided by Spencer Nyholm)**

symbionts are inside the crypts, junctions between epithelial cells probably form a physical barrier that keeps them constrained to the light organ (Nyholm and McFall-Ngai 1998). Although the *V. fischeri* genome appears to encode a Zot toxin that theoretically could disrupt tight junctions (Ruby et al. 2005), *gfp*-labeled *V. fischeri* have not been observed escaping the epithelium-lined crypts of the light organ. The squid's active daily expulsion of most light-organ lumen contents provides a physical limitation on bacterial overgrowth otherwise breaching the crypt epithelium, and it would presumably also enrich for bacteria that have the ability to avoid being expelled. As described below, *V. fischeri* appears to have a variety of pili and adhesins that may have coevolved with the host for this purpose (Browne-Silva and Nishiguchi 2008; Ruby et al. 2005).

The daily cycle of regrowth of bacteria in the light organ may give the host an additional mechanism for maintaining the specificity of its symbionts. For example, by providing or withholding certain nutrients, the growth of *V. fischeri* may be favored over other bacteria that gain access to the light organ. Such a mechanism remains speculative, and it would be difficult if not impossible to test the growth rate of different bacterial species within the physiological parameters of the light organ in the absence of other (e.g., immunological) specificity factors. It seems unlikely, however, that this could be the major factor in maintenance of specificity: the contents of the light organ appear complex and include substrates (e.g., peptides and amino acids) that could support the growth of numerous other bacteria. Although iron appears to be limiting, and iron-uptake machinery may be an important colonization factor for *V. fischeri*, many other marine bacteria have similar iron-scavenging systems. Thus, nutrient control is likely, at best, a mechanism to enrich for ongoing *V. fischeri* colonization.

Instead, it seems likely that the major control over the symbiont population and specificity is exerted by the host immune system. Naturally, this topic has been an area of great research interest, and the role of the *E. scolopes* innate immune system in the symbiosis was recently reviewed (McFall-Ngai et al. 2010). Discovery and elucidation of *E. scolopes* immune functions have been accelerated by the generation of EST libraries (described above) that profile the host transcriptome (Chun et al. 2008) and the characterization of the host light-organ proteome (Schleicher and Nyholm 2011). For example, analysis of ESTs led to the discovery of components of an immunological complement system, followed by experimental determination that complement C3 protein is expressed on the apical surface of light-organ epithelial cells (Castillo et al. 2009). Research can now address whether the complement system helps direct immunological responses that maintain specificity or constrain *V. fischeri*. There is also evidence that the squid are capable of producing antimicrobial peptides (Nyholm and McFall-Ngai 2004), which may have an immune function that is either broad-spectrum or weighted toward the control of non-*V. fischeri*.

Among the potential antimicrobial components of the squid innate immune response, the most thoroughly studied to date have been reactive oxygen species (ROS). Recent analysis of the host proteome suggested a number of highly expressed proteins are involved in producing ROS (Schleicher and Nyholm 2011). For example, the host apparently encodes a number of putative ROS-generating peroxidases, including the halide peroxidase (HPO) described below. In the same study, it was found that symbiotic host and *V. fischeri* cells contained numerous putative antioxidant proteins. For example, one of the most abundant proteins in symbiotic *V. fischeri* cells was AhpC, a predicted alkyl hydroperoxide reductase (Schleicher and Nyholm 2011). These recent data are consistent with the longstanding hypothesis that oxidative stress is a hallmark of the light-organ environment, and they provide new targets for future investigations.

Interest in *E. scolopes* ROS production began with the discovery that the squid expresses HPO in the light organ, presumably producing the antimicrobial ROS hypochlorous acid (HOCL). Peroxidase-encoding transcripts were among the most abundant found in early cDNA libraries from *E. scolopes*, and the HPO transcript was among the first expressed genes discovered from the light organ (Tomarev et al. 1993). Further studies confirmed the biochemical similarity of the squid-encoded halide peroxidase to mammalian peroxidases, including production of hypohalous acid from halide ions and $H_2O_2$, as well as its presence in the light organ (Weis et al. 1996; Small and McFall-Ngai 1999). Given the high level of chloride ions in seawater, it seems likely that the main relevant product of HPO is HOCl, an effective broad-spectrum antimicrobial used commercially as a disinfectant. The HPO gene in *E. scolopes* is more highly expressed in tissues that are exposed to bacteria, including gills as well as the light organ (Small and McFall-Ngai 1999).

Like other bacteria, *V. fischeri* is sensitive to HOCl, suggesting that HPO represents a broad-spectrum antimicrobial that keeps bacteria in check rather than a light-organ-specific mechanism for selecting *V. fischeri*. Interestingly, recent studies show that HPO is localized in the host hemocyte cells (Heath-Heckman and McFall-Ngai 2011).

Given that $H_2O_2$ is a substrate for HPO, it seems likely that this ROS is produced in the light organ as well, perhaps via a host respiratory burst. Like many bacteria, *V. fischeri* encodes a catalase (KatA) that converts $H_2O_2$ to water and oxygen, but the periplasmic location of *V. fischeri* is unusual and suggests a key role in detoxifying $H_2O_2$ originating from an external source (Visick and Ruby 1998). The relatively high catalase activity in *V. fischeri* likewise points to a role beyond coping with endogenous metabolic production of $H_2O_2$ (Visick and Ruby 1998). A *V. fischeri katA* mutant was able to colonize the *E. scolopes* light organ to wild-type levels when presented as a clonal inoculum but was outcompeted by the wild type in mixed competitive infections, indicating that catalase contributes to, but is not required for, colonization of the host (Visick and Ruby 1998). Analysis of the *V. fischeri* genome suggests that while *katA* encodes the bacterium's only catalase, there are other mechanisms for coping with oxidative damage. For example, there are three methionine sulfoxide reductase genes that presumably repair proteins damaged by $H_2O_2$ (Flores and Stabb, unpublished data). Redundancy in the *V. fischeri* response to this and other ROS may explain why mutants lacking single oxidative-response enzymes are not more severely attenuated in colonization.

Another oxidant produced by *E. scolopes* is nitric oxide (NO). Both NO and NO synthases (NOS) are produced throughout light-organ tissues, and both are found in mucus secretions that contact *V. fischeri* symbionts (Davidson et al. 2004). Although both *V. fischeri* and other species aggregate in host-derived mucus on the surface of the light organ, *V. fischeri* is somehow enriched (Nyholm et al. 2000, 2002; Nyholm and McFall-Ngai 2003). NO-scavenging molecules increased the number of either *V. fischeri* or nonsymbionts in such aggregates on the light-organ surface (Davidson et al. 2004). Although nonnative bacteria were still unable to colonize the light-organ crypts, the results suggest that NO limits bacterial growth in the aggregates and could play some role in the enrichment seen at this stage (Davidson et al. 2004).

The transcriptional response of *V. fischeri* to NO was recently elucidated (Wang et al. 2010a), leading to intriguing discoveries and new research directions. For example, a heme-independent and NO-resistant alternative oxidase gene (*aox*) is controlled by the NO-responsive regulator NsrR and is upregulated in response to NO (Dunn et al. 2010). Aox allows aerobic respiration to continue when other oxidases in the cell are inhibited by NO, which may impart a competitive advantage to *V. fischeri* over the majority of other *Vibrio* species, which lack *aox* (Dunn et al. 2010; Spiro 2010). NO production also results in the H-NOX-dependent downregulation of iron acquisition (Wang et al. 2010a). While this response may not relate directly to NO resistance, it could reflect NO being used by *V. fischeri* as an indicator of other ROS it will soon encounter, specifically $H_2O_2$. By limiting iron uptake, symbionts might limit the oxidative stress generated by Fenton chemistry when $H_2O_2$ and iron are combined (Wang et al. 2010a). Before leaving the topic of NO, it should be noted that it may play an additional symbiotic role in addition to functioning as an antimicrobial oxidant: NO has signaling functions in many higher organisms, and as described below, it may be part of the morphogenic developmental program in the light organ stimulated by *V. fischeri* (Altura et al. 2011).

In addition to producing broadly antimicrobial molecules, *E. scolopes* has a cellular immune response involving hemocyte cells (*Fig. 20.5*). These immune cells may play a key role in maintaining the specificity of the interaction with *V. fischeri* (Nyholm and McFall-Ngai 1998; Nyholm et al. 2009). As is the case in other cephalopods, *E. scolopes* appears to have a single type of hemocyte, which circulates in the blood and moves throughout the animal. Like mammalian macrophages, these hemocytes can bind, engulf, and kill bacterial cells. *E. scolopes* hemocytes have been found in the blood as well as in the light-organ crypts and the sinuses of the ciliated epithelial appendages (*Fig. 20.2c*) (Koropatnick et al. 2004; Nyholm and McFall-Ngai 1998). Within the light-organ crypts of newly hatched juveniles, the hemocytes have been observed with internal bacteria, presumably engulfed; however, in adult animals, the hemocytes were seen surrounded by densely packed *V. fischeri* cells but had not phagocytosed them (Nyholm and McFall-Ngai 1998; Nyholm et al. 2009). These data suggested that the hemocyte cells can ignore *V. fischeri* and that this may be a trait acquired as the animals develop. When removed from the squid, the macrophage-like hemocytes bound and phagocytosed *V. fischeri* less frequently than they did other marine bacteria (Nyholm et al. 2009). Binding seemed to be the key rate-limiting step in this process, as bound *V. fischeri* were as likely to be phagocytosed as another bacterium (Nyholm et al. 2009).

In an interesting twist, Nyholm et al. (2009) also found that exposure to *V. fischeri* was critical for maintaining hemocyte specificity (Nyholm et al. 2009). When squid were cured of their *V. fischeri* symbionts with antibiotics, hemocytes from these symbiont-free animals became five times more effective at binding *V. fischeri* while their affinity for *V. harveyi* or *Vibrio parahaemolyticus* was unchanged (Nyholm et al. 2009). Moreover, hemocyte binding to *V. fischeri* was similarly high in hemocytes isolated from colonized or naïve animals when the target *V. fischeri* strain was a mutant lacking the major outer membrane protein OmpU (Nyholm et al. 2009). These data suggest that *E. scolopes* hemocytes learn to discriminate *V. fischeri* from other bacteria and adapt to preferentially bind nonsymbionts through an OmpU-dependent mechanism. Interestingly, it was recently reported that *Vibrio splendidus* OmpU mediates adhesion to and invasion of oyster hemocytes (Duperthuy et al. 2011). While the two OmpU-mediated phenomena seem opposite to each other, the underlying processes involved may reveal quite parallel mechanisms once they are understood.

Another observation of *E. scolopes* hemocytes that may have widespread importance is the discovery of chitin and endogenous chitin synthesis within these immune cells

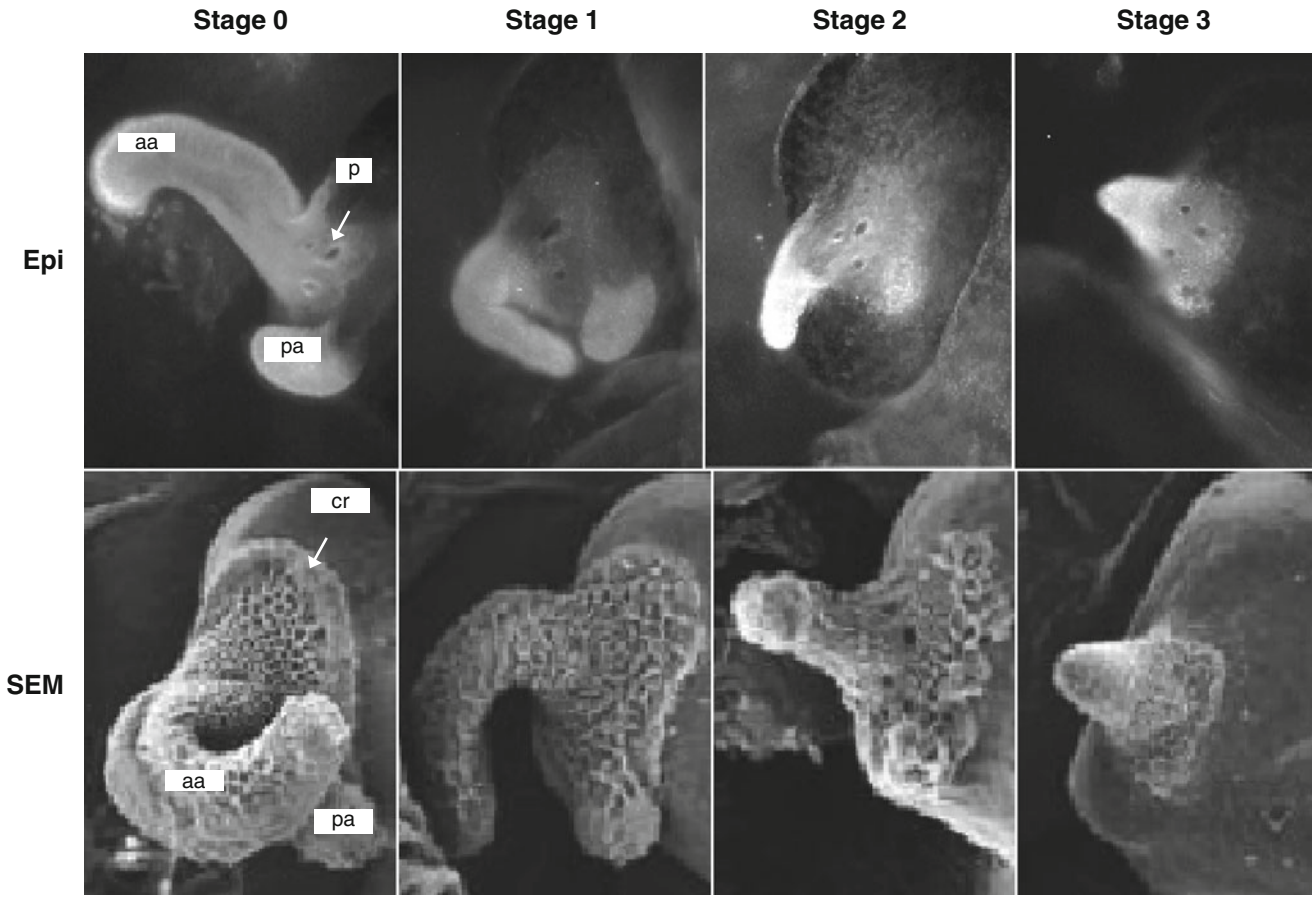


**Fig. 20.6**

**Development of the juvenile light organ. Epifluorescence (*Epi*) and scanning electron microscopy (*SEM*) images of the stages of regression of ciliated epithelial appendages. Upon exposure to peptidoglycan or symbiosis-competent *V. fischeri*, the ciliated appendages present on newly hatched squid (stage 0) undergo thinning (stage 1) and progressive shortening (stages 2 and 3), until they are lost (stage 4, not shown). *aa* anterior appendage, *pa* posterior appendage, *p* pore, and *cr* ciliated ridge (These images were previously published Adin et al. (2009))**

(Heath-Heckman and McFall-Ngai 2011). This was found to be a common property of invertebrate hemocytes that is lacking in their vertebrate counterparts (Heath-Heckman and McFall-Ngai 2011). *V. fischeri* can metabolize chitin and ferment its *N*-acetylglucosamine monomers, but it remains to be determined what, if any, role hemocyte-derived chitin plays in *E. scolopes* immunity or support of *V. fischeri* growth. From multiple perspectives, the biology of *E. scolopes* hemocytes appears worthy of further investigation likely to reveal elements unique to the *V. fischeri*-*E. scolopes* symbiosis as well as phenomena more broadly applicable to invertebrate-bacteria interactions.

## Host Development and Bacterial Signals

### Host Development

*V. fischeri* is the lone bacterial species colonizing the *E. scolopes* light organ, and infection by *V. fischeri* is required to trigger developmental changes in the host, some of which can be mimicked using bacterially derived molecules. If kept in water free of *V. fischeri*, *E. scolopes* can be raised to adulthood without the light organ becoming infected or bioluminescent (Hanlon et al. 1997). Such aposymbiotic animals are healthy and develop normally in most respects, including the lens and reflective tissue of the light organ (Claes and Dunlap 2000). However, specific developmental events fail to occur in the absence of *V. fischeri* infection, including, most dramatically, the regression of the ciliated fields on the light organ. At the time of hatching, the ciliated fields begin shedding mucus, which as described above helps facilitate infection; however, once the light organ is infected, mucus shedding ceases (Nyholm et al. 2002), and this cessation is followed by regression of the structures themselves. Over the course of 4–5 days postinfection, these infection-promoting structures are completely lost in infected animals (*Fig. 20.6*) (Montgomery and McFall-Ngai 1994; Foster and McFall-Ngai 1998; McFall-Ngai and Ruby 1991). This morphological change is accompanied by apoptosis in the epithelial cells of the ciliated fields and infiltration of host hemocytes into the sinuses of the ciliated appendages (Koropatnick et al. 2004,

2007). None of these developmental events take place in aposymbiotic animals.

*V. fischeri* also triggers more subtle developmental effects in the light organ. For example, the ducts leading from the light-organ surface to the crypts constrict (Kimbell and McFall-Ngai 2004), and the cells lining the duct become more homogenous and filled with inclusions (Claes and Dunlap 2000). The epithelial cells lining the crypts swell (Montgomery and McFall-Ngai 1994) with a proliferation of microvilli on their surfaces (Lamarcq and McFall-Ngai 1998), and there is an apparent increase in mucus secretion inside the crypts themselves (Nyholm et al. 2002). Additional *V. fischeri*-dependent molecular events, including a downregulation of NO synthase (Davidson et al. 2004), have been observed but not yet clearly linked to developmental and physiological processes (Doino Lemus and McFall-Ngai 2000; Kimbell and McFall-Ngai 2003; Chun et al. 2008). Many of these developmental events triggered by *V. fischeri*, as well as their timing, were reviewed by Nyholm and McFall-Ngai (Nyholm and McFall-Ngai 2004).

In general, most symbiont-induced developmental changes in the *E. scolopes* light organ can be rationalized in terms of this organ's two temporally distinct functions—first, to become infected with *V. fischeri* and then later to support and control symbiotic bioluminescence. For newly hatched aposymbiotic squid, acquiring symbionts from a dilute environment is a numerically daunting task that is facilitated by the ciliated cells and the mucus they secrete (Nyholm et al. 2000, 2002). However, once the squid are colonized by appropriate *V. fischeri* symbionts, the infection-promoting properties of the ciliated fields become unnecessary and could perhaps be a liability if pathogenic infections were facilitated. Thus, programmed cell death and regression of the ciliated fields and constriction of the ducts may serve to prevent further infection beyond the initial colonization with a mutualistic *V. fischeri* symbiont. The developmental events inside the crypts, cell swelling and microvillar proliferation, may serve to increase the surface area at the symbiont-host interface and promote the efficient exchange of metabolites.

The symbiont-triggered developmental events in *E. scolopes* appear to result from multiple distinct signaling pathways. Some changes are reversible if *E. scolopes* is cured of its symbionts with antibiotics (Lamarcq and McFall-Ngai 1998; Nyholm et al. 2002), whereas other events cannot be stopped once they are set in motion (Doino and McFall-Ngai 1995). For example, the swelling of crypt epithelial cells can be reversed by curing the symbionts, and microvillar proliferation on these cells does not progress and may even reverse somewhat if symbionts are cured (Lamarcq and McFall-Ngai 1998). In contrast, regression of the ciliated epithelial fields does not require persistent colonization after about 12 h postinoculation (Doino and McFall-Ngai 1995). Regression proceeds in animals that have been cured of symbionts, and the ciliated epithelial structures do not grow back. Thus, it seems that the programmed loss of infection-promoting structures is set in motion early during infection and does not require constant colonization. In one exception to this, the infection-promoting mucus secretion of the ciliated structures does reappear in animals that are infected and then cured of *V. fischeri* (Nyholm et al. 2002).

Taken together, the results of several early experiments indicate a complex pattern of light-organ development that includes symbiont-dependent and symbiont-independent programs, some of which require only transient exposure to *V. fischeri*. Moreover, although Claes and Dunlap (2000) noted that the tissues developmentally affected by *V. fischeri* all come in contact with symbiotic cells, at least some developmental events appear to involve symbiont induction remotely. Specifically, the ciliated fields on the light-organ surface are exposed to *V. fischeri*, but mutants unable to colonize the crypts still contact these cells on the surface of the light organ without triggering their regression. Once the crypts become packed with *V. fischeri* cells, regression of the ciliated appendages advances, even though symbionts in the crypts are several cell layers away.

## Bacterial Signals That Influence Host Development

The observation that infection with *V. fischeri* triggers developmental programs and morphological changes in the *E. scolopes* light organ has prompted interest in understanding the specific mechanisms and bacterial signals involved in these processes. Most research has focused on the involvement of three bacterial factors in stimulating host development: bioluminescence, LPS, and PG. Both LPS and PG can be categorized as microbe-associated molecular patterns (MAMPs), and they have intriguingly parallel roles in several host-microbe interactions, both pathogenic and mutualistic. MAMPs are relatively conserved among bacteria, and hosts ranging from plants to animals have evolved mechanisms for MAMP recognition. Although the signaling roles of LPS, PG, and bioluminescence were discovered separately, and they each have distinct influences on the host, their effects appear intertwined and difficult to deconvolute. This is well illustrated by their combined influence on the developmental program associated with regression of the ciliated fields.

It was first discovered that LPS from the bacteria triggers apoptosis in the ciliated field but not regression of this structure (Foster et al. 2000). Specifically, the lipid A moiety of LPS had this effect—an intriguing finding, given that lipid A triggers responses in other host-microbe systems. Lipid A from *V. fischeri* has several modifications including a novel acylated phosphoglycerol moiety (Phillips et al. 2011) which could contribute to specificity, and at least one of the lipid A modifying enzymes, designated HtrB1, appears to contribute to colonization efficiency early in infection (Adin et al. 2008a). Moreover, a mutant of *V. fischeri* defective for the response regulator GacA has an altered LPS structure and is impaired in stimulating apoptosis and regression of the ciliated appendages on the light organ (Whistler et al. 2007). Apoptosis in the ciliated fields of the light organ does not specifically require *V. fischeri* lipid A, as LPS and lipid A from other bacterial species will also elicit apoptosis in these cells; however, the structure of lipid A does

appear to influence its bioactivity in such assays (Foster et al. 2000). It is tempting to speculate that a distinctive lipid A structure is recognized by a host receptor(s), but it should be kept in mind that in natural infections, alterations in LPS structure could also influence colonization levels and membrane integrity, thereby affecting how much lipid A is presented to the host. Thus, structural elements of lipid A could affect either direct interactions with a host receptor or the amount of lipid A presented to host receptors.

In reporting the effects of LPS on the ciliated fields, Foster et al. (2000) noted that there must be at least one other signal and suggested potential candidates. One of these, PG, proved to be a second critical MAMP. PG stimulates mucus shedding by the ciliated epithelial fields (Nyholm et al. 2002), and it dramatically affects morphogenesis and regression of the ciliated fields (Koropatnick et al. 2004). Interestingly, it was also discovered that *V. fischeri* sheds a particular PG monomer that is usually recycled and kept within cells (Koropatnick et al. 2004). In two other Gram-negative bacteria, *Bordetella pertussis* and *Neisseria gonorrhoeae*, the same molecule is released from cells, and in each case, this molecule affects ciliated host cells. Indeed, this PG monomer is called tracheal cytotoxin (TCT), because it triggers the death of ciliated host airway cells in *Bordetella* infections, giving rise to its "whooping cough" symptoms. Although *V. fischeri* appears capable of TCT recycling, the combined activity of lytic transglycosylases apparently results in relatively large amounts of free TCT being released from cells (Adin et al. 2009). Using a mutant with decreased TCT release, Adin et al. (2009) provided evidence that the advantage of TCT shedding for *V. fischeri* may be that by triggering regression of the infection-promoting ciliated fields, an infecting strain thereby minimizes the chances of competition from later infecting *V. fischeri* strains.

Developmental changes appear to require the combined effect of the TCT and LPS signals. Koropatnick et al. (2004) found that TCT could stimulate hemocyte trafficking into the sinuses of the ciliated appendages as well as their regression, but curiously did not cause apoptosis in these cells. When TCT or PG was combined with LPS, however, the two had a synergistic effect on apoptosis and regression of the ciliated appendages that was very similar to that of a natural symbiotic infection (Koropatnick et al. 2004). Similarly, TCT and LPS together, but not singly, led to a decrease in NOS activity and NO similar to that seen in animals infected with *V. fischeri* (Altura et al. 2011). Interestingly, experiments with NOS inhibitors and NO donors suggested that NO itself may be an additional key signaling molecule in the apoptosis and morphogenesis associated with TCT and LPS exposure (Altura et al. 2011). Such two- or three-part signals may contribute to the specific recognition of the correct symbiont species.

Somewhat in contrast to these synergistic effects of LPS and TCT, one recent study suggested that TCT alone can in fact trigger apoptosis in the absence of LPS (Troll et al. 2009a). One explanation for this apparent discrepancy may be that assays measuring different stages of apoptosis were used in these two studies. TCT alone may stimulate initial elements of the apoptotic developmental program, but the combined effect of TCT and LPS likely represents the genuine symbiotic signal in a natural infection.

The recognition of MAMPs, including PG and LPS, by various hosts has been the subject of many studies, and homologs of known MAMP receptors and MAMP-responsive proteins have been found in *E. scolopes*. These MAMP-associated host genes include components of the NF-κB pathway (Goodson et al. 2005), specific PG recognition proteins (PGRPs) (Troll et al. 2009a, b), and LPS-binding proteins (Krasity et al. 2011). Although these MAMP-associated genes and proteins are orthologs of those in other organisms, they appear to have novel functions in *E. scolopes*. Notably, EsPGRP1 has an unprecedented nuclear localization, and infection by *V. fischeri* or treatment with TCT triggers loss of EsPGRP1 from host nuclei (Troll et al. 2009a). In contrast, EsPGRP2 is exported from host cells in association with light-organ mucus shedding; although similar to the dynamics of EsPGRP1, the export of EsPGRP2 is stimulated by TCT or infection with *V. fischeri* (Troll et al. 2009b). EsPGRP2 has an amidase activity capable of degrading TCT, and it is exported into the light-organ crypts colonized by *V. fischeri*, leading to the suggestion that it may attenuate this potentially toxic bacterial MAMP signal after it is received (Troll et al. 2009b).

The combined effects of TCT and LPS mimic many aspects of natural *V. fischeri* infection but as Troll et al. noted (2009a), they fail to completely duplicate it. An intriguing third signal may be the bioluminescence produced by *V. fischeri*. The squid appear capable of perceiving bioluminescence in the light organ, and components of photoreceptor-mediated signaling are present in light-organ tissue (Tong et al. 2009). Moreover, the host transcriptional profile varies depending on whether or not *V. fischeri* symbionts are bioluminescent (Chun et al. 2008). Although it is difficult to know to what extent these transcriptional changes are due to the perception of light itself or some other physiological change in dark symbionts related to the lack of luciferase activity, the presence of photoreceptors and the importance for the animal to match the intensity of its light-organ luminescence to the environment make a compelling case for light itself being perceived by the host. In any case, regression of the ciliated fields, export of EsPGRP2, and trafficking of hemocytes to the ciliated appendages early in infection are all attenuated relative to wild-type infections when squid are infected by dark mutants (McFall-Ngai et al. 2011). These differences are apparent even during infection initiation when the dark mutant presumably is colonizing at similar levels as the parent (Visick et al. 2000). Interestingly, among the host genes differentially regulated in wild-type and dark mutant infections are the PG recognition protein EsPGRP1 and a putative LPS-binding protein (LBP1) (McFall-Ngai et al. 2011). Light production induces transcription of the genes for these predicted MAMP receptors, as demonstrated by four- to fivefold lower levels of these transcripts in a (*luxA*) luminescence mutant (Chun et al. 2008). These data suggest that light production signals the host to boost production of receptors for PG and LPS.

Some of the developmental events occurring within the light organ and triggered by *V. fischeri* are not as well understood as those described above. In particular, changes in the morphology of epithelial cells lining the crypts and ducts, as well as proliferation of microvilli, have not been studied to the same extent as the developmental program associated with regression of the ciliated fields. This research focus probably reflects the relative difficulty in assaying changes in crypt and duct structure, which experimentally usually involves fixation, sectioning, and observation by TEM. It is known that crypt epithelial cell swelling requires *V. fischeri* to be bioluminescent (Visick et al. 2000), but microvillar proliferation, which is another process that can be reversed by curing symbionts, is unaffected by luminescence (Lamarcq and McFall-Ngai 1998). The swelling of epithelial cells resembles a response to hypoxic stress and could be tied to ongoing oxygen consumption by bioluminescence. Cell swelling could also represent a developmental response of the animal to light itself or to metabolic products related to the physiology of bioluminescence. Similarly, the proliferation of microvilli may relate to metabolic exchange. Although this is purely speculative, it would be consistent with the requirement for metabolically active symbionts to trigger and maintain an effect.

## Bacterial Genes and Phenotypes Involved in Colonization

### Bioluminescence and Pheromone-Dependent Regulation

As noted above, bioluminescence is the central contribution *V. fischeri* makes to this symbiosis (*Fig. 20.7a, b*), and it appears intimately involved in the host response to the bacteria as well. *V. fischeri* has long been a model for studying bacterial bioluminescence, and the biochemistry, genetics, and regulation of light production, as well as the symbiotic role of bioluminescence, have all been active research topics. These areas are interrelated, and together their study has had a widespread influence on our understanding of bacterial gene regulation and host-bacterium interactions. For example, luminescence demands a large energetic commitment, which explains why the process is tightly regulated, and control of luminescence is accomplished in part by a pheromone-mediated regulatory pathway, which has become an archetype for understanding similar regulatory mechanisms in numerous host-associated bacteria.

*V. fischeri* produces light when luciferase (LuxA and LuxB) converts $FMNH_2$, in the presence of $O_2$, and an aliphatic aldehyde (RCHO) to FMN, releasing water and an aliphatic acid (*Fig. 20.7c*) (Hastings and Nealson 1977; Tu and Mager 1995; Ziegler and Baldwin 1981). LuxD produces RCHO, which is also generated by LuxC and LuxE through recycling of the corresponding aliphatic acid (Boylan et al. 1989). LuxG and other enzymes re-reduce FMN (Zenno and Saigo 1994; Nijvipakul et al. 2008). The obvious energetic costs of this process have stimulated curiosity in its regulation and usefulness for the bacteria. These costs include Lux protein biosynthesis [LuxAB can comprise 5% of the protein in bright cells (Hastings et al. 1965)], ATP hydrolysis during RCHO recycling, and the consumption of oxygen and reducing equivalents, which theoretically competes with energy recovery from aerobic respiration.

Primarily for energetic reasons, it has long been postulated that bioluminescence should slow the growth of *V. fischeri*. Consistent with this idea, some researchers found luminescence to be negatively correlated with growth in culture (Keynan and Hastings 1961; Hastings and Nealson 1977; Dunlap et al. 1995; Pooley et al. 2004), although the undefined nature of the luminescence mutants in these experiments raised some doubts as to whether luminescence was the only variable. Recently, however, it was shown that defined mutants lacking *luxCDABEG* could outgrow an isogenic parent under certain culture conditions, such as in a carbon-limited chemostat (Bose et al. 2008).

In contrast, bioluminescence is beneficial for *V. fischeri* colonizing the *E. scolopes* light organ. To begin with, the advantage of luminescence for the host (e.g., antipredatory camouflage) would confer a fitness advantage on the bacteria, because as noted above, *V. fischeri* populations appear augmented by successful symbiosis with the squid. However, the advantage for *V. fischeri* of expressing bioluminescence in the symbiosis goes deeper, as luminescence actually promotes successful colonization (Visick et al. 2000; Bose et al. 2008). Mutants lacking bioluminescence are able to colonize the host, but do not persist as well as their parental strains.

Several explanations have been proposed for how bioluminescence might aid bacteria directly (Stabb 2005). For example, it was proposed that bioluminescence stimulates photolyase-mediated DNA repair (Czyz et al. 2003); however, photolyase mutants are unimpaired in colonizing *E. scolopes* (Walker et al. 2006). As noted above, the host can apparently detect and respond to luminescence in its light organ (Chun et al. 2008; Tong et al. 2009), and it has been suggested that the squid might impose sanctions on dark infections, thereby ensuring that it receives bioluminescence in exchange for the nutrients it provides to *V. fischeri*. Other hypotheses have posited that the real function of luminescence is either to burn oxygen or to consume excess reductant (Bourgois et al. 2001; Visick et al. 2000). The latter idea now appears inconsistent with the regulation of luminescence (Bose et al. 2007; Lyell et al. 2010), but the model that luminescence confers an advantage by consuming oxygen is intriguing: by consuming oxygen, *V. fischeri* might generate hypoxic stress in the nearby host epithelium, attenuate ROS generation by the host, or render itself more resistant to ROS. Luciferase's high affinity for $O_2$ (Km = ~35 nM) is consistent with a role in driving down ambient or intracellular oxygen levels (Bourgois et al. 2001). The ideas that the host sanctions dark infections or that the light-organ environment somehow makes bioluminescence physiologically beneficial are also both plausible and are not mutually exclusive.

The *lux* genes underlying light production are regulated tightly and induced in the symbiosis. Key enzymes and regulators for light production in *V. fischeri* are encoded in an 8-kb DNA locus (Engebrecht et al. 1983; Engebrecht and Silverman 1984)

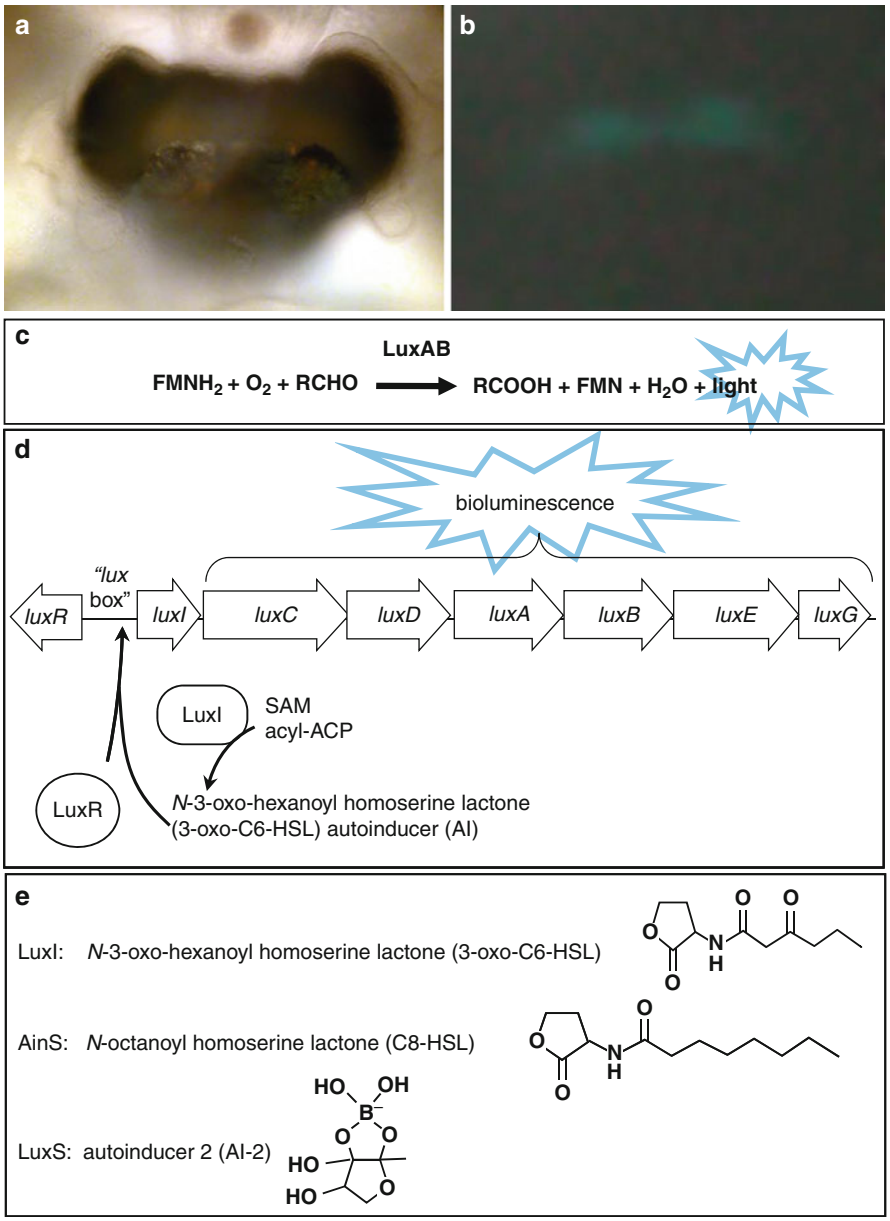


**Fig. 20.7**

**Bioluminescence control in *V. fischeri*. (a and b) Images of the same juvenile light organ taken with light and in the dark, respectively; the light emitted by the bacteria within the organ can be seen in the *center* of the image taken in the dark. (c) The substrates and products in the reaction carried out by luciferase (LuxAB) to produce light. (d) The *lux* operon and the control of *lux* transcription by AI-modified LuxR. (e) The structures of the three pheromones produced in *V. fischeri* by the three pheromone synthases LuxI, AinS, and LuxS. Panels a and b have been published previously (Stabb 2005)**

including the *luxICDABEG* operon and the divergently transcribed *luxR* gene (*Fig. 20.7d*). *luxCDABEG* encodes the enzymes for luminescence, while *luxI* encodes a pheromone synthase and *luxR* encodes a corresponding pheromone receptor. Specifically, LuxI generates *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) (*Fig. 20.7e*) (Schaefer et al. 1996), a pheromone that diffuses across the cell membrane (Kaplan and Greenberg 1985). As cell density increases, 3-oxo-C6-HSL accumulates until it reaches a critical concentration where it can bind LuxR, producing an activator that binds a "*lux* box" sequence to activate transcription of *luxICDABEG* through interactions with RNA polymerase that compensate for a poor -35 promoter element (Egland and Greenberg 1999; Fuqua et al. 1994). The result is that the *lux* genes are most highly expressed at high cell densities, when the bacteria have reached a "quorum" (Fuqua et al. 1996). Moreover, like many bacterial pheromone-mediated regulatory systems, the *lux* operon constitutes a positive feedback circuit, because the 3-oxo-C6-HSL produced by LuxI leads to increased transcription of *luxICDABEG*. Consequently, environmental regulatory inputs

to the *lux* system can be amplified, and in theory, the response could spread through a population.

It has now become clear that pheromone-mediated regulation of bioluminescence in *V. fischeri* is quite a bit more complex than this canonical model. Two other pheromones also modulate the *lux* genes (Fig. 20.7e); *N*-octanoyl homoserine lactone (C8-HSL) produced by AinS (Hanzelka et al. 1999; Kuo et al. 1994, 1996) and "AI-2," which is produced by LuxS (Lupp and Ruby 2004) and is presumably a furanosyl borate diester as it is in *Vibrio harveyi* (Chen et al. 2002). AI-2 and C8-HSL apparently function through distinct receptors that both act via LuxU and LuxO, Hfq, and a small RNA called Qrr to increase levels of LitR, which in turn increases *luxR* expression (Fidopiastis et al. 2002; Lupp et al. 2003; Miyamoto et al. 2000; Miyashiro et al. 2010). C8-HSL can also bind to and activate LuxR, although it is less effective than 3-oxo-C6-HSL and can even inhibit 3-oxo-C6-HSL-mediated stimulation (Egland and Greenberg 2000; Schaefer et al. 1996). As with LuxI and LuxR, there is also positive feedback in the AinS-AinR system (Lupp and Ruby 2004). These interconnected regulatory systems were reviewed recently elsewhere (Stabb et al. 2008).

3-oxo-C6-HSL, C8-HSL, and AI-2 all increase in concentration with increasing cell density, and textbook models of pheromone-mediated regulation typically depict these pheromones as a mechanism of cell-density-dependent regulation called quorum sensing. However, in *V. fischeri* and many other systems, it is clear that pheromone-mediated signaling is not simply a census-taking process (Dunn and Stabb 2007). Both the pheromone synthases and the pheromone receptors are also regulated in response to environmental conditions, such that high cell density may be necessary to elicit a behavior, but a "quorum" is typically not sufficient for a full response unless other conditions are met.

Context dependence of pheromone-mediated signaling is obvious in *V. fischeri* strains isolated from the light organs of *E. scolopes*. Such strains induce pheromone synthesis and luminescence in the host light organ, but they are dim and produce less pheromone in culture (Boettcher and Ruby 1990; Lee and Ruby 1994a), even at equivalent cell densities (Stabb, unpublished data). Such context-dependent *lux* expression was evident even in comparisons of different light-organ microenvironments: when *V. fischeri* was marked with both a constitutive red fluorescent protein gene and a *luxI* promoter-*gfp* reporter, dense populations of bacteria appeared in the three distinct light-organ crypt types in a similar time frame, but *lux* induction lagged in crypt 3 (Dunn et al. 2006), which is also the last crypt to mature developmentally (Montgomery and McFall-Ngai 1993). These data are consistent with induction of luminescence by a host-derived environmental cue initially absent in crypt 3. Clearly, high cell density may be necessary for pheromone-mediated induction of luminescence in *V. fischeri*, but it is not sufficient.

Bioluminescence and pheromone production by *V. fischeri* respond to the environment; however, the underlying regulatory mechanisms are still being elucidated. This is particularly true for *V. fischeri* strain ES114, which serves as a model strain typical of other *E. scolopes* symbionts (Boettcher and Ruby 1990; Lee and Ruby 1994a). Most early studies of *lux* regulation used either bright *V. fischeri* strains such as MJ1, which was isolated from a pinecone fish (Ruby and Nealson 1976), or the *lux* region cloned in *Escherichia coli*. The core circuitry of *lux* in ES114 is similar to that of MJ1 (Gray and Greenberg 1992b; Gray and Greenberg 1992a), but like most isolates from *E. scolopes*, ES114 is dim in culture.

With the caveat that there could be strain-specific differences, environmental control of *lux* in *V. fischeri* has been studied with respect to different regulators. For example, cAMP receptor protein (CRP)-mediated activation of *lux* was documented using transgenic *lux*-containing *E. coli* (Dunlap and Greenberg 1985, 1988; Dunlap and Ray 1989), and it was similarly shown that *V. fischeri* strain MJ1 regulates luminescence in response to glucose, a phenomenon that may be CRP-mediated (Friedrich and Greenberg 1983). Luminescence of MJ1 is also inhibited by iron (Haygood and Nealson 1985).

Recently, the redox-responsive ArcA/ArcB two-component regulatory system was identified as a strong repressor of luminescence (Bose et al. 2007; Lyell et al. 2010). Both *arcA* and *arcB* mutants are ~500-fold brighter in culture than ES114, and activated ArcA binds the *luxI* promoter near the "*lux* box" (Bose et al. 2007). In *E. coli*, the Arc system is activated by reducing conditions, and consistent with this mechanism, luminescence is induced by relatively oxidizing conditions (Bose et al. 2007; Stabb et al. 2008). Interestingly, however, ArcA/ArcB does not significantly repress luminescence during symbiotic colonization, and *arcA* mutants achieve nearly the same brightness in culture as wild-type cells do in the host. Thus, regulation by ArcA/ArcB could account for most of the differences in luminescence observed between cultured and symbiotic cells by invoking a model in which Arc represses *lux* in culture, but luminescence is derepressed by inactivation of Arc during initial infection (Bose et al. 2007). On the other hand, the ArcA/ArcB system could be active and repressing *lux* during initiation of the symbiosis but simply overpowered by another regulatory effect.

Other genes affecting luminescence in *V. fischeri* have been reported (Hussa et al. 2007; Visick et al. 2007; Whistler and Ruby 2003). Recently, a mutant screen revealed numerous loci involved in regulation of the *luxICDABEG* operon, either directly or indirectly (Lyell et al. 2010). That study also revealed environmental conditions that influence luminescence of wild-type *V. fischeri*. For example, mutants lacking a phosphate-uptake system or *phoQ* were brighter than wild type, leading to the demonstration that phosphate or $Mg^{2+}$ levels can influence luminescence of wild-type cells (Lyell et al. 2010). Remaining challenges include determining which environmental conditions that influence luminescence in culture also impact symbiotic luminescence and whether information about environmental conditions can be communicated among *V. fischeri* cells using pheromone signaling, as the results thus far would seem to indicate.

It should also be noted that luminescence is just one part of the pheromone-controlled regulon in *V. fischeri*, and the role(s) of other genes co-regulated by C8-HSL, 3-oxo-C-HSL, and AI-2 along with luminescence should be enlightening. LuxR controls

several genes besides the *luxICDABEG* operon (Antunes et al. 2007, 2008; Callahan and Dunlap 2000), and some of the corresponding proteins were identified in the proteome of symbiotic *V. fischeri* (Schleicher and Nyholm 2011). Especially noteworthy is the novel protein QsrP, which has no known function but is induced by LuxR and was among the most abundant proteins in symbionts (Schleicher and Nyholm 2011). Visick et al. (2000) demonstrated that the colonization deficiency of *luxR* mutants could be overcome by expressing *luxCDABEG* independently of this activator, suggesting that bioluminescence is the major (or only) LuxR-dependent colonization factor; however, other LuxR-regulated genes could have subtle influences on the symbiosis or major effects on symbiotic colonization but subtle degrees of LuxR-mediated regulation. Also, C8-HSL and AI-2 influence the expression of more genes than *luxR*, and the AinS/AinR regulon in particular is distinct from that of LuxR and important in host colonization (Lupp and Ruby 2004, 2005). Understanding the interplay of these three pheromone systems in the light-organ symbiosis, their control by the symbiotic environment, the dynamics of their signaling, and the constituents of their respective regulons should be a rich area for further research.

## Biofilm Formation and Biofilm Regulators

Studies of the initiation of symbiotic colonization revealed that an early stage involves the attachment of *V. fischeri* to the surface of the light organ in a biofilm-like aggregate (Fig. 20.2d). Subsequent studies began to probe the mechanism of that attachment. In particular, a search for genes important in the symbiosis revealed a requirement for *rscS* (regulatory of symbiotic colonization, sensor) (Visick and Skoufos 2001), which encodes a hybrid sensor kinase protein, and *syp* (Yip et al. 2005), an 18-gene symbiosis polysaccharide locus. It was subsequently determined that these genes are necessary both for the production of biofilms in culture and for the formation of the symbiotic aggregate (Yip et al. 2006) (Fig. 20.8).

Mutants lacking RscS are defective in initiating symbiotic colonization: most animals inoculated with this mutant remain uncolonized, while others exhibit a delay in initiating colonization (Visick and Skoufos 2001). This defect can be attributed to the inability of this mutant to aggregate on the surface of the light organ (Yip et al. 2006). *rscS* encodes a sensor kinase protein, a fact that immediately suggested a model for its function in symbiosis. Sensor kinases work by sensing an environmental signal, autophosphorylating on a conserved histidine residue, and serving as a phospho-donor to a downstream response regulator. The activated response regulator then carries out a response, most commonly acting as a transcription factor to increase or decrease transcription of genes. Although in many cases the genes for a sensor kinase/response regulator pair are physically linked in the chromosome, this was not the case for *rscS*. Subsequent studies revealed that RscS acts upstream of the response regulator SypG to control *syp* transcription and biofilm formation (Hussa et al. 2008).

Mutants defective for *syp* also exhibit defects in initiating symbiotic colonization. The *syp* locus comprises 18 genes (*sypA-R*), four of which encode regulatory proteins, while the remaining genes in the cluster encode proteins with predicted functions in polysaccharide biosynthesis, modification, and export (Yip et al. 2005). The genes in the *syp* locus are grouped into at least four operons (Fig. 20.8), each of which contains promoter sequences for transcription by RNA polymerase carrying the alternative sigma factor $\sigma^{54}$. Transcription from such promoters depends upon binding and activation by a $\sigma^{54}$-dependent transcriptional activator, which typically binds upstream of the promoter and provides the energy for open complex formation (Buck et al. 2000). Indeed, a conserved sequence located upstream of each of the *syp* promoters has been identified and may serve as the binding site for this key activator. The response regulator SypG is likely to serve as the activator for transcription of the *syp* locus, as this protein contains a putative $\sigma^{54}$ interaction domain in addition to its N-terminal receiver (REC) domain (containing the site of phosphorylation) and C-terminal DNA-binding domain. In support of this idea, overexpression of SypG promotes *syp* transcription in a manner that depends upon the *rpoN* gene, which encodes $\sigma^{54}$ (Yip et al. 2005).

The similarity in the symbiotic phenotypes of *rscS* and *syp* mutants led to the hypothesis that these proteins work in the same pathway, specifically, that RscS controls transcription of the *syp* locus. Indeed, overexpression of RscS led to an increase in *syp* transcription, and this induction depended on SypG (Fig. 20.8) (Hussa et al. 2008; Yip et al. 2006). Excitingly, overexpression of RscS also led to a number of unusual biofilm phenotypes (Yip et al. 2006). Whereas wild-type cells form smooth colonies on complex solid media, RscS-overexpressing cells form colonies with a wrinkled morphology (i.e., with 3D architecture), consistent with biofilm formation. In addition, RscS-overexpressing cells form a biofilm pellicle at the air-liquid interface during static growth in a minimal medium, while control cells do not. These phenotypes also depend upon SypG. Finally, consistent with the idea that RscS and Syp contribute to colonization through their role in symbiotic aggregate formation, overexpression of RscS induces the formation of very large aggregates on the light-organ surface (Fig. 20.8). Increased aggregate formation depended upon an intact *syp* locus: mutation of a representative *syp* gene, *sypN*, which encodes a putative glycosyltransferase predicted to be involved in the production of a polysaccharide, disrupted aggregate formation (Yip et al. 2006). The formation of this large aggregate is not detrimental to colonization; rather, RscS-overexpressing cells fully outcompete control cells containing an empty vector for symbiotic colonization.

The *syp* locus is controlled by several regulators besides RscS and SypG. Currently, the best understood of these additional regulators is SypE, a second response regulator encoded within the *syp* locus. SypE is an unusual response regulator in that its REC domain, containing the putative site of phosphorylation, is centrally located, rather than localized to the N-terminus as is typical of other response regulators. The REC domain is flanked

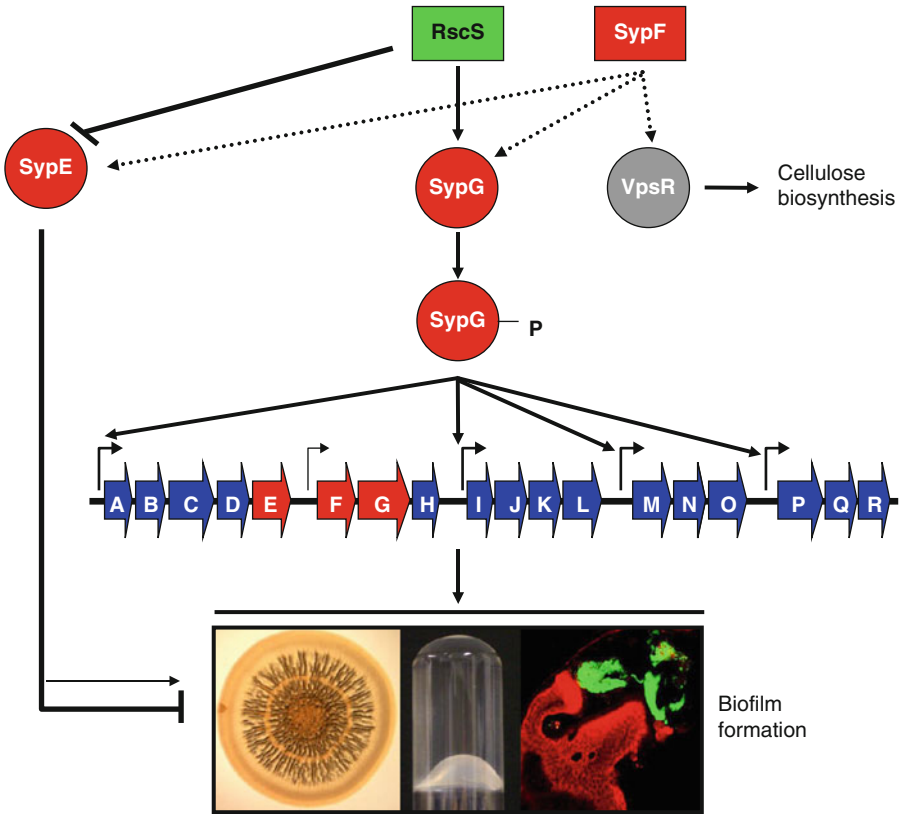


**Fig. 20.8**

**Control over biofilm formation by the *syp* regulators. The activities of two sensor kinases, RscS and SypF, influence *syp*-dependent biofilm formation. Sensor kinases respond to an environmental signal by autophosphorylating and donating a phosphoryl group to a downstream response regulator. In the case of the *syp* locus, phosphorylated SypG is predicted to activate transcription from four promoters, resulting in the production of a Syp-produced polysaccharide that promotes biofilm formation. Biofilm formation can be visualized in the laboratory by the development of wrinkled colonies and the production of pellicles at the air/liquid interface of static cultures (which can be strong enough to retain liquid when the test tube is inverted), and in symbiosis by the production of large aggregates on the surface of the light organ. RscS also appears to inactivate SypE, a response regulator that exerts a minor positive and a strong negative impact on biofilm formation. The role of SypF is poorly understood, but it appears to act upstream of SypE/SypG and VpsR to impact *syp*-dependent biofilm formation and cellulose biosynthesis, respectively (This model, which was previously published (Visick 2009), has been slightly altered by the addition of an arrow extending from SypE to biofilm formation, indicating the slight positive effect exerted by SypE on biofilm formation (Morris et al. 2011))**

by putative serine kinase (N-terminus) and serine phosphatase (C-terminus) domains (Morris and Visick 2010). SypE appears to act as both an inhibitor and an activator of biofilm formation, depending on the conditions used to promote biofilm formation (overexpression of RscS or SypG) (Hussa et al. 2008). Such phenotypes are consistent with opposing activities for the terminal domains. Indeed, it is now known that the putative serine kinase domain inhibits biofilm formation, while the putative serine phosphatase activates it (Morris et al. 2011).

These data led to a model in which RscS, in addition to activating SypG to promote *syp* transcription, inactivates SypE to permit biofilm formation. Given the nature of these proteins, Morris et al. (2011) predicted that RscS promotes phosphorylation of SypE, thereby inactivating it. Consistent with that prediction, a substitution of alanine for aspartate at the predicted site of phosphorylation results in a protein with constitutive inhibitory activity, presumably because it is unable to be phosphorylated (Morris et al. 2011). Interestingly, a *sypE* mutant does not exhibit a colonization defect, perhaps due to SypE's subtle positive role in biofilm formation. However, expression of the SypE mutant protein that cannot be phosphorylated and thus inactivated causes a severe defect in symbiotic colonization. Therefore, it appears that SypE inhibits symbiotic colonization and must be inactivated to permit colonization, presumably by RscS. The symbiotic defect of an *rscS* mutant could be attributed, in part, to its failure to inactivate SypE. Consistent with this model, an *rscS sypE* mutant colonized better than an *rscS* mutant. Thus, productive symbiosis depends upon inactivation of a negative regulator.

Another regulator encoded by the *syp* locus is SypF. Neither insertional disruption of *sypF* nor overexpression of wild-type SypF impacted biofilm formation (Darnell et al. 2008). However, overexpression of an allele with increased activity, *sypF**, led to increased biofilm formation, including wrinkled colony

formation and pellicle production. Surprisingly, these phenotypes persisted even when the two *syp* response regulators, *sypG* and *sypE*, were mutated. However, biofilm formation was eliminated when both *sypG* and a second response regulator, *vpsR*, were disrupted (Fig. 20.8). In *V. cholerae*, VpsR controls the *vps* polysaccharide locus, which is only poorly conserved in *V. fischeri* (Yildiz et al. 2001; Yildiz and Visick 2009; Darnell et al. 2008). Instead, it appears that a locus involved in cellulose biosynthesis contributes to biofilm formation induced by overexpression of SypF*. Clearly, control of biofilm formation in *V. fischeri* is complex and is not limited to a single regulator.

Many questions remain unanswered. How SypF is integrated into the regulatory pathway remains unclear, and its role in symbiotic colonization has not yet been established. The product of the *syp* locus is thought to be a polysaccharide (Yip et al. 2006), but the composition of the polysaccharide is as yet undetermined. Importantly, the environmental signals that activate RscS and the other regulators to induce biofilm formation and initiate symbiotic colonization are as yet unknown. Moreover, since symbiotic colonization is not impaired by overproduction of RscS and enhanced aggregate formation, there must be a yet-undiscovered mechanism by which aggregation is turned off to permit dispersal and migration into the pores for colonization to proceed. The discovery of the *syp* locus and its role in biofilm formation and initiating symbiotic colonization has provided important insights into host colonization by *V. fischeri* and an important model for in vivo biofilm formation and will continue to raise new questions to direct future research.

## Motility, Chemotaxis, and Their Regulation

*V. fischeri* is motile by virtue of a polar tuft of flagella (Fig. 20.9a) (Ruby and Asato 1993). Early on in the study of this symbiosis, it was determined that motility of *V. fischeri* is essential for symbiotic colonization; however, the roles of motility, flagellation, and chemotaxis continue to be intriguing and outstanding questions. Initial studies performed with uncharacterized mutants revealed that both nonflagellated and flagellated-but-nonmotile (paralyzed) mutants fail to colonize (Graf et al. 1994). However, motility *per se* is not required for the aggregation stage (Nyholm et al. 2000; Millikan and Ruby 2002), and once inside the crypt spaces, most *V. fischeri* cells become nonflagellated (Fig. 20.9b) (Ruby and Asato 1993). It is not known whether the loss of flagella is critical for colonization, especially since some bacteria do retain motility (Millikan and Ruby 2003). However, it is likely that flagella could interfere with the ability of the cells to achieve high cell density in the crypt spaces. Finally, *V. fischeri* cells rapidly regain their flagella upon release into seawater (Fig. 20.9b) (Ruby and Asato 1993).

One study suggested that excessive amounts of flagella are detrimental to initiation of colonization. Millikan and Ruby isolated three classes of hypermotile mutants with increased motility in both liquid and semisolid media (Millikan and Ruby 2002). The increased motility apparently stemmed from an increase in the number of flagella, from 1 to 3 on wild-type cells to upwards of 16 flagella on the mutants. Mutants in classes II and III also produced mucoid colonies rather than the smooth nonmucoid colony of their parent furthermore; class II mutants had a growth defect, and class III mutants exhibited defects in bioluminescence, 3-oxo-C6-HSL pheromone production, and the ability to hemagglutinate red blood cells. All three classes exhibited defects in initiating symbiotic colonization, failing to reach wild-type levels in 24 h. Class III mutants demonstrated the most severe defect, achieving only 0.1–10% of wild-type levels at 24 and 48 h postinoculation. The defect in initiation was attributed to deficiencies at the aggregation stage: wild-type cells form non-motile aggregates within 4–6 h; in contrast, the mutants retained motility. Furthermore, aggregate formation by the mutants was delayed, occurring between 8 and 12 h after inoculation, and aggregates were significantly smaller (tens of cells rather than hundreds) than those formed by the wild type (Millikan and Ruby 2002). Although the mutations in these strains were never identified, these studies nevertheless indicate the importance of regulating motility during entry into the symbiosis and beyond.

Subsequent studies and the availability of the genome sequence have confirmed the importance of motility in this symbiosis and have identified specific pathways of genetic control. Almost all genes predicted to be involved in flagellar assembly and regulation are housed together in a single locus of about 60 genes on the larger of *V. fischeri*'s two chromosomes. The functions of these genes can be readily predicted through comparisons with well-studied models such as *E. coli* and *Salmonella* (Chevance and Hughes 2008). Furthermore, comparisons with other organisms suggest that regulation of flagellar genes in *V. fischeri* is likely hierarchical. Perhaps the best comparison is with the closely related microbe *V. cholerae*, which has been extensively studied (Correa et al. 2000, 2005; Prouty et al. 2001). In that organism, the master flagellar regulator, FlrA, controls a number of flagellar genes, including additional regulators such as FlrBC and FliA. FlrBC is a predicted two-component signal transduction system that controls the next level of flagellar genes, including *flaA*, a flagellin gene; flagellin protein constitutes the major subunit assembled into the external filament of the flagellum. Indeed, a *V. fischeri flrC* mutant is non-motile and, like other non-motile mutants, exhibits a colonization defect (Hussa et al. 2007). FliA, or $\sigma^{28}$, activates another subset of flagellar genes, including four other flagellin genes (*flaB-E*). The working model is that *V. fischeri* likely controls its flagella using a hierarchy similar to that of *V. cholerae* (Fig. 20.9c) (Millikan and Ruby 2003; Prouty et al. 2001).

In *V. fischeri*, the role of FlrA was investigated in detail by Millikan and Ruby (2003). *flrA* mutants are non-motile, as predicted from FlrA's putative role as master flagellar regulator, and fail to colonize. Surprisingly, complementation with FlrA expressed from a plasmid restored normal motility in culture but not normal colonization: complemented *flrA* mutants failed to initiate colonization at the rate and with the success of wild-type cells (only 35–46% of animals became colonized by the complemented *flrA* mutant relative to 94% of

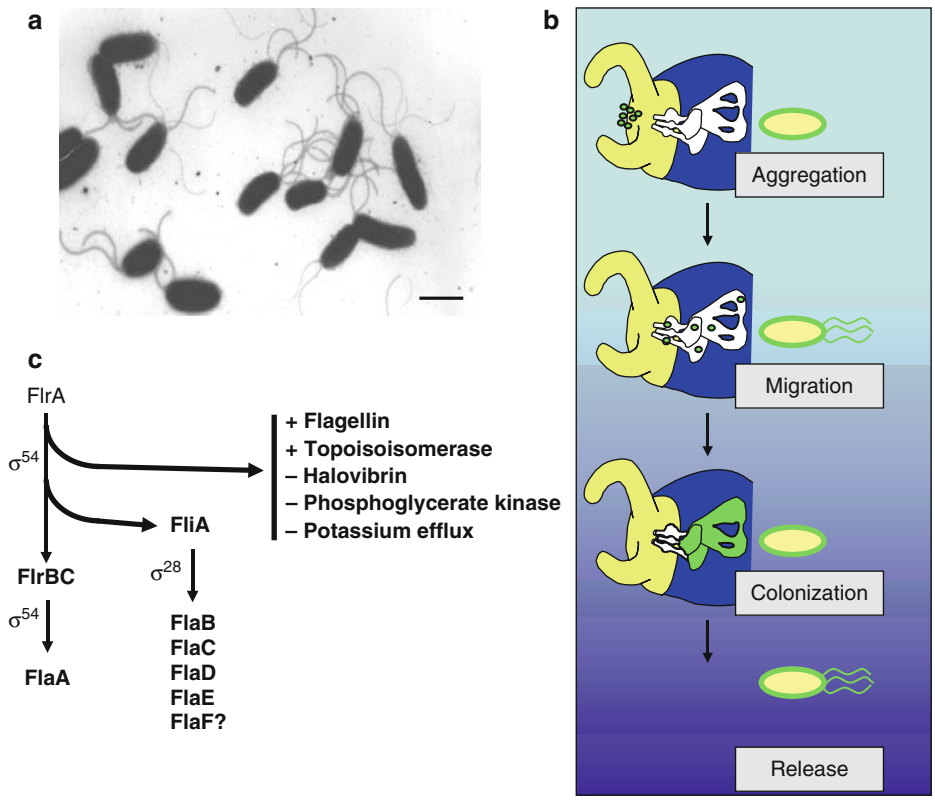


**Fig. 20.9**

**Role of motility in symbiosis. (a) *V. fischeri* contains a tuft of polar flagella. (b) Presence of flagella during different stages of colonization. Nonmotile cells are competent to aggregate, but motility is required for entry into the light-organ pores and presumably for passage to the crypts. Upon colonization, many bacteria lack flagella. Finally, following release from the light organ, *V. fischeri* cells can rapidly regrow their flagella. (c) Model for regulatory control by FlrA, a $\sigma^{54}$-dependent regulator. FlrA is predicted to induce transcription of the genes for the two-component system FlrBC, which, along with $\sigma^{54}$, controls transcription of the gene for the major flagellin protein FlaA. FlrA is also predicted to control transcription of the gene for the alternative sigma factor, FliA, which likely directs transcription of the genes for the other flagellin proteins, FlaB-E and potentially FlaF. Finally, FlrA appears to positively and negatively control the production of other proteins, although whether this effect is direct is unknown. The figure in panel A was previously published (O'Shea et al. 2005), and the scale bar represents 200 μM (The regulatory scheme depicted in panel C is modeled from reported data for *V. fischeri* and *V. cholerae* (Millikan and Ruby 2003; Prouty et al. 2001))**

wild-type-inoculated animals); they also failed to achieve wild-type levels of colonization ($1.6 \times 10^3$ colony-forming units of *flrA* mutant cells per squid relative to $9 \times 10^4$ colony-forming units of wild-type bacteria per squid). These experiments suggest that the proper level or location of FlrA is critical for symbiosis, and indicate that a trait other than motility, which was restored by multi-copy expression of *flrA* based on in vitro assays, may be responsible for the symbiotic defect in the *flrA* mutants (Millikan and Ruby 2003). In support of this idea, symbiotic aggregates produced by *flrA* mutants are diminished in size relative to those produced by the wild-type strain (tens of cells vs. hundreds of cells), despite the fact that other non-motile mutants aggregate normally (Nyholm et al. 2000). Millikan and Ruby subsequently identified four nonflagellar genes that were controlled by FlrA positively (e.g., genes encoding topoisomerase and halovibrin C) or negatively (e.g., genes encoding phosphoglycerate kinase and potassium efflux protein) (*Fig. 20.9c*). Whether FlrA controls these genes directly, and whether these FlrA-regulated genes play roles in symbiosis, remains unknown.

The FlrA protein contains a $\sigma^{54}$ interaction domain and a DNA-binding domain and thus is predicted to activate transcription of target genes in conjunction with $\sigma^{54}$. Indeed, *rpoN* mutants, which lack $\sigma^{54}$, are non-motile, fail to induce transcription of *flaA*, and fail to initiate colonization (Millikan and Ruby 2004; Wolfe et al. 2004). Like FlrA, $\sigma^{54}$ controls additional genes involved in colonization, notably luminescence and the *syp* polysaccharide locus (Wolfe et al. 2004; Yip et al. 2005). The use of this alternative sigma factor is of note, as genes under the control of $\sigma^{54}$ tend to be under tighter "on/off" regulatory control (Buck et al. 2000); whether or not this regulation is key for colonization remains to be determined.

Of *V. fischeri*'s six flagellin genes (*flaA-F*), only *flaA* appears to be directly regulated by FlrA and $\sigma^{54}$. Despite the redundancy of flagellin genes, *flaA* mutants show reduced motility with fewer motile cells, and fewer flagella per cell. In contrast, a *flaC* mutant

was seemingly unaffected for motility and flagellar elaboration. The *flaA* mutant also exhibited a symbiosis defect: loss of *flaA* delayed symbiotic colonization by about 3 h, and prevented full colonization (20–25% of that achieved by the wild-type strain) (Millikan and Ruby 2004). The mutant was also defective in competing for colonization with the wild type, and furthermore, it appeared to be preferentially expelled from the light organ. GFP-labeled cells revealed that, in contrast to the *flrA* regulatory mutant, the *flaA* flagellin mutant formed aggregates with the same approximate size as wild-type aggregates; furthermore, the *flaA* mutant entered the light organ normally. However, while the wild-type cells reached and colonized deep crypt spaces within 16 h postinoculation, *flaA* mutant cells did not colonize these sites within 20 h. This inability to reach and colonize the deep crypt spaces could account for both the delay in colonization and the preferential expulsion of the *flaA* mutant.

As suggested by the phenotype of the *flaA* mutant and numerous studies in other organisms, one prime reason for cells to be motile is to permit them to move to optimal locations. Typically, this movement is directed toward nutrients and attractants and away from toxic molecules and repellents using chemotaxis. The role of chemotaxis in symbiotic colonization by *V. fischeri* is currently an active area of investigation. One study asked what molecules serve as chemoattractants for *V. fischeri* (DeLoney-Marino et al. 2003). In tryptone-based soft agar media, *V. fischeri* forms two rings as it migrates from the spot of inoculation, indicating that it senses and responds to two different molecules that it likely consumes. The inner ring is comprised of cells sensing the amino acid serine. Cells in the outer ring sense the nucleic acid thymidine, which is present in tryptone. In addition to thymidine, *V. fischeri* can sense and respond to other ribonucleosides (adenosine, guanosine, uridine, and cytidine) as well as deoxynucleotide triphosphates. This ability of *V. fischeri* to sense nucleosides and nucleotides is unusual, although more recently it has been shown that *E. coli* and *Pseudomonas putida* can perform chemotaxis to pyrimidines (thymine and uracil in the case of *E. coli*, cytosine in the case of *P. putida*) (Liu and Parales 2008; Liu et al. 2009). In addition to serine and the building blocks of DNA and RNA, *V. fischeri* responds to a number of sugars, including *N*-acetylneuraminic acid, which is one of the sugars in the mucus secreted by the squid (DeLoney-Marino et al. 2003; Nyholm et al. 2000).

It remains unknown whether chemotaxis helps direct the bacteria into the light organ, and if so, which chemoattractants are used, but a chemotaxis-defective *cheY* mutant fails to compete effectively against wild-type cells for colonization (Hussa et al. 2007). In *E. coli* and *Salmonella*, CheY is a response regulator that modulates reversal of flagellar rotation, permitting "tumbles" that when interspersed with "runs" permit the cells to reorient and migrate toward nutrients and away from repellents. (Baker et al. 2006). Cells with defects in tumbling, such as *cheY* mutants, fail to perform normal chemotaxis; in addition, they can become trapped in "dead-end" passages present in agar-solidified media, making them appear nonmotile (Wolfe and Berg 1989). Like *E. coli* and *Salmonella* mutants, the *V. fischeri cheY* mutant exhibits smooth swimming, which may prevent it from appropriately migrating toward a stimulus. Alternatively, it may get stuck in squid-secreted mucus. Distinguishing between these possibilities requires the identification of a specific chemoreceptor(s) required for attractant recognition during symbiotic colonization. However, *V. fischeri* contains upwards of 40 such receptors, making this endeavor a challenge (Ruby et al. 2005).

One intriguing and outstanding question is: What factors direct the loss of flagella inside the light organ? Another is its corollary, What promotes flagellar regrowth in the nutrient-limited seawater? One possibility is suggested by the dependence of *V. fischeri* motility on magnesium (O'Shea et al. 2005). Cells grown in a medium lacking magnesium are poorly motile, as assessed on soft agar plates. Addition of either magnesium sulfate or magnesium chloride restores full migration. In particular, magnesium sulfate concentrations between 20 and 40 mM, similar to that found in seawater (about 50 mM), were optimal in promoting migration through soft agar. Other cations, such as calcium, could promote motility but to a lesser extent at low concentrations (5 mM); at concentrations higher than 10 mM, $CaCl_2$ was inhibitory to growth and thus difficult to assess. Subsequent analysis revealed that cells grown without magnesium elaborated few to no flagella, while those grown with magnesium contained on average two to three flagella and as many as seven or eight (O'Shea et al. 2005). It is unknown how magnesium impacts flagellar production, although it does not appear to be at the level of transcription of flagellin genes (O'Shea et al. 2006).

A search for mutants that were motile in the absence of magnesium led to the identification of two genes encoding the diguanylate cyclases MifA and MifB. These proteins are involved in the production of cyclic diguanylate (c-di-GMP), which is known to influence motility (Wolfe and Visick 2010). Disruption of either *mifA* or *mifB* increased motility in the absence of magnesium. However, neither mutant alone nor a double mutant defective for both genes exhibited the same pattern of migration in the absence of magnesium as the wild-type strain exhibits in the presence of magnesium. Thus, it remains unclear how magnesium impacts motility.

Regardless of how magnesium acts to stimulate the production of flagella, seawater contains sufficient levels of magnesium to permit *V. fischeri* to express flagella and thus be competent to colonize its squid host. Once inside, it is possible that decreased levels of magnesium and/or calcium contribute to the mechanism that promotes loss of flagella on colonizing bacteria. The levels of magnesium and calcium inside the light organ of *E. scolopes* are presently unknown.

In summary, research to date has demonstrated the importance of flagella and motility to symbiosis, but the role of motility is not simple and straightforward. In other systems, flagella can serve as mediators of attachment, a possibility that is supported by the aberrant aggregation of the hypermotile and *flrA* mutant strains. In some hosts, flagellin proteins can act as MAMPs, in much the same way as PG and LPS, which is an intriguing possibility given the signaling obvious in this symbiosis. Flagellar components can also serve as type III secretion

systems that direct bacterial proteins into the host environment, and this remains a possibility for *V. fischeri* as well. When motility or components of the flagellar system are required and when/if they are detrimental, when/if flagella or the flagellar apparatus contributes to attachment or secretion, what the precise contribution of chemotaxis and where the optimal locations of the bacteria are, and how flagella are lost and regained are all important questions that remain to be pursued. No doubt the answers will provide important insights into host colonization and colonization dynamics.

## Iron Uptake

Eukaryotic hosts generally limit the availability of iron, which is necessary for the growth and metabolism of bacteria, as a defense mechanism (reviewed in (Nairz et al. 2010)). As a result, bacteria have evolved numerous systems for the acquisition of iron. *V. fischeri*, for example, encodes a number of different proteins predicted to be involved in binding of iron or iron complexes and their associated transport systems (Ruby et al. 2005). The first study examining the role of iron uptake during the *V. fischeri*-squid symbiosis suggested that, indeed, the ability to acquire iron was an important symbiosis trait (Graf and Ruby 2000). This report relied on a transposon mutagenesis screen for mutants with altered siderophore production and focused on one that appeared completely defective in its ability to sequester iron. Indeed, growth of this strain depended on the presence of iron in the medium. Surprisingly, this mutant turned out to contain an insertion not in a siderophore biosynthesis gene, but in *glnD*, a gene known in *E. coli* to play a role in sensing nitrogen status (reviewed in (Arcondeguy et al. 2001)). Consistent with a similar role for GlnD in *V. fischeri*, the *glnD* mutant was deficient in utilizing a variety of nitrogen sources. Complementation with a wild-type copy of *glnD* restored all of the observed phenotypes to wild-type levels, supporting the novel role of GlnD in siderophore production. Finally, the *glnD* mutant exhibited a defect in colonizing juvenile squid. In support of the idea that the symbiotic phenotype was due to the iron sequestration defect, the defect was more pronounced when cells in the starting inoculum were pre-grown under conditions of limiting iron availability. Furthermore, addition of iron to seawater alleviated the defect of the *glnD* mutant (Graf and Ruby 2000). The mechanism by which GlnD impacts siderophore production remains unknown.

A more directed study investigated the role of one possible mode of acquiring iron, heme transport, in the *V. fischeri*-squid symbiosis. Bioinformatic analyses identified a locus, VF_1220-VF_1228, similar to heme-uptake loci in other Vibrios (Septer et al. 2011; Wang et al. 2010a). One operon within this locus (VF_1225-VF_1220) is predicted to encode a periplasmic heme-binding protein (HutB), an inner membrane permease (HutC), an ABC-transporter ATPase (HutD), and proteins that function in providing the energy for transport (TonB, ExbB, ExbD). Additional proteins predicted to be involved in optimal heme utilization were encoded in a divergent operon (HutW, HutX, HutZ, VF_1226-VF_1228). These genes, as well as putative hemin receptor genes VF_1234 and VF_A0331-A0333, were downregulated upon exposure of wild-type cells to NO in a manner that depended upon an intact *hnoX* gene, which encodes an NO sensor (Wang et al. 2010a). Regulatory sequences consistent with control by Fur (Ferric uptake regulator) were identified.

Consistent with the putative role of this locus, its deletion disrupted the ability of *V. fischeri* to grow with hemin ($Fe^{3+}$) as a source of iron, but did not impact growth of the cells when ferrous sulfate was supplied as an iron source (Septer et al. 2011; Wang et al. 2010a). Investigations into the transcriptional control of this locus revealed that two divergent promoters, upstream of VF_1225 and VF_1226, were upregulated in response to low iron availability. Furthermore, both promoters were repressed by Fur, as disruption of *fur* led to an increase in transcription even in the presence of iron. These data are consistent with a model in which low iron availability causes an increase in transcription of this heme-uptake locus; when iron levels rise, Fur is activated to decrease transcription of the locus (Septer et al. 2011). Thus, *V. fischeri* appears to carefully modulate transcription of this locus in response to iron availability. To assess the relevance of this phenomenon and these genes in symbiosis, two approaches were used. First, the transcriptional response of this locus in symbiosis was assessed with promoter-GFP reporters. Transcription of VF_1225 was turned on as early as 14 h postinoculation, indicating that the light-organ environment is low in iron. Second, the competence of the deletion mutant to colonize when presented in competition with the wild-type strain was evaluated. The mutant exhibited a defect in colonization, although the defect was not apparent until after several days postinoculation. Thus, these data support and extend the study by Graf and Ruby (2000) that the light-organ environment is limiting for iron, and indicate that uptake of heme is an important mechanism by which *V. fischeri* acquires iron during symbiosis. In further support of the importance of heme uptake in symbiosis, the transcriptome study by Weir et al. (2010) found that the heme-uptake genes were induced following bacterial venting. At venting, membrane "blebs" (presumably host derived) appear, and it was speculated that these contain a source of iron for the symbionts (Septer et al. 2011). Finally, a recent study investigating the proteomes of *V. fischeri* and *E. scolopes* identified host-iron-binding proteins, including transferrin, ferritin, and melanotransferrin, and symbiont heme-binding proteins (Schleicher and Nyholm 2011). Whether heme is the primary source of iron during symbiosis, and how/when iron is supplied to the bacteria, will be of interest to determine.

## GacA

Another important regulatory protein involved in symbiosis is the response regulator GacA. A *gacA* mutant was defective in initiating symbiosis: only about 50% of animals became colonized under conditions in which 100% of wild-type-inoculated animals became colonized, and animals that became colonized with the *gacA* mutant contained on average 100X fewer bacteria

(Whistler and Ruby 2003). Moreover, as mentioned above, in squid that were colonized by *gacA* mutants, the symbionts did not trigger some of the normal developmental events in the host that are elicited by wild-type *V. fischeri*, including apoptosis and cessation of mucus shedding in light-organ epithelial cells (Whistler et al. 2007). To take into account the possibility that low colonization by the *gacA* mutant might underlie these apparent non-effects on the host, comparisons were made to a *lysA* mutant, which is a lysine auxotroph that is also attenuated in colonization (Whistler et al. 2007). Using this control, the authors concluded that low colonization alone could not explain the altered signaling capacity of the *gacA* mutant.

In culture, *V. fischeri* GacA controls bioluminescence, motility, LPS structure, siderophore production, and nutrient acquisition (Whistler et al. 2007; Whistler and Ruby 2003). In rather broad terms, the *gacA* mutant is also impaired in growth, although the severity of this defect depends considerably on the culture medium (Whistler and Ruby 2003). In short, the *gacA* mutant has a pleiotropic phenotype, it is affected in a number factors known to (or likely to) affect colonization of the host, and its inability to grow to wild-type levels in the light organ is not a symbiosis-specific defect. Nonetheless, its connection to so many colonization-promoting phenotypes marks it as a global regulator of importance in the symbiosis, and the careful use of another attenuated (*lysA*) mutant as a control strongly suggests a critical symbiosis-specific signaling defect.

GacA is conserved in numerous and diverse γ-proteobacteria (although its orthologs have distinct names), where it controls a myriad of functions, including carbon-flow physiology and virulence (Lapouge et al. 2008). Certain themes relevant to a potential symbiotic role have emerged, including the control of factors involved in colonizing hosts and pathways for social behavior and intraspecies signaling (e.g., quorum sensing) (Lapouge et al. 2008). Also conserved is the mechanism of its associated regulatory cascade (Lapouge et al. 2008). The sensor kinase GacS is responsible for phosphorylating and thus activating the cognate response regulator GacA. Then GacA-P stimulates transcription of CsrB, regulatory RNAs that bind to CsrA, preventing it from binding target mRNAs and affecting their stability and/or translation. In *V. fischeri*, bioinformatic analyses revealed two such regulatory RNAs, named CsrB1 and CsrB2 (Kulkarni et al. 2006), and putative GacA-binding sites are present upstream of each of these genes. *V. fischeri* also possesses clear homologs of GacS and CsrA. Unpublished results from multiple laboratories suggest that, as predicted, CsrB1, CsrB2, CsrA, and GacS have regulatory functions in *V. fischeri*, at least some of which are interconnected. This regulatory circuit is an area of active research, particularly with respect to its effect on bioluminescence from the *lux* system and a global perspective of its regulon, which is likely to include genes involved in signals perceived by the host.

Important questions that remain include the signal or environmental cue perceived by GacS and whether this is present in the light-organ environment. The altered symbiotic phenotype of *gacA* mutants suggests the GacS/GacA system is active during colonization, although it cannot be ruled out that unphosphorylated GacA has some activity or that GacA is phosphorylated by some means other than GacS (e.g., by cross talk from another sensor kinase). The stimulus for GacS homologs in other systems has been elusive, although it may involve metabolic products of the bacteria themselves (Takeuchi et al. 2009), and could perhaps be sensed from the cytoplasmic rather than periplasmic side of GacS (Zuber et al. 2003). If so, the Gac regulon could reflect a regulatory response to symbiont physiology as it is constrained by the host environment. This possibility underscores the importance of understanding symbiont physiology and how *V. fischeri* metabolism is supported by the host. The *V. fischeri-E. scolopes* model system promises to be a rich and tractable experimental system for deciphering the widespread Gac-Csr system.

## *N*-Acetyl D-Glucosamine Repressor NagC

The bacterial transcriptome analysis of Wier et al. (2010) described above revealed that genes involved in chitin catabolism were upregulated prior to dawn (Wier et al. 2010). This finding stimulated an interest in understanding the regulation of these genes. Specifically, Miyashiro et al. (2011) searched for regulators of the exochitinase gene, VF_1598, and found that the repressor NagC negatively controls its expression and also represses the *nagA* operon (which includes *nagC* itself, VF_0806). Loss of *nagC* increased expression of these and several other genes involved in the utilization of chitin or its derivatives, the monomer *N*-acetyl-D-glucosamine (GlcNAc) or the dimer chitobiose ($GlcNAc_2$). Excitingly, in colonization assays, a *nagC* null mutant exhibited a severe defect: when the *nagC* mutant was used to inoculate squid, most squid remained uncolonized (6 of 15 became colonized within 48 h compared to 90% colonized with the wild-type control) (Miyashiro et al. 2011). In competition assays, the *nagC* mutant neither impaired wild-type colonization nor was complemented by the presence of the wild-type strain. In contrast to the *nagC* result, no defect was observed for a *nagB* mutant, which lacks a deaminase involved in releasing the amino group from GlcNAc-6P; this mutant failed to grow preferentially on GlcNAc as a carbon and nitrogen source. Thus, the symbiotic defect of the *nagC* mutant is unlikely to stem from altered metabolism of GlcNAc. However, the generation and evaluation of a *nagC nagB* double mutant would better address the symbiotic consequences of the overexpression of GlcNAc metabolism genes resulting from *nagC* disruption. Finally, exposure to GlcNAc during inoculation eliminated the competitive advantage of the wild type over the *nagC* mutant (Miyashiro et al. 2011). Together, these data indicate that NagC represses something that must be turned off for colonization to proceed normally; in the absence of NagC-mediated repression, colonization is severely impaired. It will be of great interest to determine the regulon of NagC and which regulon member(s) impair colonization when inappropriately expressed.

## Possible Role for Pili in Symbiosis

Many pathogenic associations depend upon a pilus-mediated adherence of the bacteria to the host (Pizarro-Cerda and Cossart 2006). For example, type I pili play critical roles in attachment and uptake in urinary tract infections by uropathogenic *E. coli* (Mulvey et al. 1998; Wright et al. 2007). To date, however, a critical role for pili in the *V. fischeri*-*E. scolopes* symbiosis has yet to be determined, although several studies have investigated this possibility. Interestingly, *V. fischeri* contains ten loci encoding putative pili (Ruby et al. 2005). Eight loci encode putative type IV pili, the most common type of bacterial pilus (reviewed in (Burrows 2005; Pelicic 2008; Pizarro-Cerda and Cossart 2006)). Due in part to their retractability, type IV pili play a variety of important roles in other bacteria, including adherence to various surfaces and hosts, twitching motility, and secretion of a variety of factors. Intriguingly, one of the type IV pilus loci is similar to that of the *V. cholerae* TCP (toxin co-regulated pilus), an important virulence factor in that bacterium. Although some of the *V. fischeri* genes are not clustered but are spread throughout the genome, most of the TCP genes appear present (Ruby et al. 2005). One of the other pilin loci includes a gene for MshA (mannose-sensitive hemagglutin), an adhesin located at the tip of the pilus that recognizes mannose-based receptors. Intriguingly, when a mannose analog was added to seawater, it inhibited colonization by *V. fischeri*, suggesting that a bacteria-mannose interaction may be important during initiation (McFall-Ngai et al. 1998). Of the two non-TCP loci, one appears to encode curli, an adherence factor found in the Enterobacteriaceae (Barnhart and Chapman 2006). Interestingly, the curli locus is absent in other well-studied Vibrios, including *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Ruby et al. 2005). Lastly, the large plasmid (pES100) of *V. fischeri* encodes conjugative pili (Ruby et al. 2005; Dunn et al. 2005).

Of the 10 loci, two have been characterized in some detail. One of the type IV pilus loci contains a single pilus gene (VF_A0148), encoding a PilA-like pilin protein (PilA2) predicted to be the major external subunit of the pilus (Stabb and Ruby 2003; Ruby et al. 2005). Disruption of this gene caused a small but statistically significant defect in colonization when the mutant was presented to the squid in a mixture with the wild-type strain. Potentially, the presence of one or both of the two other *pilA* genes compensates for the loss of VF_A0148. In support of this idea is the lack of adjacent pilus structural and assembly genes, suggesting that PilA2 gets assembled into a pilus that is associated with a distinct pilin protein (Ruby et al. 2005). An examination of the presence of *pilA2* in *V. fischeri* isolates revealed that it is conserved in strains isolated from other light-organ symbioses, including strains from *E. tasmanica* (from Australia) and those from *Sepiola* species (from France) (Browne-Silva and Nishiguchi 2008), further suggesting that this protein may be important in bacteria-host associations. Additional genetic characterization, including the generation of mutants that lack all three *pilA* genes and combinations thereof, will be necessary to determine the role of *pilA* in attachment and colonization.

Limited characterization of the genes encoding the conjugative pilus has also been performed. These genes (VF_B38-55) are carried on the large plasmid pES100, and they are clustered with other components of a putative conjugative transfer apparatus (Dunn et al. 2005). Indeed, experiments indicated that pES100 can direct conjugative transfer of other plasmids and is almost certainly self-transmissible (Dunn et al. 2005). Efficient transfer also required the chromosomally encoded RecA protein to be present in the donor, and this requirement was not due to homologous recombination between pES100 and the plasmid it mobilized. An exciting future direction is determining whether conjugation occurs or is even induced during colonization.

## Cellobiose

*V. fischeri* exhibits the ability to grow on cellobiose, a feature that distinguishes it from a number of other Vibrio species, including *V. parahaemolyticus*, *V. hollisae*, *V. mimicus*, and *V. cholerae*. This ability is conferred by the *cel* locus (VF_0603-VF_0608), consisting of three genes that comprise a phosphotransferase system (PTS) II (*celABC*), a putative glucokinase gene *CelK*, and a 6-phospho-β-glucosidase gene *celG* (Adin et al. 2008b). Negatively controlling this locus is a LacI-family regulator, CelI, which is encoded at the end of the operon. Loss of *celI* or the addition of cellobiose (but not other sugars) resulted in colonies that turned blue on the colorimetric substrate X-gal, a phenotype that depended upon an intact *celG*. Further investigations revealed that CelG could cleave a variety of substrates with a β-1,4 linkage and could serve both as a β-glucosidase (to cleave substrates such as cellobiose) and as a β-galactosidase (consistent with its ability to cleave X-gal); its highest activity was as a β-glucosidase, as that activity was 50-fold higher than its β-galactosidase activity. Although not a preferred substrate, the ability of CelG to cleave X-gal has implications for the use of *lacZ* as a reporter in *V. fischeri* (Adin et al. 2008b). Despite the somewhat unique ability of *V. fischeri* to use cellobiose, this ability was not required for colonization: competition experiments of various *cel* mutants with wild type revealed no competitive disadvantage of the lack of cellobiose catabolism; the exception to this was the *celI* mutant, which constitutive expressed the *cel* genes and exhibited a twofold defect in competitions with the wild-type strain for colonization. This inability of the *celI* mutant to compete could be due to the overexpression of the *cel* locus, but could not be attributed to the cellobiose catabolism, as a *celI celG* mutant exhibited a similar competitive defect (Adin et al. 2008b). Finally, a mutation in the unlinked gene *ptsI* (VF_1895/1896), which encodes the E1 component of the PTS system, exhibited a defect in cellobiose uptake as well as a severe competitive defect in colonization. However, because this strain also exhibited a growth defect, its specific role in colonization remains uncertain. These studies thus add to our knowledge

and understanding of the physiology of *V. fischeri* important to its ability to colonize its host *E. scolopes* and provide a cautionary note for those who rely on *lacZ* as a reporter.

### Other Genes

The roles in symbiosis of a number of additional genes have been investigated. Some, such as phosphoglucomutase (*pgm*), play significant but poorly understood roles in symbiosis, while others have been shown to make small contributions, and yet others have an impact on symbiosis that cannot be separated from their requirement for normal growth of *V. fischeri*. A select set of these genes is included in *Table 20.1*. As the study of *V. fischeri* symbiosis continues and the interactions between *V. fischeri* and its host become better defined, the roles of these genes may become better understood.

## Evolution of the *V. fischeri-E. scolopes* Symbiosis

Due to its experimental tractability, the *V. fischeri-E. scolopes* model has great potential to inform our understanding of evolutionary relationships between bacteria and their specific hosts. In the Hawaiian archipelago, at least two geographically isolated and genetically distinct populations of *E. scolopes* exist (Maunalua Bay and Kaneohe Bay, Oahu) (Jones et al. 2006; Kimbell et al. 2002). Furthermore, numerous related *Vibrio*-containing squids can be found around the world, including those of the same genus (e.g., *Euprymna tasmanica* from Australia and *Euprymna morsei* from Japan) as well as those from the distinct genus *Sepiola* (e.g., *Sepiola affinis* and *Sepiola robusta* from France). A wealth of questions can be addressed about symbiont specificity and evolution using phylogenetic and comparative analyses and experimental manipulation of established symbiosis models.

An early study analyzed coevolution of the bacteria and squid. Specifically, Nishiguchi et al. (1998) asked whether *V. fischeri* strains isolated from *E. scolopes* ("native" bacteria) were more successful at colonizing *E. scolopes* than strains isolated from other squid species ("nonnative" bacteria). In competition assays in which a mixture of strains was used to inoculate juvenile *E. scolopes*, the squid predominantly became colonized with native strains rather than nonnative strains (Nishiguchi et al. 1998). Furthermore, when two nonnative strains competed, the strain that was more closely related to the native symbiont was also the better colonizer of juvenile *E. scolopes*. Parallel results were obtained from competition of strains from *E. tasmanica* and *E. hyllebergi* (Nishiguchi 2002). These studies suggest that the bacteria have evolved to better colonize a specific host. The 1998 study also described evidence of parallel cladogenesis, as assessed using sequence divergence at two loci in related squids, the ITS (internal transcribed spacer between the 18S and 25S rRNA genes) and COI (cytochrome oxidase subunit I) and *gapA* (glyceraldehyde phosphate dehydrogenase) in the bacteria (Nishiguchi et al. 1998). However, a subsequent study determined that the bacterial gene sequenced was not *gapA*, but rather *epd*, and reanalysis of the data failed to support the original conclusions (Dunlap et al. 2007). Indeed, the latter study found no evidence from phylogenetic analyses for parallel evolution in either squid/bacteria or fish/bacteria symbioses. This may reflect the facultative nature of this symbiosis: *V. fischeri* is an extracellular symbiont acquired anew from the environment in each generation of squid.

In a more recent study, Wollenberg and Ruby investigated whether the symbionts from the two geographically separated populations of *E. scolopes* squid (in Maunalua Bay and Kaneohe Bay, Oahu, Hawaii) could be phenotypically distinguished (Wollenberg and Ruby 2009). Bacteria from the two populations of squid were collected over several nights and subsequently cultured to determine their phenotypes with respect to bioluminescence level, colony pigmentation, motility, growth rate, and siderophore production. Except for siderophore production, these characteristics could be used to distinguish the organisms isolated from the two squid populations in a manner that was statistically significant. These data support the idea that the squid host influences the generation of genetically distinct populations of *V. fischeri* (Wollenberg and Ruby 2009). A subsequent study, using four bacterial housekeeping genes (*recA, mdh, katA*, and *pyrC*), identified a monophyletic group ("group A") of strains that could be found at a higher frequency than other strains in Maunalua squid (Wollenberg and Ruby 2012). Group A was able to outcompete non-group A strains in colonizing Maunalua squid hosts; however, this group appeared to be at a disadvantage when free-living in the Maunalua Bay environment (Wollenberg and Ruby 2012). These findings suggested a "fitness trade-off" for growth in the host versus survival in the environment.

Studies of *Vibrio* isolates obtained elsewhere, particularly in the Mediterranean where multiple sympatric squid species reside, suggest that they are genetically diverse (Jones et al. 2006; Nyholm and Nishiguchi 2008). Thus, factors other than host availability may influence the population structure of the bacteria (Nyholm and Nishiguchi 2008). Such factors may include water flow, which can impact the distribution of the bacteria; salinity and temperature, which may exert selective pressures on the bacteria (Jones et al. 2007; Nishiguchi 2000; Soto et al. 2009); and competition from other species (Wollenberg and Ruby 2012).

The phylogenetic tree constructed by Wollenberg and Ruby (2012) revealed that *E. scolopes* symbionts form a polyphyletic clade within *V. fischeri* (Wollenberg and Ruby 2012). These data represent an expansion of a similar data set generated by Mandel et al. (2009). The Mandel study investigated host specificity by comparing the genomes of two *V. fischeri* strains, one isolated from the squid (ES114) and the other isolated from the fish *Monocentris japonica* (MJ11); the latter strain fails to colonize squid proficiently (Mandel et al. 2009; McFall-Ngai and Ruby 1991; Schuster et al. 2010). Genomic comparisons revealed that the two strains were quite similar: 91% of the genes encoded proteins with a median amino acid identity of at least 99%

**Table 20.1**

**Genes whose roles in symbiosis have been tested**

| Gene(s) | Description | Sym phenotype[a] | References |
|---|---|---|---|
| *hmp* | Flavohaemoglobin, protects against NO stress | Initiation | (Wang et al. 2010b) |
| *norV* | Flavohaemoglobin | Initiation | (Wang et al. 2010b) |
| *htrB1* | Lipid A acetyltransferase | Initiation | (Adin et al. 2008a) |
| *rscS* | Sensor kinase, promotes biofilms | Initiation | (Visick and Skoufos 2001; Yip et al. 2006) |
| *sypG* | Response regulator, promotes biofilms | Initiation | (Hussa et al. 2008; Hussa et al. 2007) |
| *syp* | Polysaccharide biosynthesis, biofilms | Initiation | (Yip et al. 2006; Yip et al. 2005) |
| *fla*[b] | Flagella biosynthesis | Initiation | (Graf et al. 1994) |
| *flrA* | Flagella biosynthesis | Initiation | (Millikan and Ruby 2003) |
| *rpoN* | Alternative sigma factor $\sigma^{54}$ | Initiation | (Wolfe et al. 2004) |
| *cheA, cheY* | Chemotaxis | Initiation | (DeLoney-Marino and Visick 2012; Mandel et al. 2012) |
| *sypE* | Serine kinase/phosphatase, inhibits biofilms | Initiation[c] | (Morris et al. 2011) |
| *ompU* | Outer membrane protein | Initiation[d] | (Aeckersberg et al. 2001; Nyholm et al. 2009) |
| *nagC* | *N*-acetyl-D-glucosamine repressor | Initiation | (Miyashiro et al. 2011) |
| *hnoX* | NO sensor | Initiation[e] | (Wang et al. 2010a) |
| *sapA* | Required for normal growth | Initiation, accommodation | (Lupp et al. 2002) |
| *ainS* | Pheromone signal synthase, luminescence | Initiation, accommodation | (Lupp et al. 2003; Lupp and Ruby 2004; Lupp and Ruby 2005) |
| *gacA* | Response regulator, various cellular functions | Initiation, accommodation[f] | (Whistler et al. 2007; Whistler and Ruby 2003) |
| *flaA* | Flagellin | Initiation, accommodation[g] | (Millikan and Ruby 2004) |
| *pgm* | Phosphoglucomutase, required for normal LPS | Accommodation | (DeLoney et al. 2002) |
| *lysA* | Lysine biosynthesis | Accommodation | (Graf and Ruby 1998; Whistler et al. 2007) |
| *thr* | Threonine biosynthesis | Accommodation | (Graf and Ruby 1998) |
| *luxI, R, C-G* | Bioluminescence | Persistence | (Visick et al. 2000; Bose et al. 2008) |
| *glnD* | Uridylyl-removing/uridylyltransferase | Persistence | (Graf and Ruby 2000) |
| *luxS* | Pheromone signal synthase, luminescence | Persistence[h] | (Lupp and Ruby 2004) |
| *ltgA ltgD ltgY* | Lytic transglycosylase, promotes release of PG | Secondary infections | (Adin et al. 2009) |
| *argG* | Arginine biosynthesis | Colonization | (Graf and Ruby 1998) |
| *cys* | Cysteine biosynthesis | Colonization | (Graf and Ruby 1998) |
| *leu* | Leucine biosynthesis | Colonization | (Graf and Ruby 1998) |
| *met* | Methionine biosynthesis | Colonization | (Graf and Ruby 1998) |
| *pro* | Proline biosynthesis | Colonization | (Graf and Ruby 1998) |
| *ser* | Serine biosynthesis | Colonization | (Graf and Ruby 1998) |
| *tatABC* | Twin arginine translocation | Competition | (Dunn and Stabb 2008b) |
| *luxO* | Response regulator, Luminescence | Competition | (Hussa et al. 2007; Lupp and Ruby 2005; Miyashiro et al. 2010) |
| *flrC* | Response regulator, motility | Competition | (Hussa et al. 2007) |
| *cheY* | Response regulator, chemotaxis | Competition | (Hussa et al. 2007) |
| *VF1909* | Response regulator NarP | Competition | (Hussa et al. 2007) |
| *vpsR* | Response regulator | Competition | (Hussa et al. 2007) |
| *VFA0698* | Response regulator CheV | Competition | (Hussa et al. 2007) |
| *VFA0179* | Response regulator | Competition | (Hussa et al. 2007) |
| *VF1988* | Response regulator PhoB | Competition | (Hussa et al. 2007) |

**Table 20.1 (continued)**

| Gene(s) | Description | Sym phenotype[a] | References |
|---|---|---|---|
| *VFA0181* | Response regulator | Competition | (Hussa et al. 2007) |
| *ntrC* | Response regulator | Competition | (Hussa et al. 2007) |
| *VF1689* | Response regulator ExpM | Competition | (Hussa et al. 2007) |
| *arcA* | Response regulator, represses luminescence | Competition | (Bose et al. 2007) |
| *cheR* | Chemotaxis regulator | Competition | (Deloney-Marino and Visick 2012) |
| *qrr* | Regulatory mRNA, destabilizes *litR* transcript | Competition | (Miyashiro et al. 2010) |
| *ptsI* | PTS enzyme EI | Competition | (Adin et al. 2008b) |
| *acs* | Acetyl coenzyme A synthetase | Competition | (Studer et al. 2008) |
| *VF1220-1228* | Haemin uptake | Competition | (Septer et al. 2011) |
| *katA* | Catalase, degrades hydrogen peroxide | Competition | (Visick and Ruby 1998) |
| *pilA* | Pilin adhesin | Competition | (Stabb and Ruby 2003) |
| *celI* | Cellobiose regulator | Competition | (Adin et al. 2008b) |
| *pepN* | aminopeptidase | Competition | (Fidopiastis et al. 2012) |
| *litR* | Transcriptional activator of luminescence | Competition[i] | (Fidopiastis et al. 2002; Miyashiro et al. 2010) |
| *fnr* | Regulator of anaerobic respiration | None detected | (Septer et al. 2010) |
| *torECA torYZ dmsABC* | Trimethylamine N-oxide reductase | None detected | (Dunn and Stabb 2008a) |
| *nsrR* | NO-responsive regulator | None detected | (Wang et al. 2010b) |
| *hvnA, hvnB* | $NAD^+$ glycohydrolase (Halovibrin) | None detected | (Stabb et al. 2001) |
| *phr* | Photolyase | None detected | (Walker et al. 2006) |
| *flaC* | Flagellin | None detected | (Millikan and Ruby 2004) |
| *celGKABC* | Cellobiose utilization | None detected | (Adin et al. 2008b) |
| *htrB2* | Lipid A acetyltransferase | None detected | (Adin et al. 2008a) |
| *msbB* | Lipid A acetyltransferase | None detected | (Adin et al. 2008a) |
| *glyA* | Glycine biosynthesis | None detected | (Graf and Ruby 1998) |
| *ampG* | PG permease | None detected | (Adin et al. 2009) |

[a]Sym phenotype refers to the symbiosis phenotype. The main categories of symbiosis phenotypes include defects in initiation, accommodation, persistence, and competition with wild-type cells. Where the defects have not been assigned to a category, the more general term of colonization is used. In some cases, multiple categories are impacted by a mutation, and only the primary defect is listed. Specific notations are made for cases in which the mutation impacts other aspects of the symbiosis, such as making the squid prone to secondary infection
[b]Uncharacterized nonmotile transposon insertion mutants
[c]*sypE* mutants that cannot be inactivated inhibit symbiotic initiation
[d]*ompU* mutants also exhibit a defect in resisting binding and uptake by hemocytes
[e]*hnoX* mutants exhibit a colonization advantage
[f]*gacA* mutants are also defective in inducing host development
[g]*flaA* mutants are also preferentially expelled from the light organ
[h]l*uxS* mutants only exhibit a phenotype when the mutation is combined with an *ainS* mutation
[i]*litR* mutants exhibit a competitive advantage

(Mandel et al. 2009). Thus, there is limited diversity within this group of bacteria, unlike in other organisms like some species within the Enterobacteriaceae, which contain large, diverse genomic islands (Welch et al. 2002).

These studies also revealed the absence of the sensor kinase gene *rscS* from the fish symbiont. Mandel et al. (2009) proposed that the absence of *rscS*, required for initiation of symbiosis by ES114 (Visick and Skoufos 2001; Yip et al. 2006), could be sufficient to account for the inability of the fish symbiont to colonize *E. scolopes* (Mandel et al. 2009). This hypothesis was experimentally addressed by introducing *rscS* into the fish symbiont. This genetically modified strain became competent to colonize squid. These experiments supported the conclusion that *rscS* serves as a specificity factor that promotes colonization of *E. scolopes* by the subset of strains that contain it. Mandel et al. (2009) subsequently investigated the origin of *rscS* in *V. fischeri* strains and determined that *rscS* likely arose in a single horizontal acquisition event from an unknown donor

prior to the introduction of *V. fischeri* into squid in the North Pacific Ocean. Subsequent transmission of *rscS* resulted in two major alleles of *rscS*, termed $rscS_A$ and $rscS_B$. Squid symbionts contained $rscS_A$, while half of the fish symbionts lacked *rscS* altogether. The half of the fish symbionts that contained *rscS* largely carried the $rscS_B$ allele; the $rscS_B$ allele was not sufficient to promote colonization of squid (Mandel et al. 2009). These data suggested that these *rscS*-containing fish symbionts descended from squid symbionts, and these bacteria either dispensed with or modified *rscS*. These experiments highlight the power of whole-genome comparisons and the utility of combining phylogenetic analyses with experimental measurements of colonization.

One major exception to the striking similarity between the ES114 and MJ11 genomes was found in the bioluminescence genes (Bose et al. 2011; Mandel et al. 2009). Although the genes and their arrangement were conserved, the proteins encoded by *luxR/I* and *luxCDABE* had only between 75% and 89.5% identity at the amino acid level, whereas most other proteins, including those whose genes flank the *lux* operon, were more than 95% identical. Moreover, the intergenic region between *luxR* and the *lux* operon showed a much greater divergence than intergenic regions of nearby genes (Bose et al. 2011). A comparison of the *luxRI* intergenic region of 18 *V. fischeri* strains revealed that only 104 of the 222 base-pair positions were conserved in all of the strains. When these sequences were used to generate a gene tree, two distinct clades were revealed, one of which encompassed all the strains classified as having highly visible luminescence. The second clade included all of the isolates from *E. scolopes*, which are non-visibly luminescent in culture, as well as isolates from *E. tasmanica* and *E. morsei* and a few planktonic isolates. To understand the significance of the diversity within this intergenic region, Bose et al. (2011) compared it to another intergenic region, that between *glpA* and *fdhA*. The latter intergenic region was conserved at 97% of the positions (289 of 298 bp). These data are consistent with the conclusion that the *lux* intergenic region is under different selective pressure—presumably the host environment—that has led to a relatively rapid evolution of bioluminescence genes and regulatory regions (Bose et al. 2011).

The above studies have been nicely complemented by an experimental evolution approach reported by Schuster et al. (2010). In this study, juvenile *E. scolopes* were exposed to two nonnative strains of *V. fischeri*: MJ11, the fish symbiont described above, and WH1, a free-living strain isolated from a region of the USA that does not harbor these squid. Although not native to *E. scolopes*, these *V. fischeri* isolates could colonize given a high inoculation dose and enough time. These strains then were serially passaged through 14 additional hatchling squid as follows. Every day, squid were rinsed and moved to fresh seawater. On the third day following inoculation, the seawater into which bacteria were vented by colonized animals was used as the inoculum for the next newly hatched squid. Thus, with the exception of the initial inoculation with cultured bacteria, these experiments were culture-independent and mimicked the natural cycle of the symbiosis through initiation, growth and persistence, venting, and persistence in the seawater. Importantly, this approach selected for improved traits at a number of different stages, rather than simply one, such as initiation. It was estimated that the bacteria in these experiments underwent a total of between 290 and 360 symbiotic generations.

During this limited experimental evolution, descendents of the two nonnative strains, WH1 and MJ11, exhibited clear phenotypic differences from their parents, notably with respect to luminescence output (Schuster et al. 2010). In contrast to the native symbiont ES114, which is non-visibly luminescent, WH1 and MJ11 produce high levels of visible light. Evolved derivatives of WH1 and MJ11 showed convergent evolution toward reduced light production. Specifically, in each of six independently evolved lines of MJ11, non-visibly luminescent isolates were observed; in four of these lines, all of the isolates examined were non-visibly luminescent. For WH1, four of the six lines yielded strains with decreased bioluminescence. Intriguingly, although the decrease in light production of the evolved WH1 strains was not substantial (approximately twofold) when light production was measured in vitro, the same strains exhibited about a 50-fold decrease in symbiotic (in vivo) bioluminescence per cell. These data supported the hypothesis that the decrease in light production was a meaningful change in the context of symbiosis. To test this idea further, Schuster et al. (2010) asked whether decreased luminescence was an advantage or disadvantage by exposing squid to two strains (from the same evolved population) that exhibited different luminescence levels (ancestral or decreased luminescence). They found that the strain with decreased luminescence outcompeted the one with ancestral luminescence levels, suggesting that this evolved phenotype may in fact be advantageous during the symbiotic life cycle of *E. scolopes*.

Together, these data indicate that decreased luminescence is one adaptation *V. fischeri* makes rapidly to become a proficient colonizer of the *E. scolopes* host. Furthermore, although it is clear that the squid select for strains that are proficient to produce light (Visick et al. 2000; Wollenberg et al. 2012), it seems likely that there is additional selection to maintain a luminescence level that is not excessive (Schuster et al. 2010). Future work that assesses the full complement of changes that occur during the experimental evolution, and a determination of which changes make important contributions to colonization proficiency, will provide great insights into the mechanisms of symbiont evolution and the selective forces that direct these processes.

## Perspectives and Future of the Field

The *V. fischeri-E. scolopes* symbiosis is a powerful experimental model for elucidating bacteria-host interactions and the impact of bacteria on host development. One strength of the system comes from the fact that research into squid biology and how bacteria influence host development informs investigations into

the roles of bacteria in symbiosis and vice versa. The result is a synergy of inquiry that has resulted in great advances in our understanding.

Another reason that work on the system has progressed so readily is the continued development of genetic tools that greatly facilitate the manipulation of the bacteria. Early work focused on the generation of *V. fischeri* mutants using such blunt instruments as transposon mutagenesis, gene replacement (with corresponding insertion of antibiotic resistance cassettes), and insertional mutagenesis. These tools were critical at the time and are still used to great advantage. However, it is now also possible to readily and rapidly generate in-frame deletions or incorporate targeted point mutations into the *V. fischeri* chromosome. Refinement of the ES114 genome database combined with whole-genome resequencing also now allows researchers to pinpoint spontaneous mutations and to track the genetic changes in evolved derivatives of the wild type. This new technology could solve old puzzles. For example, genomic sequencing of the hypermotile strains isolated by Millikan and Ruby (2002) may lead us to identify new regulators of motility and symbiosis, while sequencing the visibly bioluminescent derivative of ES114 (Dunlap et al. 1995) may uncover novel pathways of luminescence regulation. Taken together, this expanding genetic toolbox will facilitate ever more refined investigations of the roles of specific genes and their products during symbiosis. Similarly, in the future, the current construction of a comprehensive library of catalogued mutants, encompassing disruptions of nonessential genes, will provide an invaluable resource for mutant analyses.

Another critical advance has been the development of GFP both as a marker, to visualize where the bacterial cells are in the symbiosis, and as a reporter, to provide information on gene expression in symbiotic cells in real time. Our understanding of the establishment of this symbiosis stems largely from the seminal work of Nyholm et al. (2000), who first used GFP-labeled cells to visually discover aggregation and other early infection events. Subsequent work with *V. fischeri* expressing proteins with red fluorescence enabled the visualization of two infecting strains at once (Dunn et al. 2006) and provides a fluorescent standard against which GFP expression can be normalized (Miyashiro et al. 2010). Similarly, researchers studying the squid have pioneered a number of fluorescence-based microscopic methods to investigate processes such as apoptosis or specific proteins in the host. The transparent tissue of the light organ affords a rare opportunity to use fluorescence-based methods to visualize symbiotic processes in intact tissues, and the development of other fluorophores, destabilized GFP derivatives, and various animal-tissue stains should continue to be exploited to great effect.

An increased basic understanding of *V. fischeri* genetic processes has both provided tools and led to new intriguing questions. For example, the identification of a replication origin from a plasmid endogenous to *V. fischeri* permitted the construction of stable shuttle plasmids that could be readily used for a variety of experiments such as complementation or the strain-tagging with GFP, described above. The description of native *V. fischeri*

**Fig. 20.10**
**A molecular geneticist's perspective. The tube containing purified ES114 DNA (pellet at the *bottom* of the tube), used for generating the first *V. fischeri* genome, is shown with Hawaiian palm trees visible in the background**

plasmids has also raised the possibility that genetic information is being exchanged through conjugation between different strains during symbiosis. Similarly, the development of natural transformation as a tool not only provided another method for genetic manipulation of *V. fischeri* but also suggested the possibility of DNA uptake and recombination during symbiosis (Pollack-Berti et al. 2010). Taken together, one wonders whether future investigations will find that the packed confines of the light organ could be a hot spot for horizontal gene transfer.

As in many bacterial systems over the last decades, genomic approaches have transformed our lens into this symbiosis. The availability of the genome sequence of *V. fischeri* strain ES114 (*Fig. 20.10*), and subsequently that of the fish symbiont strain MJ11, made it possible to use bioinformatics to make predictions about the importance of genes in the symbiosis and to readily clone wild-type and mutant alleles of specific genes. Comparative genomic studies will become even more important as the sequences of additional genomes become available, and as the studies of symbiotic associations of *V. fischeri* with other squid species (as well as studies of other bacteria-light-organ symbioses) become more developed (Guerrero-Ferreira and Nishiguchi 2009; Nyholm and Nishiguchi 2008). Likewise, the squid EST and proteome databases have already provided insight into how the host detects and responds to *V. fischeri*, and future work is likely to expand these resources. Additional molecular technology grounded in genomics, bioinformatics, and high-throughput methods—from arrays to RNAseq to single-cell analyses—holds great promise for elucidating bacterial responses to the colonization process (and the corresponding responses of the squid). We anticipate that the next decade will see a great expansion in the availability and productive use of these approaches.

In some cases, knowledge from the system has been slow to impact the conduct of current research. For example, it has been known for a long time that juvenile squid have six pores and six

distinct crypts, and we now know that it is rare for those crypts to be highly colonized by multiple strains: in competition experiments using two differently labeled strains, most crypts contain one or the other strain but usually not both (▶ *Fig. 20.4*) (Dunn et al. 2006). Furthermore, the extent to which the different crypts expel their symbionts in the morning varies. For the most part, this information has not yet impacted our straightforward interpretation of competition experiments: if more cells of one strain are obtained following a competitive colonization experiment, then we conclude that that strain had a colonization advantage. But, did this advantage flow from superior entry into multiple crypts, superior binding within a single crypt relative to another strain, being lucky enough to colonize the right crypt, or some other reason? Follow-up experiments that visualize the colonized animals using fluorescently labeled strains will provide better insights into the requirements for a particular gene/trait.

In the early days of investigating this symbiosis, insights from other systems—and especially pathogenic systems—were used to develop models for how *V. fischeri* and *E. scolopes* interact. For example, it made sense to ask whether flagella were important for colonization, as this was the case for many pathogenic associations. As the field has developed, however, our studies have begun to provide new insights that can inform the work of others. Notably, the identification of H-NOX as a NO sensor and NO as a potential signal has clear implications for pathogenic associations (Wang et al. 2010a). Similarly, the novel nuclear localization of EsPGRP1 and the role of the symbionts in changing the localization of receptors for MAMPS have potential to impact studies of the immune response (Troll et al. 2009a, b). A third example lies in the paucity of good animal models for studying bacterial biofilms; the squid symbiosis makes a robust model, as the parallels between phenotypes observed in culture and during symbiosis are very strong, and thus the potential for informing the biofilm field is high (Morris et al. 2011; Yip et al. 2006).

Many questions remain. Although much is known about conditions and molecules that influence the activities of these organisms and their interactions, including TCT, PG, pheromones, and light itself, numerous bacterial regulators have been identified for which the signal transduction cascade remains to be elucidated. For example, the sensor kinase RscS was identified over 10 years ago, but the signal to which it responds during symbiosis remains elusive. Whether *V. fischeri* recognizes a chemotactic signal to guide it into the light organ and what drives the loss of flagella in colonizing bacteria are among the longstanding intriguing questions about colonization. Indeed, we have yet to truly understand how specificity in this symbiosis is achieved; perhaps there is no one single factor but a multitude of them, such as the ability to form a biofilm, sense a chemotactic signal, resist host defenses, and induce the right set of responses, that together result in the successful symbiont. The opportunity to be the sole occupant of a "privileged" niche is a powerful incentive, and *V. fischeri* has clearly evolved to make use of this prime opportunity for self-advancement. With the increasing sophistication of the tools, approaches, and knowledge of the system, it seems likely that many of the current secrets shortly will be revealed, but also that many more will present themselves.

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## References

Adin DM, Engle JT, Goldman WE, McFall-Ngai MJ, Stabb EV (2009) Mutations in *ampG* and lytic transglycosylase genes affect the net release of peptidoglycan monomers from *Vibrio fischeri*. J Bacteriol 191:2012–2022

Adin DM, Phillips NJ, Gibson BW, Apicella MA, Ruby EG, McFall-Ngai MJ, Hall DB, Stabb EV (2008a) Characterization of *htrB* and *msbB* mutants of the light organ symbiont *Vibrio fischeri*. Appl Environ Microbiol 74:633–644

Adin DM, Visick KL, Stabb EV (2008b) Identification of a cellobiose utilization gene cluster with cryptic β-galactosidase activity in *Vibrio fischeri*. Appl Environ Microbiol 74:4059–4069

Aeckersberg F, Lupp C, Feliciano B, Ruby EG (2001) *Vibrio fischeri* outer membrane protein OmpU plays a role in normal symbiotic colonization. J Bacteriol 183:6590–6597

Alcaide E (2003) Numerical taxonomy of *Vibrionaceae* isolated from cultured amberjack (*Seriola dumerili*) and surrounding water. Curr Microbiol 46:184–189

Altura MA, Stabb E, Goldman W, Apicella M, McFall-Ngai MJ (2011) Attenuation of host NO production by MAMPs potentiates development of the host in the squid-vibrio symbiosis. Cell Microbiol 13:527–537

Anderson RC, Mather JA (1996) Escape responses of *Euprymna scolopes* Berry, 1911 (Cephalopoda: Sepiolidae). J Moll Stud 62:543–545

Antunes LC, Ferreira RB, Lostroh CP, Greenberg EP (2008) A mutational analysis defines *Vibrio fischeri* LuxR binding sites. J Bacteriol 190:4392–4397

Antunes LC, Schaefer AL, Ferreira RB, Qin N, Stevens AM, Ruby EG, Greenberg EP (2007) Transcriptome analysis of the *Vibrio fischeri* LuxR-LuxI regulon. J Bacteriol 189:8387–8391

Arcondeguy T, Jack R, Merrick M (2001) P(II) signal transduction proteins, pivotal players in microbial nitrogen control. Microbiol Mol Biol Rev: MMBR 65:80–105

Baker MD, Wolanin PM, Stock JB (2006) Signal transduction in bacterial chemotaxis. BioEssays 28:9–22

Barnhart MM, Chapman MR (2006) Curli biogenesis and function. Annu Rev Microbiol 60:131–147

Berry SS (1912) The Cephalopoda of the Hawaiian islands. In: Bulletin of the United States Bureau of Fisheries. Government Printing Office, Washington, pp 255–362

Boettcher KJ, Ruby EG (1990) Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. J Bacteriol 172:3701–3706

Boettcher KJ, Ruby EG (1994) Occurrence of plasmid DNA in the sepiolid squid symbiont *Vibrio fischeri*. Curr Microbiol 29:279–286

Boettcher KJ, Ruby EG, McFall-Ngai MJ (1996) Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. J Comp Physiol 179:65–73

Bose JL, Kim U, Bartkowski W, Gunsalus RP, Overley AM, Lyell NL, Visick KL, Stabb EV (2007) Bioluminescence in *Vibrio fischeri* is controlled by the redox-responsive regulator ArcA. Mol Microbiol 65:538–553

Bose JL, Rosenberg CS, Stabb EV (2008) Effects of *luxCDABEG* induction in *Vibrio fischeri*: enhancement of symbiotic colonization and conditional attenuation of growth in culture. Arch Microbiol 190(2):169–183

Bose JL, Wollenberg MS, Colton DM, Mandel MJ, Septer AN, Dunn AK, Stabb EV (2011) Contribution of rapid evolution of the *luxR-luxI* intergenic region to the diverse bioluminescence outputs of *Vibrio fischeri* strains isolated from different environments. Appl Environ Microbiol 77:2445–2457

Bourgois JJ, Sluse FE, Baguet F, Mallefet J (2001) Kinetics of light emission and oxygen consumption by bioluminescent bacteria. J Bioenerg Biomembr 33:353–363

Boylan M, Miyamoto C, Wall L, Grahm A, Meighen E (1989) Lux C, D, and E genes of the *Vibrio fischeri* luminescence operon code for the reductase, transferase, and synthetase enzymes involved in aldehyde biosyntheses. Photochem Photobiol 49:681–688

Browne-Silva J, Nishiguchi MK (2008) Gene sequences of the *pil* operon reveal relationships between symbiotic strains of *Vibrio fischeri*. Int J Syst Evol Microbiol 58:1292–1299

Buck M, Gallegos M-T, Studholme DJ, Guo Y, Gralla JD (2000) The bacterial enhancer-dependent $\sigma^{54}$ ($\sigma^{N}$) transcription factor. J Bacteriol 182: 4129–4136

Burrows LL (2005) Weapons of mass retraction. Mol Microbiol 57:878–888

Callahan SM, Dunlap PV (2000) LuxR- and acyl-homoserine-lactone-controlled non-*lux* genes define a quorum sensing regulon in *Vibrio fischeri*. J Bacteriol 182:2811–2822

Castillo MG, Goodson MS, McFall-Ngai M (2009) Identification and molecular characterization of a complement C3 molecule in a lophotrochozoan, the Hawaiian bobtail squid *Euprymna scolopes*. Dev Comp Immunol 33:69–76

Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL, Hughson FM (2002) Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415:545–549

Chevance FF, Hughes KT (2008) Coordinating assembly of a bacterial macromolecular machine. Nat Rev Microbiol 6:455–465

Chun CK, Scheetz TE, Bonaldo Mde F, Brown B, Clemens A, Crookes-Goodson WJ, Crouch K, DeMartini T, Eyestone M, Goodson MS, Janssens B, Kimbell JL, Koropatnick TA, Kucaba T, Smith C, Stewart JJ, Tong D, Troll JV, Webster S, Winhall-Rice J, Yap C, Casavant TL, McFall-Ngai MJ, Soares MB (2006) An annotated cDNA library of juvenile *Euprymna scolopes* with and without colonization by the symbiont *Vibrio fischeri*. BMC Genomics 7:154

Chun CK, Troll JV, Koroleva I, Brown B, Manzella L, Snir E, Almabrazi H, Scheetz TE, Bonaldo Mde F, Casavant TL, Soares MB, Ruby EG, McFall-Ngai MJ (2008) Effects of colonization, luminescence, and autoinducer on host transcription during development of the squid-vibrio association. Proc Natl Acad Sci USA 105:11323–11328

Claes MF, Dunlap PV (2000) Aposymbiotic culture of the sepiolid squid *Euprymna scolopes*: role of the symbiotic bacterium *Vibrio fischeri* in host animal growth, development, and light organ morphogenesis. J Exp Zool 286:280–296

Correa NE, Lauriano CM, McGee R, Klose KE (2000) Phosphorylation of the flagellar regulatory protein FlrC is necessary for *Vibrio cholerae* motility and enhanced colonization. Mol Microbiol 35:743–755

Correa NE, Peng F, Klose KE (2005) Roles of the regulatory proteins FlhF and FlhG in the *Vibrio cholerae* flagellar transcription hierarchy. J Bacteriol 187:6324–6332

Czyz A, Plata K, Wegrzyn G (2003) Stimulation of DNA repair as an evolutionary drive for bacterial luminescence. Lumin: J Biol Chem Lumin 18:140–144

Darnell CL, Hussa EA, Visick KL (2008) The putative hybrid sensor kinase SypF coordinates biofilm formation in *Vibrio fischeri* by acting upstream of two response regulators, SypG and VpsR. J Bacteriol 190:4941–4950

Davidson SK, Koropatnick TA, Kossmehl R, Sycuro L, McFall-Ngai MJ (2004) NO means 'yes' in the squid-vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. Cell Microbiol 6:1139–1151

Deloney-Marino CR, Visick KL (2012) Role for *cheR* of *Vibrio fischeri* in the Vibrio-squid symbiosis. Can J Microbiol 58:29–38

DeLoney-Marino CR, Wolfe AJ, Visick KL (2003) Chemoattraction of *Vibrio fischeri* to serine, nucleosides and N-acetylneuraminic acid, a component of squid light organ mucus. Appl Environ Microbiol 69:7527–7530

DeLoney CR, Bartley TM, Visick KL (2002) Role for phosphoglucomutase in *Vibrio fischeri-Euprymna scolopes* symbiosis. J Bacteriol 184:5121–5129

Doino JA, McFall-Ngai MJ (1995) A transient exposure to symbiosis-competent bacteria induces light organ morphogenesis in the host squid. Biol Bull 189:347–355

Doino Lemus J, McFall-Ngai MJ (2000) Alterations in the proteome of the *Euprymna scolopes* light organ in response to symbiotic *Vibrio fischeri*. Appl Environ Microbiol 66:4091–4097

Dunlap PV, Ast JC, Kimura S, Fukui A, Yoshino T, Endo H (2007) Phylogenetic analysis of host-symbiont specificity and codivergence in bioluminescent symbioses. Cladistics 23:507–532

Dunlap PV, Greenberg EP (1985) Control of *Vibrio fischeri* luminescence gene expression in *Escherichia coli* by cyclic AMP and cyclic AMP receptor protein. J Bacteriol 164:45–50

Dunlap PV, Greenberg EP (1988) Control of *Vibrio fischeri lux* gene transcription by a cyclic AMP receptor protein-LuxR protein regulatory circuit. J Bacteriol 170:4040–4046

Dunlap PV, Kita-Tsukamoto K, Waterbury JB, Callahan SM (1995) Isolation and characterization of a visibly luminous variant of *Vibrio fischeri* strain ES114 from the sepiolid squid *Euprymna scolopes*. Arch Microbiol 164:194–202

Dunlap PV, Ray JM (1989) Requirement for autoinducer in transcriptional negative autoregulation of the *Vibrio fischeri luxR* gene in *Escherichia coli*. J Bacteriol 171:3549–3552

Dunn AK, Karr EA, Wang Y, Batton AR, Ruby EG, Stabb EV (2010) The alternative oxidase (AOX) gene in *Vibrio fischeri* is controlled by NsrR and upregulated in response to nitric oxide. Mol Microbiol 77:44–55

Dunn AK, Martin MO, Stabb E (2005) Characterization of pES213, a small mobilizable plasmid from *Vibrio fischeri*. Plasmid 54:114–134

Dunn AK, Millikan DS, Adin DM, Bose JL, Stabb EV (2006) New *rfp*- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux* expression in situ. Appl Environ Microbiol 72:802–810

Dunn AK, Stabb EV (2007) Beyond quorum sensing: the complexities of prokaryotic parliamentary procedures. Anal Bioanal Chem 387:391–398

Dunn AK, Stabb EV (2008a) Genetic analysis of trimethylamine N-oxide reductases in the light organ symbiont *Vibrio fischeri* ES114. J Bacteriol 190:5814–5823

Dunn AK, Stabb EV (2008b) The twin arginine translocation system contributes to symbiotic colonization of *Euprymna scolopes* by *Vibrio fischeri*. FEMS Microbiol Lett 279:251–258

Duperthuy M, Schmitt P, Garzon E, Caro A, Rosa RD, Le Roux F, Lautredou-Audouy N, Got P, Romestand B, de Lorgeril J, Kieffer-Jaquinod S, Bachere E, Destoumieux-Garzon D (2011) Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. Proc Natl Acad Sci USA 108:2993–2998

Egland KA, Greenberg EP (1999) Quorum sensing in *Vibrio fischeri*: elements of the *luxI* promoter. Mol Microbiol 31:1197–1204

Egland KA, Greenberg EP (2000) Conversion of the *Vibrio fischeri* transcriptional activator, LuxR, to a repressor. J Bacteriol 182:805–811

Engebrecht J, Nealson K, Silverman M (1983) Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. Cell 32:773–781

Engebrecht J, Silverman M (1984) Identification of genes and gene products necessary for bacterial bioluminescence. Proc Natl Acad Sci USA 81:4154–4158

Fidopiastis PM, Miyamoto CM, Jobling MG, Meighen EA, Ruby EG (2002) LitR, a new transcriptional activator in *Vibrio fischeri*, regulates luminescence and symbiotic light organ colonization. Mol Microbiol 45:131–143

Fidopiastis PM, von Boletzky S, Ruby EG (1998) A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. J Bacteriol 180:59–64

Fidopiastis PM, Rader BA, Gerling DG, Gutierrez NA, Watkins KH, Frey MW, Nyholm SV, Whistler CA (2012) Characterization of a *Vibrio fischeri* aminopeptidase and evidence for its influence on an early stage of squid colonization. J Bacteriol 194:3995–4002

Fitzgerald JM (1977) Classification of luminous bacteria from the light organ of the Australian pinecone fish, *Cleidopus gloriamaris*. Arch Microbiol 112:153–156

Foster JS, Apicella MA, McFall-Ngai MJ (2000) *Vibrio fischeri* lipopolysaccharide induces developmental apoptosis, but not complete morphogenesis, of the *Euprymna scolopes* symbiotic light organ. Dev Biol 226:242–254

Foster JS, McFall-Ngai MJ (1998) Induction of apoptosis by cooperative bacteria in the morphogenesis of host epithelial tissues. Dev Genes Evol 208:295–303

Friedrich WF, Greenberg EP (1983) Glucose repression of luminescence and luciferase in *Vibrio fischeri*. Arch Microbiol 134:87–91

Fuqua C, Winans SC, Greenberg EP (1996) Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu Rev Microbiol 50:727–751

Fuqua WC, Winans SC, Greenberg EP (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol 176:269–275

Garcia-Amado MA, Bozo-Hurtado L, Astor Y, Suarez P, Chistoserdov A (2011) Denaturing gradient gel electrophoresis analyses of the vertical distribution and diversity of *Vibrio* spp. populations in the Cariaco Basin. FEMS Microbiol Ecol 77:347–356

Goodson MS, Kojadinovic M, Troll JV, Scheetz TE, Casavant TL, Soares MB, McFall-Ngai MJ (2005) Identifying components of the NF-κB pathway in the beneficial *Euprymna scolopes*-*Vibrio fischeri* light organ symbiosis. Appl Environ Microbiol 71:6934–6946

Graf J, Dunlap PV, Ruby EG (1994) Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*. J Bacteriol 176:6986–6991

Graf J, Ruby EG (1998) Host-derived amino acids support the proliferation of symbiotic bacteria. Proc Natl Acad Sci USA 95:1818–1822

Graf J, Ruby EG (2000) Novel effects of a transposon insertion in the *Vibrio fischeri glnD* gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. Mol Microbiol 37:168–179

Gray KM, Greenberg EP (1992a) Physical and functional maps of the luminescence gene cluster in an autoinducer-deficient *Vibrio fischeri* strain isolated from a squid light organ. J Bacteriol 174:4384–4390

Gray KM, Greenberg EP (1992b) Sequencing and analysis of *luxR* and *luxI*, the luminescence regulatory genes from the squid light organ symbiont *Vibrio fischeri* ES114. Mol Mar Biol Biotechnol 1:414–419

Guerrero-Ferreira RC, Nishiguchi MK (2009) Ultrastructure of light organs of loliginid squids and their bacterial symbionts: a novel model system for the study of marine symbioses. Vie Milieu Paris 59:307–313

Hanlon RT, Claes MF, Ashcraft SE, Dunlap PV (1997) Laboratory culture of the sepiolid squid *Euprymna scolopes*: a model system for bacteria-animal symbiosis. Biol Bull 192:364–374

Hanzelka BL, Parsek MR, Val DL, Dunlap PV, Cronan JE Jr, Greenberg EP (1999) Acylhomoserine lactone synthase activity of the *Vibrio fischeri* AinS protein. J Bacteriol 181:5766–5770

Hastings JW, Nealson KH (1977) Bacterial bioluminescence. Annu Rev Microbiol 31:549–595

Hastings JW, Riley WH, Massa J (1965) The purification, properties, and chemiluminescent quantum yield of bacterial luciferase. J Biol Chem 240: 1473–1481

Haygood MG, Nealson KH (1985) Mechanisms of iron regulation of luminescence in *Vibrio fischeri*. J Bacteriol 162:209–216

Heath-Heckman EA, McFall-Ngai MJ (2011) The occurrence of chitin in the hemocytes of invertebrates. Zoology (Jena) 114:191–198

Hussa EA, Darnell CL, Visick KL (2008) RscS functions upstream of SypG to control the *syp* locus and biofilm formation in *Vibrio fischeri*. J Bacteriol 190:4576–4583

Hussa EA, O'Shea TM, Darnell CL, Ruby EG, Visick KL (2007) Two-component response regulators of *Vibrio fischeri*: identification, mutagenesis and characterization. J Bacteriol 189:5825–5838

Jones BW, Lopez JE, Huttenburg J, Nishiguchi MK (2006) Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. Mol Ecol 15:4317–4329

Jones BW, Maruyama A, Ouverney CC, Nishiguchi MK (2007) Spatial and temporal distribution of the vibrionaceae in coastal waters of Hawaii, Australia, and France. Microb Ecol 54:314–323

Jones BW, Nishiguchi MK (2004) Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). Mar Biol 144:1151–1155

Kaplan HB, Greenberg EP (1985) Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. J Bacteriol 163:1210–1214

Keynan A, Hastings JW (1961) The isolation and characterization of dark mutants of luminous bacteria. Biol Bull 121:375

Kimbell JR, McFall-Ngai MJ (2003) The squid-Vibrio symbiosis: From demes to genes. Integr Comp Biol 43:254–260

Kimbell JR, McFall-Ngai MJ (2004) Symbiont-induced changes in host actin during the onset of a beneficial animal-bacterial association. Appl Environ Microbiol 70:1434–1441

Kimbell JR, McFall-Ngai MJ, Roderick GK (2002) Two genetically distinct populations of bobtail squid, *Euprymna scolopes*, exist on the island of Oahu. Pacific Sci 56:347–355

Koropatnick TA, Engle JT, Apicella MA, Stabb EV, Goldman WE, McFall-Ngai MJ (2004) Microbial factor-mediated development in a host-bacterial mutualism. Science 306:1186–1187

Koropatnick TA, Kimbell JR, McFall-Ngai MJ (2007) Responses of host hemocytes during the initiation of the squid-Vibrio symbiosis. Biol Bull 212:29–39

Krasity BC, Troll JV, Weiss JP, McFall-Ngai MJ (2011) LBP/BPI proteins and their relatives: conservation over evolution and roles in mutualism. Biochem Soc Trans 39:1039–1044

Kulkarni PR, Cui X, Williams JW, Stevens AM, Kulkarni RV (2006) Prediction of CsrA-regulating small RNAs in bacteria and their experimental verification in *Vibrio fischeri*. Nucleic Acids Res 34:3361–3369

Kuo A, Blough NV, Dunlap PV (1994) Multiple *N*-acyl-L-homoserine lactone autoinducers of luminescence in the marine symbiotic bacterium *Vibrio fischeri*. J Bacteriol 176:7558–7565

Kuo A, Callahan SM, Dunlap PV (1996) Modulation of luminescence operon expression by *N*-octanoyl-L-homoserine lactone in *ainS* mutants of *Vibrio fischeri*. J Bacteriol 178:971–976

Lamarcq LH, McFall-Ngai MJ (1998) Induction of a gradual, reversible morphogenesis of its host's epithelial brush border by *Vibrio fischeri*. Infect Immun 66:777–785

Lamas J, Anadon R, Devesa S, Toranzo AE (1990) Visceral neoplasia and epidermal papillomas in cultured turbot *Scophthalmus maximus*. Dis Aquat Organ 8:179–187

Lapouge K, Schubert M, Allain FH, Haas D (2008) Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. Mol Microbiol 67:241–253

Lee K-H, Ruby EG (1992) Detection of the light organ symbiont, *Vibrio fischeri*, in Hawaiian seawater by using *lux* gene probes. Appl Environ Microbiol 58:942–947

Lee K-H, Ruby EG (1994a) Competition between *Vibrio fischeri* strains during initiation and maintenance of a light organ symbiosis. J Bacteriol 176: 1985–1991

Lee K-H, Ruby EG (1994b) Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl Environ Microbiol 60:1565–1571

Liu X, Parales RE (2008) Chemotaxis of *Escherichia coli* to pyrimidines: a new role for the signal transducer Tap. J Bacteriol 190:972–979

Liu X, Wood PL, Parales JV, Parales RE (2009) Chemotaxis to pyrimidines and identification of a cytosine chemoreceptor in *Pseudomonas putida*. J Bacteriol 191:2909–2916

Lupp C, Hancock RE, Ruby EG (2002) The *Vibrio fischeri sapABCDF* locus is required for normal growth, both in culture and in symbiosis. Arch Microbiol 179:57–65

Lupp C, Ruby EG (2004) *Vibrio fischeri* LuxS and AinS: Comparative study of two signal synthases. J Bacteriol 186:3873–3881

Lupp C, Ruby EG (2005) *Vibrio fischeri* utilizes two quorum-sensing systems for the regulation of early and late colonization factors. J Bacteriol 187: 3620–3629

Lupp C, Urbanowski M, Greenberg EP, Ruby EG (2003) The *Vibrio fischeri* quorum-sensing systems *ain* and *lux* sequentially induce luminescence gene expression and are important for persistence in the squid host. Mol Microbiol 50:319–331

Lyell NL, Dunn AK, Bose JL, Stabb EV (2010) Bright mutants of *Vibrio fischeri* ES114 reveal conditions and regulators that control bioluminescence and expression of the *lux* operon. J Bacteriol 192:5103–5114

Mandel MJ, Schaefer AL, Brennan CA, Heath-Heckman EA, Deloney-Marino CR, McFall-Ngai MJ, Ruby EG (2012) Squid-derived chitin oligosaccharides are a chemotactic signal during colonization by *Vibrio fischeri*. Appl Environ Microbiol 78:4620–4626

Mandel MJ, Wollenberg MS, Stabb EV, Visick KL, Ruby EG (2009) A single regulatory gene is sufficient to alter bacterial host range. Nature 458:215–218

McCann J, Stabb EV, Millikan DS, Ruby EG (2003) Population dynamics of *Vibrio fischeri* during infection of *Euprymna scolopes*. Appl Environ Microbiol 69:5928–5934

McFall-Ngai M, Brennan C, Weis V, Lamarcq L (1998) Mannose adhesin-glycan interactions in the *Euprymna scolopes-Vibrio fischeri* symbiosis. In: Le Gal Y, Halvorson HO (eds) New developments in marine biotechnology. Plenum Press, New York, pp 273–276

McFall-Ngai M, Heath-Heckman EA, Gillette AA, Peyer SM, Harvie EA (2011) The secret languages of coevolved symbioses: Insights from the *Euprymna scolopes-Vibrio fischeri* symbiosis. Semin Immunol 24:3–8

McFall-Ngai M, Montgomery MK (1990) The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda: Sepiolidae). Biol Bull 179:332–339

McFall-Ngai M, Nyholm SV, Castillo MG (2010) The role of the immune system in the initiation and persistence of the *Euprymna scolopes–Vibrio fischeri* symbiosis. Semin Immunol 22:48–53

McFall-Ngai MJ (1999) Consequences of evolving with bacterial symbionts: insights from the squid-vibrio associations. Annu Rev Ecol Syst 30:235–256

McFall-Ngai MJ, Ruby EG (1991) Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. Science 254:1491–1494

McFall-Ngai MJ, Ruby EG (1998) Sepiolids and vibrios: when first they meet. Reciprocal interactions between host and symbiont lead to the creation of a complex light-emitting organ. BioScience 48:257–265

Millikan DS, Ruby EG (2002) Alterations in *Vibrio fischeri* motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. Appl Environ Microbiol 68:2519–2528

Millikan DS, Ruby EG (2003) FlrA, a $\sigma^{54}$-dependent transcriptional activator in *Vibrio fischeri*, is required for motility and symbiotic light-organ colonization. J Bacteriol 185:3547–3557

Millikan DS, Ruby EG (2004) *Vibrio fischeri* flagellin A is essential for normal motility and for symbiotic competence during initial squid light organ colonization. J Bacteriol 186:4315–4325

Miyamoto CM, Lin YH, Meighen EA (2000) Control of bioluminescence in *Vibrio fischeri* by the LuxO signal response regulator. Mol Microbiol 36:594–607

Miyashiro T, Klein W, Oehlert D, Cao X, Schwartzman J, Ruby EG (2011) The N-acetyl-D-glucosamine repressor NagC of *Vibrio fischeri* facilitates colonization of *Euprymna scolopes*. Mol Microbiol 82:894–903

Miyashiro T, Wollenberg MS, Cao X, Oehlert D, Ruby EG (2010) A single *qrr* gene is necessary and sufficient for LuxO-mediated regulation in *Vibrio fischeri*. Mol Microbiol 77:1556–1567

Montes M, Farto R, Perez MJ, Armada SP, Nieto TP (2006) Genotypic diversity of Vibrio isolates associated with turbot (*Scophthalmus maximus*) culture. Res Microbiol 157:487–495

Montgomery MK, McFall-Ngai M (1993) Embryonic development of the light organ of the sepiolid squid *Euprymna scolopes* Berry. Biol Bull 184:296–308

Montgomery MK, McFall-Ngai M (1994) Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*. Development 120:1719–1729

Morris AR, Darnell CL, Visick KL (2011) Inactivation of a novel response regulator is necessary for biofilm formation and host colonization by *Vibrio fischeri*. Mol Microbiol 82:114–130

Morris AR, Visick KL (2010) Control of biofilm formation and colonization in *Vibrio fischeri*: a role for partner switching? Environ Microbiol 12:2051–2059

Moynihan M (1983) Notes on the behavior of *Euprymna scolopes* (Cephalopoda: Sepiolidae). Behaviour 85:25–41

Mulvey MA, Lopez-Boado YS, Wilson CL, Roth R, Parks WC, Heuser J, Hultgren SJ (1998) Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. Science 282:1494–1497

Nairz M, Schroll A, Sonnweber T, Weiss G (2010) The struggle for iron - a metal at the host-pathogen interface. Cell Microbiol 12:1691–1702

Nijvipakul S, Wongratana J, Suadee C, Entsch B, Ballou DP, Chaiyen P (2008) LuxG is a functioning flavin reductase for bacterial luminescence. J Bacteriol 190:1531–1538

Nishiguchi MK (2000) Temperature affects species distribution in symbiotic populations of *Vibrio* spp. Appl Environ Microbiol 66:3550–3555

Nishiguchi MK (2002) Host-symbiont recognition in the environmentally transmitted sepiolid squid-*Vibrio* mutualism. Microb Ecol 44:10–18

Nishiguchi MK, Ruby EG, McFall-Ngai MJ (1998) Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-vibrio symbioses. Appl Environ Microbiol 64:3209–3213

Nyholm SV, Deplancke B, Gaskins HR, Apicella MA, McFall-Ngai MJ (2002) Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion during symbiont colonization of the *Euprymna scolopes* light organ. Appl Environ Microbiol 68:5113–5122

Nyholm SV, McFall-Ngai MJ (1998) Sampling the light-organ microenvironment of *Euprymna scolopes*: description of a population of host cells in association with the bacterial symbiont *Vibrio fischeri*. Biol Bull 195:89–97

Nyholm SV, McFall-Ngai MJ (2003) Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. Appl Environ Microbiol 69:3932–3937

Nyholm SV, McFall-Ngai MJ (2004) The winnowing: establishing the squid-*Vibrio* symbiosis. Nat Rev Microbiol 2:632–642

Nyholm SV, Nishiguchi MK (2008) The evolutionary ecology of a sepiolid squid-Vibrio association: from cell to environment. Vie Milieu Paris 58:175–184

Nyholm SV, Stabb EV, Ruby EG, McFall-Ngai MJ (2000) Establishment of an animal-bacterial association: recruiting symbiotic vibrios from the environment. Proc Natl Acad Sci USA 97:10231–10235

Nyholm SV, Stewart JJ, Ruby EG, McFall-Ngai MJ (2009) Recognition between symbiotic *Vibrio fischeri* and the haemocytes of *Euprymna scolopes*. Environ Microbiol 11:483–493

O'Shea TM, DeLoney-Marino CR, Shibata S, Aizawa S-I, Wolfe AJ, Visick KL (2005) Magnesium promotes flagellation of *Vibrio fischeri*. J Bacteriol 187:2058–2065

O'Shea TM, Klein AH, Geszvain K, Wolfe AJ, Visick KL (2006) Diguanylate cyclases control magnesium-dependent motility of *Vibrio fischeri*. J Bacteriol 188:8196–8205

Ochman H, Moran NA (2001) Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. Science 292:1096–1099

Orndorff SA, Colwell RR (1980) Distribution and identification of luminous bacteria from the Sargasso Sea. Appl Environ Microbiol 39:983–987

Pelicic V (2008) Type IV pili: *e pluribus unum*? Mol Microbiol 68:827–837

Phillips NJ, Adin DM, Stabb EV, McFall-Ngai MJ, Apicella MA, Gibson BW (2011) The lipid A from *Vibrio fischeri* lipopolysaccharide: a unique structure bearing a phosphoglycerol moiety. J Biol Chem 286:21203–21219

Pizarro-Cerda J, Cossart P (2006) Bacterial adhesion and entry into host cells. Cell 124:715–727

Pollack-Berti A, Wollenberg MS, Ruby EG (2010) Natural transformation of *Vibrio fischeri* requires *tfoX* and *tfoY*. Environ Microbiol 12:2302–2311

Pooley DT, Larsson J, Jones G, Rayner-Brandes MH, Lloyd D, Gibson C, Stewart WR (2004) Continuous culture of photobacterium. Biosens Bioelectron 19:1457–1463

Prouty MG, Correa NE, Klose KE (2001) The novel $\sigma^{54}$- and $\sigma^{28}$-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. Mol Microbiol 39:1595–1609

Ramesh A, Venugopalan VK (1989) Response of enteric luminous bacteria to environmental conditions in the gut of the fish. J Appl Bacteriol 66:529–533

Ruby EG, Asato LM (1993) Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. Arch Microbiol 159:160–167

Ruby EG, Greenberg EP, Hastings JW (1980) Planktonic marine luminous bacteria: species distribution in the water column. Appl Environ Microbiol 39:302–306

Ruby EG, Morin JG (1979) Luminous enteric bacteria of marine fishes: a study of their distribution, densities, and dispersion. Appl Environ Microbiol 38:406–411

Ruby EG, Nealson KH (1976) Symbiotic association of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica*: a model of symbiosis based on bacterial studies. Biol Bull 151:574–586

Ruby EG, Urbanowski M, Campbell J, Dunn A, Faini M, Gunsalus R, Lostroh P, Lupp C, McCann J, Millikan D, Schaefer A, Stabb E, Stevens A, Visick K, Whistler C, Greenberg EP (2005) Complete genome sequence of *Vibrio fischeri*: A symbiotic bacterium with pathogenic congeners. Proc Natl Acad Sci USA 102:3004–3009

Schaefer AL, Val DL, Hanzelka BL, Cronan JE Jr, Greenberg EP (1996) Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. Proc Natl Acad Sci USA 93:9505–9509

Schleicher TR, Nyholm SV (2011) Characterizing the host and symbiont proteomes in the association between the Bobtail squid, *Euprymna scolopes*, and the bacterium *Vibrio fischeri*. PloS One 6:e25649

Schuster BM, Perry LA, Cooper VS, Whistler CA (2010) Breaking the language barrier: experimental evolution of non-native *Vibrio fischeri* in squid tailors luminescence to the host. Symbiosis 51:85–96

Septer AN, Bose JL, Dunn AK, Stabb EV (2010) FNR-mediated regulation of bioluminescence and anaerobic respiration in the light-organ symbiont *Vibrio fischeri*. FEMS Microbiol Lett 306:72–81

Septer AN, Wang Y, Ruby EG, Stabb EV, Dunn AK (2011) The haem-uptake gene cluster in *Vibrio fischeri* is regulated by Fur and contributes to symbiotic colonization. Environ Microbiol 13(11):2855–64

Shears JS (1988) The use of a sand-coat in relation to feeding and diel activity in the sepiolid squid *Euprymna scolopes*. Malacologia 29:121–133

Singley CT (1983) Euprymna scolopes. In: Boyle PR (ed) Cephalopod life cycles. Academic, London, pp 69–74

Small A, McFall-Ngai M (1999) Halide peroxidase in tissues that interact with bacteria in the host squid *Euprymna scolopes*. J Cell Biochem 72:445–457

Soto W, Gutierrez J, Remmenga MD, Nishiguchi MK (2009) Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches. Microb Ecol 57:140–150

Spiro S (2010) An alternative route to nitric oxide resistance. Mol Microbiol 77:6–10

Stabb EV (2005) Shedding light on the bioluminescence "paradox". ASM News 71:223–229

Stabb EV, Millikan DS (2009) Is the *Vibrio fischeri-Euprymna scolopes* symbiosis a defensive mutualism? In: White JF Jr, Torres MS (eds) Defensive mutualism in microbial symbiosis. Taylor and Francis, Boca Raton, pp 85–98

Stabb EV, Reich KA, Ruby EG (2001) *Vibrio fischeri* genes *hvnA* and *hvnB* encode secreted NAD(+)-glycohydrolases. J Bacteriol 183:309–317

Stabb EV, Ruby EG (2003) Contribution of *pilA* to competitive colonization of the squid *Euprymna scolopes* by *Vibrio fischeri*. Appl Environ Microbiol 69:820–826

Stabb EV, Schaefer A, Bose JL, Ruby EG (2008) Quorum signaling and symbiosis in the marine luminous bacterium *Vibrio fischeri*. In: Winans SC, Bassler BL (eds) Chemical communication among microbes. ASM Press, Washington, DC, pp 233–250

Stabili L, Gravili C, Tredici SM, Piraino S, Tala A, Boero F, Alifano P (2008) Epibiotic Vibrio luminous bacteria isolated from some hydrozoa and bryozoa species. Microb Ecol 56:625–636

Studer SV, Mandel MJ, Ruby EG (2008) AinS quorum sensing regulates the *Vibrio fischeri* acetate switch. J Bacteriol 190:5915–5923

Sugita H, Ito Y (2006) Identification of intestinal bacteria from Japanese flounder (*Paralichthys olivaceus*) and their ability to digest chitin. Lett Appl Microbiol 43:336–342

Sycuro L, Ruby EG, McFall-Ngai MJ (2006) Confocal microscopy of the light organ crypts in juvenile *Euprymna scolopes* reveals their mophological complexity and dynamic function in symbiosis. J Morphol 267:555–568

Takeuchi K, Kiefer P, Reimmann C, Keel C, Dubuis C, Rolli J, Vorholt JA, Haas D (2009) Small RNA-dependent expression of secondary metabolism is controlled by Krebs cycle function in *Pseudomonas fluorescens*. J Biol Chem 284:34976–34985

Tantillo GM, Fontanarosa M, Di Pinto A, Musti M (2004) Updated perspectives on emerging vibrios associated with human infections. Lett Appl Microbiol 39:117–126

Tomarev SI, Zinovieva RD, Weis VM, Chepelinsky AB, Piatigorsky J, McFall-Ngai MJ (1993) Abundant mRNAs in the squid light organ encode proteins with a high similarity to mammalian peroxidases. Gene 132:219–226

Tong D, Rozas NS, Oakley TH, Mitchell J, Colley NJ, McFall-Ngai MJ (2009) Evidence for light perception in a bioluminescent organ. Proc Natl Acad Sci USA 106:9836–9841

Troll JV, Adin DM, Wier AM, Paquette N, Silverman N, Goldman WE, Stadermann FJ, Stabb EV, McFall-Ngai MJ (2009a) Peptidoglycan induces loss of a nuclear peptidoglycan recognition protein during host tissue development in a beneficial animal-bacterial symbiosis. Cell Microbiol 11:1114–1127

Troll JV, Bent EH, Pacquette N, Wier AM, Goldman WE, Silverman N, McFall-Ngai MJ (2009b) Taming the symbiont for coexistence: a host PGRP neutralizes a bacterial symbiont toxin. Environ Microbiol 12:2190–2203

Tu S-C, Mager HIX (1995) Biochemistry of bacterial bioluminescence. Photochem Photobiol 62:615–624

Visick KL (2009) An intricate network of regulators controls biofilm formation and colonization by *Vibrio fischeri*. Mol Microbiol 74:782–789

Visick KL, Foster J, Doino J, McFall-Ngai M, Ruby EG (2000) *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. J Bacteriol 182:4578–4586

Visick KL, O'Shea TM, Klein A, Geszvain K, Wolfe AJ (2007) The sugar phosphotransferase system of *Vibrio fischeri* inhibits both motility and bioluminescence. J Bacteriol 189:2571–2574

Visick KL, Ruby EG (1998) The periplasmic, group III catalase of *Vibrio fischeri* is required for normal symbiotic competence and is induced both by oxidative stress and by approach to stationary phase. J Bacteriol 180:2087–2092

Visick KL, Skoufos LM (2001) Two-component sensor required for normal symbiotic colonization of *Euprymna scolopes* by *Vibrio fischeri*. J Bacteriol 183:835–842

Walker EL, Bose JL, Stabb EV (2006) Photolyase confers resistance to UV light but does not contribute to the symbiotic benefit of bioluminescence in *Vibrio fischeri* ES114. Appl Environ Microbiol 72:6600–6606

Wang Y, Dufour YS, Carlson HK, Donohue TJ, Marletta MA, Ruby EG (2010a) H-NOX-mediated nitric oxide sensing modulates symbiotic colonization by *Vibrio fischeri*. Proc Natl Acad Sci USA 107:8375–8380

Wang Y, Dunn AK, Wilneff J, McFall-Ngai MJ, Spiro S, Ruby EG (2010b) *Vibrio fischeri* flavohaemoglobin protects against nitric oxide during initiation of the squid-*Vibrio* symbiosis. Mol Microbiol 78:903–915

Wei SL, Young RE (1989) Development of symbiotic bacterial bioluminescence in a nearshore cephalopod, *Euprymna scolopes*. Mar Biol 103:541–546

Weis VM, Small AL, McFall-Ngai MJ (1996) A peroxidase related to the mammalian antimicrobial protein myeloperoxidase in the *Euprymna-Vibrio* mutualism. Proc Natl Acad Sci USA 93:13683–13688

Welch RA, Burland V, Plunkett G 3rd, Redford P, Roesch P, Rasko D, Buckles EL, Liou SR, Boutin A, Hackett J, Stroud D, Mayhew GF, Rose DJ, Zhou S, Schwartz DC, Perna NT, Mobley HL, Donnenberg MS, Blattner FR (2002) Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. Proc Natl Acad Sci USA 99:17020–17024

Whistler CA, Koropatnick TA, Pollack A, McFall-Ngai MJ, Ruby EG (2007) The GacA global regulator of *Vibrio fischeri* is required for normal host tissue responses that limit subsequent bacterial colonization. Cell Microbiol 9:766–778

Whistler CA, Ruby EG (2003) GacA regulates symbiotic colonization traits of *Vibrio fischeri* and facilitates a beneficial association with an animal host. J Bacteriol 185:7202–7212

Wier AM, Nyholm SV, Mandel MJ, Massengo-Tiasse RP, Schaefer AL, Koroleva I, Splinter-Bondurant S, Brown B, Manzella L, Snir E, Almabrazi H, Scheetz TE, Bonaldo Mde F, Casavant TL, Soares MB, Cronan JE, Reed JL, Ruby EG, McFall-Ngai MJ (2010) Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. Proc Natl Acad Sci USA 107:2259–2264

Wolfe AJ, Berg HC (1989) Migration of bacteria in semisolid agar. Proc Natl Acad Sci USA 86:6973–6977

Wolfe AJ, Millikan DS, Campbell JM, Visick KL (2004) *Vibrio fischeri* $\sigma^{54}$ controls motility, biofilm formation, luminescence and colonization. Appl Environ Microbiol 70:2520–2524

Wolfe AJ, Visick KL (2010) The second messenger cyclic di-GMP. ASM Press, Washington, DC

Wollenberg MS, Preheim SP, Polz MF, Ruby EG (2011) Polyphyly of non-bioluminescent *Vibrio fischeri* sharing a *lux*-locus deletion. Environ Microbiol 14(3):655–68

Wollenberg MS, Ruby EG (2009) Population structure of *Vibrio fischeri* within the light organs of *Euprymna scolopes* squid from Two Oahu (Hawaii) populations. Appl Environ Microbiol 75:193–202

Wollenberg MS, Ruby EG (2012) Phylogeny and fitness of *Vibrio fischeri* from the light organs of Euprymna scolopes in two Oahu, Hawaii populations. Isme J 6(2):352–62

Wright KJ, Seed PC, Hultgren SJ (2007) Development of intracellular bacterial communities of uropathogenic *Escherichia coli* depends on type 1 pili. Cell Microbiol 9:2230–2241

Yildiz FH, Dolganov NA, Schoolnik GK (2001) VpsR, a member of the response regulators of the two-component regulatory systems, is required for expression of *vps* biosynthesis genes and EPS(ETr)-associated phenotypes in *Vibrio cholerae* O1 El Tor. J Bacteriol 183:1716–1726

Yildiz FH, Visick KL (2009) *Vibrio* biofilms: so much the same yet so different. Trends Microbiol 17:109–118

Yip ES, Geszvain K, DeLoney-Marino CR, Visick KL (2006) The symbiosis regulator RscS controls the *syp* gene locus, biofilm formation and symbiotic aggregation by *Vibrio fischeri*. Mol Microbiol 62:1586–1600

Yip ES, Grublesky BT, Hussa EA, Visick KL (2005) A novel, conserved cluster of genes promotes symbiotic colonization and $\sigma^{54}$-dependent biofilm formation by *Vibrio fischeri*. Mol Microbiol 57:1485–1498

Zenno S, Saigo K (1994) Identification of the genes encoding NAD(P)H-flavin oxidoreductases that are similar in sequence to *Escherichia coli* Fre in four species of luminous bacteria: *Photorhabdus luminescens*, *Vibrio fischeri*, *Vibrio harveyi*, and *Vibrio orientalis*. J Bacteriol 176:3544–3551

Ziegler MM, Baldwin TO (1981) Biochemistry of bacterial bioluminescence. In: Current topics in bioenergetics. Academic, Chestnut Hill, pp 65–113

Zuber S, Carruthers F, Keel C, Mattart A, Blumer C, Pessi G, Gigot-Bonnefoy C, Schnider-Keel U, Heeb S, Reimmann C, Haas D (2003) GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0. Mol Plant Microbe Interact: MPMI 16:634–644

# 21 Rumen Symbioses

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## Ruminant Digestive System and the Rumen Environment

There are 155 ruminant species, six of which are domesticated: cattle, sheep, goats, buffaloes, reindeer and yaks. Chief among these are the dairy cattle and sheep, and consequently most of our knowledge is based on studies performed on these species. Ruminants are herbivores, and a fermentative digestive system in their foregut allows them to absorb and digest large amounts of plant material. This method of digesting feed is considered to be a cooperative interaction between the host and its resident microorganisms: the ruminant digestive system relies on its resident microorganisms to digest the feed and subsequently absorbs their fermentation products. Therefore, the partners are in a cooperation in which the animal provides the food and a suitable environment and the microorganisms digest the food, making it accessible to the animal (Mackie 2002). Hence, to fulfill its part of the partnership, the ruminant's digestive system has to provide optimal accommodations for its resident microorganisms and still be able to extract nutritional products from the food.

The architecture and physiology of this system have evolved over millions of years, dating back to the Jurassic era, to allow efficient digestion of plant materials (Mackie 2002). This system's effectiveness is conferred by its design, which prolongs and maximizes plant biomass exposure to specialized microorganisms that degrade the plant fibers, while providing stable and favorable conditions for their growth. The consumed feed is packed into the first two stomach chambers, collectively termed the reticulorumen. The reticulorumen environment is basically anaerobic, with a gas composition of approximately 65% $CO_2$, 27% $CH_4$, 7% $N_2$, and 0.2% $H_2$; the remaining gases include CO, $H_2S$, and transient traces of $O_2$ that are consumed very rapidly by facultative anaerobic microorganisms. The composition of this gas mixture is the outcome of the intensive microbial fermentation taking place in this compartment by the reticulorumen-resident microorganisms, which are mainly responsible for breaking down and fermenting the plant fibers (see further on) (Stewart 1986; Dehority 1991; McAllister et al. 1994; Miron et al. 2001). One of the tactics used by ruminants to better support microbial metabolism and fermentation is to regurgitate the partially digested feed (cud) and chew it again (Hoover and Miller 1991). This process, also known as rumination, decreases the size and increases the surface area of the plant fiber particles interacting with the rumen microbes. The time spent by the animal ruminating is considered to be controlled by the particle size of its plant feed: the larger the particle size, the longer the rumination period (Welch and Smith 1969; Welch 1986). The regurgitation process also mixes together the microorganisms, digesta and saliva, further increasing fiber breakdown (McAllister et al. 1994; Merchen et al. 1997). The saliva is composed of a bicarbonate and phosphate buffer at a pH of around 8 which assists in keeping the reticulorumen pH stable (Kay 1966). Salivary production increases when the animal is eating, and this in turn improves the buffering capacity of the rumen environment which has to cope with volatile fatty acids (VFAs)—the products of microbial fermentation (Counotte et al. 1979). As a result of this buffering system, the rumen pH range is normally between 5.5 and 7.0, reaching a minimum of 6 h after feeding in animals that are fed once a day (Dehority and Tirabasso 2001; Welkie et al. 2009). The rumen temperature and oxidation-reduction potential are also maintained in a constant range of 38–40 °C and 0.15–0.4 V, respectively, thus providing a stable environment for the resident microbiota (Church 1969). After the plant fibers in the feed have been degraded, the digesta passes into the next stomach chamber, the omasum, which acts as a filtering device through which only particles less than 2 mm in size can readily pass (Weimer et al. 2009). In the omasum, water and many of the inorganic mineral elements are absorbed into the bloodstream. Then the digesta moves to the true stomach, the abomasum. The abomasum is the direct equivalent of the human monogastric stomach but contains a unique enzymatic feature that is adapted to the foregut fermentation system: it secretes lysozyme—an enzyme that degrades bacterial cell walls via hydrolysis of the β-1,4 glycosidic bonds of the peptidoglycan components. Lysozyme is usually found in tears, mucus, and saliva, protecting the tissues from bacterial attack.

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In the abomasum, it acts as a digestive enzyme, lysing the bacteria coming from the rumen and enabling proper digestion of the bacterial protein synthesized by the rumen bacteria to build their cells (Jolles et al. 1984, 1989). In the abomasum, the bacterial protein and digesta are digested in much the same way as in nonruminant mammals (Merchen et al. 1997).

## Rumen Metabolism

The reticulorumen functions as a pre-gastric anaerobic fermentation chamber. It is inhabited by a high density of resident microbiota, consisting of bacteria, protozoa, archaea and fungi, which degrade the consumed plant materials (Flint 1997). In general, plant materials are composed of organic matter such as proteins, lipids, and nucleic acids and a large proportion of carbohydrate polymers such as celluloses, hemicelluloses, pectins, fructosans, starches and other polysaccharides which are mostly indigestible by the animal (Flint et al. 2008). The reticulorumen microorganisms utilize specialized enzymes and enzyme complexes to degrade these polysaccharides into monomeric or dimeric sugars and subsequently ferment them (Flint et al. 2008). The degradation and fermentation take place in a coordinated and complex manner, in which the substrate for one microorganism is the product of another (Dehority 1991; Mackie 2002). This enables the degradation of all plant materials except lignin, which is a phenolic compound present in the cell wall. The biodegradation and metabolism of plant feed by the reticulorumen microorganisms make these otherwise indigestible polymers accessible for absorption and utilization.

These indigestible polymers are converted by the reticulorumen microorganisms into two main digestible forms. The first is the microbial proteins synthesized by these microorganisms to build their cells—these proteins are digested in the abomasum (true stomach) as the microbes travel along the animal's digestive tract (Kay 1969). This digested microbial protein makes up 50–80% of the total protein absorbed by the animal (Storm and Ørskov 1983). The second is VFAs, which are fermentation end products, along with methane and carbon dioxide. VFAs are short-chain fatty acids that are absorbed by the animal in the reticulorumen and serve in important biochemical processes, such as gluconeogenesis in the case of propionate and fatty acid synthesis in the case of acetate and butyrate (Russell and Wilson 1996). Hence, the VFAs serve as an energy source for the animal. In a recent study, Weimer et al. (2009) estimated that VFAs retain approximately 70% of the energy content of the plant material's polymers.

## Rumen Microbial Composition and Interactions

As we have seen, the energy and protein used by the ruminant are a direct outcome of its resident ruminal microbes' activity. All domains of life are represented in these complex communities, and their distribution was demonstrated in a recent metagenomic study in which approximately 95% of the coding sequences were bacteria, 0.6–4% archaea, and 1.5% eukarya (Brulc et al. 2009). Each of these domains is represented in the rumen microbial ecosystem by a vast array of taxa. The general members of this microbial consortium are the subject of this section. Due to space limitations, the complex taxon diversity within each domain is not covered in this chapter. Here, we review the common traits and features of each domain, their interactions and their effects on the rumen ecosystem and ruminant host.

### Bacteria

Bacteria make up the largest domain in the rumen ecosystem and their presence is vital for the animal's well-being. They are responsible for most of the degradation and fermentation of plant mass in the rumen (Windham and Akin 1984; Akin and Benner 1988). A concentration of approximately 1,011 bacteria/ml exists in the rumen, and their populations are considered highly complex in terms of taxon identity and functionality. The coordinated and complex metabolic interactions between the different bacterial taxa enable efficient utilization of the consumed plant fiber and maximal energy consumption by the overall rumen microbiota. The composition of these bacterial populations has been the subject of intensive research using classical microbiology, culture-free methods, and next-generation sequencing techniques which have helped characterize their complexity by allowing a broad overview of the bacterial 16S rRNA gene as well as their coding capacity. As revealed by these studies, the dominant phyla in the rumen are similar to those in other enteric bacterial ecosystems, consisting of Bacteroidetes, Firmicutes, and Proteobacteria (Brulc et al. 2009; Jami and Mizrahi 2012). In a study by Stevenson and Weimer (2007), quantitative real-time PCR was used to characterize the bacterial species in two lactating cows. The dominant bacterial genus was found to be *Prevotella*, comprising 42–60% of the bacterial rRNA genes.

The rumen's bacterial populations have evolved coordinated metabolic functions, in which some taxa depend on others for their growth, complicating their cultivation and study. Thus, because most of the rumen bacteria have not been cultured, our knowledge of their metabolism is limited. Due to the high complexity of the bacterial composition and the unique functional distribution of the rumen bacterial species, only the dominant bacteria and their metabolic functions are covered in this section. Basically, the plant fibers are degraded and fermented by a consortium of bacterial taxa that interact metabolically such that the product of one taxon is utilized by another, thereby enabling sequential degradation of the plant biomass. This allows functional specialization of different bacteria for different plant mass compounds and degradation stages.

It appears that the plant cell wall, which is composed of cellulose fibers embedded in a hemicellulose matrix, is initially broken down by only a small group of cellulolytic bacteria which are capable of degrading the highly ordered and insoluble forms of cellulose. These bacteria include *Ruminococcus flavefaciens*,

*Ruminococcus albus*, and *Fibrobacter succinogenes*, which are considered to be the dominant cellulolytic species, and to a lesser extent *Butyrivibrio fibrisolvens* (Flint and Bayer 2008; Flint et al. 2008). Although these species are the best characterized ones, other uncultured bacteria might also take part in this process, such as the recently discovered and described bacterium *Cellulosilyticum ruminicola H1* isolated from yak (*Bos grunniens*) rumen, which degrades lignocellulose with multiple carbohydrate-borne fibrolytic enzymes (Cai et al. 2010). Some of these species have been shown to negatively interact with each other, with cellulose digestion decreasing when they are grown together in culture (Saluzzi et al. 1993; Odenyo et al. 1994b). This inhibitory effect is thought to be the outcome of the bacteriocins produced by some of these bacteria, such as *R. albus* and *R. flavefaciens*, to better compete for the mutual resource—cellulose (Odenyo et al. 1994a; Kalmokoff and Teather 1997; Rychlik and Russell 2002; Chen et al. 2004). Bacteriocins produced by other non-cellulolytic ruminal bacteria have been found and are thought to serve for competition in the various niches within the rumen ecosystem (Laukova 1993; Morovsky et al. 2001; Whitford et al. 2001; Cookson et al. 2004; Xavier and Russell 2009). It is important to note that evidence of quorum sensing, for both intraspecies and interspecies communication, has been documented for rumen bacteria but its exact role in the rumen ecosystem was not determined (Erickson et al. 2002; Mitsumori et al. 2003).

After the initial breakdown and solubilization of the plant fibers, the polymers are utilized by a vast array of taxa specific to the various downstream degradation stages of these polymers. This also holds true for other less recalcitrant polymers such as starch, which is utilized by *Ruminobacter amylophilus*, *Streptococcus bovis*, *Succinomonas amylolytica*, several Prevotella species, *Butyrivibrio fibrisolvens* and *Selenomonas ruminantium*, and pectin, which is utilized by *Succinivibrio dextrinosolvens* and *Lachnospira multiparus* (Krause et al. 2003). Bacterial species composition changes according to the rations and dietary ingredients consumed by the animal. This is best exemplified by the presence of starch rather than plant fiber in high-grain diets compared to the high-forage diets widely used as part of different husbandry protocols, which causes dramatic changes in the bacterial taxa. These changes are apparent at all taxonomic levels, from phylum to species (Callaway et al. 2010; Fernando et al. 2010). The energy from the plant mass is maximally extracted as even the bacterial fermentation end product lactate is utilized by other bacteria, such as *Megasphaera elsdenii* (Elsden et al. 1956) and *Veillonella alcalescens* (Johns 1951), as are other end products such as hydrogen, acetate, and succinate.

A recent metagenomic study of mammalian gut microbiota has demonstrated that in herbivore and ruminant microbiota, metabolic pathways that involve the anabolism of amino acids are more common than in carnivores. The latter's diets are much higher in protein and therefore their microbiota metagenomes are rich in protein catabolism pathways (Muegge et al. 2011). Indeed, the food entering the rumen is composed mainly of carbohydrates, and therefore most of the bacteria occupying the rumen rely on carbohydrate as their main energy source. Nevertheless, some bacterial taxa can also utilize protein, including *R. amylophilus, S. ruminantium, Prevotella ruminicola*, and *B. fibrisolvens*. The highly abundant *P. ruminicola*, which is also a hemicellulolytic species, is considered to be the most active producer of ammonia ($NH_3$) in the rumen due to its high abundance and proteolytic and deaminase activities. This function is highly important as the rumen environment is not rich in protein and ammonia serves as an important nitrogen source for the anabolism of amino acids and proteins (Stevenson and Weimer 2007).

Therefore, the ruminal bacterial populations are fine-tuned to their host ruminant's diet and change accordingly, thereby enabling fermentation of the different dietary ingredients. They also interact metabolically with each other, sometimes competing for recourses and inhabiting each other and sometimes cooperating to enable maximum energy utilization of the feed, providing the host with bacterial proteins and VFAs.

## Archaea

A large part of the Archaea population in the rumen is made up of anaerobic methanogens, as revealed by studies characterizing the microbial small subunit rRNA gene in the rumen, as well as by the aforementioned recent metagenomic study which estimated the archaea population at 0.6–3.3% of total rumen microbes (Janssen and Kirs 2008; Brulc et al. 2009). In a study characterizing methanogenic properties, Yanagita et al. (2000) calculated that 3.6% of ruminal microorganisms display autofluorescence characteristics of F420, an enzymatic cofactor found in all methanogens (Gorris and van der Drift 1994). Methanogens produce $CH_4$ by either reducing $CO_2$ or dissimilating acetate to $CH_4$ and $CO_2$ (Thauer et al. 2008). In the rumen ecosystem, the latter reaction is negligible (Hungate et al. 1970), and therefore most of the $CH_4$ is produced via reduction of $CO_2$. The electrons for the reduction of $CO_2$ can come from several chemical compounds, such as $H_2$, formate, methanol, and methylamine. In the rumen, most of the methane production results from utilization of hydrogen for carbon dioxide reduction, although formate, methanol and methylamines are also used by some methanogenic species (Hook et al. 2011).

Phylogenetically, methanogenic archaea belong to the phylum Euryarchaeota and are divided into five taxonomic orders—Methanopyrales, Methanococcales, Methanobacteriales, Methanomicrobiales, and Methanosarcinales (Balch et al. 1979). Janssen and Kirs (2008) analyzed large data sets from several culture-free studies of different ruminants, revealing that most rumen methanogens belong to three genus-level groups: *Methanobrevibacter* (61.6%) order *Methanobacteriales*, *Methanomicrobium* (14.9%) order Methanomicrobiales, and a large group of uncultured rumen archaea labeled as rumen cluster C, or RCC (15.8%) (Janssen and Kirs 2008). In a later study performed in New Zealand using denaturing gradient gel electrophoresis (DGGE) of archaeal 16S rRNA genes, the composition of archaeal communities in the rumens of farmed sheep, cattle, and red deer was investigated. Total archaeal

communities were relatively constant across species and diets and were less variable and less diverse than the bacterial communities. There were diet- and ruminant-species-based differences in archaeal community structure, but the same dominant archaea were present in all rumens. In that study, species from the genus *Methanobrevibacter* were also found dominant in all rumens. Members of the RCC archaeal group of unknown physiology were also present, accounting for an average 26.5% of the total archaea (Jeyanathan et al. 2011).

Some methanogenic archaea use cytochromes for the production of methane, whereas others use alternative complexes for this reaction. The order Methanosarcinales contains methanogens with cytochromes and can grow on the broadest range of substrates. Methanogens with cytochromes have a growth yield of 7 g/mol methane on hydrogen and carbon dioxide and have a doubling time of greater than 10 h, while methanogens without cytochromes have a growth yield of only 3 g/mol methane on hydrogen and carbon dioxide and a doubling time of 1 h (Thauer et al. 2008). Methanogens without cytochromes grow on a much lower concentration of hydrogen compared to methanogens with cytochromes, the latter needing a tenfold higher hydrogen concentration for growth (Thauer et al. 2008). This might explain the higher abundance of the former in the rumen, as they can better compete for the hydrogen produced, lowering its concentration and preventing the methanogens with cytochromes from utilizing it. The consumption of hydrogen by methanogens is highly important in the rumen as it lowers the partial pressure of rumen hydrogen, thereby enabling some endergonic metabolic reactions to become exergonic, consequently increasing energy conservation by bacterial fermentation pathways that become energetically feasible (Stams and Plugge 2009). This is the basis for the syntrophic relationship between methanogens and some of the anaerobic rumen microbes, such as those that produce hydrogen as their fermentation end product. Such is the case for *R. flavefaciens* and *R. albus*, two dominant cellulolytic rumen bacteria that have a facultative syntrophic relationship with methanogens: in the presence of methanogens, they alter their fermentation pathways to conserve the maximum amount of energy and end products (Latham and Wolin 1977; Pavlostathis et al. 1988; Stams and Plugge 2009). An effect of methanogens on the fermentation of other non-cellulolytic rumen bacteria has also been reported (Chen and Wolin 1977). Ruminal fungus fermentation and cellulolytic activities have also been reported to change in the presence of methanogens, where fermentation pathways have changed and cellulolytic activities have increased when the ruminal fungus isolates were cocultured with rumen methanogens (Joblin et al. 1990; Marvin-Sikkema et al. 1990; Teunissen et al. 1992). This was also demonstrated by Bauchop and Mountfort (1981) who studied the degradation of cellulose by rumen fungi in the presence and absence of methanogens. In that study, it was observed that after 100-h incubation, only 10% of the total cellulose was degraded by fungi alone, compared to 70% when cocultured with methanogens; at the end of the incubation period (200 h), the overall degradation of cellulose was 53% for fungi alone compared with 82% for those in coculture with methanogens (Bauchop and Mountfort 1981). Interspecies hydrogen transfer between methanogens and other rumen microbes is best demonstrated by the physical association of methanogen species with rumen protozoa. The basis of this association is the large amount of hydrogen produced by the latter eukaryotic microorganisms (Lloyd et al. 1989; Muller 1993; Hackstein and Vogels 1997). Although protozoa prey on bacteria and Archaea, methanogens have been reported to adhere to or reside within them, establishing a symbiotic relationship in which the protozoa degrade and ferment the plant material and the methanogens thermodynamically promote the protozoa's energy exploitation by utilizing the hydrogen produced in these processes (Vogels et al. 1980; Krumholz et al. 1983; Ushida and Jouany 1996; Tokura et al. 1999; Irbis and Ushida 2004; Tothova et al. 2008). Therefore, the utilization of hydrogen by methanogens is the basis of syntrophic interactions with the rumen microbial communities to maximize overall energy recovery. This notion has led to the postulation that these interactions increase overall plant fiber degradation in the rumen (Wolin 1979; Holter and Young 1992; McAllister and Newbold 2008; Janssen 2010). Feed intake and hemicellulose digestibility are also positively correlated with methane production (Holter and Young 1992; Ellis et al. 2007). Nevertheless, the production of methane in the rumen is correlated with energy loss which can reach up to 19% of the total energy stored in the feed (Czerkawski 1969; Johnson and Johnson 1995). Hence, by symbiotically interacting with other rumen microbes, methanogens enable maximal energy harvest from the feed, but by producing methane, they decrease the ruminant host's ability to extract energy from its feed.

## Protozoa

Protozoa are a diverse group of single-celled eukaryotic organisms, bound by a cuticle or pellicle. This group is often considered the simplest form of animal life (Dehority 2003).

Rumen protozoa were first observed in 1843 by Gruby and Delafond. They are unable to grow under laboratory conditions in the absence of bacteria (Fondevila and Dehority 2001a, b). Most of the protozoa in the rumen are ciliate species belonging to the phylum Ciliophora, although several flagellated species belonging to the phylum Sarcomastigophora have been described in ruminant animals as well, albeit in much lower abundance than ciliates (Ogimoto and Imai 1981). The rumen ciliates are subdivided into the orders Entodiniomorphida and Vestibuliferida, which contain 25 genera (Dehority 2003).

The rumen protozoa are highly specialized and adapted to the rumen environment. They are mostly anaerobic, although some species are known to scavenge oxygen. This latter trait is considered beneficial for ruminal communities as it protects the strictly anaerobic microorganisms, such as the cellulolytic bacterial species, and consequently increases fiber degradation (Ellis et al. 1989). The rumen protozoa can utilize a vast array of carbohydrate compounds, such as soluble sugar, starch and lignocellulose. Much of these abilities, particularly the

lignocellulolytic one, are thought to have been acquired by lateral gene transfer from their close association with the bacteria and archaea on which they prey (Ricard et al. 2006; Flint and Bayer 2008). Although the ruminal protozoa's nutritional requirement for bacteria is well established, the reasons for this requirement and the degree of specificity for the bacterial species on which they prey are not well understood. There have been several reports on protozoan species' selectivity for the bacterial species that they ingest and that grow on them (Gutierrez 1958; Gutierrez and Davis 1959; Mah 1964); however, there have also been contradictory reports showing less selective uptake and digestion of a variety of bacterial species, including nonruminal ones, by rumen protozoa, including protozoan species from the same genera as in the former reports (Coleman 1962, 1964, 1967a, b). These discrepancies may result from the different species and strains used in the studies. The reasons for ruminal protozoa's nutritional need for bacteria are also not entirely clear, except for requiring bacteria strictly as a source of nutrients. Some reports show a need for live bacteria for protozoon growth and development, which might imply an active dependence on the bacteria's metabolism (Coleman 1967b; Fondevila and Dehority 2001b). To settle these issues, better experimental systems need to be developed: currently, antibiotic compounds and boiling are used to kill the bacteria fed to protozoa, and these treatments can have an effect on the protozoa in the case of antibiotics and on the nutritional gain from the bacteria in the case of boiling.

Some rumen ciliates are considered to be associated with rumen methanogenic archaea: much of this interaction is assumed to be based on the generation of hydrogen gas by protozoan fermentation which, as mentioned above, the methanogens use as an electron donor to reduce carbon dioxide to methane, an association that results in increasing methane emission to an estimated 9–25% of total rumen methane emission (Vogels et al. 1980; Newbold et al. 1995). The nature of this association has been the subject of intensive study aimed at determining whether the methanogenic archaea are ecto- or endosymbionts and whether the associations involve specific species of ciliates and methanogenic archaea (Vogels et al. 1980; Ushida and Jouany 1996; Tokura et al. 1999; Irbis and Ushida 2004; Tothova et al. 2008). Aside from the increase in methane emission, specific ruminal ciliate species have been shown to alter the balance between the different VFAs and to increase ammonia concentrations in vitro (Ranilla et al. 2007). The characteristics of these organisms as predators of other microbes, hydrogen producers, and carbohydrate degraders and fermenters raise intriguing questions regarding their role in overall rumen metabolism and their effects on the ruminant host. These effects were examined using experimental techniques that enable working with animals that are free of protozoa, termed defaunated animals. These studies showed that the rumen ciliates are not essential for viability and growth of the host animals (Williams and Coleman 1992); this is why rumen protozoa are often considered commensal eukaryotes in the herbivore rumen. Nevertheless, in studies comparing defaunated animals to their faunated counterparts, several effects on rumen fermentation parameters and microbial composition were reported. These aspects have been studied extensively for years, as well as more recently in studies investigating the effect of presence or absence of protozoa on rumen fermentation, microbial composition, efficiency of microbial protein synthesis, and even on the host tissue. Due to their agricultural importance, these studies have been performed mostly with cows and sheep.

The effect of the presence of protozoa on the composition of rumen bacteria was investigated in cattle by Ozutsumi et al. (2005). In a comparison of 16S rRNA gene (rDNA) clonal libraries from faunated and defaunated animals, no extreme differences were found in bacterial phyla; however, a computer analysis revealed that the presence of ruminal protozoa markedly affects the composition of rumen bacteria (Ozutsumi et al. 2005). Belanche et al. (2011) examined the effect of the absence of rumen protozoa on rumen fermentation and efficiency of microbial protein synthesis in lambs on different diets and reported that the protozoa modified rumen fermentation pattern and reduced digestibility of organic matter and total cell wall (Neutral Detergent Fiber) by 2.0 and 5.1 percentage points respectively, while having only a mild effect on nitrogen flow (Belanche et al. 2011). Sultana et al. (2011) investigated the effect of protozoa on fatty acid composition profile in the rumen of cattle, indicating that protozoa contribute greatly to trans-vaccenic acid and conjugated linoleic acid production in the rumen (Sultana et al. 2011). Mosoni et al. (2011) examined the effect of long-term defaunation on the structure of the microbiota, particularly methanogenic archaea and fibrolytic bacteria. Total rumen bacterial density showed an estimated increased response to long- and short-term defaunation but without noticeable shifts in diversity. Defaunation increased the content of *R. albus* and *R. flavefaciens* but did not affect that of *F. succinogenes.* Despite a 20% reduction in methane emission, the methanogen content increased in the absence of protozoa, while the diversity of the dominant methanogenic community was not modified (Mosoni et al. 2011). Hegarty et al. (2008) studied the effects of the absence of protozoa from birth or weaning on growth and methane production in lambs. Single lambs born to defaunated ewes were heavier at birth and at weaning than lambs born to faunated ewes. Wool growth rate of defaunated lambs was 10% higher than that of faunated lambs and was increased a further 9% by a high-protein diet. There was no main effect of protozoon treatment on enteric methane production. These data indicated that while lambs without rumen protozoa have greater protein availability than faunated ruminants, there is no major effect of rumen protozoa on enteric methane production by lambs fed either a concentrate or roughage diet (Hegarty et al. 2008). Yanez-Ruiz et al. (2007) studied the effects of the absence of protozoa in the lamb rumen on animal growth, rumen fermentation, microbial diversity, and fatty acid profiles in abomasal fluid and intramuscular fat. Bacterial diversity was higher in control lambs than in their protozoon-free counterparts. Abomasal content was different with respect to fatty acid composition in the defaunated lambs. Differences were also seen in the fatty acid composition of the intramuscular fat (Yanez-Ruiz et al. 2007). These studies show

that the presence of protozoa affects rumen fermentation parameters, microbial composition, and host physiology. Therefore, the definition of commensalism—often used to refer to rumen protozoa—in which one organism benefits but the other is not affected, does not appear to apply.

### Fungi

The rumen fungi are all anaerobic, belonging to the family Neocallimasticaceae from the class Chytridiomycetes (Dehority 2003). Chytridiomycetes fungi have been recorded in various kinds of ruminants and herbivores, including sheep, cattle, camel, vicuna, deer, impala, goat, reindeer, muskox, gaur, antelope, and greater kudu (Dehority 2003), but they are only considered to occur in large numbers (up to 8% of total ruminal content) when the animals feed on low-quality forage, which increases retention time in the rumen; their numbers decrease when the animals eat high-grain diets, which reduce ruminal retention time (Russell and Rychlik 2001). These differences are thought to be due to the long life cycle of these fungi (8–32 h), which consists of a motile zoospore stage and a vegetative thallus stage (Orpin 1975) and results in their being washed out from the host's digestive tract.

In general, the metabolic activities of rumen fungi include the degradation and utilization of cellulose, hemicellulose, and starch, as well as some proteolytic activity (Dehority 2003). The metabolic interaction of ruminal fungi with other rumen microbes was studied in vitro, and they were found to be enhanced or reduced as a function of the microbial partner with which they were cocultured. The ability of some rumen fungi to degrade cellulose increased in the presence of methanogens and some bacterial species, such as *Selenomonas ruminantium, Veillonella alcalescens* and *Megasphaera elsdenii* (Marvin-Sikkema et al. 1990; Dehority 1991). Cocultivation of the rumen fungus *Neocallimastix frontalis* with *Prevotella ruminicola*, *Succinivibrio dextrinosolvens*, or *Selenomonas ruminantium* showed a synergistic interaction with regard to xylan utilization; xylan utilization decreased in cocultures containing *Lachnospira multiparus* or *Streptococcus bovis* (Williams et al. 1991). A negative interaction with respect to cellulose hydrolysis was also observed in coculture with the cellulolytic bacterial species *R. flavefaciens* and *R. albus*, which was thought to involve a polypeptide released into the supernatant that inhibits fungal cellulose hydrolysis without having any cellulolytic activity of its own (Stewart et al. 1992; Bernalier et al. 1993). Although the ruminal fungi are capable of plant mass degradation and utilization, their effect on overall ruminal fermentation is considered minor, possibly due to bacterial inhibition of their growth and activity as concluded from studies examining their in vitro effect on cellulose digestion and fermentation using antibiotics that inhibit the bacteria and fungi separately (Windham and Akin 1984; Akin and Benner 1988; Dehority and Tirabasso 2000). Nevertheless, their presence on the plant mass is thought to disrupt the lignocellulose tissues because their rhizoids penetrate the plant cell wall, thus increasing lignocellulose accessibility to other rumen microbes and contributing to overall fiber utilization (Ho et al. 1988; Akin et al. 1989).

## Acquisition of Microbial Populations After Birth

Although microbial colonization of the rumen after birth is important for understanding this ecological niche, little is known about it. In the first weeks of life, the rumen is not functional: the suckled milk does not pass through the rumen because of the esophageal groove, which is closed by reflex action, although some milk might nevertheless enter the rumen and provide nutrients for microbial populations. The occurrence of microbial communities in the rumen of young animals was researched in the early days of rumen microbiology, providing the observation of rapid colonization of the rumen by microbial taxa (Bryant et al. 1958; Bryant and Small 1960). Fonty et al. (1987) also observed that the rumen ecosystem is quickly established after birth, before the rumen itself becomes functional. In that study, the authors investigated microbial acquisition and colonization steps in the lamb rumen. The authors used five test groups which were kept with or without their mothers and with different combinations of accessibility to plant fiber and milk. Using classical microbiology methods, the authors divided the rumen microbiota into strictly anaerobic, aerobic, facultatively anaerobic, cellulolytic bacteria, methanogenic archaea and anaerobic fungi. They found that microbial colonization of the lamb's rumen is characterized by the dominance of strictly anaerobic species very soon after birth (2 days) and by the early appearance (1 week after birth) of populations of cellulolytic and methanogenic bacteria and anaerobic fungi; the aerobic and facultatively anaerobic microflora in the lambs declined rapidly as the animal matured. Surprisingly, the authors found that rather than first appearing at weaning, the cellulolytic bacteria are present in large numbers before the ingestion of solid feed, as well as in lambs fed exclusively milk. In an earlier work, Bryant et al. (1958) also observed cellulolytic bacteria in calves at the end of their first week of life. Bryant et al. (1958) also suggested that contact between the newborn lamb and its mother or other adult ruminants during the first days of life is necessary for the establishment of these cellulolytic species, since in lambs which were kept in isolation, the cellulolytic flora did not become established. The appearance of anaerobic fungi toward the end of the first week of life was also reported, possibly because the retention time in the still nonfunctional rumen is long and allows the development of fungi before the ingestion of solid food.

Minato et al. (1992) used classical microbiology methods to investigate the colonization of microbial populations in the rumen of calves under normal husbandry growth conditions. They also observed rapid colonization of the rumen immediately after birth. The first bacteria to develop in abundance were *Escherichia coli* and *Streptococci*. The number of *E. coli*, which was high in 1-day-old calves, decreased gradually to a constant

level at between 6 and 8 weeks of age, which also concurs with studies by Fonty et al. (1987) and Bryant et al. (1958) who reported a decrease in facultative anaerobic bacteria with age. The number of streptococci, which was high for the first 8 weeks of life, showed a decrease at 10 weeks of age. The number of lactobacilli, which was high in 1-day-old calves, increased until 2 weeks of age and remained constant thereafter. Amylolytic bacteria, sulfate reducers, lactate utilizers, xylan fermenters and pectin fermenters, which were scarce in 1-day-old calves, increased within 3 days after birth and then remained constant. The cellulolytic bacteria appeared in 3–5-day-old animals, and became abundant in 2–3-week-olds. The methanogenic populations appeared in 1–2-week-old calves and became abundant when the animals were approximately 3 weeks old.

Protozoa have also been reported to rapidly colonize the rumen in the first days after birth (Bryant et al. 1958; Bryant and Small 1960; Eadie and Hobson 1962). Because these microorganisms are not essential to the animal's existence, their mode of transmission could be studied in more detail. Apparently, they are transmitted from animal to animal, as when calves were separated from other ruminants they did not become faunated (Bryant et al. 1958; Bryant and Small 1960; Eadie and Hobson 1962). Transmission can occur from a mother ruminant grooming its young and passing protozoa via the saliva or via salivation in the feed or pasture (Dehority 2003). It is speculated that rumen anaerobic fungi are transmitted via the same mechanism, as they have been reported in both the feces and saliva of ruminants (Trinci et al. 1988).

## The Microbiota's Role in Energy-Utilization Efficiency in Ruminants

Cattle's ability to utilize and divert the energy stored in its feed to milk and meat production is governed by its energetic efficiency. Energetic efficiency is defined as the ratio between the energetic value of the animal and its products and the energetic value of the feed or diet; it thus includes the energy invested in maintenance, as well as the energy invested in milk and meat production (Johnson et al. 2003). In general, the energy is preferentially utilized for maintenance over production (Tolkamp 2010). The energy from the consumed feeds that is not retained in the body or milk is lost in the form of feces, urine, heat, and combustion of gases, such as methane. Different methods are used to evaluate an animal's energetic efficiency, among them the residual feed intake (RFI) method (also known as net feed efficiency method) (Koch et al. 1963). This method evaluates energetic efficiency according to the difference between the animal's actual feed intake and its estimated feed intake over a specified period of time (Koch et al. 1963; Archer et al. 1999). Animals that have low RFI values are considered to be more energetically efficient than those with high values. This method is independent of growth and body size, making it suitable for comparisons between animals (Archer et al. 1999; Moore et al. 2009).

Energetic efficiency varies considerably among breeds, as well as among different individuals from the same breed (Ferrell and Jenkins 1985; Thiessen et al. 1985; Taylor et al. 1986; Solis et al. 1988; Aharoni et al. 2006). The nature of this variation is not entirely clear, but various factors are thought to affect the animal's RFI and hence its energy utilization (Johnson et al. 2003). The presence of a moderate genetic component affecting energy utilization was demonstrated by elevating feed efficiency via successful selection of animals according to their RFI in combination with specific genomic markers (Hotovy et al. 1991; Archer et al. 1999; Herd et al. 2003; Richardson et al. 2004; Crews 2005). Differences between high- and low-RFI animals have also been reported in terms of metabolic activity, digestibility, and methane production (Richardson et al. 1996; Arthur et al. 2001; Basarab et al. 2003; Nkrumah et al. 2004, 2006). These differences can be related to various factors, one of which might be the reticulorumen microbiota, upon which the animal's digestion and absorption of feed are largely dependent. Hence, the reticulorumen microbiota may play an important role in energy harvesting from the feed, affecting the animal's energy utilization.

Some of the energy lost during the conversion can be attributed to methane production by methanogenic archaea which, as already mentioned, is one of the end products of the reticulorumen's overall metabolism. The methane gas is eructated into the atmosphere along with its retained energy, which is lost from the cow's reticulorumen. This process results in a loss of 5–19% of the energy content of the feed (Johnson and Johnson 1995) and has wide environmental implications: methane is a very potent greenhouse gas (23 times more potent than carbon dioxide) and in some countries, ruminants are responsible for up to 60% of its emission (Wuebbles and Hayhoe 2002). A recent study in which animals with different RFI values were compared reported that animals with low energetic efficiency (high RFI) exhibit significantly more methane emission (Nkrumah et al. 2006). The authors speculated that the differences in methane emission are the outcome of differences in the methanogenic populations.

In the last few years, the notion of resident microbiota affecting a host's ability to harvest energy from its food has been the topic of intense research with respect to the human gut (Ley et al. 2006; Turnbaugh et al. 2006; Turnbaugh and Gordon 2009). Those studies established a strong correlation between bacterial gut divisions and the ability to harvest energy from food, and consequently increase body weight and fat. The relationship between rumen microbiota and host feed efficiency has been examined in a few recent studies in beef cattle (Guan et al. 2008; Zhou et al. 2009). In one study, the authors used PCR-DGGE to compare bacterial reticulorumen population composition in 18 steers from different breeds with different RFI values. The authors reported the clustering of specific bacterial populations according to the animal's energy efficiency, accompanied by a correlation with VFA concentration. However, the identity, activity and coding capacity of those populations—a significant portion of the factors determining the microbial-feed efficiency relationship—were not studied (Guan et al. 2008). In a second study, the methanogenic populations of animals with different RFIs were compared

using 16S rDNA clone libraries. Efficient animals showed less species diversity, and there was a correlation between the composition of methanogens and host efficiency (Zhou et al. 2009). In two subsequent studies, the ruminal methanogenic and bacterial populations of 56 beef cattle which differed in feed efficiency and diet were analyzed using PCR-DGGE profiles (Hernandez-Sanabria et al. 2010; Zhou et al. 2010). The results indicated that the methanogenic PCR-DGGE pattern is associated with the feed efficiency of the host, with diet- and feed-efficiency-related bands being identified. However, the size of the methanogenic population did not correlate with differences in feed efficiency, diet, or metabolic measurements (Zhou et al. 2010). Bacterial PCR-DGGE bands were only related to feed-efficiency-associated traits and metabolites (Hernandez-Sanabria et al. 2010). Taken together, these suggest a link between rumen microbiota and host feed efficiency.

## Rumen Interactions

The complexity of the rumen ecosystem is also expressed in the complicated interactions among its resident microbes and between those microbes and the ruminant host. The complexity of these cooperative interactions finds its roots in the conflicting interests of the partners. The animal cannot digest its feed and, therefore, needs its resident microbes to perform this task. The animal's interest lies only in its resident microbes transforming the feed to a more digestible form with minimal effect on its nutritional and energetic value. The microbes, on the other hand, are interested in the plant fibers and their components, which are continually being supplied by the animal. Consequently, their primary interest is to use this organic material to its fullest by extracting as much energy as possible. The common plant feed resource also serves as a basis for conflict among the resident rumen microbes which are competing for it. These conflicting interests, in which each of the partners aims to use the resources to satisfy their own selfish interests, are expected to interfere with possible cooperation between the partners. This paradox, also known as "the tragedy of the commons" (Hardin 1968), is thought to shape intraspecies and interspecies cooperative relationships (Frank 1996; Herre et al. 1999). The conflicts are resolved by several strategies and interactions which include inhibition, predation, commensalism, and synergism. These interactions, described earlier in this chapter, are discussed here in terms of their effects on all involved partners, i.e., positive, negative or none.

The bacteria, representing the dominant and most important domain in the rumen ecosystem, make up the only domain that can single-handedly sustain the ruminant host's viability. The composition of the taxa in this domain changes according to the animal's diet, as different bacterial species utilize different substrates, and this in turn provides the remarkable ability to maximize and fine-tune the host's digestion and energy harvesting from its feed. The interactions within the bacterial domain are also highly complex and versatile. Most of these interactions have been documented and researched in in vitro cocultures, and they are therefore only assumed to exist in the rumen ecosystem. Competition is likely to occur in this environment, as some species compete for the same resources, such as cellulose. Therefore, as already noted, inhibition—a negative interaction between the cellulolytic species—is observed when they are cocultured in vitro. Mutualism has also been documented between bacterial species, whereby some of the species enjoy the cellulose-degradation products, fermentation products, or secondary metabolites of other species without contributing to or affecting these "producers": this can be defined as a one-way positive interaction or commensalism. Such is the case with cellodextrins, which are cellulose-degradation products that are utilized by several non-cellulolytic species such as *S. ruminantium* and *P. ruminicola* (Russell 1985). Mutual interactions in which both partners benefit are also documented for bacteria with cross-feeding or utilization of fermentation end products that allow better energy utilization (Bryant and Wolin 1975; Miura et al. 1980, 1983). Such a two-way positive interaction is nicely demonstrated by the interaction between *P. ruminicola*, which has proteolytic capabilities, and *R. albus*, which is a cellulolytic species, in which the former degrades protein and supplies $NH_3^+$ and the latter degrades cellulose and provides hydrolyzed cellulolytic products (Bryant and Wolin 1975). Thus, within the bacterial domain, all possible interactions have been documented.

As noted above, bacteria have been reported to inhibit fungal cellulose degradation and growth, exhibiting a one-way negative interaction, but synergism has also been shown between these two domains, suggesting a two-way positive interaction. Protozoa interact mainly with the other rumen microbes, and sometimes with each other, as predators exhibiting a one-way negative interaction, although cellulose degradation and the production of fermentation products are also part of their ruminal effect. Methanogenic archaea utilize the hydrogen produced by other rumen microbes as a fermentation end product for energy, which allows better energy utilization for other rumen microbes. This is a two-way positive interaction with all other domains which consequently benefits the ruminant host by improving fiber degradation within the rumen. Nevertheless, by producing methane which is eructated to the atmosphere with its retained energy, methanogenic archaea cause energy losses for the ruminant host. Therefore, the methanogenic relationship with the ruminant host is a combination of these positive and negative interactions.

As mentioned, the interactions of the resident microbes with the ruminant host harbor a fundamental conflict in terms of energy harvest from the feed, which is resolved by a remarkably creative solution. The ruminant host provides stable conditions and a continuous supply of nutrients on which the bacteria and other microbes can prosper, and extracts the maximum possible amount of energy from the feed within the limitations of the anaerobic conditions prevailing in the rumen ecosystem. The host, which is capable of aerobic respiration and gluconeogenesis, makes maximal use of the end products of fermentation by the bacteria and other rumen microbes by oxidizing them to

carbon dioxide. The energy invested in building microbes' cell components (mainly bacteria), which are mainly composed of protein, is later retrieved in the abomasum where the microbial cells are digested, serving as the animal's primary source of protein. At the same time, the rumen microbes multiply in the rumen compartment where they continue to prosper (with the limitation of flow rate as a function of microbe generation time). This architecture enables all partners to satisfy their own selfish interests while conducting mutually beneficial associations which can be defined as a two-way positive interaction.

## References


Aharoni Y, Brosh A, Kafchuk E (2006) The efficiency of utilization of metabolizable energy for milk production: a comparison of Holstein with F1 Montbeliarde 3 Holstein cows. Anim Sci 82:101–109

Akin DE, Benner R (1988) Degradation of polysaccharides and lignin by ruminal bacteria and fungi. Appl Environ Microbiol 54(5):1117–1125

Akin DE, Lyon CE, Windham WR, Rigsby LL (1989) Physical degradation of lignified stem tissues by ruminal fungi. Appl Environ Microbiol 55(3):611–616

Archer JA, Richardson EC, Herd RM, Arthur PF (1999) Potential for selection to improve efficiency of feed use in beef cattle. Aust J Agric Res 50(2):147–162

Arthur PF, Archer JA, Johnston DJ, Herd RM, Richardson EC, Parnell PF (2001) Genetic and phenotypic variance and covariance components for feed intake, feed efficiency, and other postweaning traits in Angus cattle. J Anim Sci 79(11):2805–2811

Balch WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. Microbiol Rev 43(2):260–269

Basarab JA, Price MA, Aalhus JL, Okine EK, Snelling WM, Lyle KL (2003) Residual feed intake and body composition in young growing cattle. Can J Anim Sci 83:189–204

Bauchop T, Mountfort DO (1981) Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. Appl Environ Microbiol 42(6):1103–1110

Belanche A, Abecia L, Holtrop G, Guada JA, Castrillo C, de la Fuente G, Balcells J (2011) Study of the effect of presence or absence of protozoa on rumen fermentation and microbial protein contribution to the chyme. J Anim Sci. doi:10.2527/jas.2010-3703

Bernalier A, Fonty G, Bonnemoy F, Gouet P (1993) Inhibition of the cellulolytic activity of *Neocallimastix frontalis* by *Ruminococcus flavefaciens*. J Gen Microbiol 139(4):873–880

Brulc JM, Antonopoulos DA, Miller ME, Wilson MK, Yannarell AC, Dinsdale EA, Edwards RE, Frank ED, Emerson JB, Wacklin P, Coutinho PM, Henrissat B, Nelson KE, White BA (2009) Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. Proc Natl Acad Sci USA 106(6):1948–1953

Bryant MP, Small N (1960) Observations on the ruminal microorganisms of isolated and inoculated calves. J Dairy Sci 43(5):654–667

Bryant MP, Wolin MJ (1975) Rumen bacteria and their metabolic interactions. In: Proceedings of the first intersectional congress of the International Association of Microbiology. Societies Science Council of Japan, Tokyo, vol 2, pp 297–306

Bryant MP, Small N, Bouma C, Robinsona I (1958) Studies on the composition of the ruminal flora and fauna of young calves. J Dairy Sci 41(12):1747–1767

Cai S, Li J, Hu FZ, Zhang K, Luo Y, Janto B, Boissy R, Ehrlich G, Dong X (2010) *Cellulosilyticum ruminicola*, a newly described rumen bacterium that possesses redundant fibrolytic-protein-encoding genes and degrades lignocellulose with multiple carbohydrate-borne fibrolytic enzymes. Appl Environ Microbiol 76(12):3818–3824

Callaway TR, Dowd SE, Edrington TS, Anderson RC, Krueger N, Bauer N, Kononoff PJ, Nisbet DJ (2010) Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. J Anim Sci 88(12):3977–3983

Chen M, Wolin MJ (1977) Influence of $CH_4$ production by *Methanobacterium ruminantium* on the fermentation of glucose and lactate by *Selenomonas ruminantium*. Appl Environ Microbiol 34(6):756–759

Chen J, Stevenson DM, Weimer PJ (2004) Albusin B, a bacteriocin from the ruminal bacterium *Ruminococcus albus* 7 that inhibits growth of *Ruminococcus flavefaciens*. Appl Environ Microbiol 70(5):3167–3170

Church DC (1969) Digestive physiology and nutrition of ruminants, 2nd edn. O. S. U Book Stores, Corvallis, OR

Coleman GS (1962) The preparation and survival of almost bacteria-free suspensions of *Entodinium caudatum*. J Gen Microbiol 28:271–281

Coleman GS (1964) The metabolism of *Escherichia coli* and other bacteria by *Entodinium caudatum*. J Gen Microbiol 37:209–223

Coleman GS (1967a) The metabolism of free amino acids by washed suspensions of the rumen ciliate *Entodinium caudatum*. J Gen Microbiol 47(3):433–447

Coleman GS (1967b) The metabolism of the amino acids of *Escherichia coli* and other bacteria by the rumen ciliate *Entodinium caudatum*. J Gen Microbiol 47(3):449–464

Cookson AL, Noel SJ, Kelly WJ, Attwood GT (2004) The use of PCR for the identification and characterisation of bacteriocin genes from bacterial strains isolated from rumen or caecal contents of cattle and sheep. FEMS Microbiol Ecol 48(2):199–207

Counotte GH, van't Klooster AT, van der Kuilen J, Prins RA (1979) An analysis of the buffer system in the rumen of dairy cattle. J Anim Sci 49(6):1536–1544

Crews DH Jr (2005) Genetics of efficient feed utilization and national cattle evaluation: a review. Genet Mol Res 4(2):152–165

Czerkawski JW (1969) Methane production in ruminants and its significance. World Rev Nutr Diet 11:240–282

Dehority BA (1991) Effects of microbial synergism on fibre digestion in the rumen. Proc Nutr Soc 50(2):149–159

Dehority BA (2003) Rumen microbiology. Nottingham University Press, Nottingham, UK

Dehority BA, Tirabasso PA (2000) Antibiosis between ruminal bacteria and ruminal fungi. Appl Environ Microbiol 66(7):2921–2927

Dehority BA, Tirabasso PA (2001) Effect of feeding frequency on bacterial and fungal concentrations, pH, and other parameters in the rumen. J Anim Sci 79(11):2908–2912

Eadie JM, Hobson PN (1962) Effect of the presence or absence of rumen ciliate protozoa on the total rumen bacterial count in lambs. Nature 193:503–505

Ellis JE, Williams AG, Lloyd D (1989) Oxygen consumption by ruminal microorganisms: protozoal and bacterial contributions. Appl Environ Microbiol 55(10):2583–2587

Ellis JL, Kebreab E, Odongo NE, McBride BW, Okine EK, France J (2007) Prediction of methane production from dairy and beef cattle. J Dairy Sci 90(7):3456–3466

Elsden SR, Gilchrist FM, Lewis D, Volcani BE (1956) Properties of a fatty acid forming organism isolated from the rumen of sheep. J Bacteriol 72(5):681–689

Erickson DL, Nsereko VL, Morgavi DP, Selinger LB, Rode LM, Beauchemin KA (2002) Evidence of quorum sensing in the rumen ecosystem: detection of N-acyl homoserine lactone autoinducers in ruminal contents. Can J Microbiol 48(4):374–378

Fernando SC, Purvis HT II, Najar FZ, Sukharnikov LO, Krehbiel CR, Nagaraja TG, Roe BA, Desilva U (2010) Rumen microbial population dynamics during adaptation to a high-grain diet. Appl Environ Microbiol 76(22):7482–7490

Ferrell CL, Jenkins TG (1985) Energy utilization by Hereford and Simmental males and females. Anim Prod 41:53–61

Flint HJ (1997) The rumen microbial ecosystem—some recent developments. Trends Microbiol 5(12):483–488

Flint HJ, Bayer EA (2008) Plant cell wall breakdown by anaerobic microorganisms from the mammalian digestive tract. Ann N Y Acad Sci 1125:280–288

Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA (2008) Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol 6(2):121–131

Fondevila M, Dehority BA (2001a) In vitro growth and starch digestion by *Entodinium exiguum* as influenced by the presence or absence of live bacteria. J Anim Sci 79(9):2465–2471

Fondevila M, Dehority BA (2001b) Preliminary study on the requirements of *Entodinium exiguum* and *E. caudatum* for live or dead bacteria when cultured in vitro. Reprod Nutr Dev 41(1):41–46

Fonty G, Gouet P, Jouany J-P, Senaud J (1987) Establishment of the microflora and anaerobic fungi in the rumen of lambs. J Gen Microbiol 133:1835–1843

Frank SA (1996) Host-symbiont conflict over the mixing of symbiotic lineages. Proc Biol Sci 263(1368):339–344

Gorris LG, van der Drift C (1994) Cofactor contents of methanogenic bacteria reviewed. Biofactors 4(3–4):139–145

Guan LL, Nkrumah JD, Basarab JA, Moore SS (2008) Linkage of microbial ecology to phenotype: correlation of rumen microbial ecology to cattle's feed efficiency. FEMS Microbiol Lett 288(1):85–91

Gutierrez J (1958) Observations on bacterial feeding by the rumen ciliate *Isotricha prostoma*. J Protozool 5(2):122–126

Gutierrez J, Davis RE (1959) Bacterial ingestion by the rumen ciliates *Entodinium* and *Diplodiniztm*. J Protozool 6:222–226

Hackstein JH, Vogels GD (1997) Endosymbiotic interactions in anaerobic protozoa. Antonie Van Leeuwenhoek 71(1–2):151–158

Hardin G (1968) The tragedy of the commons: the population problem has no technical solution; it requires a fundamental extension in morality. Science 162(3859):1243–1248

Hegarty RS, Bird SH, Vanselow BA, Woodgate R (2008) Effects of the absence of protozoa from birth or from weaning on the growth and methane production of lambs. Br J Nutr 100(6):1220–1227

Herd RM, Archer JA, Arthur PF (2003) Reducing the cost of beef production through genetic improvement in residual feed intake: opportunity and challenges to application. J Anim Sci 81:9–17

Hernandez-Sanabria E, Guan LL, Goonewardene LA, Li M, Mujibi DF, Stothard P, Moore SS, Leon-Quintero MC (2010) Correlation of particular bacterial PCR-denaturing gradient gel electrophoresis patterns with bovine ruminal fermentation parameters and feed efficiency traits. Appl Environ Microbiol 76(19):6338–6350

Herre EA, Knowlton N, Mueller U, Rehener S (1999) The evolution of mutualisms: exploring the paths between conflicts and cooperation. Trends Ecol Evol 14:49–53

Ho YW, Abdullah N, Jalaludin S (1988) Penetrating structure of anaerobic rumen fungi in cattle and swamp buffalo. J Gen Microbiol 134:177–181

Holter JB, Young AJ (1992) Methane prediction in dry and lactating Holstein cows. J Dairy Sci 75(8):2165–2175

Hook SE, Wright AD, McBride BW (2011) Methanogens: methane producers of the rumen and mitigation strategies. Archaea 2010:945785

Hoover WH, Miller TK (1991) Rumen digestive physiology and microbial ecology. Vet Clin North Am Food Anim Pract 7(2):311–325

Hotovy SK, Johnson KA, Johnson DE, Carstens GE, Bourdon RM, Seidel GE Jr (1991) Variation among twin beef cattle in maintenance energy requirements. J Anim Sci 69(3):940–946

Hungate RE, Smith W, Bauchop T, Yu I, Rabinowitz JC (1970) Formate as an intermediate in the bovine rumen fermentation. J Bacteriol 102(2):389–397

Irbis C, Ushida K (2004) Detection of methanogens and proteobacteria from a single cell of rumen ciliate protozoa. J Gen Appl Microbiol 50(4): 203–212

Jami E, Mizrahi I (2012) Composition and similarity of bovine rumen microbiota across individual animals. PLoS One 7:e33306

Janssen PH (2010) Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim Feed Sci Technol 160:1–22

Janssen PH, Kirs M (2008) Structure of the archaeal community of the rumen. Appl Environ Microbiol 74(12):3619–3625

Jeyanathan J, Kirs M, Ronimus RS, Hoskin SO, Janssen PH (2011) Methanogen community structure in the rumens of farmed sheep, cattle and red deer fed different diets. FEMS Microbiol Ecol 76(2):311–326

Joblin KN, Naylor GE, Williams AG (1990) Effect of *Methanobrevibacter smithii* on xylanolytic activity of anaerobic ruminal fungi. Appl Environ Microbiol 56(8):2287–2295

Johns AT (1951) Isolation of a bacterium, producing propionic acid, from the rumen of sheep. J Gen Microbiol 5(2):317–325

Johnson KA, Johnson DE (1995) Methane emissions from cattle. J Anim Sci 73(8):2483–2492

Johnson DE, Ferrell CL, Jenkins TG (2003) The history of energetic efficiency research: where have we been and where are we going? J Anim Sci 81:27–38

Jolles P, Schoentgen F, Jolles J, Dobson DE, Prager EM, Wilson AC (1984) Stomach lysozymes of ruminants. II. Amino acid sequence of cow lysozyme 2 and immunological comparisons with other lysozymes. J Biol Chem 259(18):11617–11625

Jolles J, Jolles P, Bowman BH, Prager EM, Stewart CB, Wilson AC (1989) Episodic evolution in the stomach lysozymes of ruminants. J Mol Evol 28(6):528–535

Kalmokoff ML, Teather RM (1997) Isolation and characterization of a bacteriocin (Butyrivibriocin AR10) from the ruminal anaerobe *Butyrivibrio fibrisolvens* AR10: evidence in support of the widespread occurrence of bacteriocin-like activity among ruminal isolates of *B. fibrisolvens*. Appl Environ Microbiol 63(2):394–402

Kay RN (1966) The influence of saliva on digestion in ruminants. World Rev Nutr Diet 6:292–325

Kay RN (1969) Digestion of protein in the intestines of adult ruminants. Proc Nutr Soc 28(1):140–151

Koch RM, Swiger LA, Chambers D, Gregory KE (1963) Efficiency of feed use in beef cattle. J Anim Sci 22:486–494

Krause DO, Denman SE, Mackie RI, Morrison M, Rae AL, Attwood GT, McSweeney CS (2003) Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. FEMS Microbiol Rev 27(5):663–693

Krumholz LR, Forsberg CW, Veira DM (1983) Association of methanogenic bacteria with rumen protozoa. Can J Microbiol 29(6):676–680

Latham MJ, Wolin MJ (1977) Fermentation of cellulose by *Ruminococcus flavefaciens* in the presence and absence of *Methanobacterium ruminantium*. Appl Environ Microbiol 34(3):297–301

Laukova A (1993) Antagonistic activity of the rumen bacteria, *Enterococcus faecium* and *Staphylococcus warneri*. Vet Med (Praha) 38(5):267–274

Ley RE, Turnbaugh PJ, Klein S, Gordon JI (2006) Microbial ecology: human gut microbes associated with obesity. Nature 444(7122):1022–1023

Lloyd D, Hillman K, Yarlett N, Williams AG (1989) Hydrogen production by rumen holotrich protozoa: effects of oxygen and implications for metabolic control by in situ conditions. J Protozool 36(2):205–213

Mackie RI (2002) Mutualistic fermentative digestion in the gastrointestinal tract: diversity and evolution. Integr Comp Biol 42(2):319–326

Mah RA (1964) Factors influencing the in vitro culture of the rumen ciliate *Ophryoscolex purkynei* Stein. J Protozool 11:546–552

Marvin-Sikkema FD, Richardson AJ, Stewart CS, Gottschal JC, Prins RA (1990) Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi. Appl Environ Microbiol 56(12):3793–3797

McAllister TA, Newbold CJ (2008) Redirecting rumen methane to reduce methanogenesis. Aust J Exp Agric 48:7–13

McAllister TA, Bae HD, Jones GA, Cheng KJ (1994) Microbial attachment and feed digestion in the rumen. J Anim Sci 72(11):3004–3018

Merchen NR, Elizalde JC, Drackley JK (1997) Current perspective on assessing site of digestion in ruminants. J Anim Sci 75(8):2223–2234

Minato H, Otsuka M, Shirasaka S, Itabashi H, Mitsumori M (1992) Colonization of microorganisms in the rumen of young calves. J Gen Appl Microbiol 38(5):447–456

Miron J, Ben-Ghedalia D, Morrison M (2001) Invited review: adhesion mechanisms of rumen cellulolytic bacteria. J Dairy Sci 84(6):1294–1309

Mitsumori M, Xu L, Kajikawa H, Kurihara M, Tajima K, Hai J, Takenaka A (2003) Possible quorum sensing in the rumen microbial community: detection of quorum-sensing signal molecules from rumen bacteria. FEMS Microbiol Lett 219(1):47–52

Miura H, Horiguchi M, Matsumoto T (1980) Nutritional interdependence among rumen bacteria, *Bacteroides amylophilus, Megasphaera elsdenii*, and *Ruminococcus albus*. Appl Environ Microbiol 40(2):294–300

Miura H, Horiguchi M, Ogimoto K, Matsumoto T (1983) Nutritional interdependence among rumen bacteria during cellulose digestion in vitro. Appl Environ Microbiol 45(2):726–729

Moore SS, Mujibi FD, Sherman EL (2009) Molecular basis for residual feed intake in beef cattle. J Anim Sci 87(14 Suppl):E41–E47

Morovsky M, Pristas P, Javorsky P (2001) Bacteriocins of ruminal bacteria. Folia Microbiol (Praha) 46(1):61–62

Mosoni P, Martin C, Forano E, Morgavi DP (2011) Long-term defaunation increases the abundance of cellulolytic ruminococci and methanogens but does not affect the bacterial and methanogen diversity in the rumen of sheep. J Anim Sci 89(3):783–791

Muegge BD, Kuczynski J, Knights D, Clemente JC, Gonzalez A, Fontana L, Henrissat B, Knight R, Gordon JI (2011) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. Science 332(6032):970–974

Muller M (1993) The hydrogenosome. J Gen Microbiol 139(12):2879–2889

Newbold CJ, Lassalas B, Jouany JP (1995) The importance of methanogens associated with ciliate protozoa in ruminal methane production in vitro. Lett Appl Microbiol 21(4):230–234

Nkrumah JD, Basarab JA, Price MA, Okine EK, Ammoura A, Guercio S, Hansen C, Li C, Benkel B, Murdoch B, Moore SS (2004) Different measures of energetic efficiency and their phenotypic relationships with growth, feed intake, and ultrasound and carcass merit in hybrid cattle. J Anim Sci 82(8):2451–2459

Nkrumah JD, Okine EK, Mathison GW, Schmid K, Li C, Basarab JA, Price MA, Wang Z, Moore SS (2006) Relationships of feedlot feed efficiency, performance, and feeding behavior with metabolic rate, methane production, and energy partitioning in beef cattle. J Anim Sci 84(1):145–153

Odenyo AA, Mackie RI, Stahl DA, White BA (1994a) The use of 16S rRNA-targeted oligonucleotide probes to study competition between ruminal fibrolytic bacteria: development of probes for *Ruminococcus* species and evidence for bacteriocin production. Appl Environ Microbiol 60(10):3688–3696

Odenyo AA, Mackie RI, Stahl DA, White BA (1994b) The use of 16 S rRNA-targeted oligonucleotide probes to study competition between ruminal fibrolytic bacteria: pure-culture studies with cellulose and alkaline peroxide-treated wheat straw. Appl Environ Microbiol 60(10):3697–3703

Ogimoto K, Imai S (1981) Atlas of rumen microbiology. Japan Scientific Societies Press, Tokyo, Japan

Orpin CG (1975) Studies on the rumen flagellate *Neocallimastix frontalis*. J Gen Microbiol 91(2):249–262

Ozutsumi Y, Tajima K, Takenaka A, Itabashi H (2005) The effect of protozoa on the composition of rumen bacteria in cattle using 16S rRNA gene clone libraries. Biosci Biotechnol Biochem 69(3):499–506

Pavlostathis SG, Miller TL, Wolin MJ (1988) Fermentation of insoluble cellulose by continuous cultures of *Ruminococcus albus*. Appl Environ Microbiol 54(11):2655–2659

Ranilla MJ, Jouany JP, Morgavi DP (2007) Methane production and substrate degradation by rumen microbial communities containing single protozoal species in vitro. Lett Appl Microbiol 45(6):675–680

Ricard G, McEwan NR, Dutilh BE, Jouany JP, Macheboeuf D, Mitsumori M, McIntosh FM, Michalowski T, Nagamine T, Nelson N, Newbold CJ, Nsabimana E, Takenaka A, Thomas NA, Ushida K, Hackstein JH, Huynen MA (2006) Horizontal gene transfer from bacteria to rumen ciliates indicates adaptation to their anaerobic, carbohydrates-rich environment. BMC Genomics 7:22

Richardson EC, Herd RM, Arthur PF, Wright J, Xu G, Dibley K, Oddy VH (1996) Possible physiological indicators of net feed conversion efficiency. Proc Austral Soc Anim Prod 21:901–908

Richardson EC, Herd RM, Archer JA, Arthur PF (2004) Metabolic differences in Angus steers divergently selected for residual feed intake. Aust J Exp Agric 44:441–452

Russell JB (1985) Fermentation of cellodextrins by cellulolytic and noncellulolytic rumen bacteria. Appl Environ Microbiol 49(3):572–576

Russell JB, Rychlik JL (2001) Factors that alter rumen microbial ecology. Science 292(5519):1119–1122

Russell JB, Wilson DB (1996) Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? J Dairy Sci 79(8):1503–1509

Rychlik JL, Russell JB (2002) Bacteriocin-like activity of *Butyrivibrio fibrisolvens* JL5 and its effect on other ruminal bacteria and ammonia production. Appl Environ Microbiol 68(3):1040–1046

Saluzzi L, Smith A, Stewart CS (1993) Analysis of bacterial phospholipid markers and plant monosaccharides during forage degradation by *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* in co-culture. J Gen Microbiol 139(11):2865–2873

Solis JC, Byers FM, Schelling GT, Long CR, Greene LW (1988) Maintenance requirements and energetic efficiency of cows of different breed types. J Anim Sci 66(3):764–773

Stams AJ, Plugge CM (2009) Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat Rev Microbiol 7(8):568–577

Stevenson DM, Weimer PJ (2007) Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Appl Microbiol Biotechnol 75(1): 165–174

Stewart CS (1986) Rumen function with special reference to fibre digestion. Soc Appl Bacteriol Symp Ser 13:263–286

Stewart CS, Duncan SH, Richardson AJ, Backwell C, Begbie R (1992) The inhibition of fungal cellulolysis by cell-free preparations from ruminococci. FEMS Microbiol Lett 76(1–2):83–87

Storm E, Ørskov ER (1983) The nutritive value of rumen microorganisms in ruminant. 1. Large-scale isolation and chemical composition of rumen microorganisms. Br J Nutr 50:463–470

Sultana H, Miyazawa K, Kanda S, Itabashi H (2011) Fatty acid composition of ruminal bacteria and protozoa, and effect of defaunation on fatty acid profile in the rumen with special reference to conjugated linoleic acid in cattle. Anim Sci J 82(3):434–440

Taylor SCS, Thiessen RB, Murray J (1986) Inter-breed relationship of maintenance efficiency to milk yield in cattle. Anim Prod 43:37–61

Teunissen MJ, Kets EP, Op den Camp HJ, Huis in't Veld JH, Vogels GD (1992) Effect of coculture of anaerobic fungi isolated from ruminants and non-ruminants with methanogenic bacteria on cellulolytic and xylanolytic enzyme activities. Arch Microbiol 157(2):176–182

Thauer RK, Kaster AK, Seedorf H, Buckel W, Hedderich R (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. Nat Rev Microbiol 6(8):579–591

Thiessen RB, Taylor SCS, Murray J (1985) Multibreed comparisons of British cattle. Variation in relative growth rate, relative food intake and food conversion efficiency. Anim Prod 41:193–199

Tokura M, Chagan I, Ushida K, Kojima Y (1999) Phylogenetic study of methanogens associated with rumen ciliates. Curr Microbiol 39(3):123–128

Tolkamp BJ (2010) Efficiency of energy utilisation and voluntary feed intake in ruminants. Animal 4(7):1084–1092

Tothova T, Piknova M, Kisidayova S, Javorsky P, Pristas P (2008) Distinctive archaebacterial species associated with anaerobic rumen protozoan *Entodinium caudatum*. Folia Microbiol (Praha) 53(3):259–262

Trinci AP, Lowe SE, Milne A, Theodorou MK (1988) Growth and survival of rumen fungi. Biosystems 21(3–4):357–363

Turnbaugh PJ, Gordon JI (2009) The core gut microbiome, energy balance and obesity. J Physiol 587(17):4153–4158

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444(7122):1027–1031

Ushida K, Jouany JP (1996) Methane production associated with rumen-ciliated protozoa and its effect on protozoan activity. Lett Appl Microbiol 23(2):129–132

Vogels GD, Hoppe WF, Stumm CK (1980) Association of methanogenic bacteria with rumen ciliates. Appl Environ Microbiol 40(3):608–612

Weimer PJ, Russell JB, Muck RE (2009) Lessons from the cow: what the ruminant animal can teach us about consolidated bioprocessing of cellulosic biomass. Bioresour Technol 100(21):5323–5331

Welch JG (1986) Physical parameters of fiber affecting passage from the rumen. J Dairy Sci 69(10):2750–2754

Welch JG, Smith AM (1969) Influence of forage quality on rumination time in sheep. J Anim Sci 28(6):813–818

Welkie DG, Stevenson DM, Weimer PJ (2009) ARISA analysis of ruminal bacterial community dynamics in lactating dairy cows during the feeding cycle. Anaerobe 16(2):94–100

Whitford MF, McPherson MA, Forster RJ, Teather RM (2001) Identification of bacteriocin-like inhibitors from rumen *Streptococcus* spp. and

isolation and characterization of bovicin 255. Appl Environ Microbiol 67(2):569–574

Williams AG, Coleman GS (1992) The rumen protozoa. Springer, New York

Williams AG, Withers SE, Joblin KN (1991) Xylanolysis by cocultures of the rumen fungus *Neocallimastix frontalis* and ruminal bacteria. Lett Appl Microbiol 12(6):232–235

Windham WR, Akin DE (1984) Rumen fungi and forage fiber degradation. Appl Environ Microbiol 48(3):473–476

Wolin MJ (1979) The rumen fermentation: a model for microbial interactions in anaerobic ecosystems. Adv Microb Ecol 3:49–77

Wuebbles DJ, Hayhoe K (2002) Atmospheric methane and global change. Earth Sci Rev 57:117–210

Xavier BM, Russell JB (2009) The ability of non-bacteriocin producing Streptococcus bovis strains to bind and transfer bovicin HC5 to other sensitive bacteria. Anaerobe 15(4):168–172

Yanagita K, Kamagata Y, Kawaharasaki M, Suzuki T, Nakamura Y, Minato H (2000) Phylogenetic analysis of methanogens in sheep rumen ecosystem and detection of Methanomicrobium mobile by fluorescence in situ hybridization. Biosci Biotechnol Biochem 64(8):1737–1742

Yanez-Ruiz DR, Williams S, Newbold CJ (2007) The effect of absence of protozoa on rumen biohydrogenation and the fatty acid composition of lamb muscle. Br J Nutr 97(5):938–948

Zhou M, Hernandez-Sanabria E, Guan LL (2009) Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. Appl Environ Microbiol 75(20):6524–6533

Zhou M, Hernandez-Sanabria E, Guan LL (2010) Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. Appl Environ Microbiol 76(12): 3776–3786

# 22 Symbiotic Associations Between Termites and Prokaryotes

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## Introduction

The symbiotic associations of termites with microorganisms comprise different levels of interaction, ranging from the extracorporeal cultivation of fungus gardens to the most intimate associations, where bacteria reside intracellularly in dedicated bacteriocytes. However, the majority of prokaryotic symbionts of termites are located in the intestinal tract, where they are free-swimming, attached to the gut epithelium, or associated with the intestinal protozoa (*Fig. 22.1*). Although it is suggestive that the gut microbiota of termites is directly or indirectly involved in the digestion of lignocellulose or has other nutritional implications, the exact nature of the associations and possible benefits for the partners of each particular symbiosis are often far from clear. Therefore, this chapter will use the term "symbiosis" in its broader sense, as originally defined by Anton

E. Rosenberg et al. (eds.), *The Prokaryotes – Prokaryotic Biology and Symbiotic Associations*, DOI 10.1007/978-3-642-30194-0_20,

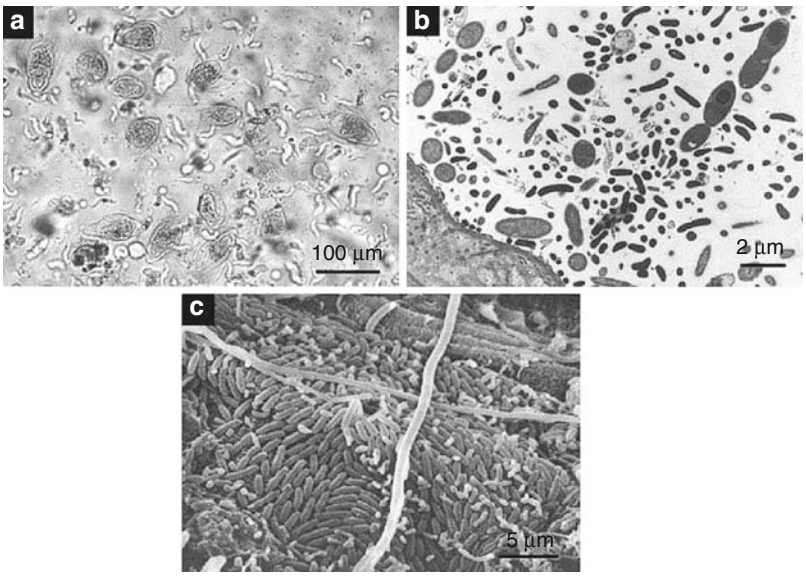


**Fig. 22.1**
**Examples of microbial symbionts in the hindgut of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae), a wood-feeding lower termite. (a) Preparation of anaerobic protozoa from the hindgut of a worker larva, showing the large hypermastigote flagellate *Trichonympha agilis*, filled with wood particles, and numerous smaller flagellates (mainly oxymonads, *Dinenympha* spp.). Differential interference contrast photomicrograph taken by U. Stingl. (b) Transverse section through the peripheral hindgut, showing the diverse bacterial microbiota associated with the thin cuticle of the hindgut wall (*bottom left*). Transmission electron micrograph provided by J. A. Breznak. (c) Preparation of the hindgut wall, showing the dense colonization of the cuticle by numerous rod-shaped and filamentous bacterial morphotypes. Scanning electron micrograph provided by J. A. Breznak (Reproduced from Brune 2003)**

de Bary (1878). A definitive classification of the associations into the different categories of symbiosis, such as mutualism, parasitism, or commensalism, would require a level of understanding that is yet to be reached.

In view of the enormous body of literature on the intestinal microbiota of termites and its role in lignocellulose digestion, the subject cannot be covered exhaustively. This chapter will attempt to summarize the current state of knowledge on the prokaryotic communities within the intestinal tracts of termites, the major populations and their metabolic activities, and their interactions. In addition, it will focus on the gut as a microbial habitat. The chapter will touch only briefly on the intestinal flagellates, which are most important in the phylogenetically lower termites, the exosymbiotic fungi in fungus-cultivating Macrotermitinae, and the intracellular bacteria in termite tissues. For details on these subjects and for many other aspects of the termite gut symbiosis, the reader will be referred to the pertinent review articles.

## Symbiotic Digestion

Termites, like other insects thriving on a lignocellulosic diet, possess a pronounced gut microbiota housed in specially adapted regions of the alimentary tract (*Fig. 22.2*). The symbionts convert a substantial portion of the dietary components to microbial fermentation products, which are then eventually resorbed by the intestinal epithelia. It is generally assumed that the intestinal symbioses provide metabolic capacities that are otherwise not available to the host. For reviews, see Breznak and Brune (1994), Kane (1997), Brune (1998, 2003), Bignell (2000), Breznak (2000), Brune and Friedrich (2000), and Ohkuma (2003).

The symbiotic digestion of lignocellulose by termites is a complex series of events involving both the host and its gut microbiota (*Fig. 22.3*). While the events in the foregut and midgut seem to be mainly due to host activities, the digestive processes in the hindgut are largely controlled by the symbionts. Many aspects of lignocellulose digestion are common to all termites, but there are also several noteworthy differences between the phylogenetically lower and higher taxa.

## Fiber Degradation

The degradation of plant cell walls requires the synergistic action of many different enzymes and, in the case of lignified substrates, also a mechanism to break up the lignocellulose complex (Breznak and Brune 1994). Microorganisms, i.e., bacteria, protozoa, and fungi, are the most efficient cellulose and hemicellulose degraders in nature, and fungi and certain actinomycetes are also the only organisms that have developed a strategy for the chemical breakdown of lignin (Béguin and Aubert 1994; Jeffries 1994). Not surprisingly, termites and other animals have made use of these capacities by employing microbial symbionts in the digestion of lignocellulosic food (Martin 1983).

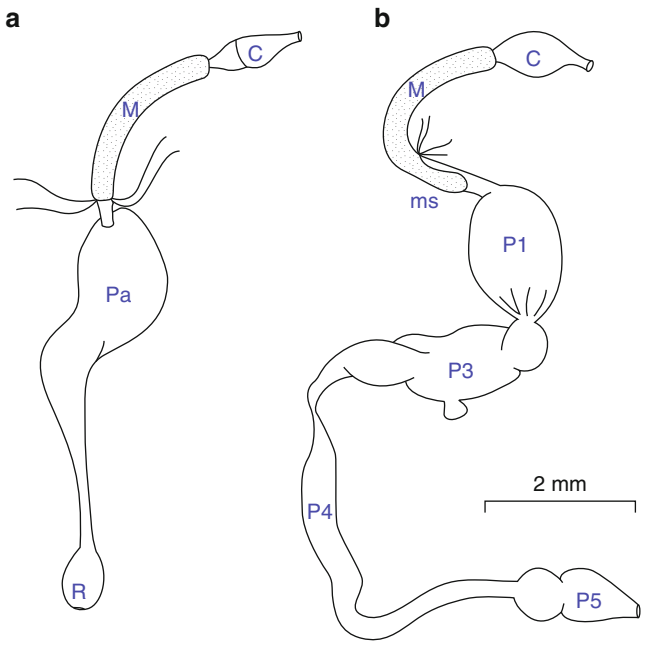


**Fig. 22.2**
**Structure of the intestinal tract of *Reticulitermes* species (a) and *Cubitermes* species (b). All lower termites harbor the gut microbiota in a single, strongly dilated hindgut paunch (*Pa*) that tapers out via the colon into the rectum (*R*). In most higher termites, especially the soil-feeding species, the hindgut is more elongated and has additional dilatations. Abbreviations: crop *C*, midgut *M*, mixed segment *ms*, and proctodeal segments *P1–P5***

## Role of Intestinal Protozoa

The presence of protozoa in termite guts was recognized very early, although they were initially considered to be parasites (e.g., Leidy 1881; Koidzumi 1921). The American protozoologist L. R. Cleveland recognized that the wood-feeding lifestyle in the evolutionarily "lower" termites is based on a mutualistic association with their intestinal protozoa (Cleveland 1925a, 1926). In a series of elegant experiments, he established that the ability of lower termites to live on a diet of wood or cellulose depends on the digestive capacities of their intestinal flagellates. His pioneering work, published in 1923–1928, paved the way for many later studies (see reviews by Honigberg (1970), Inoue et al. (2000), and Brune and Stingl (2005)).

About 15 years later, Hungate elucidated the biochemical basis for this symbiosis (Hungate 1939, 1943). He showed that the gut flagellates depolymerize and ferment lignocellulose to short-chain fatty acids, which are resorbed and oxidized by the host [reviewed by Hungate (1955) and Breznak and Brune (1994)]. The importance of protozoa for the metabolic processes in the hindgut of lower termites is most impressively evidenced by their enormous numbers, which may constitute more than one-third of the body mass in *Zootermopsis* species (Katzin and Kirby 1939).

There is a large body of literature on the decomposition of wood and cellulose by termite gut flagellates [for references, see O'Brien and Slaytor (1982), Breznak and Brune (1994), and Inoue et al. (2000)]. Apparently, the different flagellate species are nutritionally specialized, and each species might fill a specific niche in lignocellulose digestion (Yoshimura et al. 1996;

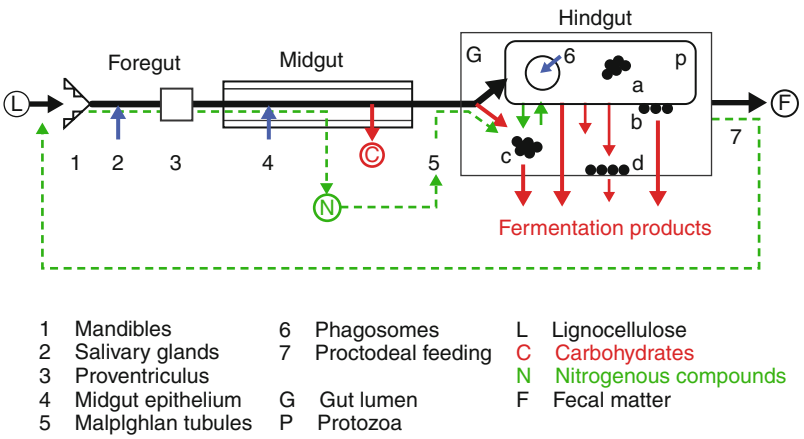


**Fig. 22.3**
**Major events in the symbiotic digestion of lignocellulose by wood-feeding lower termites. The *black lines* show the path of the insoluble material whose lignin-rich residues are released as feces, whereas the *red lines* represent soluble degradation products, which are eventually resorbed by the host. The *green lines* indicate the cycling of nitrogenous compounds. *Blue arrows* mark the sites where cellulolytic enzymes are secreted. *Lower-case letters* refer to the different groups of bacteria, which are either endobionts (*a*) or epibionts (*b*) of the protozoa, suspended in the gut lumen (*c*) or attached to the gut wall (*d*). The scheme has been simplified for the sake of clarity; not all possible interactions are shown (Adapted from Brune 2003)**

Inoue et al. 2000). Most of the endoxylanase and β-xylosidase activity in the lower termite *Reticulitermes speratus* is located in the anterior hindgut and is lost upon defaunation (removal of protozoa by ultraviolet irradiation; Inoue et al. 1997), and the effects of artificial diets on the composition of the protozoan community corroborate that different gut flagellates are involved in xylan and cellulose degradation (Inoue et al. 1997; Cook and Gold 2000).

The protozoa possess their own cellulase genes, which fall into different glycosyl hydrolase families (Ohtoko et al. 2000; Nakashima et al. 2002a; Watanabe et al. 2002; Li et al. 2003; Inoue et al. 2005) and may even exploit host cellulases that are secreted in the anterior gut regions (Li et al. 2003). There is no evidence that the prokaryotic symbionts of the gut flagellates (see the section ❯ "Interactions Between Prokaryotes and Protozoa" in this chapter) confer cellulolytic activity to their hosts.

## The Role of Fungi

The role of fungi in the digestion of lignocellulose by termites is less clear. There are termites that can thrive on sound wood, but many species show a strong preference for decaying wood colonized by saprophytic fungi, which may either precondition the wood for digestion or provide metabolic products important for termite nutrition (Sands 1969; Rouland 2000; Cornelius et al. 2002). Schäfer et al. suggested that the yeasts and other fungi present in the guts of the lower termites *Zootermopsis angusticollis* and *Neotermes castaneus* are involved in hemicellulolytic degradation (Schäfer et al. 1996).

Higher termites of the subfamily Macrotermitinae have established a unique exosymbiosis with basidiomycetes of the genus *Termitomyces*, which are maintained on predigested plant litter in so-called fungus gardens within the nests. The symbiosis has rendered fungus-cultivating termites independent of the intestinal protozoa, which probably allowed for the obvious diversification in their diet (Sands 1969; Rouland 2000). The specificity of this symbiosis, whose enormous evolutionary success is impressively evidenced by the huge nests of fungus-cultivating termites populating the grasslands of Africa, is documented by several instances of coevolution between the termites and their fungal partners, indicating both horizontal and vertical transmission of the fungal symbionts (Aanen et al. 2002; Katoh 2002; Rouland-Lefevre et al. 2002; Taprab et al. 2002).

The association with the lignin-degrading fungus enables the fungus-cultivating termites to utilize lignocellulose nearly completely, as reflected in the small volume of their final feces (Darlington 1994). The key activities attributed to the fungal partner in this mutualistic symbiosis are extensive delignification of the substrate (Hyodo et al. 1999, 2000; Johjima et al. 2003) and the conversion of plant fiber to fungal biomass, as proposed earlier in the *lignin degradation hypothesis* of Grassé and Noirot (1958). Evidence for an activity within the gut of fungal cellulases ingested by the termites together with the fungus comb material (Abo-Khatwa 1978; Martin and Martin 1978, 1979) gave rise to the *acquired enzyme hypothesis* of Martin (1983). However, claims that the fungal cellulases are essential for cellulose digestion in the termite gut remain controversial (Slaytor 1992; Bignell et al. 1994; Crosland et al. 1996), especially in view of the recently discovered ability of termites to produce their own cellulases (see the section ❯ "The Role of Host Enzymes" in this chapter).

## The Role of Host Enzymes

Since phylogenetically higher termites (family: Termitidae) lost their gut flagellates in the course of termite evolution, it was initially assumed that either ingested fungal enzymes (see the section ❯ "The Role of Fungi" in this chapter) or prokaryotic symbionts took over the function of the cellulolytic protozoa. However, there is still no clear evidence that bacteria play a major role in cellulose degradation in any of the termites investigated (see the section ❯ "Cellulolytic Bacteria" in this chapter), which may find its explanation in the recently discovered ability of termites to produce their own cellulases.

In all insects, the digesta are exposed to a variety of digestive enzymes secreted by the salivary glands and the midgut epithelium (Terra 1990). Cook (1943) had already demonstrated that termites are able to absorb sugars directly, and evidence is accumulating that termites secrete a full complement of enzymes necessary for the digestion of plant structural polysaccharides, including cellulose, into the midgut (e.g., Rouland et al. 1989; Slaytor 1992; Rouland 2000). The presence of protease and lysozyme activities has been documented for several termites, which indicates that also microbial cells can be digested (Rohrmann and Rossman 1980; Fujita et al. 2001; Fujita and Abe 2002; Fujita et al. 2002). Although experimental evidence is scarce, one can safely assume that – as in other insects – most of the easily digestible material has been mobilized and resorbed by the time the digesta reach the end of the midgut (❯ *Fig. 22.3*).

The persisting dogma that higher animals do not possess their own cellulases has been unequivocally refuted by the demonstration of endoglucanase genes in the termite genome and their expression in the cells of the midgut epithelium and in the salivary glands (reviewed by Watanabe and Tokuda 2001). Even in lower termites, host cellulases secreted by the salivary glands and complement (and surpass) the cellulolytic activities of the intestinal protozoa in the hindgut (Nakashima et al. 2002b; Tokuda et al. 2004). There is evidence that glycosyl hydrolase family 9 (GHF9) cellulases present in the genomes of termites are ancient and widespread in Metazoa (Lo et al. 2003b; Davison and Blaxter 2005).

## Soil-Feeding Termites

The majority of termite species are humivorous, yet little is known about the exact nature of the dietary components exploited by these ecologically important soil macroinvertebrates (Brauman et al. 2000). Besides fragments of plant tissue,

fungal hyphae, and numerous microorganisms, their diet consists largely of undefined humic material intimately associated with the mineral soil matrix (Donovan et al. 2001). While the aromatic component of humus was initially assumed to be the principal substrate of soil-feeding termites (Noirot 1992; Bignell 1994), feeding trials with soil-feeding *Cubitermes* spp. have shown that peptidic soil components – free or polymerized into humic model compounds – are preferentially digested and mineralized (Ji et al. 2000; Ji and Brune 2001, 2005).

The anterior hindgut of soil-feeding termites is extremely alkaline (Bignell and Eggleton 1995; Brune and Kühl 1996), which favors the extraction of organic matter from the soil (Brune 1998; Kappler and Brune 1999). Microbial biomass and its structural components are assimilated more efficiently than cellulose, which supports the hypothesis that soil microorganisms and the nitrogenous components of humus are an important dietary resource for humivorous soil macroinvertebrates (Ji et al. 2000; Ji and Brune 2001).

## Host Nutrition

Irrespective of their contribution to polymer degradation, the majority of prokaryotes in termite guts are probably involved in the fermentation of the soluble products released into the gut (see the section "Microbial Fermentations" in this chapter), which are derived either directly from the food by the digestive enzymes (see the section "The Role of Host Enzymes" in this chapter) or by the fermentative activity of the intestinal protozoa (see the section "Role of Intestinal Protozoa" in this chapter). The major products of the hindgut metabolism are acetate and, to a smaller extent, other short-chain fatty acids (mainly propionate and butyrate), which accumulate in the hindgut fluid and are eventually resorbed by the hindgut epithelium (*Fig. 22.3*). Termites – like other insects – cannot use acetate as a substrate for gluconeogenesis, but as long as the digestive processes in the midgut release sufficient amounts of soluble sugars and amino acids, this is not a problem. Breznak calculated that acetate produced by the hindgut microbiota of *Reticulitermes flavipes* would suffice to support the respiratory activity of the host (Breznak 2000).

Besides being difficult to degrade, lignocellulose is also an extremely nutrient-poor substrate. While non-lignified plant cells are usually rich in protein and other nitrogenous compounds, the C-to-N ratio of sound wood is up to 100-fold higher than that of the insect body (La Fage and Nutting 1978). Moreover, a lignocellulosic diet lacks most of the essential nutrients required by animals, such as amino acids, vitamins, and sterols. Many microorganisms are capable of fixing dinitrogen, assimilating nitrate and ammonia, or synthesizing those amino acids and vitamins essential for the host. Animals, including termites, have developed means of exploiting these biosynthetic capacities, which include – in the simplest case – the digestion of the intestinal symbionts.

Wood-feeding termites, especially those feeding on sound wood, have an extreme shortage of nutrients in their diet, and the digestion of microbial biomass acquired in the course of anal trophallaxis supplies them with high-quality nutrients (Machida et al. 2001). The gut microbiota supplies essential precursors for the biosynthesis, e.g., of methyl-branched hydrocarbons (Guo et al. 1991), and might play a role in nestmate recognition (Matsuura 2001). To date, the lack of knowledge on the individual components of the prokaryotic microbiota and their metabolic capacities and activities in situ still makes it difficult to define the essential functions and understand the complex interactions.

## The Gut Microenvironment

The intestinal tract of insects is organized into three major gut regions: a short foregut, a midgut (which is the main site of digestion), and a usually short hindgut (proctodeum). The hindguts of all termites, however, have immensely increased in length and volume over the course of evolution (*Fig. 22.2*). In the more primitive, lower termites, the hindgut is still relatively simple, consisting of a dilated "hindgut paunch" that tapers out into the colon and ends in the rectal compartment (Noirot 1995). While this organization has been retained in the fungus-cultivating termites (Termitidae: Macrotermitinae), all other lineages of higher termites show a trend toward a further elongation and additional compartmentalization of the hindgut (Noirot 2001), which is most pronounced in the soil-feeding representatives. The gut morphologies of lower and higher termites and the significance of the adaptations for the digestive process have been reviewed exhaustively (Noirot 1995, 2001).

The proctodeal dilatations increase the residence time of the digesta, thereby prolonging the exposure to the activities of the intestinal microbiota. Moreover, host factors and microbial activities give rise to physicochemical gradients that create distinct microenvironmental conditions in each gut compartment. This has been shown for oxygen, hydrogen, redox potential, and intestinal pH and has to be expected also for any other metabolite when source and sink are spatially separated (Brune and Friedrich 2000), especially since the microbiota is not randomly distributed within the gut (see the section "Spatial Organization" in this chapter).

## Physicochemical Gradients

### Redox Conditions and Oxygen Status

The general concept of termite guts as anoxic habitats had been based on several pieces of circumstantial evidence (outlined by Veivers et al. 1980): (1) the oxygen sensitivity of the intestinal protozoa already recognized by Cleveland (1925a, b); (2) the demonstration of a fermentative metabolism of cellulose by these flagellates (Hungate 1939, 1943) and the high concentrations of microbial fermentation products in the hindgut of all termites investigated; and (3) the presence of oxygen-sensitive or strictly anaerobic processes, such as nitrogen fixation and

methanogenesis (Breznak et al. 1973; Breznak 1975). Also, the subsequent isolation of anaerobic bacteria from termite guts (see the section "Isolates and Major Metabolic Activities" in this chapter) supported the general assumption that the principle of symbiotic digestion in termite guts was analogous to that in the rumen.

Slaytor and coworkers were the first to question the anoxic status of termite guts. Following the color reaction in the hindgut of redox indicator dyes fed to *Nasutitermes exitiosus* and *Coptotermes lacteus*, they initially claimed that the hindgut paunch was "aerobic," since methylene blue remained oxidized (Eutick et al. 1976). In a later study, however, using a more refined technique, they obtained $E_h$ values between –230 mV and –270 mV in the hindgut paunch of these and seven other termite species (Veivers et al. 1980). The initial error had been caused by the color of the reduced dye within the gut being obscured by that of the oxidized dye, which had also impregnated the oxic gut epithelium.

Moreover, Slaytor and coworkers demonstrated that the vitality of *Nasutitermes exitiosus* and *Coptotermes lacteus* depended on the presence of their prokaryotic gut microbiota (Eutick et al. 1978b) and that bacteria play an important role in maintaining the low redox potential of the hindgut paunch (Veivers et al. 1982), which led to the postulation that they maintain anoxic conditions by removing oxygen from the hindgut.

Bignell (1984) pointed out that arthropods are relatively small animals with surface-to-volume ratios higher than those in practically all vertebrates and that they are likely to reach equilibrium with their environment unless efficient permeability barriers for oxygen are established or oxygen is sequestered by the animal or by intestinal microorganisms. In a series of studies employing oxygen microsensors, Brune and coworkers clarified the situation (Brune et al. 1995a; Ebert and Brune 1997; Schmitt-Wagner and Brune 1999) by demonstrating that the steep gradient in oxygen partial pressure between the oxic gut epithelium and the anoxic gut contents drives a continuous influx of oxygen into the hindgut (*Fig. 22.4*). In all termites investigated, oxygen penetrated 50–200 µm into the periphery of the hindgut lumen, leaving only the central portion of the dilated compartments anoxic (Ebert and Brune 1997; Brune 1998; Kappler and Brune 1999; Schmitt-Wagner and Brune 1999).

The maintenance of anoxia in the termite hindgut is not a trivial issue. Since the removal of oxygen in the gut periphery is fueled by the fermentative processes in the hindgut lumen, there must be a lower size limit for arthropods with a symbiotic digestion. However, even the smallest of all termites investigated to date (*Anoplotermes pacificus*, Termitidae: Apicotermitinae) seems to possess a symbiotic gut microbiota, although the spectrum of fermentation products in the hindgut differs from that of other termites (Bauer et al. 2000).

Fine-scaled redox measurements with platinum microelectrodes in wood- and soil-feeding termites (Ebert and Brune 1997; Kappler and Brune 2002) have shown that the redox potential in the gut mirrors the oxygen gradients (*Fig. 22.5*),

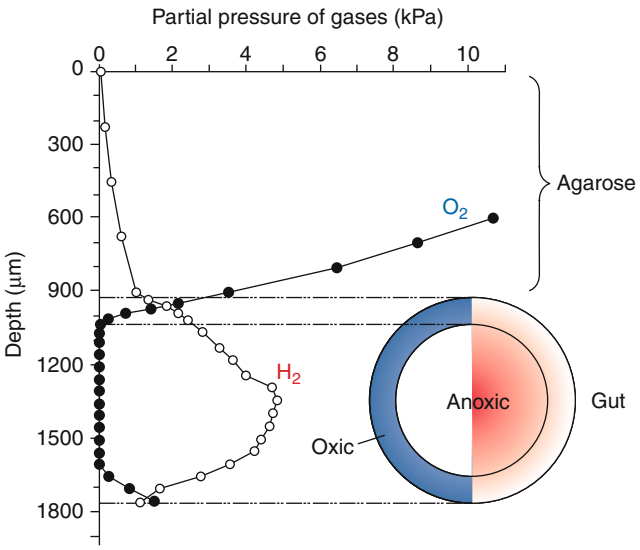


**Fig. 22.4**
**Radial gradients of oxygen (●) and hydrogen (○) in an agarose-embedded hindgut (*paunch region*) of *Reticulitermes flavipes* worker larva. A schematic cross-section through the paunch illustrates the relative sizes of the oxic and anoxic zones (Modified after Brune 1998)**

and also the absolute values are in good agreement with the previously published data based on feeding experiments with redox dyes (Veivers et al. 1980). The most negative redox potentials are found in the regions of high hydrogen partial pressure, although in soil-feeding termites of the genus *Cubitermes*, parameters other than oxygen or hydrogen partial pressure seem to control the redox status of the intestinal contents (Kappler and Brune 2002). There is evidence for ferric iron reduction in the gut of soil-feeding and wood-feeding species, which may be a microbial process, possibly mediated by the presence of humic acids or other phenolic polymers (Kappler and Brune 2002; Vu et al. 2004).

## Hydrogen Partial Pressure

Despite the massive hydrogen production by the intestinal protozoa (Odelson and Breznak 1985b), the hydrogen emission rates of termites are relatively low (Odelson and Breznak 1983; Ebert and Brune 1997; Sugimoto et al. 1998; Schmitt-Wagner and Brune 1999). Originally, it had been assumed that the situation in the intestinal tract of termites was similar to that in other methanogenic habitats, where low hydrogen partial pressures result from a tight coupling between hydrogen-producing and hydrogen-consuming processes (Breznak 1994; Breznak and Brune 1994). However, hydrogen microsensor measurements revealed that the situation in termites is quite different, giving rise to steep radial gradients of hydrogen toward the gut epithelium and enormous differences in hydrogen partial pressure along the gut axis (Ebert and Brune 1997;

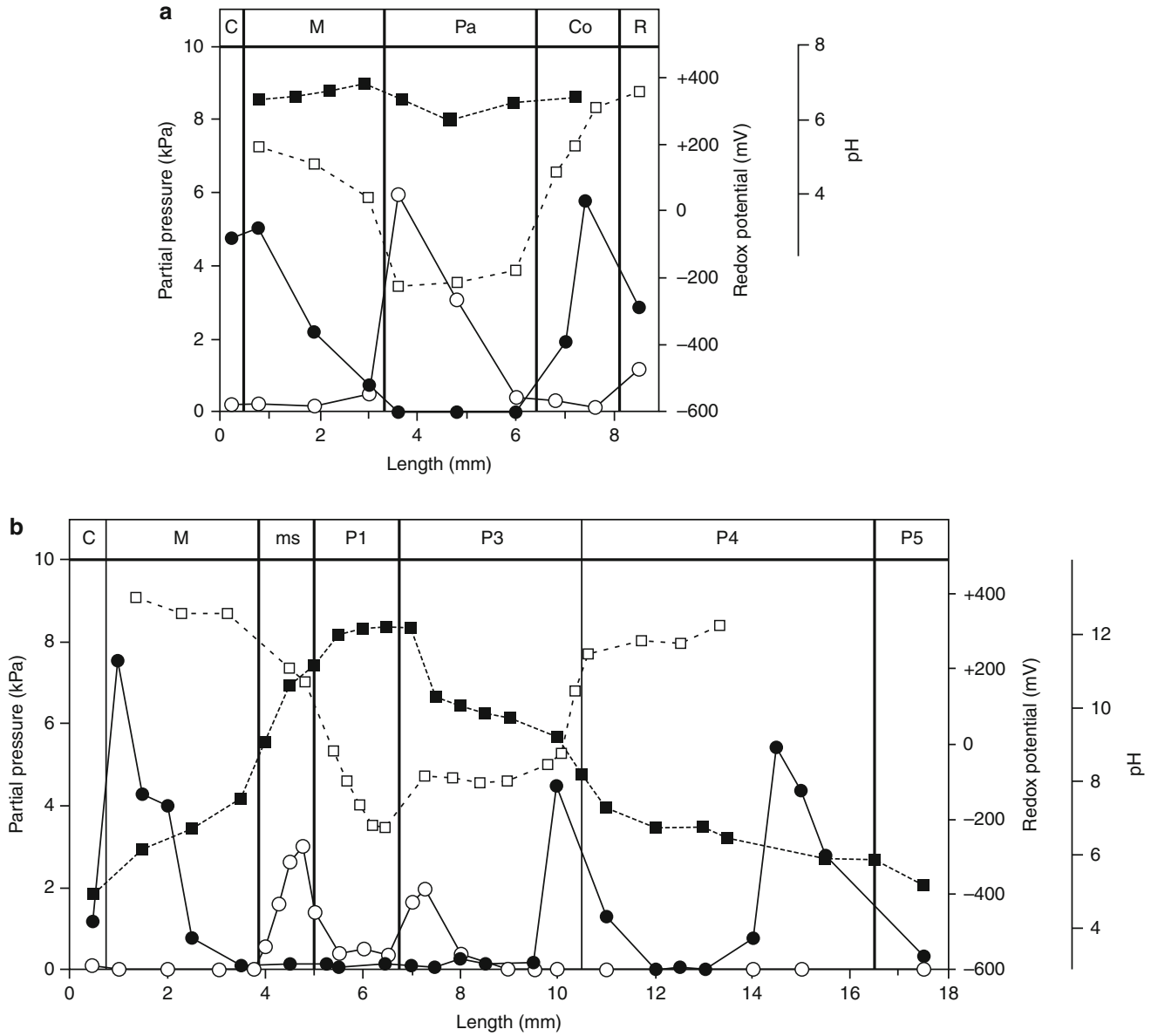


**Fig. 22.5**

**Profiles of physicochemical conditions along the gut axis of *Reticulitermes flavipes* (a) and *Cubitermes orthognathus* (b). Oxygen (•) and hydrogen (○) partial pressures, intestinal pH (■), and apparent redox potential (□) (against a standard hydrogen reference electrode [SHE]) were measured with the respective microsensors. Guts were embedded in agarose-solidified Ringer's solution. The borders between the different gut regions (see legend to *Fig. 22.2*) are indicated by the vertical lines (Data from Brune et al. (1995a), Brune and Kühl (1996), Ebert and Brune (1997), Schmitt-Wagner and Brune (1999), and Kappler and Brune (2002))**

Schmitt-Wagner and Brune 1999; Kappler and Brune 2002), which gave rise to the hypothesis that the spatial organization of the hydrogen-producing and hydrogen-consuming populations controls the hydrogen partial pressure in different gut regions.

## Intestinal pH

The intestinal pH in the hindgut of most phylogenetically lower termites seems to be around neutral (Eutick et al. 1976; Bignell and Anderson 1980; Veivers et al. 1980; Brune et al. 1995a). Kovoor (1967) was the first to report an alkaline region in the anterior hindgut of wood-feeding termites; this observation was later extended also to soil-feeding species (Bignell and Anderson 1980). Since then, a large body of data has accumulated (Bignell 1994; Bignell and Eggleton 1995), documenting a tendency toward strong alkalinity in the anterior hindgut of all higher termites except the Macrotermitinae (Anklin-Mühlemann et al. 1995).

While the initial measurements (performed mostly by spotting pooled, disrupted samples of individual gut regions on pH

indicator paper) still lacked accuracy and resolution, studies with pH microsensors allowed alkaline regions to be precisely located (Brune et al. 1995a; Brune and Kühl 1996). The latter measurements are not biased by homogenization and documented that guts of soil-feeding termites are even more alkaline than reported previously. The most alkaline values (pH 11–12.5) were found among soil-feeding Termitinae and represent the highest values ever encountered in biological systems (Brune and Kühl 1996). In all species tested, the pH of the gut contents increases sharply from circumneutral in the midgut to highly alkaline between the midgut–hindgut junction and the first proctodeal dilation (P1), which coincides exactly with the location of the mixed segment (*Fig. 22.5*), a morphologically unique gut region present in all higher termites except the Macrotermitinae (Noirot 2001).

## Gut Compartmentation and Microhabitats

Each gut compartment provides various microhabitats differing in many environmental parameters (see the section "Physico-chemical Gradients" in this chapter). The small size of the guts results in large surface-to-volume ratios (Brune 1998), and the epithelial surfaces provide ample attachment sites for gut microorganisms, which are thus protected from washout (Bignell 1984). Additional compartmentalization is created by the protozoa inhabiting the hindgut lumen of lower termites.

### Midgut Epithelium, Ectoperitrophic Space

As in other insects, the midgut epithelium is not protected by a cuticle, but a peritrophic membrane separates the epithelial surface from the digesta (Terra 1990). The ectoperitrophic space harbors a distinct bacterial microbiota, which can be intimately associated with the microvilli of the brush border (Breznak and Pankratz 1977). In addition, the so-called mixed segment in many Termitidae, a region where midgut and hindgut epithelia overlap (Noirot 2001), is a microhabitat that harbors a specific bacterial microbiota. Kovoor (1968) described a "pure culture of spore-forming fusiform bacteria" in the mixed segment of *Microcerotermes edentatus*, located outside of the peritrophic membrane in a posterior pocket formed by the mesenteric side. Also, Potts and Hewitt (1973) observed a prominent flora of "thin long filaments with terminal spores" in the mixed segment of the harvester termite, *Trinervitermes trinervoides* (Nasutitermitidae), located in the ectoperitrophic space posterior to the Malpighian tubules. Other authors described dense populations of different but also relatively uniform microorganisms in the mixed segment of *Nasutitermes exitiosus* (Czolij et al. 1985) and of soil-feeding Termitinae (*Procubitermes aburiensis* and *Cubitermes severus*; Bignell et al. 1980a, 1983). Recently, Tokuda et al. (2000) demonstrated that the bacteria populating the mixed segment of *Nasutitermes takasagoensis* are phylogenetically within the radiation of the Clostridiales (see the section "Clostridiales" in this chapter). Electron microscopy confirmed their close association with the mesenteric epithelium, suggesting that there is some kind of interaction with the gut tissue (Tokuda et al. 2001).

### Hindgut Cuticle

Electron-microscopy studies revealed intimate associations of microorganisms with the cuticle of the hindgut epithelium in all termites investigated (Breznak and Pankratz 1977; To et al. 1980; Czolij et al. 1985; Yara et al. 1989; *Fig. 22.1*). Bacteria are associated with cup-like indentations on the epithelial surface of the hindgut in *Reticulitermes flavipes* (Breznak and Pankratz 1977). Although *Mastotermes darwiniensis* and *Nasutitermes exitiosus* possess similar structures, they seem not to be associated with microorganisms (Czolij et al. 1984). In certain soil-feeding termites, cuticular spines protrude from the hindgut wall into the lumen of the P4 compartment and form additional attachment sites for the gut microbiota (*Procubitermes aburiensis*; Bignell et al. 1980b).

### Hindgut Protozoa

The protozoa in the hindgut of lower termites occupy the bulk of the hindgut volume (Katzin and Kirby 1939) and represent an enormous surface area in the hindgut (Berchtold et al. 1999). Pierantoni (1936) was first to point out the association of gut flagellates with bacteria; since then, ectobiotic and endocytobiotic bacteria have been found on and in almost every flagellate investigated. For example, the hypermastigote flagellate *Joenia annectens*, a symbiont in the hindgut of *Kalotermes flavicollis*, is densely colonized by prokaryotic microorganisms (Hollande and Valentin 1969). The body is covered with rod-shaped bacteria, and the nucleus and the cytoplasm contain various types of endocytobiotic bacteria (Radek et al. 1992; Patricolo et al. 2001). Also the oxymonadid flagellate *Streblomastix strix*, a hindgut symbiont of *Zootermopsis* species, is associated with several, morphologically distinct types of bacteria that are orderly arranged end-to-end on six or seven longitudinal vanes, lending *S. strix* a stellate appearance in transverse section (Leander and Keeling 2004). Adhesion of bacteria to the flagellate surfaces is based on different mechanisms and facilitated by special surface structures (e.g., Radek et al. 1996; Radek and Tischendorf 1999; Rother et al. 1999; Patricolo et al. 2001). The literature has been recently reviewed by Radek (1999). Possible significance of these associations of prokaryotes with hindgut flagellates is discussed in a different section (see the section "Interactions Between Prokaryotes and Protozoa" in this chapter).

## Prokaryotic Gut Symbionts

In view of the variety of microhabitats and microenvironmental conditions in the intestinal tracts of termites (see the section

"The Gut Microenvironment" in this chapter), it is not astonishing to find an equally large diversity among the microorganisms colonizing the gut. The amount of diversity indicated already by the morphological and ultrastructural features of the microbiota is greatly exceeded by that encountered at the phylogenetic level.

## Morphological Diversity

Already the observation of gut preparations with a phase-contrast light microscope reveals a wide variety of prokaryotic life forms. Several comprehensive studies of the bacterial gut microbiota of termites using transmission electron microscopy have provided detailed accounts of the morphological diversity of gut microorganisms for several termite species from different families. In addition to the abundant protozoan fauna in all so-called lower termites (Yamin 1979), at least 20–30 different bacterial morphotypes have been distinguished among the microorganisms colonizing the intestinal tract of *Reticulitermes flavipes* and *Coptotermes formosanus* (Rhinotermitidae; Breznak and Pankratz 1977) and *Pterotermes occidentis* (Kalotermitidae; To et al. 1980).

The so-called higher termites (Termitidae) lack intestinal flagellates, but the morphology of their prokaryotic microbiota appears to be equally diverse. The hindgut of the wood-feeding *Nasutitermes exitiosus* (Termitidae: Nasutitermitinae) contains almost 30 different bacterial morphotypes (Czolij et al. 1985), and also the hindgut microbiota of the fungus-growing termite *Odontotermes formosanus* (Termitidae: Macrotermitinae) comprises at least 20 different morphotypes (Yara et al. 1989). Numerous bacterial morphotypes, including many filamentous forms, colonize the intestinal epithelia and the ectoperitrophic space of the soil-feeding termite *Procubitermes aburiensis* (Termitidae: Termitinae; Bignell et al. 1980a).

Although the morphological features usually do not allow the affiliation of a bacterium to a specific taxon, members of the termite gut microbiota have conspicuous forms or other morphological features that are of (albeit limited) taxonomic value or conspicuous forms that seem to occur in different species of termites. One example is the thin, spore-forming filaments described by Leidy (1881) as "Arthromitus" species, which occur in many invertebrates, including termites (Leidy 1881; Margulis et al. 1990). Also, many of the spirochetal forms are so large and conspicuous that they can be morphologically distinguished (Breznak 1984). On the basis of the detailed morphological features visualized by transmission electron microscopy, Margulis and coworkers (Bermudes et al. 1988; Wier et al. 2000) proposed a number of new species for the larger spirochetes.

## Phylogenetic Diversity and Community Structure

In the recent years, the microbiota in the intestinal tracts of termites has been investigated also with molecular tools (see Ohkuma (2002) for a review). Most studies employed the 16S rRNA gene as a molecular marker. As in most other environments, phylogenetic diversity of the intestinal microbiota is enormous, and there is still little overlap between the phylotypes recovered with cultivation-independent techniques and the isolates obtained by cultivation (see the section "Isolates and Major Metabolic Activities" in this chapter). Molecular fingerprinting has been used to compare the structure of gut communities and to follow temporal changes. The bias inherent in all polymerase chain reaction (PCR)-based approaches has been addressed by backing the results with independent methods, such as fluorescence in situ hybridization (FISH).

### Bacterial Diversity

König and coworkers were among the first to use the 16S rRNA-based approach to identify the phylogenetic position of uncultivated spirochetes in the gut of *Mastotermes darwiniensis* (Berchtold et al. 1994; Berchtold and König 1996); a parallel study of Paster et al. (1996) was aimed at characterizing the spirochetes in the gut of *Nasutitermes lujae*. At about the same time, Ohkuma and coworkers attempted to characterize the full diversity of archaea and bacteria in the intestinal tract of *Reticulitermes speratus* and *Cryptotermes domesticus* using a similar strategy (Ohkuma et al. 1995; Ohkuma and Kudo 1996, 1998).

Although the cultivation-independent approach documented the presence of many new, hitherto uncultivated phylotypes in the intestinal tracts of termites (for a review, see Kudo et al. 1998), these early studies lacked resolution since only small numbers of clones were investigated. Later studies documented that diversity coverage of the clone libraries was far from exhaustive, even if larger numbers of clones were used. The most comprehensive assessment of molecular diversity and bacterial community structure in termite guts to date involved the gut microbiota of *Reticulitermes* species. Hongoh et al. analyzed 14 clone libraries (96 clones each) of the bacterial 16S rRNA genes in the hindgut of the Japanese termite species *Reticulitermes speratus* to characterize phylogenetic diversity and to address the bias introduced by different primer combinations and PCR conditions (Hongoh et al. 2003a, b). Yang et al. (2005) performed a similar analysis with more than 500 clones from the European termite species *Reticulitermes santonensis*, focusing on the differences between the bacterial communities in the four major intestinal habitats: the midgut, the wall of the hindgut paunch, the hindgut fluid, and the intestinal protozoa (see the section "Gut Compartmentation and Microhabitats" in this chapter).

The intestinal community of the two *Reticulitermes* species is quite similar, comprising representatives of several bacterial phyla (*Fig. 22.6*). Both termite species harbor Gram-positive bacteria (mainly clostridia, streptococci, and Mycoplasmatales-related clones), members of the Bacteroidetes, spirochetes, and a number of Proteobacteria, albeit at slightly different ratios. A large number of clones fall into the so-called termite

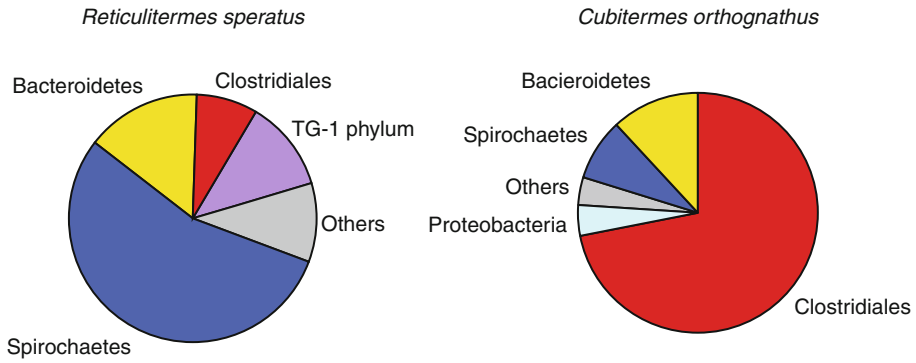


**Fig. 22.6**
**Relative abundance of the major bacterial phyla in clone libraries of 16S rRNA genes from the hindgut of the wood-feeding, lower termite *Reticulitermes speratus* and the soil-feeding, higher termite *Cubitermes orthognathus* (Data from Hongoh et al. (2003a) and Schmitt-Wagner et al. (2003b))**

group 1 (TG-1) phylum, which were most abundant in *Reticulitermes santonensis* (Yang et al. 2005); spirochetal clones were less abundant in this termite but accounted for approximately half of the analyzed clones in *Reticulitermes speratus* (Hongoh et al. 2003b).

The situation in soil-feeding termites is quite different. In a study analyzing bacterial diversity in the highly compartmentalized intestinal tract of *Cubitermes orthognathus* (Schmitt-Wagner et al. 2003a, b), the authors combined clone analysis with FISH and a molecular fingerprinting analysis, which not only substantiated the data obtained by the individual approaches but also allowed differences in community structure between the different gut compartments to be investigated. In contrast to the situation in *Reticulitermes speratus*, the bacterial clone libraries contained no clones from the TG-1 phylum and only few spirochetal clones. In the anterior gut sections, most clones represented Firmicutes. In the posterior gut sections, clones belonging to the Bacteroidetes and different subgroups of the Proteobacteria gained some numerical significance. A study of the bacterial microbiota in the P1 compartment of several higher termites extended the presence of a compartment-specific microbiota and a predominance of Firmicutes in the highly alkaline gut regions also to representatives of other feeding guilds (Thongaram et al. 2005).

All PCR bias notwithstanding, these large datasets, together with the numerous clones obtained from other termite species (for references, see Ohkuma 2003), allow a reasonably accurate picture of the dominant phylogenetic groups to be drawn. Most clones obtained in the different studies represent lineages of microorganisms that were exclusively recovered from the intestinal tract of termites. The termite specificity of these lineages was underscored by the finding that the closest relatives of the bacterial clones within each lineage were usually derived also from the most-closely related termites, supporting the concept of coevolution between gut microbiota and host (Yang et al. 2005).

### Spirochaetes

Not astonishingly, in view of the numerical predominance of this morphologically diverse and conspicuous group in most wood-feeding termites (Breznak 2002), the majority of the clones obtained with bacteria-specific primers from the hindgut of *Reticulitermes speratus* represent spirochetes (Hongoh et al. 2003a). Already the first molecular studies had indicated that termite gut spirochetes represent a lineage phylogenetically distinct from other Spirochaetes (Berchtold and König 1996; Ohkuma and Kudo 1996; Paster et al. 1996). Better diversity coverage was achieved by Lilburn et al. (1999), who targeted the intestinal spirochetes of *Reticulitermes flavipes* with spirochete-specific primers. They demonstrated that the 12–15 spirochete morphotypes in *Reticulitermes flavipes* (Breznak and Pankratz 1977) were paralleled by 21 different spirochete phylotypes, which formed two major clusters of treponemes, one of them containing only clones of termite origin (Lilburn et al. 1999). *Treponema*-related clones have also been recovered from a variety of other termite species (Lilburn et al. 1999; Ohkuma et al. 1999a). In several cases, the ectosymbiotic association of certain phylotypes with flagellate protozoa has been documented using FISH with group-specific oligonucleotide probes (Berchtold and König 1996; Iida et al. 2000; Noda et al. 2003).

## Endomicrobia

In their first survey of the bacterial diversity in *Reticulitermes speratus*, Ohkuma and Kudo (1996) obtained a number of clones whose sequences were only distantly related to other bacteria and which were subsequently recognized as a novel bacterial phylum (Hugenholtz et al. 1998). Also many clones in the comprehensive libraries subsequently obtained with *Reticulitermes speratus* (Hongoh et al. 2003a) and *Reticulitermes santonensis* (Yang et al. 2005) belong to this lineage, indicating

that members of the TG-1 phylum represent a hitherto uncultivated but numerically dominant group of prokaryotes in the gut of *Reticulitermes* species.

Using a full-cycle molecular approach, combined with transmission electron microscopy, Stingl et al. (2005) showed that the TG-1 bacteria in *Reticulitermes* species are endosymbionts that colonize – exclusively and in high abundance – the cytoplasm of the larger flagellate species. The symbionts were specific for their respective host flagellate and were provisionally classified in the candidate genus "*Endomicrobium*." Members of the TG-1 phylum, for which the name "Endomicrobia" has been proposed, are phylogenetically quite diverse and seem to be present in and also restricted to the guts of those insects (lower termites and wood-feeding cockroaches of the genus *Cryptocercus*) that are in mutualistic association with such cellulose-fermenting flagellates (Stingl et al. 2005).

## Firmicutes

In clone libraries of lower termites, clones affiliated with the Clostridia are abundant and fall into apparently termite-specific lineages (Hongoh et al. 2003a; Yang et al. 2005). In the higher termite *Cubitermes orthognathus*, they dominated the clone library of the alkaline hindgut sections (Schmitt-Wagner et al. 2003b), which was confirmed using FISH with cluster-specific probes and supported by molecular fingerprints of the different gut compartments (Schmitt-Wagner et al. 2003a).

One of the clostridial clusters from the *Cubitermes orthognathus* clone libraries falls into the *Clostridium propionicum* group (Schmitt-Wagner et al. 2003b). Interestingly, this cluster comprises also clones from the termite *Nasutitermes takasagoensis*, which were localized in the mixed segment between the midgut epithelium and the peritrophic membrane using FISH (Tokuda et al. 2000). Other clones are affiliated with homoacetogenic isolates (see the section ❯ "Homoacetogenic Bacteria" in this chapter).

Clone libraries and molecular fingerprints indicated that Clostridia dominate also the bacterial microbiota in the most alkaline hindgut compartment (P1) of *Termes comis*, *Pericapritermes latignathus*, and a *Microcerotermes* species (all Termitinae), whereas Bacilli dominate the P1 of a *Speculitermes* species (Apicotermitinae) (Thongaram et al. 2005). Many of the clones derived from the P1 region form phylogenetic clusters that are unique to termites and are often related to clones obtained from the other insects with alkaline digestive tracts, which suggests that they represent lineages of alkaliphilic bacteria (Schmitt-Wanger et al. 2003; Thongaram et al. 2005).

## Bacteroidetes

Clones affiliated with the Bacteroidetes were recovered from the guts of numerous termite species (Ohkuma et al. 2002; Schmitt-Wagner et al. 2003b). There is an enormous diversity of such phylotypes in *Reticulitermes* species (Hongoh et al. 2003a; Yang et al. 2005). Most clones are only distantly related to described taxa and often form monophyletic clusters with clones recovered from the gut of other termite species. While some of the phylotypes seem to be associated with the hindgut cuticle (Yang et al. 2005), others represent epibionts of protozoa (see the section ❯ "Interactions Between Prokaryotes and Protozoa" in this chapter).

## Other Groups

Clone libraries of *Reticulitermes* species contained clones related to the Mycoplasmatales in a distinct and apparently termite-specific lineage (Hongoh et al. 2003a; Yang et al. 2005) that were abundant in the protozoan fraction of *Reticulitermes santonensis* (Yang et al. 2005) and comprised also a clone obtained from a symbiont of the termite gut flagellate *Koruga bonita* from *Mastotermes darwiniensis* by single-cell PCR (Fröhlich and König 1999). Among the Lactobacillales, most clones were affiliated with the genus *Streptococcus* and were mainly from the midgut clone library. Most clones affiliated with the Proteobacteria formed distinct, termite-specific lineages in the α-subgroup (only distantly related to other lineages of the Rickettsiales) or in the β-subgroup (most closely related to *Dechlorimonas agitatus* or to fermenting bacteria of the genus *Propionivibrio*; Brune et al. 2002). Clones belonging to the δ-subgroup were rare but virtually identical to the sequences of *Desulfovibrio desulfuricans* and of a sulfate-reducing isolate from *Reticulitermes santonensis* (Kuhnigk et al. 1996).

Only a single clone among the >100 clones retrieved from the hindgut of the soil-feeding termite *Cubitermes orthognathus* was affiliated with the Planctomycetales (Schmitt-Wagner et al. 2003b). However, a large fraction of the cells in the posterior hindgut of the closely related *Cubitermes ugandensis* hybridized with a mixture of FISH probes targeting this phylum. This severe underestimation of this phylum in the clone libraries is probably caused by the inadequacy of the commonly used Bacteria-specific PCR primers to amplify the 16S rRNA genes of planctomycetes (Derakshani et al. 2001) and underlines the importance of backing the results of PCR-based analyses with an independent method. Although FISH analysis indicates that more than one-third of the bacteria in the second hindgut compartment (P3 segment) of *Cubitermes ugandensis* may be planctomycetes (Schmitt-Wagner et al. 2003b), their metabolic function remains obscure.

# Archaeal Diversity

Molecular phylogenetic profiling of the microbial communities by dot-blot hybridization with domain-specific probes has indicated that archaea represent 0.1–2.6 % of small subunit (SSU) rRNA extracted from the guts of 24 nutritionally and taxonomically diverse termite species (Brauman et al. 2001).

Interestingly, the relative abundance of archaea seems to be related to the host diet. The percentage of archaeal 16S rRNA among prokaryotic 16S rRNA in the gut of soil-feeding termite species (1.4–3.1 %) was significantly higher than in wood-feeding and litter-feeding termite species (0.1–1.7 %). This is in agreement with the methane emission rates, which are generally higher among soil-feeding termite species (Brauman et al. 1992), and it has been speculated that the majority of the archaea in termite guts are methanogens (Brauman et al. 2001).

## Methanogenic Archaea

Partial sequences of the genes encoding for 16S rRNA and for subunit A of the methyl coenzyme M reductase (*mcrA*) of methanogens indicated that the methanogens in *Reticulitermes speratus* belong to the order Methanobacteriales (Ohkuma et al. 1995) and are closely related but not identical to the *Methanobrevibacter* species isolated from *Reticulitermes flavipes* (Leadbetter and Breznak 1996; Leadbetter et al. 1998; also, see the subsection "Methanogenic Archaea" in section "Hydrogen Metabolism"). Later studies concentrated on the 16S rRNA genes and confirmed the presence of Methanobacteriales in *Reticulitermes speratus* (Shinzato et al. 1999), *Cryptotermes domesticus* (Ohkuma and Kudo 1998; Shinzato et al. 2001), *Hodotermopsis sjöstedti* (Ohkuma et al. 1999c), *Neotermes koshunensis*, *Reticulitermes kanmonensis*, *Coptotermes formosanus*, and *Mastotermes darwiniensis* (Shinzato et al. 2001). All sequences cluster within the radiation of the genus *Methanobrevibacter*, but the sequences from termites differ from those of known methanogens, forming unique lineages in the phylogenetic trees. A single clone related to Methanomicrobiales was recovered from *Reticulitermes speratus* (Shinzato et al. 1999).

Dot-blot hybridization indicated that Methanobacteriales constitute one-third to more than one-half of the archaea in the guts of almost all termite species studied (Brauman et al. 2001). By contrast, Methanosarcinales seem to be present only in the guts of about half of the termite species, apparently forming the dominant group of methanogens in 4 of the 24 species studied and accounting for the total archaeal signal in the fungus-growing species *Macrotermes subhyalinus* (Brauman et al. 2001). Additionally, the clones retrieved from the guts of the phylogenetically higher termites *Nasutitermes takasagoensis*, *Odontotermes formosanus*, and *Pericapritermes nitobei* clustered mostly among the Methanomicrobiales and Methanosarcinales (Ohkuma et al. 1999c). In a detailed study of archaeal diversity in the gut of the soil-feeding higher termite, *Cubitermes orthognathus*, most archaeal clones were affiliated with Methanobacteriales, Methanomicrobiales, and Methanosarcinales, and a few clones had their closest relatives among the Methanococcales (Friedrich et al. 2001). Similar results were obtained in a study with *Cubitermes* fungifaber, which also corroborated that there is little overlap between the communities of methanoarchaea present in the gut and in the food soil (Donovan et al. 2004). In contrast, the similarities between the methanoarchaeal communities of congeneric termites are substantial, and many clones obtained from the intestinal tract of termites cluster with clones retrieved from other insects. However, a purely vertical transmission of the methanogenic gut microbiota is not supported (Donovan et al. 2004).

*Methanobrevibacter* spp. in *Reticulitermes* are associated with the hindgut wall (Leadbetter and Breznak 1996; Leadbetter et al. 1998) and, in the gut of *Reticulitermes speratus* and *Hodotermopsis sjoestedti*, attached to the flagellated protist species *Dinenympha* and *Microjoenia* (Tokura et al. 2000): there are indications that the lineages attached to the flagellates are phylogenetically different from those associated with the gut epithelium. Fröhlich and König (1999) retrieved single cells of endosymbiotic methanogens from the anaerobic flagellate *Pentatrichomonoides scroa* occurring in the hindgut of *Mastotermes darwiniensis* that were affiliated with the genus *Methanobrevibacter*.

Methanogens are among the few groups of organisms for which one can infer metabolic information from the 16S rRNA gene sequence. FISH with an archaea-specific probe revealed that archaea are largely restricted to the gut sections P3 and P4 in *Cubitermes ugandensis*, which is in agreement with the distribution of $F_{420}$-fluorescent cells and methane emission rates along the gut axis of *Cubitermes* species (Schmitt-Wagner and Brune 1999). Cells hybridizing with the archaea-specific probe presented 1.6 % and 3.8 % of the DAPI-stained cells in the P3 and P4 section, respectively (Schmitt-Wagner et al. 2003b), but since many of the abundant and morphologically diverse $F_{420}$-fluorescent microorganisms in these gut sections were filamentous forms and appeared to be fragmented or destroyed during homogenization, they were likely underestimated by the FISH analysis.

## Non-methanogenic Archaea

In a dot-blot analyses of many termite species, the total combined value of the subgroup-specific probes was much lower than that of the Archaea domain probe, indicating that termite guts may contain (possibly non-methanogenic) archaeal populations whose 16S-like rRNAs do not hybridize with probes for methanoarchaeal subgroups employed in this study (Brauman et al. 2001).

Shinzato et al. (1999) provided the first evidence for the presence of Thermoplasmales in the intestinal tract of *Reticulitermes speratus*. Friedrich et al. (2001) obtained numerous clones of Thermoplasmales and a few clones related to the Thermococcales from the gut of the soil-feeding termite *Cubitermes orthognathus* and also documented for the first time the presence of Crenarchaeota in an intestinal tract. Donovan et al. (2004) retrieved several clones related to the haloalkaliphile genus *Natronococcus* from *Cubitermes fungifaber*, which is quite intriguing in view of the extreme alkalinity of the anterior hindgut of *Cubitermes* species (Brune and Kühl 1996).

### Spatial Organization

As mentioned above, termite guts are axially and radially structured, providing numerous microhabitats with different physicochemical microenvironments (see the section "The Gut Microenvironment" in this chapter). Not astonishingly, therefore, the distribution of gut microbiota is not random but seems to be spatially organized. Detailed descriptions of the spatial arrangement of the intestinal prokaryotes in situ (Breznak and Pankratz 1977; To et al. 1978, 1980; Czolij et al. 1985; Yara et al. 1989), together with numerous other observations of certain morphotypes in particular regions of the gut (see the section "Gut Compartmentation and Microhabitats" in this chapter), indicate that many microhabitats harbor characteristic microbial populations.

Until recently, most of such evidence was based purely on morphological data, and the line of evidence is far from complete. With the advent of molecular tools, however, it became possible to address not only the diversity of the termite gut microbiota but also the spatial distribution of individual phylotypes or phylogenetic groups. A study employing whole-cell hybridization in homogenates of different gut regions and in situ hybridization of gut cryosections with group-specific oligonucleotide probes provided the first results documenting differences in microbial community structure between different regions of the hindgut of *Mastotermes darwiniensis* at the level of phylogenetically defined microbial groups (Berchtold et al. 1999). Other studies used FISH to document the association of gut protozoa with certain phylotypes of hitherto uncultivated spirochetes (Berchtold and König 1996; Iida et al. 2000; Noda et al. 2003), Bacteroidetes (Wenzel et al. 2003; Stingl et al. 2004), members of the TG-1 phylum (Stingl et al. 2005), or methanogenic archaea (Tokura et al. 2000).

Also, PCR-based approaches allow one to resolve differences in the community structure of different gut regions or gut compartments (Friedrich et al. 2001; Schmitt-Wagner et al. 2003b; Yang et al. 2005). Costs and effort involved in sequencing and phylogenetic analysis limit investigations based on clone analysis, but molecular fingerprinting allows expansion of the investigation of diversity and – observing the necessary cautions inherent to all PCR-based techniques – community structure to include a larger number of samples. Terminal-restriction-fragment-length polymorphism (T-RFLP) analysis in the higher, soil-feeding termite *Cubitermes orthognathus* documented that the different archaeal and bacterial populations are not randomly distributed along the gut and that the prokaryotic communities in the individual gut segments differ considerably with respect to diversity and abundance (Friedrich et al. 2001; Schmitt-Wagner et al. 2003a). By contrast, the bacterial community structure in homologous compartments in three different species of *Cubitermes* was quite similar, indicating the existence of gut-segment-specific communities (Schmitt-Wagner et al. 2003a).

## Isolates and Major Metabolic Activities

The major metabolic activities of the gut microbiota have been outlined (Breznak 2000; Slaytor 2000; Tholen and Brune 2000; *Fig. 22.7*), but there are still considerable gaps in our knowledge that underline the need for a refined concept (Tholen and Brune 2000). Numerous attempts have been made to characterize the prokaryotic gut microbiota of termites by isolating microorganisms that either were numerically abundant or possessed a metabolic potential considered important in the metabolism of the hindgut. Many of these efforts have yielded results of uncertain significance, either because the methods were not fully described or because no quantitation of bacteria was made relative to the total number of microorganisms present. As there is little overlap between the existing isolates and the 16S rRNA genes obtained in the molecular studies (see the section "Phylogenetic Diversity and Community Structure" in this chapter), it is apparent that many of the microorganisms responsible for the major metabolic activities remain to be cultivated.

### Numerically Predominant Isolates

Most cultivable heterotrophic bacteria in the hindgut of *Reticulitermes flavipes* are *Streptococcus* and *Enterococcus* species, followed by *Bacteroides* species and representatives of the Enterobacteriaceae, mostly *Citrobacter* species and *Enterobacter cloacae* (Schultz and Breznak 1978; Tholen et al. 1997). Coccoid lactic acid bacteria also dominated among the isolates obtained from the hindguts of the lower termites *Mastotermes darwiniensis* and *Cryptotermes primus* (Eutick et al. 1978a) and the higher termites *Nasutitermes arborum*, *Thoracotermes macrothorax*, and *Anoplotermes pacificus* (Bauer et al. 2000). While *Enterobacter* species were found to dominate among the isolates from the rhinotermitid species *Heterotermes ferox*, *Coptotermes acinaciformis*, *Coptotermes lacteus*, and *Schedorhinotermes intermedius* (Eutick et al. 1978a), most isolates from termitid species *Nasutitermes exitiosus*, *Nasutitermes graveolus*, and *Nasutitermes walkeri* were staphylococci. Isolates from these genera have been recovered also in earlier studies (e.g., Mannesmann and Piechowski 1989).

Although some of these studies were at best semiquantitative and many employed only aerobic techniques, the pattern of bacterial species cultivated from each host species is remarkably constant. Most importantly, the majority of the isolates obtained from termite guts are either aerobes or aerotolerant anaerobes. The absence of obligate anaerobes among the isolates in those studies that did not attempt to apply a methodology appropriate for the successful cultivation of such bacteria is not astonishing. However, even in the studies that explicitly used the Hungate technique and employed reduced media for cultivation, the fraction of obligate anaerobes was always smaller than that of aerotolerant anaerobes and aerobes (Schultz and Breznak 1978; Tholen et al. 1997). It is not clear whether this phenomenon is caused by the oxygen status of the termite gut (see the section "Redox Conditions and Oxygen Status" in this chapter) or the

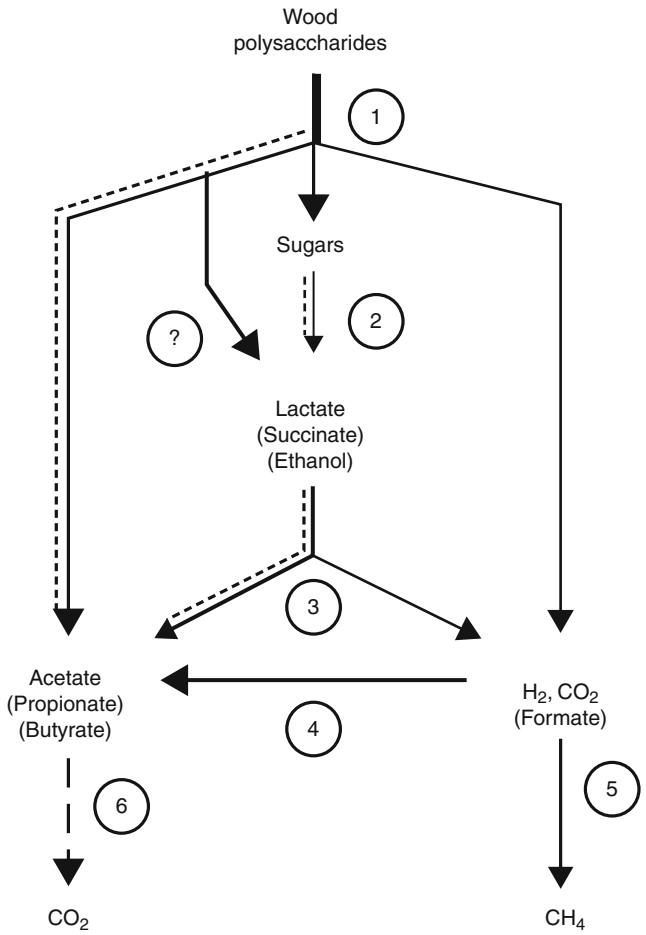


**Fig. 22.7**
**Schematic presentation of the metabolic processes involved in the fermentative degradation of polysaccharides in the hindgut of *Reticulitermes flavipes*. The *dashed lines* indicate metabolic fluxes which seem to be strongly influenced by the continuous influx of oxygen into the gut periphery. *Line thickness* indicates the relative importance of the process. The major metabolic groups are gut flagellates (*1*), primary (*2*) and secondary (*3*) fermenting bacteria, homoacetogens (*4*), and methanogens (*5*); it remains to be clarified whether the flagellates are also a major source of lactate (*?*). Short-chain fatty acids are oxidized by the host (*6*) (Adapted from Brune 2003)**

strong cultivation bias against certain groups of bacteria (see the section "Cultivation Bias" in this chapter).

## Cultivation Bias

A comparison of the viable counts of heterotrophic bacteria to the direct microscopic counts of the microorganisms in the hindgut of *Reticulitermes flavipes* indicates that about 90 % of the microbial cells have escaped cultivation (Schultz and Breznak 1978; Tholen et al. 1997). Viable counts obtained in a similar study attempting to characterize the major gut bacteria of nine species of termites indicate an even larger cultivation bias (Eutick et al. 1978a).

When the isolates obtained in these studies are compared to the results of the cultivation-independent characterization (see the section "Phylogenetic Diversity and Community Structure" in this chapter), there are enormous discrepancies between the frequencies of the phylogenetic groups dominating the clone libraries (*Fig. 22.6*) and the species recovered by cultivation. Nevertheless, many isolates are unique to the termite gut habitat, and their characterization has provided valuable information on metabolic properties and other physiological features relevant for the colonization of this particular habitat (*Table 22.1*). To increase cultivation efficiency, it will be necessary to develop new cultivation strategies that take into account the special environmental conditions within the gut, in particular, the steep physicochemical gradients (see the section "The Gut Microenvironment" in this chapter) and the metabolic interactions among the microbiota (see the section "Microbe–Microbe Interactions" in this chapter).

Like many arthropods, termites harbor filamentous bacterial morphotypes with refractile inclusions resembling endospores. These bacteria are usually attached to the hindgut wall (*Fig. 22.8*) and were first described in 1849 as "Arthromitus" by Leidy (1849, 1881). The filaments have not been cultivated, but Margulis et al. (1998) have proposed that they represent a different life stage of aerobic, rod-shaped bacteria closely related to *Bacillus cereus*, a species group that occurs ubiquitously in soil. However, their conclusions were based merely on the isolation of such bacteria from the boiled intestines of ten species of soil arthropods containing "Arthromitus"-like filaments and on earlier reports on the isolation of B. cereus from arthropod guts. A phylogenetic identity of the filaments with the isolates has not been confirmed with molecular methods. In this context, it should be noted that other authors had previously identified the segmented filamentous bacteria in the gut of mice, rats, and chickens as a new lineage of Clostridiales, based on a 16S rRNA gene sequence analysis (Snel et al. 1994). The same group has proposed "Candidatus Arthromitus" as a provisional generic name for the segmented filamentous bacteria falling into this lineage (Snel et al. 1995) – unfortunately without verifying whether they are indeed related to the morphologically similar filaments in arthropods originally described by Leidy.

## Lignocellulose Degradation

There are numerous reports on the presence in termite guts of enzyme activities acting on different cellulose and hemicellulose preparations. The enzyme activities in foregut and midgut are most likely of host origin or are ingested fungal cellulases that remain active within the gut (see the section "Fiber Degradation" in this chapter), whereas most activities in the hindgut are probably due to the microbiota. In the lower termites, it is also important to differentiate between protozoan and bacterial origin.

**Table 22.1**
**Described species of prokaryotes unique to the intestinal tract of termites**

| Group species | Termite species[a] | Unusual feature | References |
|---|---|---|---|
| Firmicutes | | | |
| *Acetonema longum* | *Pterotermes occidentis* (K) | Homoacetogenic | Kane and Breznak (1991) |
| *Bacillus oleronius* | *Reticulitermes santonensis* (R) | Degrades aromatic compounds | Kuhnigk et al. (1995) |
| *Clostridium mayombei* | *Cubitermes speciosus* (T) | Homoacetogenic | Kane et al. (1991) |
| *Clostridium termitidis* | *Nasutitermes lujae* (T) | Cellulolytic | Hethener et al. (1992) |
| *Isoptericola variabilis* (formerly *Cellulosimicrobium variabile*) | *Mastotermes darwiniensis* (M) | Cellulolytic | Bakalidou et al. (2002), Stackebrandt et al. (2004) |
| *Sporobacter termitidis* | *Nasutitermes lujae* (T) | Homoacetogenic | Grech-Mora et al. (1996) |
| *Sporomusa aerivorans* | *Thoracotermes macrothorax* (T) | Homoacetogenic | Boga and Brune (2003) |
| *Sporomusa termitida* | *Nasutitermes nigriceps* (T) | Homoacetogenic | Breznak et al. (1988) |
| *Sporotomaculum hydroxybenzoicum* | *Cubitermes speciosus* (T) | Degrades aromatic compounds | Brauman et al. (1998) |
| Proteobacteria | | | |
| *Desulfovibrio intestinalis* | *Mastotermes darwiniensis* (M) | Sulfate-reducing | Fröhlich et al. (1999) |
| *Desulfovibrio termitidis* | *Heterotermes indicola* (R) | Sulfate-reducing | Trinkerl et al. (1990) |
| Fusobacteria | | | |
| *Sebaldella termitidis* (formerly *Bacteroides termitidis*) | *Reticulitermes lucifugus* (R) | Uricolytic | Collins and Shah (1986) |
| Bacteroidetes | | | |
| *Candidatus* Vestibaculum illigatum[b] | *Neotermes cubanus* (K) | Epibiont of *Staurojoenina* sp. | Stingl et al. (2004) |
| Spirochaetes | | | |
| *Treponema azotonutricium* | *Zootermopsis angusticollis* (Z) | Nitrogen-fixing | Graber et al. (2004) |
| *Treponema primitia* | *Zootermopsis angusticollis* (Z) | Homoacetogenic | Graber et al. (2004) |
| "Endomicrobia" | | | |
| *Candidatus* Endomicrobium trichonymphae[b] | *Reticulitermes santonensis* (R) | Endobiont of *Trichonympha agilis* | Stingl et al. (2005) |
| *Candidatus* Endomicrobium pyrsonymphae[b] | *Reticulitermes santonensis* (R) | Endobiont of *Pyrsonympha vertens* | Stingl et al. (2005) |
| Methanobacteriales | | | |
| *Methanobrevibacter curvatus* | *Reticulitermes flavipes* (R) | Methanogenic | Leadbetter and Breznak (1996) |
| *Methanobrevibacter cuticularis* | *Reticulitermes flavipes* (R) | Methanogenic | Leadbetter and Breznak (1996) |
| *Methanobrevibacter filiformis* | *Reticulitermes flavipes* (R) | Methanogenic | Leadbetter et al. (1998) |

[a]Termites belong to the families Kalotermitidae *K*, Mastotermitidae *M*, Rhinotermitidae *R*, Termitidae *T*, and Termopsidae *Z*
[b]*Candidatus* taxon: not cultivated but well characterized with respect to morphology, ultrastructure, phylogeny, and specific location

## Cellulolytic Bacteria

Cellulolytic prokaryotes have been isolated from the guts of lower and higher termites on numerous occasions (for references, see Breznak (1975), Breznak and Brune (1994), and Wenzel et al. (2002)). However, many of these efforts either were unsuccessful or have yielded positive results of uncertain significance, either because the methods were not fully described or because the population size had not been established (Schultz and Breznak 1978). Therefore, the contribution of bacteria to cellulose degradation in the termite gut has always been a matter of debate (see the section "Fiber Degradation" in this chapter).

Interestingly, successful attempts to isolate cellulolytic bacteria usually employed oxic cultivation conditions; only a few anaerobic strains have been reported. Hungate isolated an anaerobic actinomycete, "*Micromonospora propionici*," from *Amitermes minimus* (Hungate 1946). *Clostridium termitidis* was isolated from the gut of the wood-feeding termite *Nasutitermes lujae* (Hethener et al. 1992). All attempts to isolate anaerobic cellulolytic bacteria from the gut of *Reticulitermes flavipes* were negative (Schultz and Breznak 1978). No

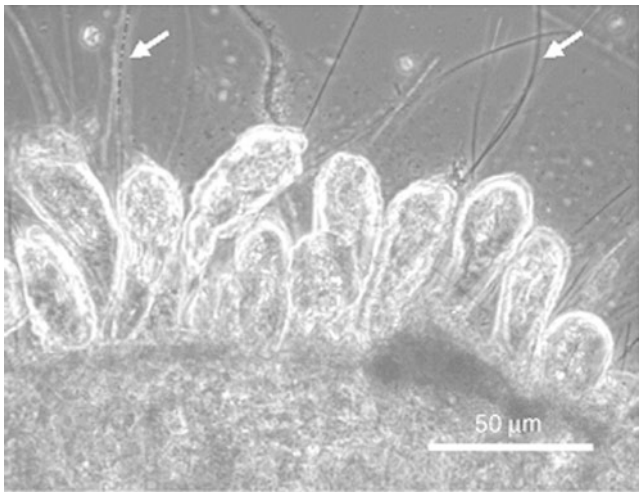


**Fig. 22.8**
**Phase-contrast photomicrograph of the hindgut wall of *Reticulitermes santonensis*, colonized by *Pyrsonympha flagellates* and "Arthromitus"-like filaments (*arrows*) (Reprinted from Yang et al. 2005)**

cellulose-degrading bacteria were present among the numerically predominant isolates recovered from nine species of termites representing all major families, using both aerobic and anaerobic techniques (Eutick et al. 1978a). No anaerobic cellulolytic bacteria were isolated from the soil-feeding termite *Cubitermes speciosus* using serial dilutions of gut homogenates and the Hungate technique (Brauman et al. 1990b).

By contrast, numerous studies have yielded aerobic bacteria, albeit of often dubious numerical significance. Frequent isolates, found in numerous studies, were assigned to *Serratia marcescens* and *Bacillus cereus* (e.g., Thayer 1976). Strains of both species, isolated from *Reticulitermes hesperus*, formed soluble, and in the case of *Bacillus cereus*, also cell-bound, carboxymethylcellulases (Thayer 1978). *Serratia marcescens* seems to be a common but minor inhabitant of the intestinal tract of insects that on occasion can become pathogenic (see the section "Pathogens" in this chapter).

On the basis of the few studies where the number or density of cellulolytic bacteria in the gut had been determined (e.g., Paul et al. 1993; Wenzel et al. 2002), it appears that bacteria are not very relevant for cellulose digestion. The most abundant among 23 groups of cellulolytic isolates from *Zootermopsis angusticollis* were closely affiliated with *Bacillus megaterium*, *Bacillus cereus*, or *Paenibacillus polymyxa* (Wenzel et al. 2002). Many of the cellulolytic isolates are species known to occur also in soil and other habitats and may represent transient permanent populations of microorganisms ingested with the food, while others are apparently specific for the intestinal tracts of insects (*Table 22.1*).

*Isoptericola variabilis* (formerly *Cellulosimicrobium variabile*; Stackebrandt et al. 2004) and other closely related bacteria were isolated from the hindgut of *Mastotermes darwiniensis* and various other termites (Bakalidou et al. 2002) and form significant populations also in the gut of other insects (Cazemier et al. 2003).

Cellulolytic activity has been detected also among numerous actinomycetes isolated from the gut of several soil-feeding Termitidae (Pasti and Belli 1985); some of the isolates were also lignolytic (Pasti et al. 1990). Although an unusual association of soil-feeding termites (Termitidae, Termitinae) with actinomycete-like bacteria has been documented (Bignell et al. 1979, 1980b) and facultatively aerobic actinomycetes have been isolated from the gut, parent soil, and mound materials of the termites *Procubitermes aburiensis* and *Cubitermes severus* (Bignell et al. 1991), their significance in the degradation of lignocellulose remains to be established.

## Hemicellulolytic Bacteria

Compared to the information on cellulose degradation, our understanding of the degradation of the hemicellulose component of lignocellulose in termite guts is rather meager. Xylanase activity has been observed in midgut and hindgut of several termites, including wood-feeding, soil-feeding, and fungus-cultivating species (Breznak and Brune 1994; Rouland 2000). In the fungus-cultivating *Macrotermes bellicosus*, there is evidence that the source of xylanase activity may be a symbiotic fungus (Matoub and Rouland 1995), but analogous to the host cellulases, a host origin of the midgut xylanases remains to be scrutinized.

In lower termites, the gut flagellates also seem to play a major role in the degradation of xylan (see the section "Role of Intestinal Protozoa" in this chapter). However, also bacteria and yeasts might be involved in the metabolism of hemicelluloses. Aerobic and facultatively anaerobic hemicellulose-degrading bacteria and yeasts were isolated from the guts of several wood-feeding termites, with xylan-degrading bacteria ($10^6$–$10^7$ per ml) dominating in *Mastotermes darwiniensis* and xylan-degrading yeasts ($10^7$–$5 \times 10^8$ cells per ml) in *Zootermopsis angusticollis* and *Neotermes castaneus* (Schäfer et al. 1996). Gram-positive isolates belonged to the genera *Bacillus* and *Paenibacillus* or to the Actinobacteria, while Gram-negative strains were affiliated with the genera *Pseudomonas*, *Acinetobacter*, and *Ochrobactrum* or with the enterobacteria.

Several strains of alkaliphilic bacteria, which were isolated from the extremely alkaline P1 compartment the soil-feeding *Sinocapritermes mushae* and *Amitermes longignathus* and represent a novel lineage of *Paenibacillus*, express alkalitolerant xylanase activity with a high pH optimum (Ohkuma et al. 2003; Thongaram et al. 2003). An unusual xylanase, distantly related to xylanases of bacteria and fungi colonizing the bovine rumen (GHF11), has been discovered in a genomic library to microbial DNA extracted from the intestinal tract of an unspecified higher termite belonging to the subfamily Nasutiermitinae (Brennan et al. 2004).

### Lignin-Degrading Bacteria

The degradation of lignin involves the "enzymatic combustion" of the highly recalcitrant polyphenolic resin by peroxidases. Despite a few claims to the contrary, there is currently no conclusive evidence that bacteria lacking extracellular peroxidative activity solubilize or degrade polymeric core lignin significantly, and lignin degradation by the gut microbiota has been questioned (Breznak and Brune 1994; Kuhnigk et al. 1994). A $^{13}$C-nuclear magnetic resonance (NMR) analysis of the feces of the wood-feeding termites *Microcerotermes parvus* and *Coptotermes formosanus* indicated a preferential loss of polysaccharide during gut passage, whereas lignin accumulated and was modified only when its O-aromatic-C and O-methyl-C components were modified (Hopkins et al. 1998; Hyodo et al. 1999).

However, extensive delignification occurs in the fungal gardens of fungus-cultivating termites (Hyodo et al. 2000) and has to be expected also in any wood colonized by lignolytic fungi. Many lignin-derived aromatic compounds (representing the major subunits and lignin–carbohydrate linkages found in lignins and their depolymerization products) can be degraded by aerobic bacteria, and numerous bacteria degrading lignin monomers or certain dimeric lignin model compounds have been isolated from various wood-feeding termites (Kuhnigk et al. 1994; Kuhnigk and König 1997; Harazono et al. 2003). They comprised a wide range of Gram-positive bacteria and Proteobacteria, with strict aerobes dominating in *Nasutitermes nigriceps* and enterobacteria in the lower termites. An aerobic isolate able to degrade hydroxybenzoic acids was described as a new species, *Bacillus oleronius* (Kuhnigk et al. 1995).

All attempts to enumerate bacteria degrading aromatic compounds indicated that termite guts contain significant numbers of aerobic bacteria capable of degrading aromatic rings, whereas anaerobic degradation of the aromatic nucleus appears to be of little significance (Brune et al. 1995b; Kuhnigk and König 1997). Under anoxic conditions, only ring and side-chain modification seem to be of importance (Kuhnigk et al. 1994; Brune et al. 1995b). Some isolates from termite guts involved in anaerobic modification or degradation of aromatic compounds were novel taxa (*Table 22.1*).

The digestibility of lignocellulose is improved if the phenylpropanoic acid residues esterified to the hemicellulose chains and the diferulic ester linkages between the hemicellulose chains in plant cell walls are hydrolyzed, since this increases the solubility of the macromolecules and reduces steric hindrance of hydrolytic enzymes (Jeffries 1994). A strain of *Clostridium xylanolyticum* (producing hydroxycinnamoyl esterases) that hydrolyzes and then hydrogenates ferulic and *p*-coumaric acid residues has been isolated from the gut of the grass-feeding termite *Tumulitermes pastinator* (McSweeney et al. 1999). Several termites have been shown to possess also activities cleaving non-phenolic β-O-4 type-lignin model compounds representing the major linkage within the lignin macromolecule (Hirai et al. 2000).

Lignin-solubilizing actinomycetes have been isolated from the gut of soil-feeding termites (Pasti et al. 1990), and all isolates were *Streptomyces* strains. Moreover, in lower termites, the only isolate decolorizing Remazol Brilliant Blue and Azure B was a *Streptomyces* strain (Kuhnigk and König 1997). Since isolation and phylogenetic characterization of actinomycetes from termite guts indicated that the actinomycete flora of termites depends largely on their geographical origin (Watanabe et al. 2003), it is not clear whether such isolates simply represent spores ingested with the food.

### Oxygen Reduction

The bacteria and protozoa colonizing the gut periphery, especially those directly associated with the gut epithelium (*Fig. 22.8*), are exposed to the continuous influx of oxygen (Brune et al. 2000). Oxygen microprofiles indicate that the anoxic status of the termite hindguts must be attributed to the oxygen consumption of the microbiota located in the gut periphery (see the section "Redox Conditions and Oxygen Status" in this chapter), which can represent a substantial fraction of the respiratory activity of the host (Brune et al. 1995a). This conclusion is supported by the predominance of facultatively or even obligately aerobic isolates in all cultivation-based studies.

However, there are several indications that not all oxygen-consuming activities in termite guts are of a respiratory nature. In the extremely alkaline gut regions, the high rates of oxygen consumption might be partly attributable also to chemical reactions, such as the autoxidation of phenolic residues in lignin or humic substances (see Kappler and Brune 1999). On the other hand, radiotracer analysis of the in situ metabolism in the hindgut of *Reticulitermes flavipes* demonstrated that the high oxygen fluxes also significantly influence fermentation processes in the hindgut (Tholen and Brune 2000). This is supported by the oxygen reduction potential found in obligate anaerobes such as lactic acid bacteria (see the section Lactic Acid Bacteria in this chapter), homoacetogenic bacteria (see the section "Homoacetogenic Bacteria" in this chapter), and sulfate-reducing bacteria (see the section "Sulfate-reducing Bacteria" in this chapter).

### Microbial Fermentations

In lower termites, the bulk of the fermentative activity should be caused by the intestinal protozoa, which immediately phagocytize the wood particles entering the hindgut. The identity of the substrates of the prokaryotic microbiota is far from clear. Since most of the prokaryotic symbionts probably do not participate in fiber degradation, presumably they ferment either soluble products entering the hindgut from the midgut or intermediates released by the protozoa (*Fig. 22.7*). The composition of short-chain fatty acids and other fermentation products in the

hindgut fluid of termites indicates that a variety of microbial fermentations occur simultaneously (Schultz and Breznak 1979; Odelson and Breznak 1983; Anklin-Mühlemann et al. 1995; Tholen and Brune 2000). However, the exact nature and amount of the monomeric substrates entering the hindgut are not known, and the spectrum of fermentation products formed by the protozoa might be much larger than initially assumed (Tholen and Brune 2000).

The apparent absence of pyruvate dehydrogenase activity in termite tissues (O'Brien and Breznak 1984) and several other observations prompted the suggestion that the termite releases pyruvate into the hindgut to be converted to acetate and reabsorbed by the host (Slaytor et al. 1997; Slaytor 2000). However, recent findings documented that termite mitochondria possess a pyruvate dehydrogenase complex, although the difference in activity suggests differences in pyruvate metabolism between lower and higher termites (Itakura et al. 1999, 2003).

### Lactobacillales

Coccoid lactic acid bacteria predominate among the carbohydrate-utilizing bacteria isolated from the hindguts of termites of the families Mastotermitidae, Kalotermitidae, and Rhinotermitidae (Eutick et al. 1978a; Schultz and Breznak 1978; Tholen et al. 1997; Bauer et al. 2000) and have been isolated also from several representatives of the Termitidae (Eutick et al. 1978a; Bauer et al. 2000). The most abundant isolates obtained from the hindguts of *Reticulitermes flavipes* and *Thoracotermes macrothorax* belong to the genera *Enterococcus* and *Lactococcus* (Bauer et al. 2000). However, the low frequency of such clones in cultivation-independent studies indicates that lactic acid bacteria might be present only in moderate numbers (Hongoh et al. 2003a; Schmitt-Wagner et al. 2003b; Yang et al. 2005).

Physiological characterization of the isolates has revealed high potential rates of $O_2$ reduction in the presence of fermentable substrates (Tholen et al. 1997; Bauer et al. 2000), which might represent an adaptation to variable oxygen tensions and could explain why lactococci and enterococci are regularly encountered in the intestinal tracts of termites and other insects and possibly restricted to specific compartments of the intestinal tract (Yang et al. 2005).

### Clostridiales

Compared to their abundance in bacterial clone libraries derived from termite guts, clostridial isolates are underrepresented in cultivation-based studies. Several isolates have been described as new species and seem to be unique to termite guts (*Table 22.1*), but their numerical significance is either low or has not been established. Bacteria distantly related to *Clostridium oroticum*, detected in an alkaline enrichment culture derived from gut homogenates of *Pericapritermes latignathus*, may prove to be first exception, since they were represented also in a clone library obtained from the P1 compartment (Thongaram et al. 2005).

### Bacteroidetes

Besides the coccoid lactic acid bacteria, a relatively large proportion of the isolates from the hindgut of *Reticulitermes flavipes* have been identified as *Bacteroides* species (Schultz and Breznak 1978). The strains fall into two groups: aeroduric anaerobes fermenting lactate to propionate and acetate (Schultz and Breznak 1979) and anaerobic strains forming butyrate and isobutyrate as characteristic products from complex media (Schultz and Breznak 1978). The only described *Bacteroides* species isolated from termite guts, *Bacteroides termitidis* (Sebald) from *Reticulitermes lucifugus*, was phylogenetically misplaced (Paster et al. 1985) and recently reclassified among the Fusobacteria in the genus *Sebaldella* (Collins and Shah 1986). A recent isolate, obtained from the hindgut of *Reticulitermes speratus* by dilution plating of gut suspensions, has a 16S rRNA gene sequence identical to that of a group of clones recovered exclusively from termite guts, representing a lineage of bacteria within the radiation of the Bacteroides subgroup but only distantly related to the genus *Bacteroides* (Ohkuma et al. 2002).

The uricolytic activity of *Bacteroides* isolates from termite guts (Potrikus and Breznak 1980) suggests a potential role in nitrogen cycling (see the section "Nitrogen Recycling" in this chapter). Many of the clones from termite guts clustering among the Bacteroidetes fall into a lineage comprising epibionts of protozoa (see the section "Interactions Between Prokaryotes and Protozoa" in this chapter).

## Hydrogen Metabolism

The enormous accumulation of hydrogen at the gut center and the steep radial hydrogen gradients in the gut periphery of several termites (Ebert and Brune 1997; Schmitt-Wagner and Brune 1999) indicate that molecular hydrogen is a key intermediate in the microbial food chain. Hydrogen-dependent $CO_2$ reduction by methanogens and homoacetogens is probably the most important hydrogen sink in termite hindguts. To understand the metabolism in termite guts, it is important to identify the hydrogen-producing and hydrogen-consuming populations and their functional interactions (see the section "Microbe–Microbe Interactions" in this chapter).

### Hydrogen-Producing Microorganisms

On the basis of the few pure culture studies available, it is generally assumed that the polysaccharides of the wood particles taken up by the gut flagellates are oxidized to acetate and $CO_2$, and the reducing equivalents are released as $H_2$ (see Breznak and Brune (1994) and Brune and Stingl (2005); *Fig. 22.7*). This is

in agreement with the enormous accumulation of hydrogen in the hindgut of *Reticulitermes flavipes* (Ebert and Brune 1997; *Fig. 22.4*). In view of the wide variety of clostridial clones retrieved from *Reticulitermes* species (Hongoh et al. 2003a; Yang et al. 2005), bacterial fermentations may also contribute to the intestinal $H_2$ production. Such assumptions are substantiated by the considerable amounts of $H_2$ produced in certain gut regions of soil-feeding *Cubitermes* species (Schmitt-Wagner and Brune 1999), a group of termites that lack gut flagellates but contain a similar assemblage of clostridial clones (Schmitt-Wagner et al. 2003b). The cellulolytic bacterium *Clostridium termitidis* isolated from *Nasutitermes lujae* (Hethener et al. 1992) is affiliated with a number of clones from *Cubitermes orthognathus* guts (Schmitt-Wagner et al. 2003b), but nothing is known about the physiology of the bacteria represented by these 16S rRNA genes.

## Homoacetogenic Bacteria

Reductive acetogenesis from $H_2$ and $CO_2$ in termite gut homogenates was first demonstrated by Breznak and Switzer (1986). In the following years, the presence of homoacetogenic activities was established for a large number of termite species from all major feeding guilds, including representatives of wood-feeding, fungus-cultivating, and soil-feeding termites (Breznak and Kane 1990; Brauman et al. 1992; Williams et al. 1994). Although reductive acetogenesis in gut homogenates of soil-feeding termites was always outcompeted as a hydrogen sink by methanogenesis (Brauman et al. 1992; Breznak 1994), microinjection of $H^{14}CO_3^-$ into intact hindguts of soil-feeding *Cubitermes* spp. has identified a high potential for reductive acetogenesis (Tholen and Brune 1999), which indicates that the contribution of reductive acetogenesis to the overall electron flow in the guts of soil-feeding termites might be larger than expected. Possibly, part of the explanation lies in the ability of homoacetogens to grow mixotrophically on $H_2$ and other substrates (Breznak and Switzer Blum 1991; Breznak 1994).

Although reductive acetogenesis has been demonstrated to occur in a large number of termite species from all major feeding guilds (Brauman et al. 1992), only seven species of homoacetogenic bacteria from termites have been described to date (*Table 22.1*). *Sporomusa termitida* (isolated from *Nasutitermes nigriceps*; Breznak et al. 1988), *Sporomusa aerivorans* (isolated from *Thoracotermes macrothorax*; Boga et al. 2003), and *Acetonema longum* (isolated from *Pterotermes occidentis*; Kane and Breznak 1991) are three spore-forming representatives in the *Sporomusa* group of Gram-positive bacteria that are characterized by a Gram-negative cell wall. All isolates are capable of $H_2$-dependent reduction of $CO_2$ to acetate and possess a large potential for hydrogen-dependent oxygen reduction (Boga and Brune 2003).

*Clostridium mayombei* has been isolated from *Cubitermes speciosus* (Kane et al. 1991) and belongs to the *Clostridium lituseburense* group. A very unusual homoacetogen (*Sporobacter termitidis*, isolated from *Nasutitermes lujae*) clusters in the *Clostridium leptum* group together with numerous clones of 16S rRNA genes retrieved from the anterior hindgut of *Cubitermes orthognathus* (Schmitt-Wagner et al. 2003b). It grows exclusively by the disproportionation of methyl groups derived from methoxylated aromatic compounds but not on $H_2 + CO_2$ or other typical substrates of homoacetogens, and it methylates sulfide and cysteine if these compounds are present in the medium (Grech-Mora et al. 1996).

Recently, numerous spirochetal strains have been isolated from the gut of *Zootermopsis angusticollis* (Leadbetter et al. 1999). They belong to the *Treponema* branch of Spirochaetes that contains mainly sequences obtained from termite guts (Lilburn et al. 1999), and many are capable of $H_2$-dependent acetogenesis from $CO_2$ (Leadbetter et al. 1999). One of the homoacetogenic isolates, which has been described as a new species, *Treponema primitia* (Graber et al. 2004), utilizes a wide range of substrates and is capable of mixotrophic growth, i.e., the simultaneous utilization of $H_2$ and organic substrates (Graber and Breznak 2004). These represent important clues for the unknown metabolic function of the enormous populations of spirochetes colonizing the hindgut lumen of termites and the surfaces of many intestinal protozoa (Breznak 2002).

Generally, there seems to be a strong cultivation bias against homoacetogens from termite guts, and their numerical abundance and contribution to reductive acetogenesis in situ are not clear. Recently, sequences clustering with the formyltetrahydrofolate synthase (FTHFS) homologues of termite gut spirochetes were found to dominate the diversity of genes in the hindgut of *Zootermopsis angusticollis* coding for FTHFS (Salmassi and Leadbetter 2003). FTHFS is a key enzyme in reductive acetogenesis, which makes the FTHFS gene a good functional marker for homoacetogenic bacteria (Leaphart and Lovell 2001; Leaphart et al. 2003). Quite likely therefore homoacetogenic spirochetes are responsible for the large potential activities of $H_2$-dependent acetogenesis encountered in most wood-feeding termites (Breznak 1994, 2002).

All investigated homoacetogens isolated from termite guts catalyzed hydrogen-dependent oxygen reduction (Boga and Brune 2003); the isolate *Sporomusa aerivorans* has the highest known oxygen-reducing capacity among obligately anaerobic bacteria other than sulfate-reducing bacteria of the genus *Desulfovibrio* (see the section "Sulfate-Reducing Bacteria" in this chapter), which possibly indicates an adaptation to the oxygen status of the gut environment. Although the location of homoacetogens relative to the oxygen gradient remains to be established, it is rather unlikely that the homoacetogens in *Reticulitermes flavipes* contribute substantially to the removal of oxygen diffusing into the gut: in contrast to methanogenesis, the in situ rates of reductive acetogenesis in this termite species are not affected by oxygen (Tholen and Brune 2000).

**Fig. 22.9**
**Termites entrapped in a block of copal (young amber) from the Andean uplift region of Boyaca Province, Colombia (Pleistocene). The photograph shows gas bubbles around the termites, presumably methane that escaped from the body after the termites had been engulfed by the resin**

## Methanogenic Archaea

Methane formation by termites, first documented by Breznak (Breznak 1975; Odelson and Breznak 1983), has received considerable attention owing to its implication for the global methane budget (Sanderson 1996). Methane formation is restricted to the archaea; therefore, the methane production by all termites investigated indicates that methanogenic archaea are present in virtually all termites (Brauman et al. 1992). Furthermore, the gas bubbles around termites entrapped in amber (*Fig. 22.9*), which have been attributed to continuing methanogenesis in the fresh resin, are paleontological evidence for the presence of methanogenic archaea in termite guts.

The autofluorescence of coenzyme $F_{420}$ allows easy visualization of methanogens by epifluorescence microscopy. They are either located free in the hindgut lumen, attached to the hindgut cuticle, or associated with other microorganisms (Breznak and Brune 1994). In *Zootermopsis*, methanogens are almost exclusively associated with the gut flagellates (Lee et al. 1987). On the basis of their $F_{420}$-like autofluorescence, filamentous bacteria colonizing the cuticular spines in the fourth proctodeal segment of soil-feeding termites (Bignell et al. 1980a) also appear to be methanogens (Schmitt-Wagner et al. 2003b).

Despite their relative abundance and phylogenetic diversity in termite guts (see the section "Archaeal Diversity" in this chapter), to date, only a few methanogens from *Reticulitermes flavipes* have been isolated in pure culture. The isolates represent new species in the genus *Methanobrevibacter* (*Table 22.1*), grow best with $H_2$ and $CO_2$, and form large populations that are attached to the luminal side of the gut epithelium or adhere to other prokaryotes colonizing the hindgut wall (Leadbetter and Breznak 1996; Leadbetter et al. 1998).

Already the location in the microoxic gut periphery and the presence of catalase activity in *Methanobrevibacter cuticularis* indicated a considerable oxygen tolerance (Leadbetter and Breznak 1996). This was substantiated by the finding that – despite a general inability among Methanobacteriales to synthesize heme – the wetwood isolate *Methanobrevibacter arboriphilus* expresses a catalase when the medium is supplemented with hemin (Shima et al. 2001). The documentation of a $F_{420}H_2$ oxidase in *M. arboriphilus* (Seedorf et al. 2004) finally provided also a biochemical basis for the high rates of $H_2$-dependent $O_2$ reduction exhibited by *Methanobrevibacter* species colonizing the periphery of the termite hindgut (A. Tholen and A. Brune, unpublished results) and their apparent ability to cope with the continuous influx of oxygen (Leadbetter and Breznak 1996; Ebert and Brune 1997).

## Sulfate-Reducing Bacteria

Sulfate-reducing bacteria have been isolated from the intestinal tracts of many different termite species (Brauman et al. 1990a, b; Trinkerl et al. 1990; Kuhnigk et al. 1996; Fröhlich et al. 1999). All isolates are members of the genus *Desulfovibrio* and seem to form substantial populations in the gut of certain termites. The relevance of sulfate reduction under in situ conditions is not clear, but the sulfate-reducing bacteria in termite guts might partake also in the removal of oxygen or play a role in syntrophic fermentations (Kuhnigk et al. 1996). Like other *Desulfovibrio* species isolated from sediments (for references, see Cypionka 2000), all strains isolated from termite guts show extremely high rates of $O_2$ reduction in the presence of $H_2$ (Kuhnigk et al. 1996; Fröhlich et al. 1999).

## Nitrogen Transformations

The diet of the termites ranges from sound wood to lignocellulosic plant materials in various stages of humification, including soil and animal dung. Owing to the high C-to-N ratio of sound wood, many xylophagous termites are strongly limited by nitrogen (Collins 1983). They conserve nitrogen by recycling – a strategy that has been termed "carbon elimination" (Higashi et al. 1992). Another way to improve the C/N balance of the colony is through nitrogen fixation.

This section will summarize the present knowledge on these processes and the microorganisms involved. For details, the reader is referred to the comprehensive review of Breznak (2000).

## Nitrogen Recycling

The best way to deal with a rare resource is conservation and recycling. Termites, like other insects, secrete uric acid and urea, the waste products of nucleic acid and protein metabolism, via

the Malpighian tubules into the intestinal tract (Terra 1990; ◉ *Fig. 22.3*). Potrikus and Breznak (1981) demonstrated that termites lack uricase and that uric acid is recycled by anaerobic bacteria in the hindgut of *Reticulitermes flavipes*, including *Streptococcus* and *Citrobacter* species and *Bacteroides* (now *Sebaldella*) *termitidis* (Potrikus and Breznak 1980).

Despite the low nitrogen content of the diet, nitrogen recycling creates high ammonia concentrations in the hindgut of wood-feeding termites, which allows the maintenance of an active gut microbiota and thus ensures high rates of carbon mineralization. The concentration of ammonia in the paunch of *Nasutitermes walkeri* is in the range of 3 mM (Slaytor and Chappell 1994). The efficient assimilation of ammonium into microbial biomass (and possibly also resorption of ammonium in the posterior hindgut) avoids the loss of nitrogen via the feces (Breznak 2000). The transformation to high-quality microbial protein also leads to an upgrading of any low-quality nitrogen in the diet.

The nitrogen cycle is closed by the digestion of microbial cells. Since termites cannot access the microbes in the hindgut directly, worker larvae solicit hindgut contents from their nestmates. This behavior, termed "proctodeal trophallaxis," is unique to this group of social insects and increases in frequency with the level of nitrogen limitation (Machida et al. 2001). Digestion of the hindgut contents and resorption of the nitrogenous products probably take place in the foregut and midgut (◉ *Fig. 22.3*), as indicated by lysozyme and protease activities in these gut regions (Fujita et al. 2001; Fujita and Abe 2002; Fujita 2004). The efficiency of nitrogen conservation within the colony is increased further by the consumption of exuviae and dead nestmates.

### Nitrogen Fixation

Though nitrogen cycling is efficient in termites, termite colony growth is limited by the net nitrogen taken in with food (Breznak 2000). Therefore, many termites show a preference for lignocellulosic substrates that are colonized by fungi and therefore have a decreased C-to-N ratio (Amburgey et al. 1980; Cornelius et al. 2002). Termites living on sound wood, however, rely on the exclusive capacity of their prokaryotic gut microbiota to fix atmospheric nitrogen.

After the first convincing demonstrations of $N_2$ fixation in termites using the acetylene reduction assay (Benemann 1973; Breznak et al. 1973) and the incorporation of $^{15}N$ into biomass by termites incubated under an $^{15}N_2$-enriched atmosphere (Bentley 1984), a large body of data on nitrogen fixation in termite guts has accumulated. A few aspects will be mentioned; for more details, the reader is again referred to the excellent review of this subject by Breznak (2000).

Nitrogen fixation is widespread among termites, although the rates differ considerably. Nitrogenase activity in certain *Nasutitermes* spp. would be sufficient to double the nitrogen content of a colony within a few years (Breznak 2000), and stable isotope analysis has revealed that 30–60 % of the nitrogen in *Neotermes koshunensis* workers is derived via this pathway (Tayasu et al. 1994).

However, the nitrogen content of the diet increases with humification of the organic matter, and peptides, amino sugars, and microbial biomass are potential sources of nutrition for soil-feeding *Cubitermes* spp. (Ji et al. 2000; Ji and Brune 2001). The natural abundance of the $^{15}N$ isotope indicates that dietary nitrogen and not atmospheric nitrogen fixation is an important nitrogen source in humivorous termite species (Tayasu et al. 1997; Tayasu 1998). The enormous ammonium concentrations in feces and nests (constructed from feces) of soil-feeding termites (Ndiaye et al. 2004; R. Ji and A. Brune, unpublished results) suggest that termites in this feeding guild are not nitrogen limited.

There are also many indications that the nitrogen fixation rates cannot easily be extrapolated to the colony level or ecosystem level. Apart from methodological aspects, there are interspecific differences, intraspecific variations, seasonal patterns, and effects of laboratory maintenance (Curtis and Waller 1995, 1998) to consider. Additionally, the reduced oxygen partial pressure in subterraneous nests and galleries has to be taken into account (Curtis and Waller 1996). The problems are discussed in detail by Breznak (2000).

Dinitrogen-fixing bacteria isolated from termite guts include enterobacteria (French et al. 1976; Potrikus and Breznak 1977; Eutick et al. 1978a), sulfate-reducing bacteria (Kuhnigk et al. 1996), and spirochetes (Leadbetter et al. 1999; Graber et al. 2004). Molecular approaches, using the *nifH* gene as a functional marker, have revealed a much wider spectrum of potentially $N_2$-fixing microorganisms, including clostridia, Proteobacteria, and methanogenic archaea, comprising several sequence clusters unique to termites (Ohkuma et al. 1996, 1999b). However, a gene-expression study at the community level has revealed that only a few *nifH* homologs, probably representing alternative nitrogenases (*anf* genes), were actually transcribed under in situ conditions in the gut of *Neotermes koshunensis* (Noda et al. 1999). The preferential expression of *anf* genes might be related to an insufficient molybdenum (Mo) supply with the lignocellulosic diet (Ohkuma 2002). The number of unrelated *nifH* homologs in a single spirochete species and the variety of apparently spirochete-related *nifH* homologs in hindgut clone libraries indicate that spirochetes are potentially important nitrogen-fixing microorganisms in termite guts (Lilburn et al. 2001).

## Symbiotic Interactions

The enormous complexity of this subject emerges from the large number of species present within the gut, the different organizational levels of the potential partners, their metabolic capacities and topological orientation, and the numerous possibilities for metabolic interactions especially within anaerobic

communities. In addition, symbiotic interactions can be extremely specific with respect to the partners involved or may simply consist of a cross-feeding of metabolites or nutrients between populations that are not even in direct contact with each other.

## Microbe–Microbe Interactions

Trophic cascades and cross-feeding of metabolites can form a network of interactions between the individual populations of any microbial community. In termite guts, the resulting uneven distribution of sources and sinks of microbial metabolites within the system gives rise to metabolic gradients (see the section ❯ "Physicochemical Gradients" in this chapter), which are indicators of the location of microbial activities in situ. Additionally, the spatial organization of different populations, including intimate associations between prokaryotes and protozoa, underlines the importance of studying the nature of the respective interactions.

### Interactions Among Prokaryotes

At least three metabolically different groups of microorganisms are involved in the metabolism of hydrogen in the gut of lower termites: the protozoa, which produce $H_2$ as a product of their fermentative metabolism, and methanogens and homoacetogens, which reduce $CO_2$ to methane or acetate, respectively (see the section ❯ "Hydrogen Metabolism" in this chapter). It is generally assumed that hydrogenotrophic methanogens outcompete homoacetogens owing to their higher affinity for the common substrate (Cord-Ruwisch et al. 1988). Nevertheless, both metabolic groups occur simultaneously in the hindgut of termites. In *Reticulitermes flavipes*, where methanogens are mostly restricted to the gut periphery (Leadbetter and Breznak 1996), in situ rates of reductive acetogenesis surpass those of methanogenesis considerably (Tholen and Brune 2000), whereas in *Zootermopsis angusticollis*, where methanogens are located mostly inside the gut protozoa (Lee et al. 1987), methanogenesis appears to be the major hydrogenotrophic process (Brauman et al. 1992).

The methanogens in termites seem to be hydrogen limited in situ, as indicated by the stimulation of methane emission by externally supplied $H_2$ (Odelson and Breznak 1983; Messer and Lee 1989; Ebert and Brune 1997; Schmitt-Wagner and Brune 1999). Also, the per cell rates of methanogenesis determined in vitro with *Methanobrevibacter cuticularis*, multiplied by the viable counts of methanogens in *Reticulitermes flavipes*, are much higher than the methane emission rate of living termites (Leadbetter and Breznak 1996). The relative rates of methane and hydrogen emission by different termites vary considerably (Sugimoto et al. 1998), and evidence is accumulating that the spatial organization of the hydrogen-producing and hydrogen-consuming microorganisms determines the competition and coexistence of different populations (Ebert and Brune 1997; Tholen and Brune 2000).

Although reductive acetogenesis is outcompeted as a hydrogen sink by methanogenesis in gut homogenates of soil-feeding termites (Brauman et al. 1992), intact guts of soil-feeding *Cubitermes* spp. show a large potential for reductive acetogenesis (Tholen and Brune 1999). Taking into account the possibility of an intercompartmental transfer of $H_2$ (Schmitt-Wagner and Brune 1999), as has been demonstrated between the midgut and hindgut compartments of cockroaches (Lemke et al. 2001), the contribution of reductive acetogenesis to the overall electron flow in the guts of soil-feeding termites might be larger than expected.

A cross-feeding of hydrogen could also be the basis for the interaction between the prokaryotic epibionts on filamentous bacteria in the gut of *Reticulitermes flavipes* (Breznak and Pankratz 1977), some of which seem to be methanogenic archaea (Leadbetter and Breznak 1996). Schultz and Breznak (1979) had demonstrated a cross-feeding of lactate between *Lactococcus lactis* and a propionigenic *Bacteroides* species, to consolidate the predominance of these species among the cultivable bacteria in the hindgut fluid of *Reticulitermes flavipes* with the absence of lactate accumulation. Microinjection of $^{14}C$-labeled lactate confirmed that lactate is an important intermediate under in vivo conditions but raised several new questions regarding the source of lactate and the role of oxygen in shifting the product spectrum from propionate to acetate (Tholen and Brune 2000).

An example for cross-feeding of nutrients has been provided by Graber and Breznak (2005), who made a convincing case that *Treponema primitia*, a homoacetogenic spirochete in the gut of *Zootermopsis angusticollis* that requires folate for growth, benefits from the excretion of 5-formyltetrahydrofolate by other community members (*Lactococcus lactis* and *Serratia grimesii*).

### Interactions Between Prokaryotes and Protozoa

Most of the flagellate protozoa in the guts of lower termites are associated with prokaryotic microorganisms, either as epibionts on their cell surface or as endobionts in the cytoplasm and in the nucleus. The abundance and variety of such associations are documented by numerous electron-microscopy studies (for references, see Honigberg (1970), Radek (1999), Inoue et al. (2000), and Dolan (2001)), but virtually, nothing is known about the physiological basis of these associations, especially the metabolic interactions (Brune and Stingl 2005).

Cook (1932) was the first to observe the emission of a gas other than $CO_2$ by *Termopsis nevadensis* (syn. *Zootermopsis nevadensis*) and speculated that intestinal protozoa could produce hydrogen, methane, or possibly a mixture of both. Many decades later, termites were shown to emit both hydrogen and methane (Breznak 1975; Odelson and Breznak 1983), and axenic cultures of *Trichomitopsis termopsidis* (a gut flagellate from *Zootermopsis*) were shown to produce only hydrogen after

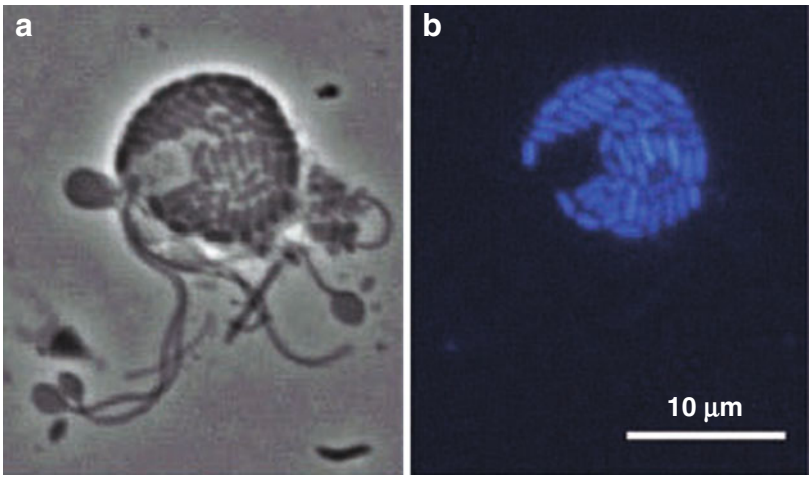


**Fig. 22.10**
**Phase-contrast (a) and epifluorescence (b) photomicrographs of a small trichomonad in *Schedorhinotermes lamanianus* and its attached epibionts, showing the typical $F_{420}$-autofluorescence of methanogenic archaea (Photomicrographs taken by M. Pester)**

methanogenic symbionts were eliminated by treating the cultures with bromoethanesulfonate, an inhibitor of methanogenesis (Odelson and Breznak 1985a, b).

Methanogenic symbionts of protozoa can be easily visualized by epifluorescence microscopy (*Fig. 22.10*). Lee et al. (1987) reported that methanogenic bacteria in the hindgut of *Zootermopsis angusticollis* are associated only with the small flagellated protozoa *Trichomitopsis termopsidis*, *Tricercomitus termopsidis*, and *Hexamastix termopsidis*, whereas they were not observed in the large, hypermastigid protozoa. On the basis of the results of various treatments that selectively eliminated or affected certain protozoa from the gut of this termite, Messer and Lee (1989) concluded that the large protozoa of the genus *Trichonympha* were the most important hydrogen source in the hindgut, whereas the methanogenic symbionts of *Trichomitopsis termopsidis* produced most of the methane.

The large gut flagellates are often colonized by epibiotic bacteria. The presence of special attachment sites on the cell envelope of the flagellates (e.g., Tamm 1980; Radek et al. 1992, 1996; Dolan and Margulis 1997; Stingl et al. 2004) indicates a tight association. In some cases, the epibionts seem to be responsible for the locomotion of the host.

The polymastigote flagellate *Mixotricha paradoxa*, which occurs exclusively in the gut of *Mastotermes darwiniensis*, uses its four flagella only for steering. The cell is propelled by the many thousands of spirochetes that cover most of the body surface. The spirochetes are attached to projecting brackets of the cell surface in a manner that allows the helical movement of the individual cells to travel in metachronal waves along the cell surface of the host, resulting in locomotion (Cleveland and Grimstone 1964). The epibiotic spirochetes were recently identified as members of the *Treponema* cluster by 16S rRNA gene sequence analysis (Wenzel et al. 2003). In addition, a second, rod-shaped epibiont is intimately associated with the cell surface by specific attachment sites (*Fig. 22.11*); it is affiliated with other uncultivated Bacteroidales.

The devescovinid flagellate *Caduceia versatilis* (d'Ambrosio et al. 1999) carries two different, rod-shaped and filamentous, epibionts. In this case, the host is propelled by the self-synchronizing movement of the flagella of several thousand rod-shaped bacteria, which are deeply embedded into its cell surface (Tamm 1982). The epibionts of *Staurojoenina* flagellates (*Fig. 22.12*), recently assigned to the candidate taxon "*Vestibaculum illigatum*" (Stingl et al. 2004), have a similar morphology, although in this flagellate, motility is not due to the bacteria but to their own flagella. Nevertheless, "*Vestibaculum illigatum*" falls into the same cluster of Bacteroidales as the rod-shaped epibiont of *Mixotricha paradoxa* (Stingl et al. 2004), a lineage that also contains numerous clones obtained from other termites (Ohkuma et al. 2002; Yang et al. 2005).

The larger flagellate species in the gut of *Reticulitermes* species harbor numerous prokaryotic endobionts within their cytoplasm (Bloodgood et al. 1974; Bloodgood and Fitzharris 1976). The endobionts of *Trichonympha* and *Pyrsonympha* species in *Reticulitermes santonensis* (*Fig. 22.13*) belong to the candidate phylum "Endomicrobia," which seem to occur exclusively in termite gut flagellates (Stingl et al. 2005). To date, there is no indication of their possible function. An involvement in hydrogen metabolism is unlikely since the oxymonadid *Pyrsonympha* species are phylogenetically distant from the hypermastigid flagellates (Moriya et al. 2003; Stingl and Brune 2003) and do not seem to possess hydrogenosomes (see Brune and Stingl 2005).

## Microbe–Host Interactions

Although the host provides a favorable habitat for the intestinal microbiota and thrives on their metabolic products, there is not

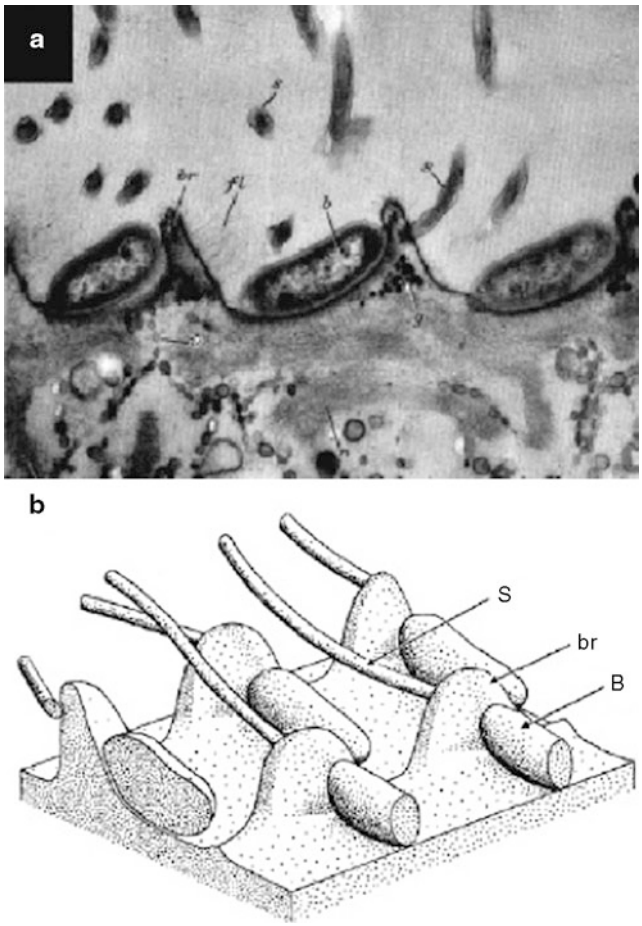


**Fig. 22.11**
**Transmission electron micrograph (a) of the epibiotic bacteria on the cell surface of *Mixotricha paradoxa*, a large flagellate occurring exclusively in the gut of *Mastotermes darwiniensis* that lives in a motility symbiosis with prokaryotes. The schematic presentation (b) illustrates the regular arrangement of the spirochetal (*S*) and rod-shaped (*B*) epibionts and the special attachment brackets (*br*) at the cell surface (Reproduced from Cleveland and Grimstone 1964)**

always a necessity or clear indication for any specific interactions. However, the intimate associations of bacteria with microvilli of the midgut epithelium or with the epithelial cups in the hindgut cuticle (Breznak and Pankratz 1977) indicate a closer integration of certain bacteria with the host tissues. Another example suggesting interactions with the gut tissue are the clostridia-related bacteria located in the ectoperitrophic space between the midgut wall and the peritrophic membrane in the mixed segment, closely associated with the mesenteric epithelium (Tokuda et al. 2000, 2001).

### Pathogens

A few reports indicate that the gut microbiota of termites is not always beneficial. *Serratia marcescens*, which has been isolated from the guts of *Reticulitermes hesperus* (Thayer 1976) and *Mastotermes darwiniensis* (Kuhnigk et al. 1994), causes septicemia in *Coptotermes formosanus* (Osbrink et al. 2001). *Serratia marcescens* seems to form part of the normal microflora of insects since it can be isolated from both healthy and diseased specimens. Usually nonpathogenic when present in the digestive tract in small numbers, it multiplies rapidly once it enters the hemocoel and causes death in 1–3 days [for references, see Lysenko (1985) and Sikorowski and Lawrence (1998)]. Also, the fungi associated with *Reticulitermes flavipes* include both cellulolytic species and potential pathogens (Zoberi and Grace 1990). It is not clear whether entomopathogenic fungi form a part of the natural gut community or are only found among diseased insects (Rath 2000).

### Intracellular Symbionts

Termites, like many other insects, are also associated with intracellular prokaryotes that are vertically transmitted via the ovaries (Breznak 2004). Flavobacteria of the genus *Blattabacterium* reside in specialized cells (bacteriocytes) of cockroaches and the most primitive termite, *Mastotermes darwiniensis* (Bandi et al. 1995). The close phylogenetic relationship between endosymbionts from *Mastotermes darwiniensis* and members of the wood-feeding cockroach genus *Cryptocercus* supports the hypothesis that termites evolved from subsocial, wood-dwelling cockroaches (Lo et al. 2003). All other termites examined carried endocytoplasmic bacteria that are affiliated with the *Wolbachia* group and are located in nonspecialized fat body cells (Bandi et al. 1997). Endonuclear bacteria have been observed in the trophocytes of *Reticulitermes lucifugus* and *Kalotermes flavicollis* (Grandi et al. 1997).

## Mutualists or Commensals?

In the hindgut symbiosis, the host creates a rather constant environment for its symbionts; provides a continuous supply of substrates; and, by the transfer of the microorganisms to other nestmates, ensures their propagation within the ecosystem. In the symbiosis between lower termites and fiber-digesting flagellates, both partners are indispensable and the mutual advantage is obvious. However, in the case of most prokaryotic gut symbionts, the symbionts appear to be of little advantage to the host.

If the benefit of the association is unidirectional, a symbiont would be classified as a commensal, and the host might even benefit from its elimination (Brune 2003). Unfortunately, it is not easy to eliminate specific prokaryotes from the intestinal microbial community of termites selectively, and it is difficult to distinguish between the direct and indirect consequences of their elimination. Removal of the spirochetes from the gut of *Nasutitermes exitiosus* by feeding metronidazole or exposing the termites to pure oxygen kills the termites almost as rapidly as the complete removal of all bacteria by antibiotics (Eutick et al. 1978b).

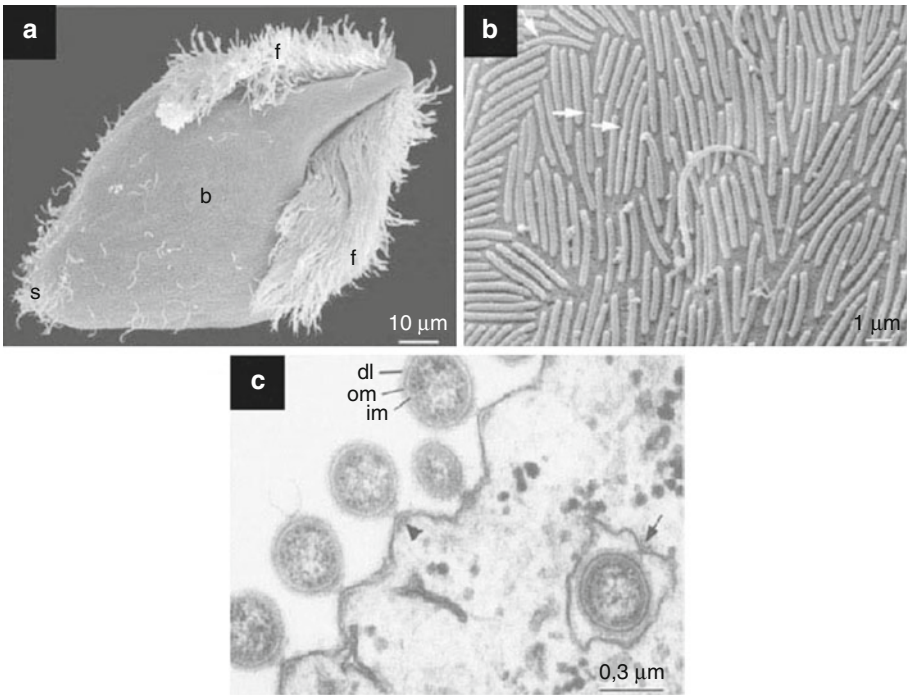


**Fig. 22.12**
**Scanning (a, b) and transmission (c) electron micrographs of *Staurojoenina* sp. from *Neotermes cubanus*. (a) Overview of a cell, showing two of the four flagellar tufts (*f*), numerous bacterial rods (b), and occasional spirochetes (*s*) attached to the surface. (b) Close-up of the cell surface, showing single spirochetes between the ectobiotic rods. *Arrows point* to early stages of cell division. (c) Cross-section of the epibiotic rods. In addition to an inner membrane (*im*) and outer membrane (*om*), the cell is surrounded by a diffuse layer (*dl*). Electron-dense material supports the plasma membrane of the flagellate below the attachment sites (*arrowhead*). *The arrow points* to a phagocytized rod-shaped bacterium with remnants of attachment complex (Reprinted from Stingl et al. 2004)**

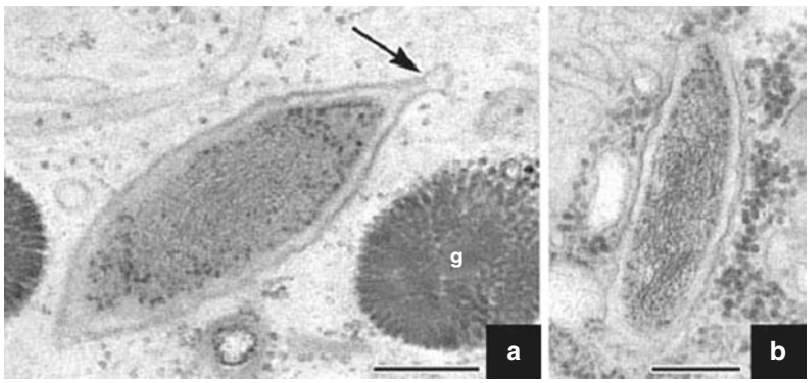


**Fig. 22.13**
**Transmission electron micrographs of ultrathin sections of *Trichonympha agilis* (a) and *Pyrsonympha vertens* (b) showing the ultrastructure of the endosymbiotic bacteria in the candidate genus "*Endomicrobium*," which are very abundant in the cytoplasm of these flagellate species. Cells are surrounded by two membranes; the outermost membrane forms tube-like elongations at the tapered cell poles (*arrow* in a). *g* glycogen. Bars = 0.2 µm (Reprinted from Stingl et al. 2005)**

In view of the constant and massive inoculation with microorganisms in their diet, it is clear that termites cannot keep their gut sterile. The molecular diversity studies have provided ample evidence for a specific microbiota, composed of lineages that occur exclusively in termites (Yang et al. 2005). The absence of a "normal gut microflora" would allow the uncontrolled proliferation of ingested foreign microorganisms and increase the danger of colonization by potential pathogens. *Serratia*

**Table 22.2**
**Free energy of important reactions involved in symbiotic digestion**[a]

| Reaction | $\Delta G^{\circ\prime}$ (kJ/mol)[b] | Relative change[c] (%) |
|---|---|---|
| (1) Glucose + 6 $O_2$ → 6 $CO_2$ + 6 $H_2O$ | −2,872 | 100 |
| (2) Glucose + 2 $H_2O$ → 2 Acetate$^-$ + 2 $H^+$ + 2 $CO_2$ + 4 $H_2$ | −216 | 7.5 |
| (3) 4 $H_2$ + 2 $CO_2$ → Acetate$^-$ + $H^+$ + 2 $H_2O$ | −95 | 3.3 |
| (4) 3 Acetate$^-$ + 3 $H^+$ + 6 $O_2$ → 6 $CO_2$ + 6 $H_2O$ | −2,561 | 89.2 |
| (5) 4 $H_2$ + $CO_2$ → $CH_4$ + 2 $H_2O$ | −131 | 4.6 |
| (6) 2 Acetate$^-$ + 2 $H^+$ + 4 $O_2$ → 4 $CO_2$ + 4 $H_2O$ | −1,707 | 59.5 |
| (7) $CH_4$ + 2 $O_2$ → $CO_2$ + 2 $H_2O$ | −818 | 28.4 |

[a]For explanations, see text
[b]Gibbs free energy under standard conditions at pH 7 is calculated after Thauer et al. (1977)
[c]Free energy change relative to the aerobic oxidation of glucose (Reaction 1)

*marcescens* failed to colonize the gut of normal *Coptotermes lacteus*, but transient colonization occurred when the protozoa and spirochetes were killed by exposure to pure oxygen (Veivers et al. 1982).

Under oxygen-limited conditions, the energy loss caused by the activity of the gut microbiota is relatively small (*Table 22.2*). The hypothetical, nonsymbiotic oxidation of cellulose to $CO_2$ would allow the termite to exploit 100 % of the free energy of the reaction (1). A homoacetogenic degradation of one glucose equivalent to three acetate molecules by the gut microbiota (Reactions 2, 3) releases only 10.8 % of the free energy contained in the substrate; the rest is still available for the host (Reaction 4), which also benefits from the nutritionally valuable microbial biomass. However, the situation is quite different if the $H_2$ formed in the microbial fermentations is converted to methane (Reactions 2, 5). Although the amount of free energy released in methanogenesis is not much larger than that released in reductive acetogenesis, the host receives only two-thirds of the acetate available under homoacetogenic conditions (Reaction 6); 28.4 % of the free energy in the substrate remains in methane, which cannot be exploited by the host (Reaction 7) and is lost to the environment.

Such energetic considerations suggest an obvious advantage if a termite uses reductive acetogenesis as the hydrogen-consuming process, and why especially the more highly evolved termites (Termitidae) show a tendency toward increasing methane emission rates is still a mystery (Brauman et al. 1992; Bignell et al. 1997). Nevertheless, considering the added value of metabolic properties such as nitrogen fixation, ammonia assimilation, and provision with vitamins, the advantages for the host may be well worth the investment. The exploitation of the nutritive resources contained in the microbial biomass requires the digestion of the gut symbionts, which is realized by proctodeal trophallaxis (Machida et al. 2001), a behavioral trait that was probably fundamental to both the establishment of the gut microbial community and the evolution of sociality in termites (Nalepa et al. 2001).

## Conclusions

Termite guts are minute but efficient bioreactors for the conversion of lignocellulose to short-chain fatty acids and microbial biomass. However, termite guts are not simply anoxic fermentors but axially and radially structured environments with physicochemically distinct microhabitats, and we are just beginning to understand the complex interactions within the intestinal microbial communities. Microbial diversity in the termite gut is enormous, and the existing isolates represent only a negligible fraction of the untapped diversity of prokaryotes in the guts of the more than 2,600 described species of termites (Kambhampati and Eggleton 2000).

## References


Aanen DK, Eggleton P, Rouland-Lefevre C, Guldberg-Froslev T, Rosendahl S, Boomsma JJ (2002) The evolution of fungus-growing termites and their mutualistic fungal symbionts. Proc Natl Acad Sci USA 99:14887–14892

Abo-Khatwa N (1978) Cellulase of fungus-growing termites: a new hypothesis on its origin. Experientia 34:559–60

Amburgey TL, Johnson GN, Etheridge JL (1980) A method to mass-produce decayed-wood termite bait blocks. J Georgia Entomol Soc 16:112–115

Anklin-Mühlemann R, Bignell DE, Veivers PC, Leuthold RH, Slaytor M (1995) Morphological, microbiological and biochemical studies of the gut flora in the fungus-growing termite *Macrotermes subhyalinus*. J Insect Physiol 41:929–940

Bakalidou A, Kämpfer P, Berchtold M, Kuhnigk T, Wenzel M, König H (2002) *Cellulosimicrobium variabile* sp. nov., a cellulolytic bacterium from the hindgut of the termite *Mastotermes darwiniensis*. Int J Syst Evol Microbiol 52:1185–1192

Bandi C, Sironi M, Damiani G, Magrassi L, Nalepa CA, Laudani U, Sacchi L (1995) The establishment of intracellular symbiosis in an ancestor of cockroaches and termites. Proc R Soc Lond Ser B Biol Sci 259:293–299

Bandi C, Sironi M, Nalepa CA, Corona S, Sacchi L (1997) Phylogenetically distant intracellular symbionts in termites. Parassitologia 39:71–75

Bauer S, Tholen A, Overmann J, Brune A (2000) Characterization of abundance and diversity of lactic acid bacteria in the hindgut of wood-and soil-feeding termites by molecular and culture-dependent techniques. Arch Microbiol 173:126–173

Béguin P, Aubert J-P (1994) The biological degradation of cellulose. FEMS Microbiol Rev 13:25–58

Benemann JR (1973) Nitrogen fixation in termites. Science 181:164–165

Bentley BL (1984) Nitrogen fixation in termites: fate of newly fixed nitrogen. J Insect Physiol 30:653–655

Berchtold M, König H (1996) Phylogenetic analysis and in-situ identification of uncultivated spirochetes from the hindgut of the termite *Mastotermes darwiniensis*. Syst Appl Microbiol 19:66–73

Berchtold M, Ludwig W, Koenig H (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, Chatzinotas A, Schönhuber W, Brune A, Amann R, Hahn D, König H (1999) Differential enumeration and in situ localization of micro-organisms in the hindgut of the lower termite *Mastotermes*

*darwiniensis* by hybridization with rRNA-targeted probes. Arch Microbiol 172:407–416

Bermudes D, Chase D, Margulis L (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.; hollandina gen. nov., nom. rev.*; Hollandina pterotermitidis* sp. nov., nom. rev.; and *Clevelandina reticulitermitidis* gen. nov., sp. nov. Int J Syst Bacteriol 38:291–302

Bignell DE (1984) The arthropod gut as an environment for microorganisms. In: Anderson JM, Rayner ADM, Walton DWH (eds) Invertebrate-microbial interactions. Cambridge University Press, Cambridge, UK, pp 205–227

Bignell DE (1994) Soil-feeding and gut morphology in higher termites. In: Hunt JH, Nalepa CA (eds) Nourishment and evolution in insect societies. Westview Press, Boulder, pp 131–158

Bignell DE (2000) Introduction to symbiosis. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbiosis, ecology. Kluwer, Dordrecht, pp 189–208

Bignell DE, Anderson JM (1980) Determination of pH and oxygen status in the guts of lower and higher termites. J Insect Physiol 26:183–188

Bignell DE, Eggleton P (1995) On the elevated intestinal pH of higher termites (Isoptera: Termitidae). Insect Soc 42:57–69

Bignell DE, Oskarsson H, Anderson JM (1979) Association of actinomycete-like bacteria with soil-feeding termites (Termitidae, Termitinae). Appl Environ Microbiol 37:339–342

Bignell DE, Oskarsson H, Anderson JM (1980a) Distribution and abundance of bacteria in the gut of a soil-feeding termite *Procubitermes aburiensis* (Termitidae, Termitinae). J Gen Microbiol 117:393–403

Bignell DE, Oskarsson H, Anderson JM (1980b) Specialization of the hindgut wall for the attachment of symbiotic microorganisms in a termite *Procubitermes aburiensis*. Zoomorphology 96:103–112

Bignell DE, Oskarsson H, Anderson JM, Ineson P, Wood TG (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

Bignell DE, Anderson JM, Crosse R (1991) Isolation of facultatively aerobic actinomycetes from the gut, parent soil and mound materials of the termites *Procubitermes aburiensis* and *Cubitermes severus*. FEMS Microbiol Ecol 85:151–160

Bignell DE, Slaytor M, Veivers PC, Mühlemann R, Leuthold RH (1994) Functions of symbiotic fungus gardens in higher termites of the genus Macrotermes: evidence against the acquired enzyme hypothesis. Acta Microbiol Immunol Hung 41:391–401

Bignell DE, Eggleton P, Nunes L, Thomas KL (1997) Termites as mediators of carbon fluxes in tropical forests: budgets for carbon dioxide and methane emissions. In: Watt AB, Stork NE, Hunter MD (eds) Forests and insects. Chapman and Hall, London, pp 109–134

Bloodgood RA, Fitzharris TP (1976) Specific association of prokaryotes with symbiotic flagellate protozoa from the hindgut of the termite Reticulitermes and the wood-eating roach. Cryptocercus Cytobios 17:103–122

Bloodgood RA, Miller KR, Fitzharris TP, Mcintosh JR (1974) The ultrastructure of Pyrsonympha and its associated microorganisms. J Morphol 143:77–106

Boga HI, Brune A (2003) Hydrogen-dependent oxygen reduction by homoacetogenic bacteria isolated from termite guts. Appl Environ Microbiol 69:779–786

Boga HI, Ludwig W, Brune A (2003) *Sporomusa aerivorans* sp. nov., an oxygen-reducing homoacetogenic bacterium from the gut of a soil-feeding termite. Int J Syst Evol Microbiol 53:1397–1404

Brauman A, Koenig JF, Dutreix J, Garcia JK (1990a) Characterization of two sulfate-reducing bacteria from the gut of the soil-feeding termite, *Cubitermes speciosus*. Ant v Leeuwenhoek 58:271–275

Brauman A, Labat M, Garcia JL (1990b) Preliminary studies on the gut microbiota of the soil-feeding termite: *Cubitermes speciosus*. In: Lésel R (ed) Microbiology in poecilotherms. Elsevier, Amsterdam, pp 73–77

Brauman A, Kane MD, Labat M, Breznak JA (1992) Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. Science 257:1384–1387

Brauman A, Müller JA, Garcia JL, Brune A, Schink B (1998) Fermentative degradation of 3-hydroxybenzoate in pure culture by a novel strictly anaerobic bacterium, *Sporotomaculum hydroxybenzoicum* gen. nov., sp. nov. Int J Syst Bacteriol 48:215–221

Brauman A, Bignell DE, Tayasu I (2000) Soil-feeding termites: biology, microbial associations and digestive mechanisms. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbiosis, ecology. Kluwer, Dordrecht, pp 233–259

Brauman A, Dore J, Eggleton P, Bignell D, Breznak JA, Kane MD (2001) Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits. FEMS Microbiol Ecol 35:27–36

Brennan Y, Callen WN, Christoffersen L, Dupree P, Goubet F, Healey S, Hernandez M, Keller M, Li K, Palackal N, Sittenfeld A, Tamayo G, Wells S, Hazlewood GP, Mathur EJ, Short JM, Robertson DE, Steer BA (2004) Unusual microbial xylanases from insect guts. Appl Environ Microbiol 70:3609–3617

Breznak JA (1975) Symbiotic relationships between termites and their intestinal microbiota. Symp Soc Exp Biol 29:559–580

Breznak JA (1984) Hindgut spirochetes of termites and *Cryptocercus punctulatus*. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriolog, vol 1. Williams and Wilkins, Baltimore, pp 67–70

Breznak JA (1994) Acetogenesis from carbon dioxide in termite guts. In: Drake HL (ed) Acetogenesis. Chapman and Hall, New York, pp 303–330

Breznak JA (2000) Ecology of prokaryotic microbes in the guts of wood- and litter-feeding termites. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbiosis, ecology. Kluwer, Dordrecht, pp 209–231

Breznak JA (2002) Phylogenetic diversity and physiology of termite gut spirochetes. Integr Comp Biol 42:313–318

Breznak JA (2004) Invertebrates – insects. In: Bull AT (ed) Microbial biodiversity and bioprospecting. ASM Press, Washington, DC, pp 191–203

Breznak JA, Brune A (1994) Role of microorganisms in the digestion of lignocellulose by termites. Ann Rev Entomol 39:453–487

Breznak JA, Kane MD (1990) Microbial $H_2/CO_2$ acetogenesis in animal guts: nature and nutritional significance. FEMS Microbiol Rev 87:309–314

Breznak JA, Pankratz HS (1977) In situ morphology of the gut microbiota of wood-eating termites [Reticulitermes flavipes (Kollar) and Coptotermes formosanus Shiraki]. Appl Environ Microbiol 33:406–426

Breznak JA, Switzer JM (1986) Acetate synthesis from $H_2$ plus $CO_2$ by termite gut microbes. Appl Environ Microbiol 52:623–630

Breznak JA, Switzer Blum J (1991) Mixotrophy in the termite gut acetogen, *Sporomusa termitida*. Arch Microbiol 156:105–110

Breznak JA, Brill WJ, Mertins JW, Coppel HC (1973) Nitrogen fixation in termites. Nature 244:577–580

Breznak JA, Switzer JM, Seitz HJ (1988) Sporomusa termitida sp. nov., an $H_2/CO_2$-utilizing acetogen isolated from termites. Arch Microbiol 150:282–288

Brune A (1998) Termite guts: the world's smallest bioreactors. Trends Biotechnol 16:16–21

Brune A (2003) Symbionts aiding digestion. In: Resh VH, Cardé RT (eds) Encyclopedia of insects. Academic, New York, pp 1102–1107

Brune A, Friedrich M (2000) Microecology of the termite gut: structure and function on a microscale. Curr Opin Microbiol 3:263–269

Brune A, Kühl M (1996) pH profiles of the extremely alkaline hindguts of soil-feeding termites (Isoptera: Termitidae) determined with microelectrodes. J Insect Physiol 42:1121–1127

Brune A, Stingl U (2005) Prokaryotic symbionts of termite gut flagellates: phylogenetic and metabolic implications of a tripartite symbiosis. In: Overmann J (ed) Molecular basis of symbiosis. Springer, New York

Brune A, Emerson D, Breznak JA (1995a) The termite gut microflora as an oxygen sink: microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl Environ Microbiol 61:2681–2687

Brune A, Miambi E, Breznak JA (1995b) Roles of oxygen and the intestinal microflora in the metabolism of lignin-derived phenylpropanoids and other monoaromatic compounds by termites. Appl Environ Microbiol 61:2688–2695

Brune A, Frenzel P, Cypionka H (2000) Life at the oxic-anoxic interface: microbial activities and adaptations. FEMS Microbiol Rev 24:691–710

Brune A, Ludwig W, Schink B (2002) Propionivibrio limicola sp. nov., a fermentative bacterium specialized in the degradation of hydroaromatic compounds, reclassification of *Propionibacter pelophilus* as *Propionivibrio pelophilus* comb. nov. and amended description of the genus Propionivibrio. Int J Syst Evol Microbiol 52:441–444

Cazemier AE, Verdoes JC, Reubsaet FAG, Hackstein JHP, van der Drift C, Op den Camp HJM (2003) Promicromonospora pachnodae sp. nov., a member of the (hemi)cellulolytic hindgut flora of larvae of the scarab beetle *Pachnoda marginata*. Ant v Leeuwenhoek 83:135–148

Cleveland LR (1925a) The effects of oxygenation and starvation on the symbiosis between the termite, Termopsis, and its intestinal flagellates. Biol Bull 48:309–327

Cleveland LR (1925b) Toxicity of oxygen for protozoa in vivo and in vitro: animals defaunated without injury. Biol Bull 48:455–468

Cleveland LR (1926) Symbiosis among animals with special reference to termites and their intestinal flagellates. Quart Rev Biol 1:51–64

Cleveland LR, Grimstone AV (1964) The fine structure of the flagellate *Mixotricha paradoxa* and its associated micro-organisms. Proc R Soc Lond Ser B Biol Sci 159:668–686

Collins NM (1983) The utilization of nitrogen resources by termites (Isoptera). In: Lee JA, McNeill S, Rorison ICH (eds) Nitrogen as an ecological factor. Blackwell Scientific, Oxford, UK, pp 381–412

Collins MD, Shah HN (1986) Reclassification of *Bacteroides termitidis* Sebald (Holdeman and Moore) in a new genus Sebaldella, as *Sebaldella termitidis* comb. nov. Int J Syst Bacteriol 36:349–350

Cook SF (1932) The respiratory gas exchange in *Termopsis nevadensis*. Biol Bull 63:246–257

Cook SF (1943) Nonsymbiotic utilization of carbohydrates by the termite *Zootermopsis angusticollis*. Physiol Zool 16:123–128

Cook TJ, Gold RE (2000) Effects of different cellulose sources on the structure of the hindgut flagellate community in *Reticulitermes virginicus* (Isoptera: Rhinotermitidae). Sociobiology 35:119–130

Cord-Ruwisch R, Seitz HJ, Conrad R (1988) The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. Arch Microbiol 149:350–357

Cornelius ML, Daigle DJ, Connick WJ Jr, Parker A, Wunch K (2002) Responses of Coptotermes formosanus and *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) to three types of wood rot fungi cultured on different substrates. J Econ Entomol 95:121–128

Crosland MWJ, Chan LK, Buswell JA (1996) Symbiotic fungus and enzymatic digestion in the gut of the termite, *Macrotermes barneyi* (Light) (Isoptera: Termitidae). J Entomol Sci 31:132–137

Curtis AD, Waller DA (1995) Changes in nitrogen fixation rates in termites (Isoptera: Rhinotermitidae) maintained in the laboratory. Ann Entomol Soc Am USA 88:764–767

Curtis AD, Waller DA (1996) The effects of decreased $pO_2$ and increased $pCO_2$ on nitrogen fixation rates in termites (Isoptera: Rhinotermitidae). J Insect Physiol 42:867–872

Curtis AD, Waller DA (1998) Seasonal patterns of nitrogen fixation in termites. Funct Ecol 12:803–807

Cypionka H (2000) Oxygen respiration by Desulfovibrio species. Ann Rev Microbiol 54:827–848

Czolij R, Slaytor M, Veivers PC, O'Brien RW (1984) Gut morphology of Mastotermes darwiniensis Froggatt (Isoptera: Mastotermitidae). Int J Insect Morphol Embryol 13:337–355

Czolij R, Slaytor M, O'Brien RW (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

D'Ambrosio U, Dolan M, Wier AM, Margulis L (1999) Devescovinid trichomonad with axostyle-based rotary motor ("Rubberneckia"): taxonomic assignment as *Caduceia versatilis* sp. nov. Eur J Protistol 35:327–337

Darlington JPEC (1994) Nutrition and evolution in fungus-growing termites. In: Hunt JH, Nalepa CA (eds) Nourishment and evolution in insect societies. Westview Press, Boulder, pp 105–130

Davison A, Blaxter M (2005) Ancient origin of glycosyl hydrolase family 9 cellulase genes. Mol Biol Evol 22:1273–1284

de Bary A (1878) Ueber Symbiose Bericht der Versammlung. Deutscher Naturforscher und Aerzte, pp 121–126

Derakshani M, Lukow L, Liesack W (2001) Novel bacterial lineages at the (sub) division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. Appl Environ Microbiol 67:623–631

Dolan MF (2001) Speciation of termite gut protists: the role of bacterial symbionts. Int Microbiol 4:203–208

Dolan M, Margulis L (1997) Staurojoenia and other symbionts in Neotermes from San Salvador Island. Bahamas Symbiosis 22:229–239

Donovan SE, Eggleton P, Bignell DE (2001) Gut content analysis and a new feeding group classification of termites. Ecol Entomol 26:356–366

Donovan SE, Purdy KJ, Kane MD, Eggleton P (2004) Comparison of Euryarchaea strains in the guts and food-soil of the soil-feeding termite *Cubitermes fungifaber* across different soil types. Appl Environ Microbiol 70:3884–3892

Ebert A, Brune A (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 ML, O'Brien RW, Slaytor M (1976) Aerobic state of gut of *Nasutitermes exitiosus* and *Coptotermes lacteus*, high and low caste termites. J Insect Physiol 22:1377–1380

Eutick ML, O'Brien RW, Slaytor M (1978a) Bacteria from the gut of Australian termites. Appl Environ Microbiol 35:823–828

Eutick ML, Veivers PC, O'Brien RW, Slaytor M (1978b) Dependence of the higher termite *Nasutitermes exitiosus* and the lower termite *Coptotermes lacteus* on their gut flora. J Insect Physiol 24:363–368

French JRJ, Turner GL, Bradbury JF (1976) Nitrogen fixation by bacteria from the hindgut of termites. J Gen Microbiol 95:202–206

Friedrich MW, Schmitt-Wagner D, Lueders T, Brune A (2001) Axial differences in community structure of Crenarchaeota and Euryarchaeota in the highly compartmentalized gut of the soil-feeding termite *Cubitermes orthognathus*. Appl Environ Microbiol 67:4880–4890

Fröhlich J, König H (1999) Rapid isolation of single microbial cells from mixed natural and laboratory populations with the aid of a micromanipulator. Syst Appl Microbiol 22:249–257

Fröhlich J, Sass H, Babenzien HD, Kuhnigk T, Varma A, Saxena S, Nalepa C, Pfeiffer P, König H (1999) Isolation of *Desulfovibrio intestinalis* sp. nov. from the hindgut of the lower termite *Mastotermes darwiniensis*. Can J Microbiol 45:145–152

Fujita A (2004) Lysozymes in insects: what role do they play in nitrogen metabolism? Physiol Entomol 299:305–310

Fujita A, Abe T (2002) Amino acid concentration and distribution of lysozyme and protease activities in the guts of higher termites. Physiol Entomol 27:76–78

Fujita A, Shimizu I, Abe T (2001) Distribution of lysozyme and protease, and amino acid concentration in the guts of a wood-feeding termite, Reticulitermes speratus (Kolbe): possible digestion of symbiont bacteria transferred by trophallaxis. Physiol Entomol 26:116–123

Fujita A, Minamoto T, Shimizu I, Abe T (2002) Molecular cloning of lysozyme-encoding cDNAs expressed in the salivary gland of a wood-feeding termite, *Reticulitermes speratus*. Insect Biochem Molec Biol 32:1615–1624

Graber JR, Breznak JA (2004) Physiology and nutrition of *Treponema primitia*, an $H_2/CO_2$-acetogenic spirochete from termite hindguts. Appl Environ Microbiol 70:1307–1314

Graber JR, Breznak JA (2005) Folate cross-feeding supports symbiotic homoacetogenic spirochetes. Appl Environ Microbiol 71:1883–1889

Graber JR, Leadbetter JR, Breznak JA (2004) Description of *Treponema azotonutricium* sp. nov. and *Treponema primitia* sp. nov., the first spirochetes isolated from termite guts. Appl Environ Microbiol 70:1307–1314

Grandi G, Guidi L, Chicca M (1997) Endonuclear bacterial symbionts in two termite species: an ultrastructural study. J Submicrosc Cytol Pathol 29:281–292

Grassé P-P, Noirot C (1958) Le meule des termites champignonnistes et sa signification symbiotique. Ann Sci Nat Ser 11, Zool Biol Animale 11:113–128

Grech-Mora I, Fardeau ML, Patel BKC, Ollivier B, Rimbault A, Prensier G, Garcia JL, Garnier-Sillam E (1996) Isolation and characterization of *Sporobacter termitidis* gen. nov., sp. nov., from the digestive tract of the wood-feeding termite *Nasutitermes lujae*. Int J Syst Bacteriol 46:512–518

Guo L, Quicili DR, Chase J, Blomquist GJ (1991) Gut tract microorganisms supply the precursors for methyl-branched hydrocarbon biosynthesis in the termite, *Zootermopsis nevadensis*. Insect Biochem 21:327–333

Harazono K, Yamashita N, Shinzato N, Watanabe Y, Fukatsu T, Kurane R (2003) Isolation and characterization of aromatics-degrading microorganisms from the gut of the lower termite *Coptotermes formosanus*. Biosci Biotechnol Biochem 67:889–892

Hethener P, Brauman A, Garcia JL (1992) *Clostridium termitidis* sp. nov., a cellulolytic bacterium from the gut of the wood-feeding termite, *Nasutitermes lujae*. Syst Appl Microbiol 5:52–58

Higashi M, Abe T, Burns TP (1992) Carbon-nitrogen balance and termite ecology. Proc R Soc Lond Ser B Biol Sci 249:303–308

Hirai H, Shinzato N, Nakagawa A, Watanabe Y, Kurane R (2000) Degradation of lignin model compounds by various termites [in Japanese]. Mokuzai Gakkaishi 46:63–67

Hollande A, Valentin J (1969) Appareil de Golgi, pinocytose, lysosomes, mitochondries, bactéries symbiontiques, atractophores et pleuromitose chez les Hypermastigines du genre Joenia: Affinités entre. Joenidae et Trichomonadines Protistologica 5:39–86

Hongoh Y, Ohkuma M, Kudo T (2003a) Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera: Rhinotermitidae). FEMS Microbiol Ecol 44:231–242

Hongoh Y, Yuzawa H, Ohkuma M, Kudo T (2003b) Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. FEMS Microbiol Lett 221:299–304

Honigberg BM (1970) Protozoa associated with termites and their role in digestion. In: Krishna K, Weesner FM (eds) Biology of termites, vol 2. Academic, New York, pp 1–36

Hopkins DW, Chudek JA, Bignell DE, Frouz J, Webster EA, Lawson T (1998) Application of $^{13}C$ NMR to investigate the transformations and biodegradation of organic materials by wood-and soil-feeding termites, and a coprophagous litter-dwelling dipteran larva. Biodegradation 9:423–431

Hugenholtz P, Goebel BM, Pace NR (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 180:4765–4774

Hungate RE (1939) Experiments on the nutrition of Zootermopsis. III: The anaerobic carbohydrate dissimilation by the intestinal protozoa. Ecology 20:230–245

Hungate RE (1943) Quantitative analyses of the cellulose fermentation by termite protozoa. Ann Entomol Soc Am 36:730–739

Hungate RE (1946) Studies on cellulose fermentation. II: an anaerobic cellulose-decomposing actinomycete, *Micromonospora propionici*, n. sp. J Bacteriol 51:51–56

Hungate RE (1955) Mutualistic intestinal protozoa. In: Hutner SH, Lwoff A (eds) Biochemistry and physiology of protozoa, vol 2. Academic, New York, pp 159–199

Hyodo F, Azuma J, Abe T (1999) Estimation of effect of passage through the gut of a lower termite, *Coptotermes formosanus* Shiraki, on lignin by solid-state CP MAS $^{13}C$-NMR. Holzforschung 53:244–246

Hyodo F, Inoue T, Azuma JI, Tayasu I, Abe T (2000) Role of the mutualistic fungus in lignin degradation in the fungus-growing termite Macrotermes gilvus (Isoptera: Macrotermitinae). Soil Biol Biochem 32:653–658

Iida T, Ohkuma M, Ohtoko K, Kudo T (2000) Symbiotic spirochetes in the termite hindgut: Phylogenetic identification of ectosymbiotic spirochetes of oxymonad protists. FEMS Microbiol Ecol 34:17–26

Inoue T, Murashima K, Azuma JI, Sugimoto A, Slaytor M (1997) Cellulose and xylan utilization in the lower termite *Reticulitermes speratus*. J Insect Physiol 43:235–242

Inoue T, Kitade O, Yoshimura T, Yamaoka I (2000) Symbiotic associations with protists. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbiosis, ecology. Kluwer, Dordrecht, pp 275–288

Inoue T, Moriya S, Ohkuma M, Kudo T (2005) Molecular cloning and characterization of a cellulase gene from a symbiotic protist of the lower termite Coptotermes formosanus. Gene 349:67–75

Itakura S, Tanaka H, Enoki A (1999) Occurrence and metabolic role of the pyruvate dehydrogenase complex in the lower termite Coptotermes formosanus (Shiraki). Insect Biochem Molec Biol 29:625–633

Itakura S, Tanaka H, Enoki A, Chappell DJ, Slaytor M (2003) Pyruvate and acetate metabolism in termite mitochondria. J Insect Physiol 49:917–926

Jeffries TW (1994) Biodegradation of lignin and hemicelluloses. In: Ratledge C (ed) Biochemistry of microbial degradation. Kluwer, Dordrecht, pp 233–277

Ji R, Brune A (2001) Transformation and mineralization of $^{14}C$-labeled cellulose, peptidoglycan, and protein by the soil-feeding termite *Cubitermes orthognathus*. Biol Fertil Soils 33:166–174

Ji R, Brune A (2005) Digestion of peptidic residues in humic substances by an alkali-stable and humic-acid tolerant proteolytic activity in the gut of soil-feeding termites. Soil Biol Biochem 37:1648–1655

Ji R, Kappler A, Brune A (2000) Transformation and mineralization of synthetic $^{14}C$-labeled humic model compounds by soil-feeding termites. Soil Biol Biochem 32:1281–1291

Johjima T, Ohkuma M, Kudo T (2003) Isolation and cDNA cloning of novel hydrogen peroxide-dependent phenol oxidase from the basidiomycete *Termitomyces albuminosus*. Appl Microbiol Biotechnol 61:220–225

Kambhampati S, Eggleton P (2000) Taxonomy and phylogenetics of Isoptera. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbiosis, ecology. Kluwer, Dordrecht, pp 1–23

Kane MD (1997) Microbial fermentation in insect guts. In: Mackie RI, White BA (eds) Gastrointestinal microbiology, vol 1. Chapman and Hall, New York, pp 231–265

Kane MD, Breznak JA (1991) Acetonema longum gen. nov. sp.nov., an $H_2/CO_2$ acetogenic bacterium from the termite, *Pterotermes occidentis*. Arch Microbiol 156:91–98

Kane MD, Brauman A, Breznak JA (1991) *Clostridium mayombei* sp. nov., an $H_2/CO_2$ acetogenic bacterium from the gut of the African soil-feeding termite, *Cubitermes speciosus*. Arch Microbiol 156:99–104

Kappler A, Brune A (1999) Influence of gut alkalinity and oxygen status on mobilization and size-class distribution of humic acids in the hindgut of soil-feeding termites. Appl Soil Ecol 13:219–229

Kappler A, Brune A (2002) Dynamics of redox potential and changes in redox state of iron and humic acids during gut passage in soil-feeding termites (Cubitermes spp.). Soil Biol Biochem 34:221–227

Katoh H, Miura T, Maekawa K, Shinzato N, Matsumoto T (2002) Genetic variation of symbiotic fungi cultivated by the macrotermitine termite *Odontotermes formosanus* (Isoptera: Termitidae) in the Ryukyu Archipelago. Molec Ecol 11:1565–1572

Katzin LI, Kirby H (1939) The relative weight of termites and their protozoa. J Parasitol 25:444–445

Koidzumi M (1921) Studies on the intestinal protozoa found in the termites of Japan. Parasitology 13:235–305

Kovoor J (1967) Le pH intestinal d'un termite supérieur, *Microcerotermes edentatus* (Was., Amitermitinae). Insect Soc 14:157–160

Kovoor J (1968) L'intestin d'un termite supérieur (Microcerotermes edentatus, Wasman, Amitermitinae). Histophysiologie et flore bacterienne symbiotique. Bull Biol Fr Belg 102:45–84

Kudo T, Ohkuma M, Moriya S, Noda S, Ohtoko K (1998) Molecular phylogenetic identification of the intestinal anaerobic microbial community in the hindgut of the termite, *Reticulitermes speratus*, without cultivation. Extremophiles 2:155–161

Kuhnigk T, König H (1997) Degradation of dimeric lignin model compounds by aerobic bacteria isolated from the hindgut of xylophagous termites. J Basic Microbiol 37:205–211

Kuhnigk T, Borst E-M, Ritter A, Kämpfer P, Graf A, Hertel H, König H (1994) Degradation of lignin monomers by the hindgut flora of xylophagous termites. Syst Appl Microbiol 17:76–85

Kuhnigk T, Borst EM, Breunig A (1995) *Bacillus oleronius* sp. nov., a member of the hindgut flora of the termite *Reticulitermes santonensis* (Feytaud). Can J Microbiol 41:699–706

Kuhnigk T, Branke J, Krekeler D, Cypionka H, König H (1996) A feasible role of sulfate-reducing bacteria in the termite gut. Syst Appl Microbiol 19:139–149

La Fage JP, Nutting WL (1978) Nutrient dynamics of termites. In: Brian MV (ed) Production ecology of ants and termites. Cambridge University Press, Cambridge, UK, pp 165–232

Leadbetter JR, Breznak JA (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 JR, Crosby LD, Breznak JA (1998) Methanobrevibacter filiformis sp. nov., a filamentous methanogen from termite hindguts. Arch Microbiol 169:287–292

Leadbetter JR, Schmidt TM, Graber JR, Breznak JA (1999) Acetogenesis from $H_2$ plus $CO_2$ by spirochetes from termite guts. Science 283:686–689

Leander BS, Keeling PJ (2004) Symbiotic innovation in the oxymonad *Streblomastix strix*. J Euk Microbiol 51:291–300

Leaphart AB, Lovell CR (2001) Recovery and analysis of formyltetrahydrofolate synthetase gene sequences from natural populations of acetogenic bacteria. Appl Environ Microbiol 67:1392–1395

Leaphart AB, Friez MJ, Lovell CR (2003) Formyltetrahydrofolate synthetase sequences from salt marsh plant roots reveal a diversity of acetogenic bacteria and other bacterial functional groups. Appl Environ Microbiol 69:693–696

Lee MJ, Schreurs PJ, Messer AC, Zinder SH (1987) Association of methanogenic bacteria with flagellated protozoa from a termite hindgut. Curr Microbiol 15:337–341

Leidy J (1849) Proc Acad Nat Sci (Phila) 4:225–233

Leidy J (1881) The parasites of the termites. J Acad Nat Sci (Phila), 2nd Ser 8:425–447

Lemke T, van Alen T, Hackstein JHP, Brune A (2001) Cross-epithelial hydrogen transfer from the midgut compartment drives methanogenesis in the hindgut of cockroaches. Appl Environ Microbiol 67:4657–4661

Li L, Fröhlich J, Pfeiffer P, König H (2003) Termite gut symbiotic archaezoa are becoming living metabolic fossils. Eukar Cell 2:1091–1098

Lilburn TG, Schmidt TM, Breznak JA (1999) Phylogenetic diversity of termite gut spirochaetes. Environ Microbiol 1:331–345

Lilburn TG, Kim KS, Ostrom NE, Byzek KR, Leadbetter JR, Breznak JA (2001) Nitrogen fixation by symbiotic and free-living spirochetes. Science 292:2495–2498

Lo N, Bandi C, Watanabe H, Nalepa C, Beninati T (2003a) Evidence for co-cladogenesis between diverse dictyopteran lineages and their intracellular endosymbionts. Molec Biol Evol 20:907–913

Lo N, Watanabe H, Sugimura M (2003b) Evidence for the presence of a cellulase gene in the last common ancestor of bilaterian animals. Proc R Soc Lond Ser B Biol Sci 270(Suppl 1):S69–S72

Lysenko O (1985) Non-sporeforming bacteria pathogenic to insects: incidence and mechanisms. Ann Rev Microbiol 39:673–695

Machida M, Kitade O, Miura T, Matsumoto T (2001) Nitrogen recycling through proctodeal trophallaxis in the Japanese damp-wood termite *Hodotermopsis japonica* (Isoptera, Termopsidae). Insect Soc 48:52–56

Mannesmann R, Piechowski B (1989) Verteilungsmuster von Gärkammerbakterien einiger Termitenarten. Mater Org 24:161–178

Margulis L, Olendzenski A, Afzelius BA (1990) Endospore-forming filamentous bacteria symbiotic in termites: ultrastructure and growth in culture of Arthromitus. Symbiosis 8:95–116

Margulis L, Jorgensen JZ, Dolan S, Kolchinsky R, Rainey FA, Lo SC (1998) The Arthromitus stage of *Bacillus cereus*: intestinal symbionts of animals. Proc Natl Acad Sci USA 95:1236–1241

Martin MM (1983) Cellulose digestion in insects. Comp Biochem Physiol 75A:313–324

Martin MM, Martin JS (1978) Cellulose digestion in the midgut of the fungus-growing termite *Macrotermes natalensis*: the role of acquired digestive enzymes. Science 199:1453–1455

Martin MM, Martin JS (1979) The distribution and origins of the cellulolytic enzymes of the higher termite, Macrotermes natalensis. Physiol Zool 52:11–21

Matoub M, Rouland C (1995) Purification and properties of the xylanases from the termite *Macrotermes bellicosus* and its symbiotic fungus Termitomyces sp. Comp Biochem Physiol 112B:629–635

Matsuura K (2001) Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. Oikos 92:20–26

McSweeney CS, Dulieu A, Webb RI, Del Dot T, Blackall LL (1999) Isolation and characterization of a Clostridium sp. with cinnamoyl esterase activity and unusual cell envelope ultrastructure. Arch Microbiol 172:139–149

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

Moriya S, Dacks JB, Takagi A, Noda S, Ohkuma M, Doolittle WF, Kudo T (2003) Molecular phylogeny of three oxymonad genera: Pyrsonympha, Dinenympha and Oxymonas. J Eukar Microbiol 50:190–197

Nakashima K, Watanabe H, Azuma JI (2002a) Cellulase genes from the parabasalian symbiont *Pseudotrichonympha grassii* in the hindgut of the wood-feeding termite *Coptotermes formosanus*. Cell Mol Life Sci 59:1554–1560

Nakashima K, Watanabe H, Saitoh H, Tokuda G, Azuma J-I (2002b) Dual cellulose-digesting system of the wood-feeding termite, *Coptotermes formosanus* Shiraki. Insect Biochem Molec Biol 32:777–784

Nalepa CA, Bignell DE, Bandi C (2001) Detritivory, coprophagy, and the evolution of digestive mutualisms in Dictyoptera. Insect Soc 48:194–201

Ndiaye D, Lensi R, Lepage M, Brauman A (2004) The effect of the soil-feeding termite *Cubitermes niokoloensis* on soil microbial activity in a semi-arid savanna in West Africa. Plant Soil 259:277–286

Noda S, Ohkuma M, Usami R, Horikoshi K, Kudo T (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

Noda S, Ohkuma M, Yamada A, Hongoh Y, Kudo T (2003) Phylogenetic position and in situ identification of ectosymbiotic spirochetes on protists in the termite gut. Appl Environ Microbiol 69:625–633

Noirot C (1992) From wood-to humus-feeding: an important trend in termite evolution. In: Billen J (ed) Biology and evolution of social insects. Leuven University Press, Leuven, pp 107–119

Noirot C (1995) The gut of termites (isoptera): comparative anatomy, systematics, phylogeny I: lower termites. Ann Soc Entomol Fr (NS) 31:197–226

Noirot C (2001) The gut of termites (isoptera) Comparative anatomy, systematics, phylogeny. II: higher termites (termitidae). Ann Soc Entomol Fr (NS) 37:431–471

O'Brien RW, Breznak JA (1984) Enzymes of acetate and glucose metabolism in termites. Insect Biochem 14:639–643

O'Brien RW, Slaytor M (1982) Role of microorganisms in the metabolism of termites Australian. J Biol Sci 35:239–262

Odelson DA, Breznak JA (1983) Volatile fatty acid production by the hindgut microbiota of xylophagous termites. Appl Environ Microbiol 45:1602–1613

Odelson DA, Breznak JA (1985a) Cellulase and other polymer-hydrolyzing activities of Trichomitopsis termopsidis, a symbiotic protozoan from termites. Appl Environ Microbiol 49:622–626

Odelson DA, Breznak JA (1985b) Nutrition and growth characteristics of *Trichomitopsis termopsidis*, a cellulolytic protozoan from termites. Appl Environ Microbiol 49:614–621

Ohkuma M (2002) Symbiosis in the termite gut: culture-independent molecular approaches. In: Seckbach J (ed) Symbiosis: mechanisms and model systems. Kluwer, Dordrecht, pp 717–730

Ohkuma M (2003) Termite symbiotic systems: efficient bio-recycling of lignocellulose. Appl Microbiol Biotechnol 61:1–9

Ohkuma M, Kudo T (1996) Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. Appl Environ Microbiol 62:461–468

Ohkuma M, Kudo T (1998) Phylogenetic analysis of the symbiotic intestinal microflora of the termite *Cryptotermes domesticus*. FEMS Microbiol Lett 164:389–395

Ohkuma M, Noda S, Horikoshi K, Kudo T (1995) Phylogeny of symbiotic methanogens in the gut of the termite *Reticulitermes speratus*. FEMS Microbiol Lett 134:45–50

Ohkuma M, Noda S, Usami R, Horikoshi K, Kudo T (1996) Diversity of nitrogen-fixation genes in the symbiotic intestinal microflora of the termite *Reticulitermes speratus*. Appl Environ Microbiol 62:2747–2752

Ohkuma M, Iida T, Kudo T (1999a) Phylogenetic relationships of symbiotic spirochetes in the gut of diverse termites. FEMS Microbiol Lett 181:123–129

Ohkuma M, Noda S, Kudo T (1999b) Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. Appl Environ Microbiol 65:4926–4934

Ohkuma M, Noda S, Kudo T (1999c) Phylogenetic relationships of symbiotic methanogens in diverse termites. FEMS Microbiol Lett 171:147–153

Ohkuma M, Noda S, Hongoh Y, Kudo T (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

Ohkuma M, Shimizu H, Thongaram T, Kosono S, Moriya K, Trakulnaleamsai S, Noparatnaraporn N, Kudo T (2003) An alkaliphilic and xylanolytic Paenibacillus species isolated from the gut of a soil-feeding termite. Microb Environ 18:145–151

Ohtoko K, Ohkuma M, Moriya S, Inoue T, Usami R, Kudo T (2000) Diverse genes of cellulase homologues of glycosyl hydrolase family 45 from the symbiotic protists in the hindgut of the termite *Reticulitermes speratus*. Extremophiles 4:343–349

Osbrink WLA, Williams KS, Connick WJ Jr, Wright MS, Lax AR (2001) Virulence of bacteria associated with the formosan subterranean termite (Isoptera: Rhinotermitidae) in New Orleans, La. Environ Entomol 30:443–448

Paster BJ, Ludwig W, Weisburg WG, Stackebrandt E, Hespell RB, Hahn CM, Reichenbach H, Stetter KO, Woese CR (1985) A phylogenetic grouping of the bacteroides, cytophagas, and certain flavobacteria. Syst Appl Microbiol 6:34–42

Paster BJ, Dewhirst FE, Cooke SM, Fussing V, Poulsen LK, Breznak JA (1996) - Phylogeny of not-yet-cultured spirochetes from termite guts. Appl Environ Microbiol 62:347–352

Pasti MB, Belli ML (1985) Cellulolytic activity of actinomycetes isolated from termite (Termitidae) gut. FEMS Microbiol Lett 26:107–112

Pasti MB, Pometto AL III, Nuti MP, Crawford DL (1990) Lignin-solubilizing ability of actinomycetes isolated from termite (Termitidae) gut. Appl Environ Microbiol 56:2213–2218

Patricolo E, Villa L, Arizzi M (2001) TEM observations on symbionts of Joenia annectens (Flagellata Hypermastigida). J Nat Hist 35:471–480

Paul J, Sarkar A, Varma AK (1986) In vitro studies of cellulose digesting properties of *Staphylococcus saprophyticus* isolated from termite gut. Curr Sci 55:710–714

Paul J, Saxena S, Varma A (1993) Ultrastructural studies of the termite (*Odontotermes obesus*) gut microflora and its cellulolytic properties. World J Microbiol Biotechnol 9:108–112

Pierantoni U (1936) La simbiosi fisiologica nei termitidi xilophagi e nei loro flagellati intestinali. Arch Zool Ital 22:135–173

Potrikus CJ, Breznak JA (1977) Nitrogen-fixing *Enterobacter agglomerans* isolated from guts of wood-eating termites. Appl Environ Microbiol 33:392–399

Potrikus CJ, Breznak JA (1980) Anaerobic degradation of uric acid by gut bacteria of termites. Appl Environ Microbiol 40:125–132

Potrikus CJ, Breznak JA (1981) Gut bacteria recycle uric acid nitrogen in termites: a strategy for nutrient conservation. Proc Natl Acad Sci USA 78:4601–4605

Potts RC, Hewitt PH (1973) The distribution of intestinal bacteria and cellulase activity in the harvester termite *Trinervitermes trinervoides* (Nasutitermitidae). Insect Soc 20:215–220

Radek R (1999) Flagellates, bacteria, and fungi associated with termites: diversity and function in nutrition – a review. Ecotropica 5:183–196

Radek R, Tischendorf G (1999) Bacterial adhesion to different termite flagellates: ultrastructural and functional evidence for distinct molecular attachment modes. Protoplasma 207:43–53

Radek R, Hausmann K, Breunig A (1992) Ectobiotic and endocytobiotic bacteria associated with the termite flagellate Joenia annectens. Acta Protozool 31:93–107

Radek R, Rösel J, Hausmann K (1996) Light and electron microscopic study of the bacterial adhesion to termite flagellates applying lectin cytochemistry. Protoplasma 193:105–122

Rasmussen RA, Khalil MAK (1983) Global production of methane by termites. Nature 301:704–705

Rath AC (2000) The use of entomopathogenic fungi for control of termites. Biocontrol Sci Technol 10:563–581

Rohrmann GF, Rossman AY (1980) Nutrient strategies of *Macrotermes ukuzii* (Isoptera: Termitidae). Pedobiologia 20:61–73

Rother A, Radek R, Hausmann K (1999) Characterization of surface structures covering termite flagellates of the family Oxymonadidae and ultrastructure of two oxymonad species, *Microrhopalodina multinucleata* and Oxymonas sp. Eur J Protistol 35:1–16

Rouland C (2000) Symbiosis with fungi. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbiosis, ecology. Kluwer, Dordrecht, pp 289–306

Rouland C, Chararas C, Renoux J (1989) Les osidases digestives présentes dans l'intestin moyen, l'intestin postérieur et les glandes salivaires du termite humivore *Crenetermes albotarsalis*. CR Acad Sci Paris Série III 308:281–285

Rouland-Lefevre C, Diouf MN, Brauman A, Neyra M (2002) Phylogenetic relationships in Termitomyces (family Agaricaceae) based on the nucleotide sequence of ITS: a first approach to elucidate the evolutionary history of the symbiosis between fungus-growing termites and their fungi. Molec Phylogenet Evol 22:423–429

Sacchi L, Nalepa CA, Lenz M, Bandi C, Corona S, Grigolo A, Bigliardi E (2000) Transovarial transmission of symbiotic bacteria in *Mastotermes darwiniensis* (Isoptera: Mastotermitidae): ultrastructural aspects and phylogenetic implications. Ann Entomol Soc Am 93:1308–1313

Salmassi TM, Leadbetter JR (2003) Molecular aspects of $CO_2$-reductive acetogenesis in cultivated spirochetes and the gut community of the termite *Zootermopsis angusticollis*. Microbiology 149:2529–2537

Sanderson MG (1996) Biomass of termites and their emissions of methane and carbon dioxide: a global database. Global Biogeochem Cycles 10:543–557

Sands WA (1969) The association of termites and fungi. In: Krishna K, Weesner FM (eds) Biology of termites, vol 1. Academic, New York, pp 495–524

Schäfer A, Konrad R, Kuhnigk T, Kämpfer P, Hertel H, König H (1996) Hemicellulose-degrading bacteria and yeasts from the termite gut. J Appl Bacteriol 80:471–478

Schmitt-Wagner D, Brune A (1999) Hydrogen profiles and localization of methanogenic activities in the highly compartmentalized hindgut of soil-feeding higher termites (Cubitermes spp.). Appl Environ Microbiol 65:4490–4496

Schmitt-Wagner D, Friedrich M, Wagner B, Brune A (2003a) Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (Cubitermes spp.). Appl Environ Microbiol 69:6018–6024

Schmitt-Wagner D, Friedrich M, Wagner B, Brune A (2003b) Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (Cubitermes spp.). Appl Environ Microbiol 69:6007–6017

Schultz JE, Breznak JA (1978) Heterotrophic bacteria present in hindguts of wood-eating termites [Reticulitermes flavipes (Kollar)]; Appl Environ Microbiol 35:930–936

Schultz JE, Breznak JA (1979) Cross-feeding of lactate between *Streptococcus lactis* and Bacteroides sp. isolated from termite hindguts. Appl Environ Microbiol 37:1206–1210

Seedorf H, Dreisbach A, Hedderich R, Shima S, Thauer RK (2004) $F_{420}H_2$ oxidase (FprA) from *Methanobrevibacter arboriphilus*, a coenzyme $F_{420}$-dependent enzyme involved in $O_2$ detoxification. Arch Microbiol 182:126–137

Shima S, Sordel-Klippert M, Brioukhanov A, Netrusov A, Linder D, Thauer RK (2001) Characterization of a heme-dependent catalase from *Methanobrevibacter arboriphilus*. Appl Environ Microbiol 67:3041–3045

Shinzato N, Matsumoto T, Yamaoka I, Oshima T, Yamagishi A (1999) Phylogenetic diversity of symbiotic methanogens living in the hindgut of the lower termite *Reticulitermes speratus* analyzed by PCR and in situ hybridization. Appl Environ Microbiol 65:837–840

Shinzato N, Matsumoto T, Yamaoka I, Oshima T, Yamagishi A (2001) Methanogenic symbionts and the locality of their host lower termites. Microb Environ 16:43–47

Sikorowski PP, Lawrence AM (1998) Transmission of Serratia marcescens (Enterobacteriaceae) in adult *Heliothis virescens* (Lepidoptera: Noctuidae) laboratory colonies. Biol Control 12:50–55

Slaytor M (1992) Cellulose digestion in termites and cockroaches: what role do symbionts play? Comp Biochem Physiol 103B:775–784

Slaytor M (2000) Energy metabolism in the termite gut and its gut microbiota. In: Abe T, Bignell DE, Higashi M (eds) Termites: evolution, sociality, symbiosis, ecology. Kluwer, Dordrecht, pp 307–332

Slaytor M, Chappell DJ (1994) Nitrogen metabolism in termites. Comp Biochem Physiol 107:1–10

Slaytor M, Veivers PC, Lo N (1997) Aerobic and anaerobic metabolism in the higher termite *Nasutitermes walkeri* (Hill). Insect Biochem Molec Biol 27:291–303

Snel J, Blok HJ, Kengen HMP, Ludwig W, Poelma FGJ, Koopman JP, Akkermans ADL (1994) Phylogenetic characterization of Clostridium related segmented filamentous bacteria in mice based on 16S ribosomal RNA analysis. Syst Appl Microbiol 17:172–179

Snel J, Heinen PP, Blok HJ, Carman RJ, Duncan AJ, Allen PC, Collins MD (1995) Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "*Candidatus Arthromitus*". Int J Syst Bacteriol 45:780–782

Stackebrandt E, Schumann P, Cui XL (2004) Reclassification of Cellulosimicrobium variabile Bakalidou et al. 2002 as *Isoptericola variabilis* gen. nov., comb. nov. Int J System Evol Microbiol 54:685–688

Stingl U, Brune A (2003) Phylogenetic diversity and whole-cell hybridization of oxymonad flagellates from the hindgut of the wood-feeding lower termite *Reticulitermes flavipes*. Protist 154:147–155

Stingl U, Maass A, Radek R, Brune A (2004) Symbionts of the gut flagellate Staurojoenina sp. from *Neotermes cubanus* represent a novel, termite-associated lineage of Bacteroidales: description of "Candidatus Vestibaculum illigatum. Microbiology 150:2229–2235

Stingl U, Radek R, Brune A (2005) "Endomicrobia": cytoplasmic symbionts of termite gut protozoa form a separate phylum of prokaryotes. Appl Environ Microbiol 71:1473–1479

Sugimoto A, Inoue T, Tayasu I, Miller L, Takeichi S, Abe T (1998) Methane and hydrogen production in a termite-symbiont system. Ecol Res 13:241–257

Tamm SL (1980) The ultrastructure of prokaryotic-eukaryotic cell junctions. J Cell Sci 44:335–352

Tamm SL (1982) Flagellated epibiotic bacteria propel a eucaryotic cell. J Cell Biol 94:697–709

Taprab Y, Ohkuma M, Johjima T, Maeda Y, Moriya S, Inoue T, Suwanarit P, Noparatnaraporn N, Kudo T (2002) Molecular phylogeny of symbiotic basidiomycetes of fungus-growing termites in Thailand and their relationship with the host. Biosci Biotechnol Biochem 66:1159–1163

Tayasu I (1998) The use of carbon and nitrogen isotope ratios in termite research. Ecol Res 13:377–387

Tayasu I, Sugimoto A, Wada E, Abe T (1994) Xylophagous termites depending on atmospheric nitrogen. Naturwissenschaften 81:229–231

Tayasu I, Abe T, Eggleton P, Bignell DE (1997) Nitrogen and carbon isotope ratios in termites: an indicator of trophic habit along the gradient from wood-feeding to soil-feeding. Ecol Entomol 22:343–351

Terra WR (1990) Evolution of digestive systems of insects. Ann Rev Entomol 35:181–200

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180

Thayer DW (1976) Facultative wood-digesting bacteria from the hind-gut of the termite *Reticulitermes hesperus*. J Gen Microbiol 95:287–296

Thayer DW (1978) Carboxymethylcellulase produced by facultative bacteria from the hind-gut of the termite *Reticulitermes hesperus*. J Gen Microbiol 106:13–18

Tholen A, Brune A (1999) Localization and in situ activities of homoacetogenic bacteria in the highly compartmentalized hindgut of soil-feeding higher termites (Cubitermes spp.). Appl Environ Microbiol 65:4497–4505

Tholen A, Brune A (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

Tholen A, Schink B, Brune A (1997) The gut microflora of *Reticulitermes flavipes*, its relation to oxygen, and evidence for oxygen-dependent acetogenesis by the most abundant Enterococcus sp. FEMS Microbiol Ecol 24:137–149

Thongaram T, Kosono S, Ohkuma M, Hongoh Y, Kitada M, Yoshinaka T, Trakulnaleamsai S, Noparatnaraporn N, Kudo T (2003) Gut of higher termites as a niche for alkaliphiles as shown by culture based and culture-independent studies. Microb Environ 18:152–159

Thongaram T, Hongoh Y, Kosono S, Ohkuma M, Trakulnaleamsai S, Noparatnaraporn N, Kudo T (2005) Comparison of bacterial communities in the alkaline gut segment among various species of higher termites. Extremophiles 9:229–238

To L, Margulis L, Cheung ATW (1978) Pillotinas and hollandinas: distribution and behaviour of large spirochaetes symbiotic in termites. Microbios 22:103–133

To LP, Margulis L, Chase D, Nutting WL (1980) The symbiotic microbial community of the Sonoran desert termite: *Pterotermes occidentis*. Biosystems 13:109–137

Tokuda G, Yamaoka I, Noda H (2000) Localization of symbiotic clostridia in the mixed segment of the termite *Nasutitermes takasagoensis* (Shiraki). Appl Environ Microbiol 66:2199–2207

Tokuda G, Nakamura T, Murakami R, Yamaoka I (2001) Morphology of the digestive system in the wood-feeding termite *Nasutitermes takasagoensis* (Shiraki) [Isoptera: Termitidae]; Zool Sci 18:869–877

Tokuda G, Lo N, Watanabe H, Arakawa G, Matsumoto T, Noda H (2004) Major alteration of the expression site of endogenous cellulases in members of an apical termite lineage. Mol Ecol 13:3219–3228

Tokura M, Ohkuma M, Kudo T (2000) Molecular phylogeny of methanogens associated with flagellated protists in the gut and with the gut epithelium of termites. FEMS Microbiol Ecol 33:233–240

Trinkerl M, Breunig A, Schauder R, König H (1990) Desulfovibrio termitidis sp. nov., a carbohydrate-degrading sulfate-reducing bacterium from the hindgut of a termite. System Appl Microbiol 13:372–377

Veivers PC, O'Brien RW, Slaytor M (1980) The redox state of the gut of termites. J Insect Physiol 26:75–77

Veivers PC, O'Brien RW, Slaytor M (1982) Role of bacteria in maintaining the redox potential in the hindgut of termites and preventing entry of foreign bacteria. J Insect Physiol 28:947–951

Vu AT, Ngyen NC, Leadbetter JR (2004) Iron reduction in the metal-rich guts of wood-feeding termites. Geobiology 2:239–247

Watanabe H, Tokuda G (2001) Animal cellulases. Cell Mol Life Sci 58:1167–1178

Watanabe H, Nakashima K, Saito H, Slaytor M (2002) New endo-beta-1,4-glucanases from the parabasalian symbionts, *Pseudotrichonympha grassii* and *Holomastigotoides mirabile* of *Coptotermes termites*. Cell Mol Life Sci 59:1983–1992

Watanabe Y, Shinzato N, Fukatsu T (2003) Isolation of actinomycetes from termites' guts. Biosci Biotechnol Biochem 67:1797–1801

Wenzel M, Schönig I, Berchtold M, Kämpfer P, König H (2002) Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis*. J Appl Microbiol 92:32–40

Wenzel M, Radek R, Brugerolle G, König H (2003) Identification of the ectosymbiotic bacteria of *Mixotricha paradoxa* involved in movement symbiosis. Eur J Protistol 39:11–24

Wier A, Ashen J, Margulis L (2000) *Canaleparolina darwiniensis*, gen. nov., sp. nov., and other pillotinaceous spirochetes from insects. Int Microbiol 3:213–223

Williams CM, Veivers PC, Slaytor M, Cleland SV (1994) Atmospheric carbon dioxide and acetogenesis in the termite *Nasutitermes walkeri* (Hill). Comp Biochem Physiol 107A:113–118

Yamin MA (1979) Termite flagellates. Sociobiology 4:1–119

Yang H, Schmitt-Wagner D, Stingl U, Brune A (2005) Niche heterogeneity determines bacterial community structure in the in the termite gut (*Reticulitermes santonensis*). Environ Microbiol 7(7):916–932

Yara K, Jahana K, Hayashi H (1989) In situ morphology of the gut microbiota of the fungus-growing termite *Odontotermes formosanus* (Termitidae: Macrotermitinae). Sociobiology 15:247–260

Yoshimura T, Fujino T, Ito T, Tsunoda K, Takahashi M (1996) Ingestion and decomposition of wood and cellulose by the protozoa in the hindgut of *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) as evidenced by polarizing and transmission electron microscopy. Holzforschung 50:99–104

Zoberi MH, Grace JK (1990) Fungi associated with the subterranean termite *Reticulitermes flavipes* in Ontario. Mycologia 82:289–294

# 23 Marine Chemosynthetic Symbioses

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## Introduction

Bacteria and marine eukaryotes often coexist in symbioses that significantly influence the ecology, physiology, and evolution of both partners. De Bary (1879) defined symbiosis as "the living together of differently named organisms," implying that the term encompasses both positive (e.g., mutualism) and negative (e.g., parasitism) associations. Many researchers now view symbiotic interactions as those that persist over the majority of the lifespan of the organisms involved and that provide benefits to each partner beyond those obtained in the absence of association. This chapter describes such symbioses, specifically those between marine invertebrate and protist hosts and chemosynthetic bacterial symbionts.

These bacteria, which cluster primarily within the Gammaproteobacteria (*Fig. 23.1*), are chemoautotrophs or methanotrophs. In both chemoautotrophic and methanotrophic symbioses, the hosts, through an astonishing array of physiological and behavioral adaptations, provide the symbiont access to the substrates (i.e., electron donors and acceptors) necessary for the generation of energy and bacterial biomass. In exchange, a portion of the carbon fixed by the symbiont is used, either directly or indirectly, for host energy and biosynthesis. These symbioses thereby increase the metabolic capabilities, and therefore the number of ecological niches, of both the host and the bacterial symbiont.

In those symbioses for which the electron donor has been explicitly identified, sulfide and other reduced inorganic sulfur compounds (e.g., thiosulfate) fuel energy generation by the chemoautotrophic symbionts, serving as electron sources for oxidative phosphorylation. In these symbioses, the ATP produced in electron transport fuels autotrophic $CO_2$ fixation via the Calvin cycle. In contrast, bacteria in marine invertebrate-methanotroph symbioses use methane ($CH_4$) as an energy, electron, and carbon source. Unlike their protist or metazoan hosts, chemoautotrophs and methanotrophs share the ability to use reduced inorganic compounds or methane for energy generation and carbon dioxide or methane for carbon fixation and utilization. On the basis of these unique biosynthetic capacities, notably the ability to synthesize $C_3$ compounds from $C_1$ compounds, we refer collectively to these bacterial symbionts as "chemosynthetic."

Given the sulfide-rich habitats in which chemoautotrophic symbioses occur, researchers infer that the bacterial symbionts oxidize reduced inorganic sulfur compounds to obtain energy and reducing power for autotrophic carbon fixation. While some endosymbionts (such as those in the protobranchs *Solemya velum* and *S. reidi*; Cavanaugh 1983; Anderson et al. 1987) utilize thiosulfate ($S_2O_3^{\mu}$), an intermediate in sulfide oxidation, hydrogen sulfide is inferred to be the preferred energy source in a variety of symbioses (see review in Van Dover 2000). But for many symbioses, the actual energy source has not been identified definitively; rather, only an autotrophic metabolism has been confirmed. Indeed, chemosynthetic bacteria utilizing other energy sources (e.g., hydrogen or ammonia) could also serve similar nutritional roles in symbiotic associations. In this review, bacterial symbionts that have been shown to use reduced sulfur compounds ($H_2S$, $HS^{\mu}$, $S^{\mu 2}$, $S_2O_3^{\mu}$, $S^{o}$) for energy metabolism are referred to as thioautotrophs, while the more general term "chemoautotroph" is used to describe symbionts for which data supporting autotrophic $CO_2$ fixation exist but for which the lithotrophic energy source is unknown.

E. Rosenberg et al. (eds.), *The Prokaryotes – Prokaryotic Biology and Symbiotic Associations*, DOI 10.1007/978-3-642-30194-0_21,

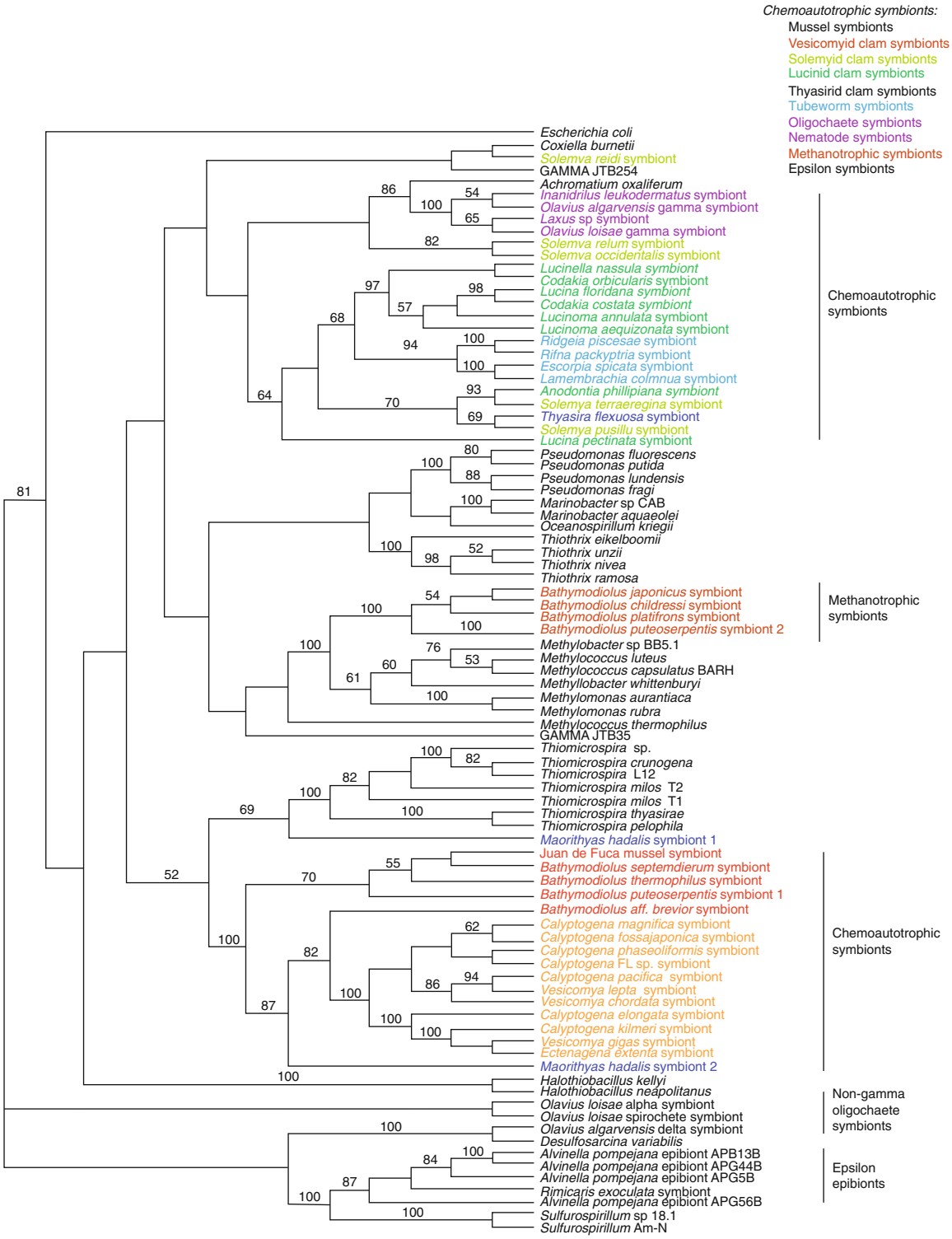


**Fig. 23.1**

**Phylogeny showing the strict consensus of 46 trees obtained via parsimony analyses of 16S rRNA gene sequences (1,456 bp) from symbiotic and free-living bacteria. Results greater than 50 % from a 500 replicate bootstrap analysis are reported above respective branches. Chemosynthetic symbiont taxa are *color* coded (see key on Figure)**

## History

The discovery of deep-sea hydrothermal vents and the flourishing ecosystems associated with them significantly advanced scientific understanding of chemosynthetic symbioses. Oceanographers in the research submersible ***Alvin*** discovered hydrothermal vents along the Galapagos Rift in 1977. In stark contrast to perceptions of the deep benthos as a cold, food-limited habitat incapable of supporting substantial biomass, hydrothermal vents are oases, characterized by high concentrations of free-living microorganisms and dense aggregations of invertebrates (Lonsdale 1977; Grassle 1985; Van Dover 2000). Researchers first argued that vent invertebrates achieved high densities by filtering organic matter, which was presumably transported to vent sites in hydrothermally driven convection cells (Lonsdale 1977). A second hypothesis suggested that the invertebrate community fed directly on locally dense populations of free-living chemoautotrophic bacteria (Lonsdale 1977; Corliss et al. 1979).

However, studies of the giant tubeworm, *Riftia pachyptila* (*Fig. 23.2*), whose lack of mouth and gut precludes suspension feeding, suggested that sulfide-oxidizing endosymbiotic bacteria might contribute substantially to the vent food web. Cavanaugh et al. (1981) proposed that symbiotic chemosynthetic bacteria occurred in *R. pachyptila*. Microscopic and biochemical evidence indicated Gram-negative bacteria were present, packed within the tubeworm trophosome (Cavanaugh 1981; *Fig. 23.3*), a highly vascularized organ in the tubeworm trunk, in which activities of enzymes involved in sulfide oxidation and carbon fixation were also detected (Felbeck 1981). In addition, Rau (1981) used stable isotope signatures to show a nonphotosynthetic carbon source for *R. pachyptila*, implying a role for chemoautotrophy in tubeworm metabolism. Following confirmation of a chemosynthetic endosymbiosis within the giant tubeworm, researchers questioned the putative reliance on filter feeding by other vent invertebrates. Ultimately, anatomical, enzymological, and isotopic analyses revealed the presence of sulfur-oxidizing bacterial symbionts either within the tissues (endosymbiotic) or attached to the surfaces (episymbiotic) of most vent taxa, including vesicomyid clams, mytilid mussels, shrimp, and polychaete worms (Cavanaugh 1994; Nelson and Fisher 1995; Van Dover 2000).

Recognizing the ubiquity of chemoautotrophic symbioses at hydrothermal vents, researchers searched for and discovered similar symbiotic associations in other marine habitats, including coastal and subtidal reducing sediments (e.g., Felbeck et al. 1981; Southward et al. 1981; Southward 1982; Cavanaugh 1983; Giere 1985; Bauer-Nebelsick et al. 1996), brine and hydrocarbon seeps (Sibuet and Olu 1998), and whale skeletons (Bennett et al. 1994; Smith and Baco 2003), thereby extending the host taxa to include solemyid and lucinid bivalves, pogonophoran tubeworms, echinoids, and ciliates. In addition, methanotrophic bacteria were detected in a marine sponge (Vacelet et al. 1995), a pogonophoran tubeworm (Schmaljohann and Flügel 1987), and in vent and seep mussels, sometimes co-occurring with sulfur-oxidizing chemoautotrophs in a "dual symbiosis" (Childress et al. 1986; Cavanaugh et al. 1987; Cavanaugh 1993).


**Fig. 23.2**
**Symbiont-containing host organisms from hydrothermal vents and cold seeps. (a) *Calyptogena magnifica* shell (Courtesy of Emilio Jorge Power). (b) Filamentous bacteria on sulfide deposits from the East Pacific Rise. (c) *Bathymodiolus childressi* from the Gulf of Mexico (Courtesy of the National Oceanic and Atmospheric Administration). (d) *Riftia pachyptila* at the East Pacific Rise**

## Habitat Chemistry

The seemingly disparate ecological niches where these symbioses are found all possess a chemical gradient, or chemocline, which chemosynthetic bacteria exploit for energy production. Chemoclines form where reduced, high-energy compounds such as sulfide or methane (typically produced in anoxic habitats, including vent fluids and sediments) underlie an oxic water column. Chemosynthetic microorganisms must bridge the oxic-anoxic interface to access both the reduced compounds (e.g., $H_2S$) used as an energy source and the oxygen to which electrons are shuttled in aerobic energy metabolism.

The source of reduced compounds used in chemoautotrophic energy metabolism differs among habitats. In marine

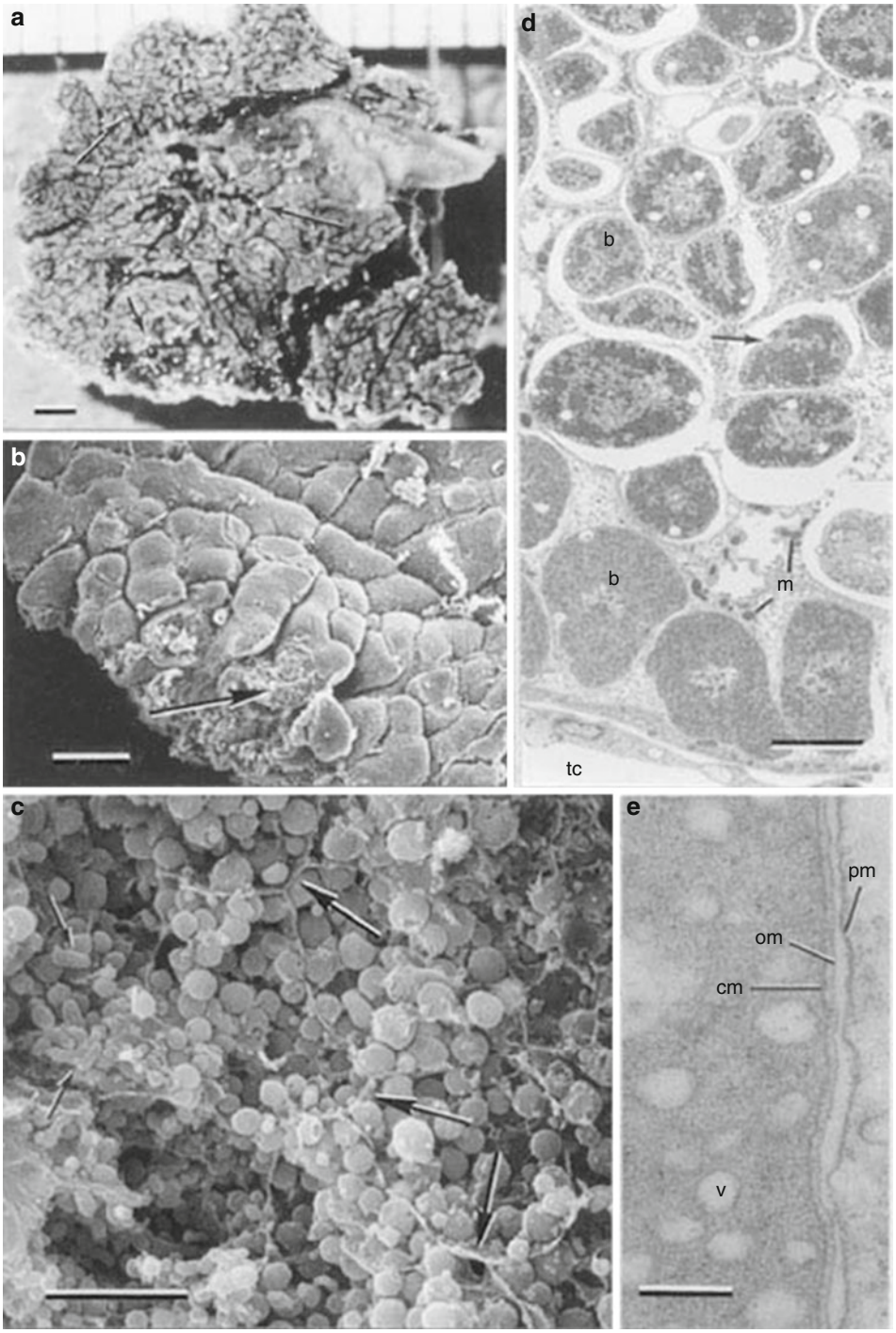


**Fig. 23.3**

***Riftia pachyptila*** **Jones (a–c: Galapagos Rift; d, e: 21°N, East Pacific Rise). (a) Photograph showing elemental sulfur crystals (*arrows*) scattered throughout trophosome (Courtesy of M. L. Jones). (b) Scanning electron micrograph showing lobules of trophosome; *arrow* indicates area of c (*below*) where surface epithelium was removed to reveal symbionts within trophosome. (c) Same, higher magnification, showing symbionts within trophosome; note spherical cells as well as rod-shaped cells (*small arrows*); *large arrows* indicate likely host cell membranes. (d) Cross section of portion of trophosome lobule showing variable fine structure of symbionts, including membrane-bound vesicles in many cells; all symbionts contained within membrane-bound vacuoles, either singly or in groups of two or more; *arrow*: dividing bacterium; *b* bacteria, *m* mitochondria, *tc* trunk coelomic cavity. (e) Same, higher magnification, showing cell envelope of symbiont (resembling that of Gram-negative bacteria), intracytoplasmic vesicles, and peribacterial membrane; *v* vesicle, *cm* symbiont cytoplasmic membrane, *om* symbiont outer membrane, *pm* peribacterial membrane. Scale bars: a, 1 μm; b, 250 μm; c, 10 μm; d, 3 μm; and e, 0.2 μm (From Cavanaugh (1985), with permission)**

sediments microbial sulfate reduction (in which $SO_4^{\mu 2}$ is used as an electron acceptor during the oxidation of organic matter) dominates, and the sulfide utilized by thioautotrophic symbioses (e.g., involving lucinid clams or solemyid protobranchs) in these habitats is of biogenic origin. In contrast, sulfide at hydrothermal vents is produced by the geothermal reduction of seawater sulfate and by the interaction between geothermally heated water and sulfur-containing rocks (e.g., basalt; Alt 1995; Elderfield and Schultz 1996; Rouxel et al. 2004). Seawater that percolates into the developing crust becomes heated and reacts with oceanic basalt, becoming enriched with metals and sulfide and charged with volcanic gases such as methane and carbon dioxide. Heated vent water then exits with concentrations of reduced compounds orders of magnitude higher than in ambient seawater. Hydrothermal effluent is hot (temperatures up to 350–400 °C), acidic (pH ∼ 3) and anoxic and can contain 3–12 mmol/kg of $H_2S$, 25–100 μmol/kg of $CH_4$, and 0.05–1 mmol/kg of $H_2$, as well as 360–1,140 μmol/kg of Mn and 750–6,500 μmol/kg of Fe (Elderfield and Schultz 1996). As it exits the seafloor and mixes with the ambient bottom oxygenated seawater (pH, ca. 8; temperature = 1.8 °C; $[O_2]$, ca. 110 μM), metallic sulfides precipitate out resulting in "black smokers" (reviewed in Elderfield and Schultz 1996). Vent organisms are typically found clustered around more diffuse or low flow vents, which are caused by ambient seawater mixing in the shallow subsurface with vent fluid. These vents are characterized by a higher pH (ca. 6), lower temperatures (1.8 to ca. 40 °C), and, consequently, lower concentrations of reduced chemicals (Van Dover 2000).

The relative acidity of vent fluid (black smokers—pH, ca. 3; diffuse flow vents—pH, ca. 6) significantly impacts the concentration of inorganic chemicals available to chemosynthetic symbioses. For example, carbon dioxide ($CO_2$), bicarbonate ($HCO_3^{\mu}$), and carbonate ($CO_3^{\mu 2}$), the three distinct chemical species of the dissolved inorganic carbon (DIC) pool, vary in relative abundance depending on pH; $pK_a$ values for these compounds are 6.4 for $CO_2$:$HCO_3^{\mu}$ and 10.3 for $HCO_3^{\mu}$:$CO_3^{\mu 2}$ at 25 °C (Stumm and Morgan 1996). Thus, $CO_2$, which diffuses freely across biological membranes and is the DIC species fixed by chemoautotrophic symbionts utilizing the Calvin cycle, is readily available at vents (Cavanaugh and Robinson 1996; Goffredi et al. 1997b). In addition, sulfide exists at three levels of dissociation ($H_2S$, $HS^{\mu}$, and $S^{\mu 2}$) depending on pH, with $pK_a$ values of 7.0 for $H_2S$:$HS^{\mu}$ and 12.9 for $HS^{\mu}$:$S^{\mu 2}$ at 25 °C (Stumm and Morgan 1996). Therefore, in the relatively acidic vent fluids, sulfide occurs predominantly as $H_2S$. In such effluent, total sulfide ($H_2S$, $HS^{\mu}$, and $S^{\mu 2}$) concentration correlates positively with temperature (Johnson et al. 1988); conversely, higher temperatures (>30 °C) may facilitate reactions between sulfide and other dissolved elements (such as iron) that reduce free sulfide availability (Luther et al. 2001). The chemical environment (i.e., concentrations of $CO_2$, $O_2$, $H_2S$, $CH_4$, $H^+$, and dissolved metals) is therefore expected to significantly influence the ecology and evolution of chemosynthetic symbioses.

## Methods for Studying Chemosynthetic Symbioses

To date, the bacteria involved in these symbiotic associations have not yet been isolated and grown in pure culture—perhaps because the unique environment encountered in situ by chemosynthetic symbionts has not been recreated or because a reduced genome, characteristic of many endosymbionts, has precluded growth outside of the host. Symbiotic bacteria are therefore studied indirectly, using methods to assess their physiology, ecology, and phylogeny within the context of the intact symbiosis. Traditionally, researchers identify chemosynthetic symbioses using a combination of microscopy (light, confocal, scanning, and transmission electron), which provides visual information on the location, morphology, and ultrastructure of symbionts, and enzyme assays, which detect and quantify the activity of key proteins involved in chemoautotrophic (e.g., ribulose 1,5-bisphosphate carboxylase-oxygenase) or methanotrophic (e.g., methanol dehydrogenase) metabolism. In addition, tracing the incorporation of radiolabeled substrates (e.g., carbon dioxide, methane, and nitrogen species) within the host helps define the physiology of the host-bacteria partnership. Such physiological assays, in conjunction with the analyses of stable isotope signatures of symbiont-containing and symbiont-free host tissue, provide valuable insight into the trophic dynamics of symbiont-based communities. Molecular approaches, such as polymerase chain reaction (PCR)-based gene probing, 16S rRNA gene analysis, and fluorescent in situ hybridization (FISH), are increasingly used to characterize the systematic relationships of symbiont and host species (e.g., Distel et al. 1995; Peek et al. 1998; Dubilier et al. 1999) and the metabolism and gene flow of the bacterial symbionts (Robinson et al. 1998; Lee et al. 1999; Millikan et al. 1999; Podar et al. 2002). Molecular techniques have also been used to detect symbiont transmission modes (Cary and Giovannoni 1993; Krueger 1996) as well as symbiont abundance (Polz and Cavanaugh 1995).

## Summary

This chapter reviews symbiotic associations between chemosynthetic bacteria and marine invertebrate and protist hosts. A bias toward symbioses between chemoautotrophic bacteria and hydrothermal vent invertebrates is evident, primarily because our knowledge of marine bacterial symbioses stems largely from studies of vent fauna done in the 27 years following the discovery of these unique organisms. But despite this impressive body of research, much about these marine symbioses remains to be revealed. In conjunction with several earlier reviews that provide a thorough and thoughtful treatment of symbioses occurring at hydrothermal vents and cold seeps (Fisher 1990; Felbeck and Distel 1991; Childress and Fisher 1992; Cavanaugh 1994; Nelson and Fisher 1995), the following chapter presents an overview of the ecology, physiology, and evolution of chemosynthetic symbioses.

## Host Diversity

Chemosynthetic bacteria are known to associate with a diversity of invertebrate hosts (six phyla) as well as with ciliate protists (Table 23.1 and references therein). To date, the majority of the symbionts characterized via 16S rRNA phylogenetic analyses fall within the Gammaproteobacteria division (Fig. 23.1; discussed further below). The intimacy of these associations varies among taxa. The bacterial partners may be episymbionts living on the surface of the host (e.g., on shrimp, nematodes, sponges, limpets, and ciliates; Figs. 23.4–23.6) or endosymbionts living either extracellularly within host tissue (e.g., in oligochaetes; Fig. 23.7) or in specialized host cells and organs (e.g., in bivalves and vestimentiferan tubeworms; Figs. 23.2, 23.3, and 23.8). In intracellular endosymbioses, the symbionts are housed within specialized host cells called "bacteriocytes" and are contained within a host-derived membrane-bound vacuole (Cavanaugh 1983, 1994; Fisher 1990). Host morphology clearly suggests a nutritional benefit from these intimate associations. Indeed, as in the giant vent tubeworms, the digestive system of many endosymbiont-containing marine invertebrates is either reduced (e.g., in coastal solemyid protobranchs) or absent altogether (e.g., in oligochaetes and vestimentiferan and pogonophoran tubeworms), consistent with host dependence on the symbiont for part or all of its nutrition.

All of the members of the tubeworm family Siboglinidae examined to date, including the vestimentiferan and the smaller pogonophoran tubeworms, contain intracellular symbionts. Most of these symbionts are inferred to be chemoautotrophic, but methanotrophs have been found in one host species (*Siboglinum poseidoni*; Schmaljohann and Flügel 1987). The vent tubeworm *Riftia pachyptila* and other vestimentiferan and pogonophoran tubeworm species possess a unique morphological adaptation to accommodate their symbionts. Tubeworm bacteria reside within a lobular and highly vascularized organ (the trophosome) that occupies most of the tubeworm trunk and functions specifically to house bacteria (Cavanaugh 1981; Felbeck 1981; Figs. 23.3 and 23.9). The symbiosis is obligate for these worms, as they are mouthless and gutless as adults and depend on their internal bacteria for their nutrition.

Chemosynthetic symbioses are widespread within the Mollusca and have been detected in five bivalve and two gastropod families (Table 23.1). In mollusk symbioses, the bacteria occur only in the gills; bacteria have been found within gill epithelial cells of solemyid protobranchs (Cavanaugh 1983; Krueger et al. 1996; Figs. 23.10–23.13), lucinid clams (Cavanaugh 1983; Felbeck 1983), thyasirid clams (Felbeck et al. 1981; Cavanaugh 1983; Arp et al. 1984), vesicomyid clams (Boss and Turner 1980; Rau 1981; Arp et al. 1984; Fig. 23.8), mytilid mussels (Fiala-Médioni 1984; Le Pennec and Hily 1984; Figs. 23.2 and 23.14), and provannid gastropods (Stein et al. 1988; Windoffer and Giere 1997). Within certain mollusk families (e.g., Solemyidae, Lucinidae, and Thyasiridae), all species examined form symbioses with chemoautotrophic bacteria. In other families, such as the Mytilidae, chemoautotrophic symbionts have been detected only in members of the subfamily Bathymodiolinae, which are found exclusively in the deep sea. Further, dual symbioses involving both methanotrophs and chemoautotrophs are restricted to species of deep-sea bathymodioline mussels collected from methane seeps and hydrothermal vents (Cavanaugh 1994; Nelson and Fisher 1995; Van Dover 2000; Fig. 23.14).

Chemosynthetic bacteria also occur as episymbionts on marine invertebrates (Table 23.1; Fig. 23.4). These symbionts include the Epsilonproteobacteria that cover the cuticle of ***Rimicaris*** shrimp, dominant members of the metazoan fauna at vents on the Mid-Atlantic Ridge (MAR; Polz et al. 1998) and the Central Indian Ridge (CIR; Van Dover et al. 2001; Van Dover 2002), and the surfaces of alvinellid polychaetes (Desbruyères et al. 1985; Cary et al. 1997). Chemosynthetic episymbionts also associate with nematodes (Weiser 1959; Ott et al. 1991; Polz et al. 1992, 1994) and ciliates (e.g., Fenchel and Finlay 1989; Bauer-Nebelsick et al. 1996). In addition, methanotrophic episymbionts have been found living on a deep-sea sponge (Vacelet et al. 1995, 1996). Vent limpet-bacteria associations seem to be an intermediate between epi- and endosymbioses; bacteria exist partially embedded in the limpet gill epidermis and may be endocytosed or fed on by the host (de Burgh and Singla 1984; Bates et al. 2004; Fig. 23.5). Some epibiont communities, like those residing on the ***Rimicaris*** shrimp and the nematode *Laxus* sp., are dominated by a single phylotype (Polz et al. 1994; Polz and Cavanaugh 1995), while others are quite diverse (Polz et al. 1999; Campbell et al. 2003). But given that morphological plasticity often belies the phylogenetic identity of symbionts (Polz et al. 1999; Giere and Krieger 2001), symbiont diversity estimates are only appropriate when putative symbiont phylotypes are confirmed using hybridization methods (e.g., FISH).

## Symbiont Diversity

### Morphology and Ultrastructure

Symbiont morphology varies among functional types (chemoautotroph vs. methanotroph), among phylotypes within the same functional group, and among individuals in a population of a single phylotype. The symbionts all have a Gram-negative cell envelope but range from small (ca. 0.25 µm diameter) coccoid endosymbionts within mussel gills (Cavanaugh 1985; Dubilier et al. 1998) to large (>10 µm length) rod-shaped and filamentous episymbionts on vent shrimp (Hentschel et al. 1993; Polz and Cavanaugh 1995; Fig. 23.4). Some bathymodioline mussels host two metabolically distinct symbionts: small (<0.5 µm) chemoautotrophs and larger (1.5–2.0 µm) methanotrophic bacteria possessing stacked intracytoplasmic membranes, which are typical of type I methanotrophs (e.g., Childress et al. 1986; Cavanaugh et al. 1987, 1992; Fiala-Médioni et al. 2002;

**Table 23.1**

**List of invertebrate taxa hosting chemoautotrophic or methanotrophic bacterial symbionts[a]**

| Group | Common name | Symbiont-containing tissue | Location | Habitat | Symbiont type | References |
|---|---|---|---|---|---|---|
| Protozoa | | | | | | |
| Class Ciliata | Ciliate | NA | Epibiotic | Cold seeps, mangrove swamp | Chemoautotroph | Bauer-Nebelsick et al. (1996) |
| | | | | | | Ott et al. (1998) |
| | | | | | | Buck et al. (2000) |
| | | | | | | Fenchel and Finlay (1989) |
| Porifera | | | | | | |
| Class Demospongiae | Sponge | NA | Extracellular | Cold seeps | Methanotroph | Vacelet et al. (1995, 1996) |
| Family Cladorhizidae | | | | | | |
| Nemata | | | | | | |
| Subfamily Stilbonematinae | Nematode | Cuticle | Epibiotic | Reducing sediments | Chemoautotroph | Schiemer et al. (1990) |
| | | | | | | Polz et al. (1992) |
| Mollusca | | | | | | |
| Class Bivalvia | | | | | | |
| Subclass Protobranchia | | | | | | |
| Family Solemyidae | Clam | Gills | Intracellular | Reducing sediments, hydrothermal vents,[b] cold seeps[c] | Chemoautotroph | Cavanaugh (1983) |
| | | | | | | Fisher and Childress (1986) |
| | | | | | | Conway et al. (1989) |
| Subclass Heterodonta | | | | | | |
| Family Lucinidae | Clam | Gills | Intracellular | Reducing sediments, cold seeps[c] | Chemoautotroph | Giere (1985) |
| | | | | | | Schweimanns and Felbeck (1985) |
| Family Thyasiridae | Clam | Gills | Intracellular | Reducing sediments, cold seeps[c] | Chemoautotroph | Dando and Southward (1986) |
| | | | | | | Herry and Le Pennec (1987) |
| Family Vesicomyidae | Clam | Gills | Intracellular | Hydrothermal vents, cold seeps | Chemoautotroph | Cavanaugh (1983) |
| | | | | | | Rau (1981) |
| Subclass Pteriomorphia | | | | | | |
| Family Mytilidae | Mussel | Gills | Extracellular | Hydrothermal vents, cold seeps | Chemoautotroph and/or methanotroph | Childress et al. (1986) |
| | | | | | | Cavanaugh et al. (1987) |
| | | | Intracellular | | | Fisher et al. (1988) |
| | | | | | | Cavanaugh et al. (1992) |
| Class Gastropoda | | | | | | |
| Family Provannidae | Snail | Gills | Intracellular | Hydrothermal vents | Chemoautotroph | Stein et al. (1988) |
| | | | | | | Endow and Ohta (1989) |

**Table 23.1 (continued)**

| Group | Common name | Symbiont-containing tissue | Location | Habitat | Symbiont type | References |
|---|---|---|---|---|---|---|
| Family Lepetodrilidae | Limpet | Gills | Epibiotic | Hydrothermal vents | Chemoautotroph | de Burgh and Singla (1984) |
| | | | | | | Fox et al. (2002) |
| | | | | | | Bates et al. (2004) |
| Annelida[d] | | | | | | |
| Class Polychaeta | Worm | Dorsal surface | Epibiotic | Hydrothermal vents | Chemoautotroph | Desbruyères et al. (1983, 1985) |
| Family Alvinellidae | | | | | | Cary et al. (2003) |
| Family Siboglinidae (Vestimentiferan and pogonophoran) | Tubeworm | Trophosome | Intracellular | Deep-sea hydrothermal vents, cold seeps, reducing sediments, fjords | Chemoautotroph | Cavanaugh et al. (1981) |
| | | | | | | Felbeck (1981) |
| | | | | | | Southward et al. (1981) |
| | | | | | | Brooks et al. (1987) |
| | | | | | | Schmaljohann and Flügel (1987) |
| | | | | | | Southward and Southward (1988) |
| | | | | | | de Burgh et al. (1989) |
| Class Clitellata | | | | | | |
| Subfamily Phallodrilinae | Oligochaete | Subcuticular | Extracellular | Coralline sands | Chemoautotroph[e] | Felbeck et al. (1983) |
| | | | | | | Giere (1981, 1985) |
| | | | | | | Giere and Langheld (1987) |
| Arthropoda | | | | | | |
| Class Crustacea | | | | | | |
| Family Alvinocarididae | Shrimp | Carapace | Epibiotic | Hydrothermal vents | Chemoautotroph | Van Dover et al. (1988) |
| | | | | | | Polz and Cavanaugh (1995) |
| | | | | | | Polz et al. (1999) |
| Echinodermata | | | | | | |
| Class Echinoidea | Sea urchin | Gut | Extracellular | Reducing sediments | Chemoautotroph[f] | Temara et al. (1993) |
| | | | | | | Brigmon and De Ridder (1998) |

Abbreviation: *NA* not applicable

[a]Chemosynthetic status of symbionts inferred from ultrastructural, physiological, enzymatic, and molecular data

[b]A solemyid protobranch, *Acharax alinae*, has been described from the Lau Basin hydrothermal vents, but the symbiosis has not been characterized (Metivier and Voncosel 1993)

[c]Solemyid clams have been collected from cold seeps in the eastern Pacific, and lucinid and thyasirid clams have been collected from cold seeps in the Gulf of Mexico, Sagami Bay, and Barbados Prism, though the presence of symbionts has not been formally described (Sibuet and Olu 1998)

[d]Though it is now accepted that the pogonophoran and vestimentiferan worms are not separate phyla but members of phylum Annelida, they are listed as separate groups for identification purposes

[e]The oligochaete *Olavius algarvensis* has been shown to have an additional symbiont which is a sulfur-reducing bacterium and member of the delta Proteobacteria (Dubilier et al. 2001). *Olavius loisae* has been shown to host an alpha proteobacterium and a spirochete as well as a chemoautotroph (Dubilier et al. 1999)

[f]This symbiont has been described as *Thiothrix*-like on the basis of morphology, physiology and immunological assays (Temara et al. 1993; Brigmon and de Ridder 1998)

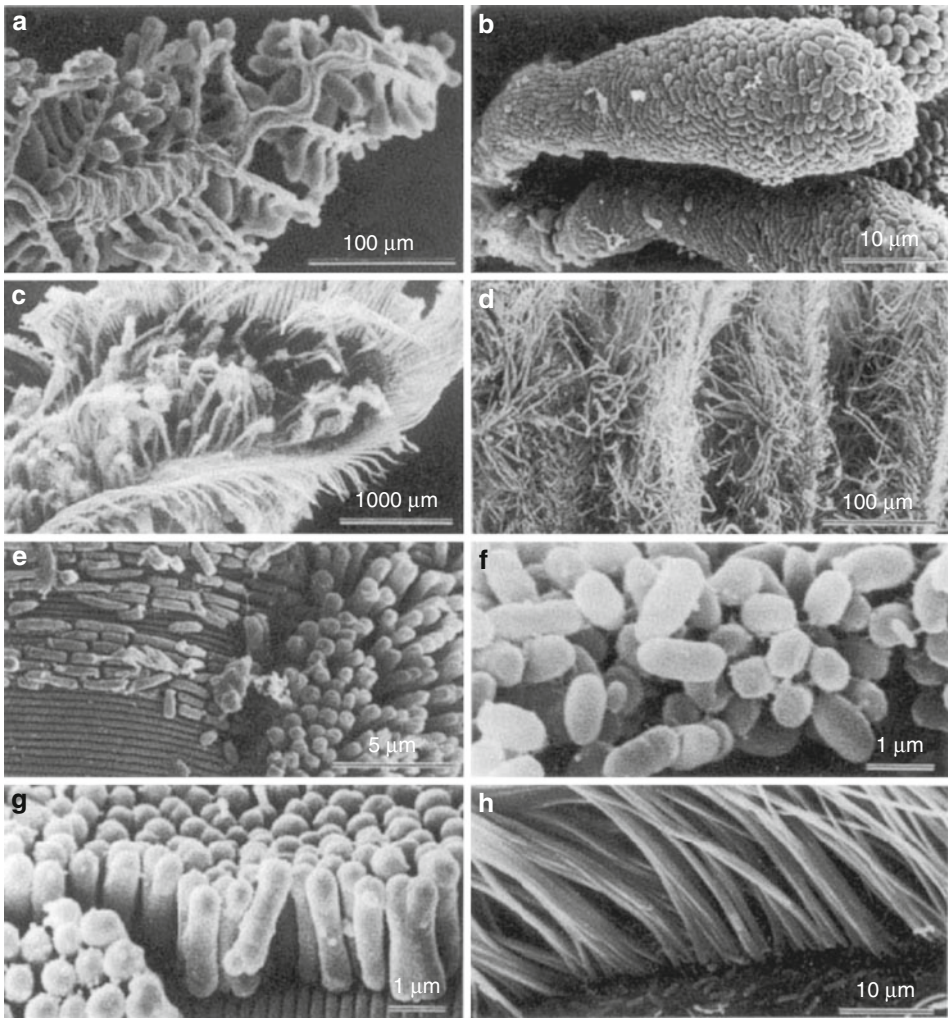


**Fig. 23.4**
**Scanning electron micrographs showing the morphological diversity of ectosymbiotic bacteria on the colonial ciliate *Zoothamnium niveum* (a, b), the shrimp *Rimicaris exoculata* (c, d), and the nematodes within the subfamily Stilbonematinae (e–h). (a) Entire ciliate colony with zooids attached to a common stem and (b) bacterial epigrowth on an individual zooid. (c) Shrimp appendage covered by dense arrays of filamentous bacteria and (d) detail of the hairlike bacterial covering. Epigrowth on different species of nematodes showing (e) irregular epigrowth of two morphological types on *Robbea* sp.; (f) coccoid bacteria forming multilayers on *Stibonema* sp.; (g) upright standing, longitudinally dividing rods on *Laxus oneistus*; and (h) dense array of nonseptate filaments that can reach up to 100 mm in length on *Eubostrichus dianae* (From Polz et al. (2000), with permission)**

Pimenov et al. 2002; ◉ *Fig. 23.14*). Morphological diversity can also occur throughout monospecific populations within a single host animal. For example, populations of sulfur-oxidizing chemoautotrophic symbionts in the tubeworm *Riftia pachyptila* contain distinct morphotypes that vary in abundance depending on location within the trophosome lobule (Bright et al. 2000); small, rod-shaped bacteria occur primarily in the innermost zone of the lobule nearest the host's axial blood vessel, while small and large cocci (1.6–10.7 µm diameter) occupy zones nearer the periphery of the trophosome (Bright et al. 2000). Such variability may relate to differences in the life cycle stage and metabolism among symbiont cells (Bright et al. 2000).

## Symbiont Phylogeny

While chemoautotrophic symbionts have consistently evaded culture, the suite of cellular and molecular methods used to characterize these bacteria has revealed startling evolutionary trends. Investigators have successfully sequenced 16S rRNA genes from symbiont-containing tissue and subsequently confirmed the symbiont origin of these sequences via hybridization with symbiont-specific probes. In contrast to the wide diversity of host taxa involved in these symbioses, chemosynthetic symbionts cluster primarily within a single bacterial division, the Gammaproteobacteria, on the basis of 16S rRNA gene sequences (Distel and Cavanaugh 1994; Dubilier et al. 1999;

McKiness 2004). Such analyses have also shown that most host species typically form a relationship with a unique symbiont phylotype. But this clearly is not always the case. For example, while strain level variation may occur, vestimentiferan tubeworms belonging to the genera ***Riftia***, ***Tevnia***, and ***Oasisia*** appear to share a single, or very similar, symbiont phylotype based on 16S rRNA gene sequences (Feldman et al. 1997; Laue and Nelson 1997; Di Meo et al. 2000; Nelson and Fisher 2000; McMullin et al. 2003), as do some species of tropical lucinid clams (Durand and Gros 1996; Durand et al. 1996).

Recently, McKiness (2004) reported phylogenetic analyses of 16S rRNA gene sequences from chemosynthetic symbionts within the Gammaproteobacteria. This study represented the most comprehensive analysis of chemoautotrophic symbionts to date. It included 39 symbiont sequences and over 30 sequences from free-living bacteria representatives of chemoautotrophs, methanotrophs, and marine bacterioplankton. Here, an expanded phylogenetic analysis is presented that includes the Epsilonproteobacteria symbionts of shrimp and alvinellid worms (Fig. 23.1). This consensus tree illustrates the strong level of resolution afforded by the 16S rRNA gene and shows that almost all of the chemosynthetic symbionts for which sequence data are available cluster into two main clades. The first clade includes symbionts of lucinid and thyasirid clams, solemyid protobranchs, tubeworms, nematodes, and oligochaetes, and the second clade includes the mytilid mussel and vesicomyid clam symbionts.

Though the first clade as a whole has relatively low bootstrap support, smaller clusters within the first clade are strongly supported. For example, the monophyletic cluster of nematode and oligochaete symbionts has 100 % bootstrap support. Similarly, the vestimentiferan tubeworm symbiont clade also has high bootstrap support, corroborating prior evidence that these worms share a single or very similar symbiont phylotype, which is consistent with environmental transmission of symbionts (Feldman et al. 1997; Laue and Nelson 1997; Di Meo et al. 2000; Nelson and Fisher 2000; McMullin et al. 2003; see the section "Ecology and Evolution" in this chapter). In contrast, the clam symbionts exhibit a more complicated relationship. The lucinid clam symbionts form a paraphyletic group; some are sister to the tubeworm symbionts, while others group with thyasirid and solemyid symbionts. The solemyid symbionts show similarly complicated relationships, as they are polyphyletic and scattered throughout the first clade. *Solemya velum* and *S. occidentalis* symbionts cluster with the nematode and oligochaete symbionts, while *S. terraeregina* and *S. pusilla* symbionts cluster with lucinid and thyasirid clam symbionts. Thus, this disjointed distribution does not suggest cospeciation between host taxa and symbionts in this first clade and indicates that there were multiple initiations of symbiosis within the solemyid and lucinid clams.

The position of the *S. reidi* symbiont within this first main group is curious; this symbiont falls at the base of this first main group, clustering with an intracellular pathogen, *Coxiella*

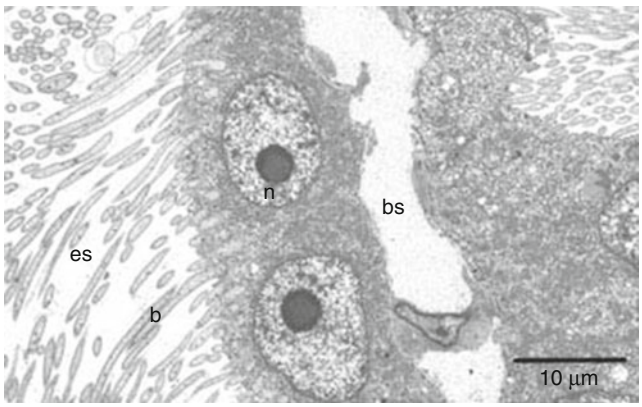


**Fig. 23.5**
***Lepetodrilus fucensis*. Transverse section of gill tissue from the hydrothermal vent limpet showing episymbiotic filamentous bacteria partially embedded in the host epithelium. *b* bacteria, *es* extracellular space, *n* nucleus, *bs* blood space (Courtesy of Amanda Bates)**

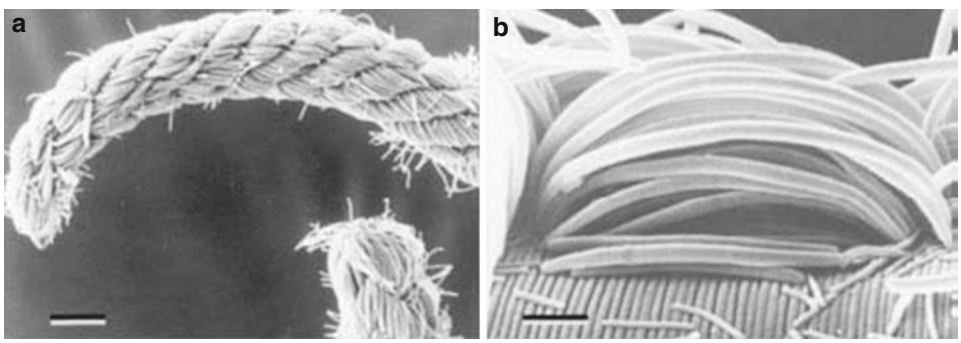


**Fig. 23.6**
***Eubostrichus* cf. *parasitiferus*. Scanning electron micrographs showing the symbiotic bacteria on the surface of the nematode. (a) Anterior (*bottom*) and posterior (*top*) end with symbionts arranged in a characteristic helix. (b) Higher magnification. Bacteria are attached with both ends to the worm's cuticle. Note the increasing length of the cells from proximal to distal along the worm's surface. Scale bars: a, 20 µm; b, 2 µm (From Polz et al. (1992), with permission)**

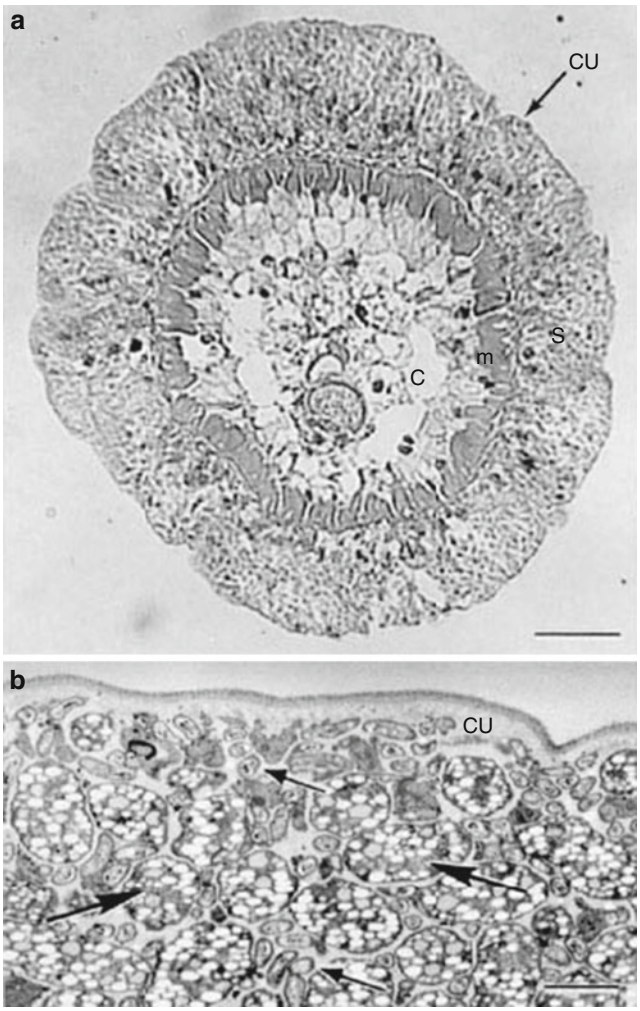


**Fig. 23.7**
***Inanidrilus leukodermatus*. (a) Light micrograph of a cross section of an oligochaete worm. (b) Transmission electron micrograph of symbiont-containing region just below the cuticle. Note smaller and larger symbiont morphotypes (*smaller* and *larger arrows*, respectively). *c* coelomic cavity, *m* muscle tissue, *s* symbiont-containing region between cuticle and epidermis, *cu* cuticle. Scale bars: a, 20 μm; b, 2 μm (From Dubilier et al. (1995), with permission)**

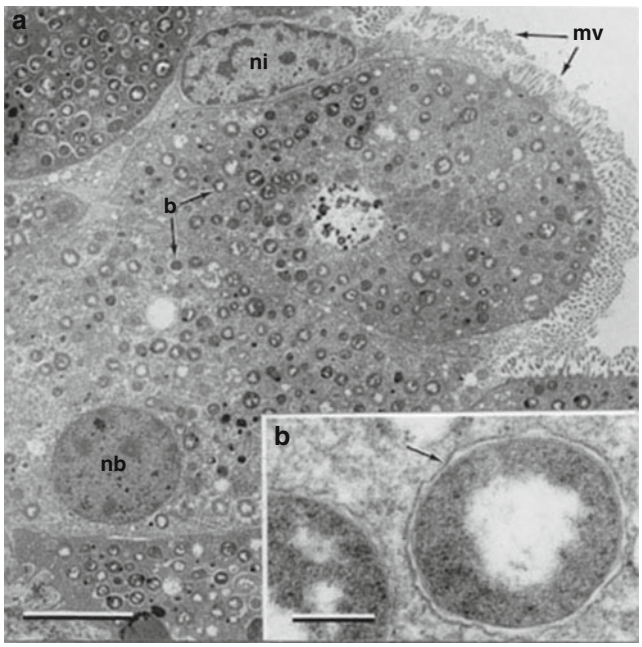


**Fig. 23.8**
***Calyptogena magnifica* Boss and Turner (21°N East Pacific Rise). (a) Transmission electron micrograph of slightly oblique transverse section of gill filament, showing coccoid-shaped bacteria within gill bacteriocyte and intercalary cells lacking symbionts; *b* bacteria, *mv* microvilli (of both cell types), *nb* nucleus of bacteriocyte, *ni* nucleus of intercalary cell. (b) Same, higher magnification, transverse section of coccoid-shaped symbionts, showing cell ultrastructure typical of Gram-negative bacteria and peribacterial membrane (*arrow*). Scale bars: a, 5 μm; b, 0.25 μm (From Cavanaugh (1985), with permission)**

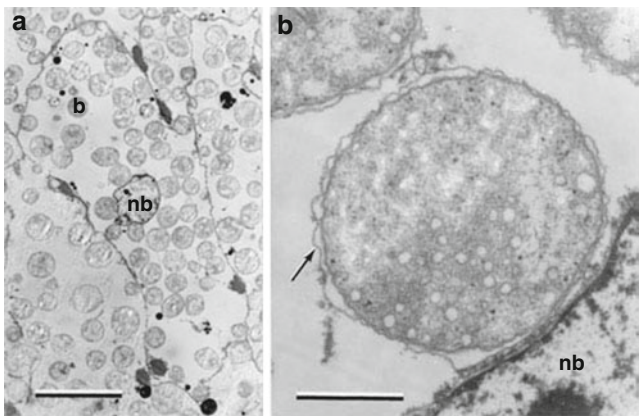


**Fig. 23.9**
***Escarpia spicata* Jones (San Clemente Fault). (a) Transmission electron micrograph, portion of trophosome lobule showing numerous coccoid- to ovoid-shaped bacterial symbionts, some of which appear intracellular. (b) Same, higher magnification, showing bacterial cell envelope (resembling that of Gram-negative bacteria) and intracytoplasmic membrane-bound vesicles; arrow: peribacterial membrane; *b* bacteria, *nb* nucleus of bacteriocyte. Scale bars: a, 10 μm; b, 1 μm (From Cavanaugh (1985), with permission)**

*burnetti*, and an environmental clone from a Japanese cold seep, Gamma JTB254 (discussed further below). This symbiont sequence has held a basal position in other analyses (see Bayesian analysis in McKiness 2004) and provokes questions concerning the nature of symbiosis in protobranch bivalves. As additional sequences become available, it will be necessary to reassess the position of the *S. reidi* symbiont with respect to other chemoautotrophic symbionts.

The second clade of symbionts, which includes the mytilid mussel and vesicomyid clam symbionts from vents and cold seeps, shows 100 % bootstrap support (support for the mussel and vesicomyid clades being 70 % and 99 %, respectively). The symbiont from the Central Indian Ridge mussel, ***Bathymodiolus***

**Fig. 23.10**
***Solemya* sp. (*right hand*) collected from deep-sea vent sites (2,380 m depth) along the subduction zone off Oregon and *Solemya velum* (*left hand*) collected from subtidal reducing sediments (<1 m depth, mean low tide) of Massachusetts eelgrass beds (Photo courtesy of Dr. Ruth D. Turner)**

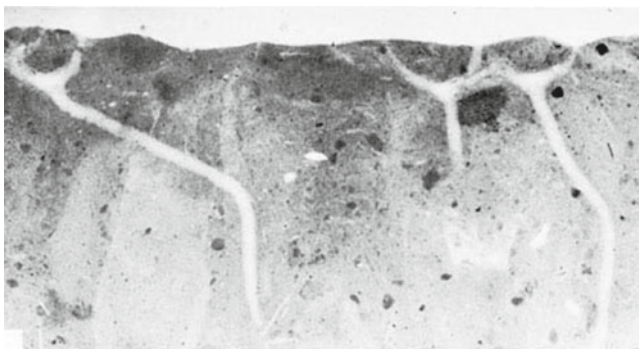

PLATE 3. SOLEMYA

**Fig. 23.11**
***Solemya velum*. Characteristic Y-shaped burrows dug by the coastal protobranch clam to bridge the oxic-anoxic interface and access both reduced sulfur (from below) and oxygen (from above) (From Stanley (1970), with permission)**

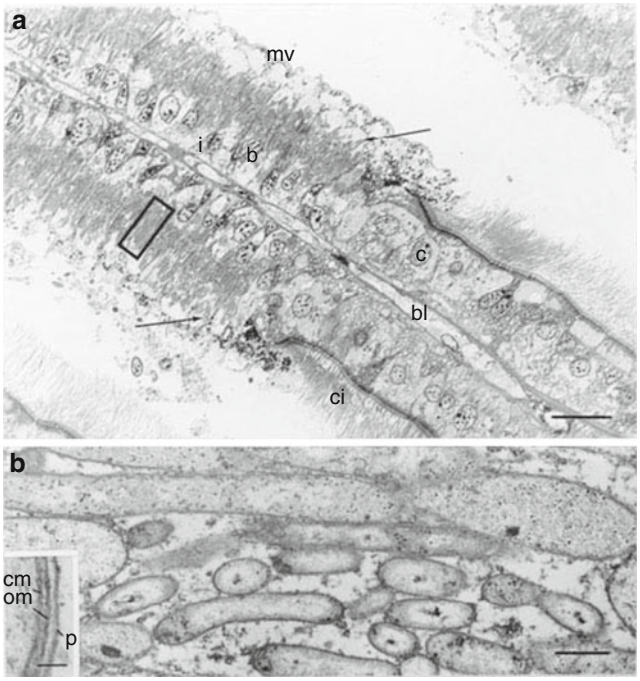


**Fig. 23.12**
***Solemya borealis*. (a) Transverse section of gill filaments showing intracellular rod-shaped bacteria (*arrows*, *rectangle*). Bacteriocytes are confined to the region proximal to the ciliated edge of the gill and are flanked by symbiont-free intercalary cells that appear to comprise the microvillar surface of the gill filament. Light micrograph. *b* bacteriocyte nucleus, *c* ciliated cell nucleus, *i* intercalary cell nucleus, *bl* blood space, *ci* cilia, *mv* microvilli. (b) Higher magnification of symbionts showing cell ultrastructure typical of Gram-negative. Inset: detail of symbiont cell envelope and peribacterial membrane. *p* peribacterial membrane, *cm* cell membrane, *om* outer membrane. Scale bars: a, 20 μm; b, 1 μm; *inset*, 0.05 μm (From Conway et al. (1992), with permission)**

aff. ***brevior***, falls at the base of the vesicomyid clam symbionts, with 82 % bootstrap support. In contrast to the first symbiont clade (◉ *Fig. 23.1*, top), evidence suggests that an ancestral symbiont initiated symbioses with both the vesicomyid clams and the bathymodioline mussels and predated the split between these bivalve lineages. On the basis of 16S rRNA gene sequence data, the divergence of the clam and mussel symbionts has been dated to 125–300 million years ago (Mya). This is corroborated by the fossil record, which dates the bathymodioline mussel hosts to 150 Mya and the vesicomyid clams to 100 Mya (Distel 1998).

The symbionts of the thyasirid clam *Maorhithyas hadalis* occupy unique positions in this phylogenetic framework. On the basis of 16S rRNA sequence data and in situ hybridization, Fujiwara et al. (2001) described two different symbionts within this clam. One of the symbionts shows evolutionary relatedness to the bathymodioline mussel and vesicomyid clam symbionts, occurring basal to the clade containing these bacteria, and the other symbiont clusters with the free-living *Thiomicrospira* spp. This free-living "symbiont" phylotype, however, may be a contaminant. Difficulties with in situ hybridization have precluded attempts to describe the microdistribution of the two symbiont types (Fujiwara and Uematsu 2002), bringing into question the phylogenetic identity of the clam symbionts.

The phylogenetic positions of the methanotrophic endosymbionts and the filamentous epibionts are shown also in ◉ *Fig. 23.1*. The methanotrophic symbionts characterized to date all belong to the Gammaproteobacteria, forming a clade with 100 % bootstrap support. The sister group of this clade consists of free-living type I methanotrophs (*Methylococcus*,

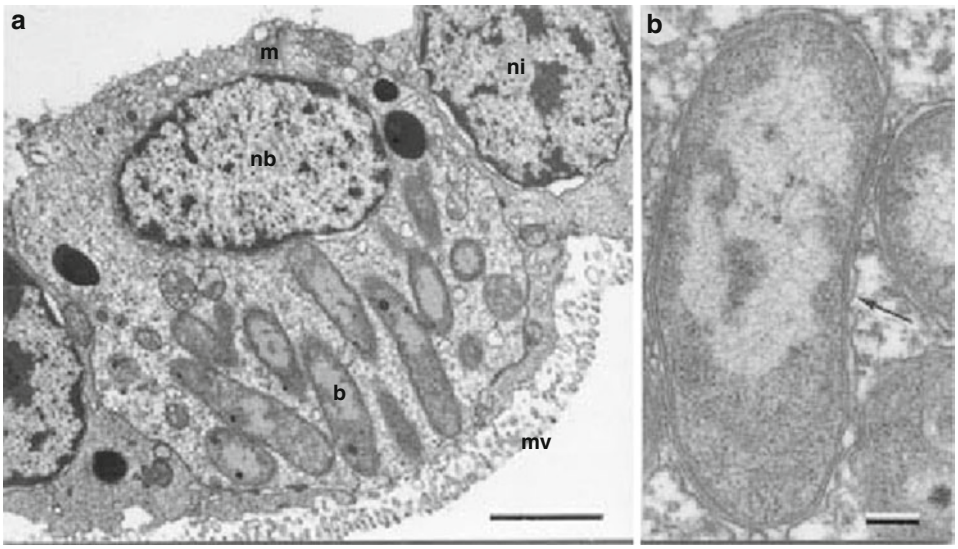


**Fig. 23.13**

***Solemya velum*. (a) Transmission electron micrograph, transverse section of gill filament, showing rod-shaped bacteria within gill bacteriocyte and intercalary cells lacking symbionts; *b* bacteria, *mv* microvilli, *nb* nucleus of bacteriocyte, *ni* nucleus of intercalary cell. (b) Same, higher magnification, transverse section of rod-shaped bacterium, showing cell ultrastructure typical of Gram-negative bacteria and peribacterial membrane (*arrows*). Scale bars: a, 3 μm; b, 0.2 μm (From Cavanaugh (1985), with permission)**

*Methylobacter*, and *Methylomonas* spp.). Given their monophyly, the mussel symbionts apparently arose from a common ancestor. But the question of whether these symbionts subsequently cospeciated with their hosts remains unanswered.

The episymbionts in this analysis include the sulfur-oxidizing Epsilonproteobacteria found on the Mid-Atlantic Ridge shrimp *Rimicaris exoculata* and the eastern Pacific polychaete *Alvinella pompejana*. Interestingly, the shrimp epibiont clusters with the polychaete epibionts despite the fact that *R. exoculata* occurs on the Mid-Atlantic Ridge, while alvinellid polychaetes inhabit vents in the eastern Pacific Ocean.

Free-living microorganisms can potentially provide insight into the ancestral form of endosymbionts. For instance, the evolution of insect endosymbionts (e.g., *Wolbachia* and *Buchnera* spp.) is commonly studied by comparative analyses with free-living, closely related microbes (Wernegreen 2002; Moran 2003). But until recently, the chemoautotrophic symbiont clades have not included any free-living bacteria. *Figure 23.1* includes two species of bacteria that are not chemoautotrophic symbionts (*Coxiella burnetti* and *Achromatium oxaliferum*) and an environmental clone (JTB254). All three of these sequences fall within the first symbiont clade. *Coxiella burnetii* is an intracellular pathogenic bacterium (Woldehiwet 2004) and the Gamma JTB254 clone was recovered from a deep-sea cold seep in the Japan Trench (Li et al. 1999). They both fall in a cluster with the *S. reidi* symbiont. Both *C. burnetii* and the *S. reidi* symbionts are able to maintain an intracellular existence in eukaryotic hosts. *Coxiella burnetii*, however, is capable of growth in animal cell lines (e.g., Woldehiwet 2004) and pathogenically infects a wide range of hosts (Niemczuk and Kondracki 2004; Watanabe 2004; Woldehiwet 2004). In addition, *A. oxaliferum*, a freshwater sulfur-oxidizing bacterium, falls out with the nematode and oligochaete symbionts clade. *Achromatium oxaliferum* occurs in freshwater sediments along the redox zone where it has access to sulfide and oxygen (Head et al. 1996; Glockner et al. 1999; Gray et al. 1999). As cultivation methods improve and sequences are added to the 16 rRNA gene database, other free-living bacteria that are closely related to symbionts will likely be identified. Indeed, recent studies incorporating 16S rRNA gene sequences from unidentified environmental clones into phylogenetic analyses of free-living and symbiotic bacteria suggest that chemosynthetic symbionts may in fact resolve into three distinct clades (N. Dubilier, personal communication; Duperron et al. 2004). Free-living relatives of chemosynthetic symbionts should reveal much about the ecological and evolutionary constraints on the symbiont as well as about the potential for gene loss during the transition from the free-living to the symbiotic state.

## Symbiont Characterization

### Enzyme Activities

Researchers routinely demonstrate chemoautotrophy or methanotrophy in symbionts by the activity or presence of diagnostic enzymes. Indeed, given the inability to culture chemoautotrophic symbionts, detection of such enzymes is often the only evidence used to infer symbiont metabolism. This characterization often involves physiological assays using tissue or purified protein extract, immunodetection, or PCR-based gene probing. Such studies were initially conducted on the

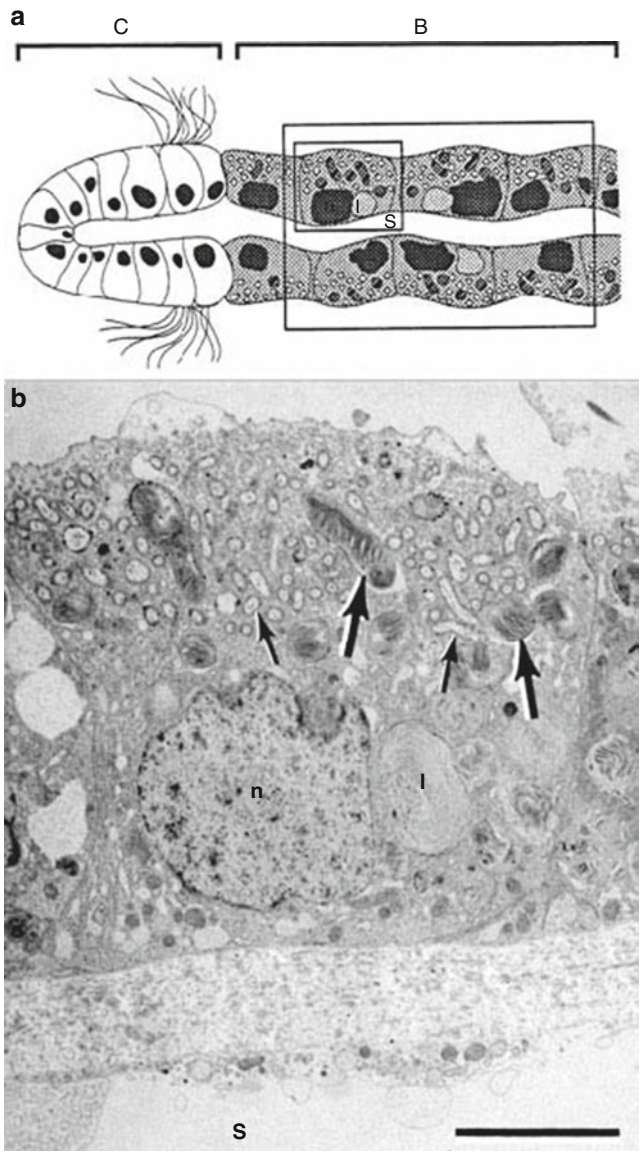


**Fig. 23.14**

***Bathymodiolus puteoserpentis*. Transverse section of Mid-Atlantic Ridge (MAR) mytilid gill filament, showing symbiont-containing gill epithelial cells (bacteriocytes). (a) Diagram of gill filament. Bacteriocytes are confined to the region proximal to the ciliated border of the gill. *Small box* shows positions of Figs. 21.14b. (b) symbiont-containing bacteriocyte region and (C) symbiont-free ciliated region. (b) Transmission electron micrograph. Large and small symbionts (*large* and *small arrows*, respectively) are located in the apical region of the cells, while nuclei and lysosomal residual bodies occupy the region closest to the blood sinus. Note centrally stacked intracytoplasmic membranes in large symbionts. *1* lysosomal residual body, *n* bacteriocyte nucleus, and *s* blood sinus. Scale bar: 5 μm (From Distel et al. (1995), with permission)**

tubeworm *Riftia pachyptila*. For example, Felbeck (1981) assayed tubeworm trophosome tissue for the activities of key enzymes of the Calvin cycle, the $CO_2$-fixing enzyme, ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO), and phosphoribulose kinase (PRK) as well as enzymes associated with the oxidation of reduced inorganic sulfur compounds. The activity or presence of RubisCO has subsequently been used to diagnose symbiont autotrophy in a diversity of host species, including all of the chemoautotroph-harboring invertebrates listed in *Table 23.1* (excluding alvinellid polychaetes; see below): shallow water solemyid and lucinid bivalves, vent and seep tubeworms and bivalves (including mussels hosting both methanotrophic and chemoautotrophic symbionts), nematodes, oligochetes, shrimp, sea urchins, and ciliates (Felbeck et al. 1981; Cavanaugh 1983; Cavanaugh et al. 1988; Polz et al. 1992; Johnson et al. 1994; Nelson et al. 1995; Bauer-Nebelsick et al. 1996; Krieger et al. 2000; Elsaied et al. 2002; Fiala-Médioni et al. 2002). While RubisCO has not been detected in the alvinellid epibionts, genes encoding citrate lyase, a key enzyme of the reductive tricarboxylic acid (TCA) cycle, recently have been detected via analyses of symbiont DNA sequences, suggesting that the Epsilonproteobacteria symbionts of alvinellid worms fix carbon via this pathway (Campbell et al. 2003).

Enzymes involved in chemosynthetic energy generation have also been used to characterize these symbionts. Although the sulfur metabolism enzymes are not unique to sulfur oxidation, certain enzymes such as ATP sulfurylase, when detected in high activities, have been used to infer sulfur-based chemolithotrophy (Felbeck 1981; Fisher et al. 1993; Laue and Nelson 1994). As methane monoxygenase, the enzyme that catalyzes the first step in the oxidation of methane in aerobic methanotrophs, is notoriously labile (Prior and Dalton 1985; Cavanaugh 1993), methanol dehydrogenase (MeDH), the enzyme that catalyzes the second oxidation step (i.e., methanol to formaldehyde) and is known to occur only in methylotrophs, has been used extensively to diagnose methanotrophy. MeDH has been detected in gill extracts of mussels hosting methanotrophs or both methanotrophs and thioautotrophs (Cavanaugh et al. 1992; Fisher et al. 1993; Robinson et al. 1998; Fiala-Médioni et al. 2002; Pimenov et al. 2002; Barry et al. 2002) and in a deep-sea sponge (Vacelet et al. 1996). Such enzymatic evidence strongly suggests methanotrophy, particularly when coupled with ultrastructural observations showing symbionts with the complex intracytoplasmic membranes that are characteristic of type I methanotrophs.

## Stable Isotope Signatures

### Carbon Isotopes

In addition to enzymology, stable isotope data provided some of the first evidence in support of chemoautotrophy in marine invertebrate-bacteria symbioses (e.g., Rau and Hedges 1979; Spiro et al. 1986) and continue to be useful in assessing symbiont metabolism and tracking energy and carbon transfer in chemosynthetic symbioses (Colaco et al. 2002; Levin and Michener 2002; Van Dover 2002; Robinson et al. 2003; Scott et al. 2004). Because enzymes involved in distinct carbon fixation pathways discriminate differently against the use of the heavier carbon isotope ($^{13}C$), the stable carbon isotope ratio comparing $^{13}C$ to

$^{12}C$ ($\delta^{13}C$) can be used to help distinguish different autotrophic metabolisms. For example, whereas $\delta^{13}C$ values of marine phytoplankton typically vary between µ18‰ and µ28‰ (Fry and Sherr 1984; Gearing et al. 1984; Goericke et al. 1994), carbon derived chemosynthetically at vents is either considerably lighter (enriched in $^{12}C$), with $\delta^{13}C$ values from µ27‰ to µ35‰, or heavier (depleted in $^{12}C$), with values from µ9‰ to µ16‰ (Childress and Fisher 1992; Robinson and Cavanaugh 1995; Robinson et al. 2003). Depending on the source of methane, symbioses between mussels and methanotrophic bacteria may be even more depleted in $^{13}C$, with $\delta^{13}C$ ranging from µ37‰ to µ78‰ (Cavanaugh 1993; Nelson and Fisher 1995; Barry et al. 2002).

Because consumers generally retain the carbon isotopic signature of their food (i.e., "you are what you eat"; DeNiro and Epstein 1979), comparisons between $\delta^{13}C$ signatures of symbiont-containing host tissue and symbiont-free host tissue can be used to study the transfer of symbiont-derived carbon to the host. For example, $\delta^{13}C$ values (µ30.8‰–µ35.8‰) in symbiont-containing gill tissue from the western Pacific vent mussel *Bathymodiolus brevior* were significantly lower than values from symbiont-free foot tissue, suggesting that *B. brevior* supplements its diet via filter feeding on photosynthetically derived carbon (Dubilier et al. 1998). In contrast, other studies show a high dependence on symbiont carbon by host species, including coastal solemyid protobranchs (Fisher and Childress 1986; Conway and Capuzzo 1991), thyasirid clams (Dando and Spiro 1993; Fiala-Médioni et al. 1993), and vestimentiferan tubeworms (Kennicutt et al. 1992), as well as suggest differences in the contribution of methanotrophs and chemoautotrophs to host carbon in mussels containing dual symbioses (Cavanaugh 1993; Trask and Van Dover 1999; Fiala-Médioni et al. 2002; Yamanaka et al. 2003).

Stable carbon isotope signatures have also been used to detect chemosynthetic symbioses in fossil bivalves of the clam family Lucinidae, whose extant members all host chemosynthetic symbionts. CoBabe (1991), by determining the $\delta^{13}C$ values of organic matrix material extracted from lucinid fossils dating to ca. 120,000 ya, showed that fossilized lucinid (*Epilucina* sp.) shells ($\delta^{13}C$ = µ25‰) were about 5‰ lighter than values from other bivalves collected in the same deposit (Pt. Loma, CA). Also, the organic matter of lucinid fossils was similar to that from modern samples, implying that the fossil organic matrix did not decay or change significantly over time. These results, along with strong evidence showing that shell $\delta^{13}C$ values are reasonable proxies for tissue values in extant species, suggest that the fossil lucinid hosted chemosynthetic symbionts ca. 120,000 ya (CoBabe 1991). Thus, stable carbon isotope analysis may be an effective tool for tracing the evolution of chemosynthetic symbioses in the fossil record.

One of the main factors affecting stable carbon isotope signatures of chemoautotroph-invertebrate symbioses appears to be the form of RubisCO used by the symbionts. While $\delta^{13}C$ values for vent bivalves hosting sulfide-oxidizing symbionts cluster between µ27‰ and µ35‰ and resemble values for free-living chemoautotrophic bacteria, values for vent tubeworms, shrimp episymbionts, and many free-living bacterial mats at vents are significantly heavier, ranging from µ9‰ to µ16‰ (Childress and Fisher 1992; Van Dover and Fry 1994; Robinson and Cavanaugh 1995; Cavanaugh and Robinson 1996; Robinson et al. 2003). The difference between these groups relates to the form of RubisCO used to fix $CO_2$ by the symbionts, with form I RubisCO occurring in most members of the isotopically lighter group and form II in all members of the heavier tubeworm group (Robinson and Cavanaugh 1995). Corroborating this hypothesis, Robinson et al. (2003) showed that the kinetic isotope effect (ε value), the relative rate of $^{13}CO_2$ to $^{13}CO_2$ fixation ($^{12}k/^{13}k$) and a measure of discrimination against $^{13}C$ by the purified RubisCO enzyme in vitro, is significantly lower for form II RubisCO from *Riftia pachyptila* symbionts (ε = 19.5‰) than for the form I enzyme (ε = 22–30‰). Such variation may have arisen from evolution under differing concentrations of $CO_2$ and $O_2$ (Robinson et al. 2003).

But isotopic discrimination by RubisCO does not fully account for $^{13}C$-enrichment in these symbioses. For example, to explain the discrepancy in *R. pachyptila* biomass $\delta^{13}C$ values, Scott (2003) used a mass balance model to show that steep gradients in [$CO_2$] among symbiont, host, and environmental pools may drive $^{13}C$-enrichment. RubisCO, which occurs in the symbiont cytoplasm, preferentially fixes $^{12}CO_2$, leaving $^{13}CO_2$ behind. If fixation is rapid, $CO_2$ equilibration between the isotopically lighter host cytoplasm and the isotopically heavier symbiont cytoplasm cannot occur, causing RubisCO to draw from a more enriched $^{13}CO_2$ pool and accounting for the relatively heavy $\delta^{13}C$ of tubeworm biomass. Further, stable carbon isotope values are also affected by the $\delta^{13}C$ of the environmental carbon pool (Fisher 1995; Colaco et al. 2002). Scott et al. (2004) demonstrated that the "light" $\delta^{13}C$ values of the symbionts of the coastal protobranch clam *Solemya velum* are explained not only by the kinetic isotope effect of symbiont form I RubisCO (ε = 24.5‰) but also by the $\delta^{13}C$ value of the $CO_2$ in the sediment.

Similarly, the source of methane production can significantly impact the isotopic signature of methanotroph symbioses (Fisher 1995; MacAvoy et al. 2002). The $\delta^{13}C$ of methane varies considerably depending on whether it is produced thermogenically ($\delta^{13}C$ of $CH_4$ > µ45‰) or biologically by methanogens ($\delta^{13}C$ of $CH_4$ < µ60‰; Lilley et al. 1993; Fisher 1995), and this variability is reflected in the $\delta^{13}C$ values of chemosynthetic symbioses (Fisher 1995). Therefore, in instances where the $\delta^{13}C$ of the source methane is unknown, conclusions about the contribution of methanotrophic symbionts to host $\delta^{13}C$ values should be interpreted with caution (Fisher 1995). In addition, interpreting $\delta^{13}C$ signatures may be especially problematic for dual symbioses in which both methanotrophic and thioautotrophic symbionts co-occur in the same host cell. In these symbioses, the $\delta^{13}C$ values of the symbionts and the host reflect a mix of methanotrophic and thioautotrophic metabolism (Fisher 1995). These signatures are potentially confounded in instances when the thioautotrophic symbionts use the $CO_2$ respired by the methanotrophs, resulting in a second discrimination against an already light pool of $CO_2$ and an anomalously light tissue $\delta^{13}C$ value (Fisher 1993).

Thus, while $\delta^{13}C$ values often provide the first evidence that chemoautotrophic or methanotrophic symbioses occur in certain animal species, researchers must recognize that $\delta^{13}C$ is inherently responsive to physical, environmental, and enzymatic factors. Stable carbon isotope signatures therefore should not be used apart from other corroborating evidence (e.g., physiological and enzyme activity assays and genetic characterization) to identify carbon fixation pathways or methane oxidation in chemosynthetic symbioses (Fisher 1995; Scott 2003; Scott et al. 2004).

#### Sulfur and Nitrogen Isotopes

In addition to carbon isotopes, stable isotopes of sulfur and nitrogen are also used to study sources and metabolism of these elements in symbioses. The extent to which different sources of reduced sulfur—geothermal production in vent fluid or microbial sulfate reduction in bottom sediment—support thioautotrophic metabolism has been inferred from the $\delta^{34}S$ value of biological samples. Such analyses revealed hydrothermally derived sulfide as the dominant sulfide source for deep-sea vent symbioses (Fry et al. 1983; Yamanaka et al. 2003). In contrast, symbiotic bacteria within a shallow water vestimentiferan tubeworm, *Lamellibrachia satsuma* (Miura et al. 2002), and the protobranch, *Solemya velum* (Conway et al. 1989), rely predominantly on sulfide derived from microbial sulfate reduction.

Similarly, $\delta^{15}N$ values, because they vary predictably and largely between producer and consumer trophic levels (increase of ca. 3.4 per level), are particularly useful markers for studying aquatic food web interactions (Minagawa and Wada 1984). In general, $\delta^{15}N$ values of chemoautotrophic organisms are significantly lighter (<0‰; Van Dover and Fry 1994) than values for photosynthetic organisms (>6‰; see Michener and Schell (1994) and Fisher (1995)). Researchers have used this discrepancy and the predictable trophic level fractionation of $^{15}N$ to show host reliance on symbiont-derived organic matter in a number of symbioses including the coastal clams *Solemya velum* and *S. borealis* (Conway et al. 1989, 1992) and in vent mussels from the Mid-Atlantic Ridge (MAR) and the Galapagos Rift (Fisher et al. 1988; Trask and Van Dover 1999). In addition, $\delta^{15}N$ values have been used extensively in conjunction with $\delta^{13}C$ values to show the flow of chemosynthetically derived organic matter through vent food webs, including those on the MAR (Vereshchaka et al. 2000; Colaco et al. 2002), the Central Indian Ridge (Van Dover 2002), and the Galapagos Rift (Fisher et al. 1994). As with $\delta^{13}C$ data, $\delta^{15}N$ values vary considerably among sites; $\delta^{15}N$ may depend in part on the $\delta^{15}N$ of the dissolved inorganic nitrogen (DIN) pool, the proportions and $\delta^{15}N$ values of different components ($NH_4^+$, $NO_3^{\mu 2}$, $NO_2^{\mu}$, and urea) in the DIN pool (Waser et al. 1998; Colaco et al. 2002), the uptake kinetics of different DIN assimilation pathways (Waser et al. 1998; Krueger 1996), and, as shown for vent shrimp (Vereshchaka et al. 2000) and mussels (Trask and Van Dover 1999), the ontogenetic stage of the host. Therefore, as noted above with stable carbon isotopes, in the absence of additional enzymatic, genetic, and environmental data, caution must be used when comparing $\delta^{15}N$ values from different habitats and species.

## Ecophysiology

Symbioses between chemosynthetic bacteria and marine invertebrates must acquire all of the substrates necessary for chemosynthetic metabolism: reduced sulfur or methane, oxygen, dissolved inorganic carbon (DIC, as $CO_2$ or $CH_4$), and other nutrients (e.g., nitrogen and phosphorus) for use in biosynthesis. In particular, to support energy generation, these symbioses must obtain substrates from both oxic and anoxic environments. To meet these demands, the host-symbiont association relies on specialized biochemistry, physiology, and behavior. These adaptations are best studied in thioautotrophic endosymbioses and are discussed primarily within this context below.

### Spanning the Oxic-Anoxic Interface

Access to both oxygen and reduced chemicals is necessary for aerobic respiration by chemosynthetic symbionts. Specifically, thioautotrophs shuttle electrons from reduced sulfur (e.g., sulfide) to a terminal electron acceptor during oxidative phosphorylation, generating a proton gradient that drives ATP synthesis. Though some thioautotrophic symbionts (such as those in the tubeworm *Riftia pachyptila* (Hentschel and Felbeck 1993) and the clam *Lucinoma aequizonata* (Hentschel et al. 1993)) may use nitrate as an electron acceptor during periods of anoxia, most thioautotrophic symbionts typically use molecular oxygen for respiration. Similarly, methanotrophs must obtain oxygen for respiration as well as methane for both energy generation (via methane oxidation) and carbon assimilation (Anthony 1982).

This dual requirement for oxygen and reduced compounds poses unique problems for thioautotrophs and methanotrophs. First, these organisms must obtain energy substrates from mutually exclusive environments—oxygen is absent or at very low levels in the anoxic zones from which sulfide or methane is typically obtained. Second, sulfide, the predominant energy source for thioautotrophy, spontaneously reacts with oxygen to form less-reduced sulfur compounds ($S^0$, $S_2O_3^{\mu 2}$, or $SO_4^{\mu 2}$; Zhang and Millero 1993), thereby decreasing the availability of substrates for thioautotrophy. Though such abiotic oxidation may be several orders of magnitude slower than biological sulfide oxidation (Millero et al. 1987; Johnson et al. 1988), thioautotrophic symbioses must still compete with oxygen for free sulfide. Also, in habitats containing both sulfide and methane, abiotic oxidation of sulfide may limit the oxygen available for methanotrophy. These limitations force free-living thioautotrophs and methanotrophs into microaerophilic zones at the interface, or chemocline, between oxic (e.g., water column) and anoxic (e.g., vent fluid and sediment pore water) habitats. Such free-living bacteria demonstrate unique

mechanisms to support life at the oxic-anoxic interface; these adaptations may be behavioral (e.g., tracking the chemocline via gliding by *Beggiatoa*), anatomical (e.g., keeping cells in the chemocline via "veil" formation by *Thiovulum* or creation of a filamentous sulfur matrix by *Arcobacter*), biochemical (e.g., internal or external sulfur deposition that serves as an electron source or sink when sulfide or oxygen is limiting, as by *Beggiatoa* and *Arcobacter*), or developmental (e.g., resting stage formation by methanotrophs; ❯ *Table 23.2* and references therein).

Symbiosis thus may be viewed as an adaptation to simultaneously obtain sulfide (or methane) and oxygen from anoxic-oxic interfaces, allowing thioautotroph or methanotroph symbionts, via association with a eukaryotic host, to circumvent many of the problems of sulfide acquisition (Cavanaugh 1985). Similarly to free-living sulfur bacteria, thioautotrophic symbioses use specialized behavioral, anatomical, or physiological mechanisms, either to spatially or temporally bridge sulfidic and oxic zones or to simultaneously sequester sulfide and oxygen (Cavanaugh 1994; Fisher 1996; Polz et al. 2000). For instance, the cold seep vestimentiferan tubeworm *Lamellibrachia* cf. *luymesi* acquires oxygen via its anterior plume while extending a posterior section of its tube (the root) deep into the sediment to acquire sulfide (Julian et al. 1999; Freytag et al. 2001). Similar burrowing tactics occur in some species of symbiont-containing thyasirid clams, which possess a superextensile foot (up to 30 times the length of the shell) that burrows into the sediment to access hydrogen sulfide (Dufour and Felbeck 2003), and in protobranchs of the genus *Solemya*, which dig Y-shaped burrows in reducing sediments to allow simultaneous pumping of oxygenated water from above and sulfide-rich pore water from below (Stanley 1970; Cavanaugh 1983; ❯ *Fig. 23.11*). Also, shrimp, nematodes, and oligochaetes migrate vertically along the oxygen-sulfide gradient or between separate oxic and anoxic zones, thereby enabling their symbionts to simultaneously access both energy substrates or to store reduced sulfur compounds for later oxidation (Polz et al. 2000).

The vent tubeworm *Riftia pachyptila* possesses a remarkable biochemical adaptation to simultaneously acquire sulfide and oxygen. *R. pachyptila* produces coelomic and vascular hemoglobins that, in contrast to most invertebrate and vertebrate hemoglobins, can bind oxygen in the presence of sulfide (Arp et al. 1985, 1987; Childress et al. 1991; Zal et al. 1996). *R. pachyptila* appears to preferentially take up $HS^{\mu}$ from the surrounding fluid, despite a large $H_2S$ gradient from tubeworm blood to the environment (Goffredi et al. 1997a). The $HS^{\mu}$ diffuses across the plume of the worm (Goffredi et al. 1997a) and then binds reversibly and independently of $O_2$ at two free cysteine residues, each located on a distinct globin type (Zal et al. 1997, 1998; Bailly et al. 2002). These residues are well conserved in both symbiont-containing and symbiont-free annelids from sulfidic environments but are absent in annelids from sulfide-free habitats (Bailly et al. 2002, 2003). Bailly et al. (2003) suggest that the sulfide-binding function may have been lost via positive selection, if the sulfide-binding cysteine residues react disadvantageously with other blood components in the absence of sulfide.

**Table 23.2**
**Adaptations of thioautotrophs and methanotrophs for life at oxic-anoxic interfaces[a]**

| Adaptation | Example |
|---|---|
| Attachment | *Thiothrix* |
| Motility, chemotaxis | *Beggiatoa* and *Thioploca* |
| Elemental sulfur deposition | *Beggiatoa* and *Thiothrix* |
| Nitrate and sulfur storage | *Thiomargarita* and *Thioploca* |
| Create own interface | *Thiovulum* |
| Filamentous sulfur production | *Arcobacter* sp. |
| Resting cysts | Methanotrophs |
| Associate with eukaryote | Thioautotroph and methanotroph symbionts |

[a]From Anthony (1982), Jørgensen and Postgate (1982), Cavanaugh (1985), Schulz et al. (1999), and Wirsen et al. (2002)

Extracellular hemoglobins that simultaneously bind sulfide and oxygen are absent in most other marine invertebrates that host sulfide-oxidizing symbionts (Weber and Vinogradov 2001); such organisms have evolved other mechanisms for regulating sulfide toxicity and delivery. For instance, the vesicomyid clam *Calyptogena magnifica* synthesizes a di-globular, nonheme molecule that readily binds free sulfide within the blood serum, perhaps via zinc residues (Arp et al. 1984; Zal et al. 2000). Also, several thioautotroph-containing species, including the vent mussel *Bathymodiolus thermophilus* and the coastal clam *Solemya velum*, appear to mediate detoxification in part by storage of sulfur in amino acids (e.g., taurine and thiotaurine; Conway and Capuzzo 1992; Pruski et al. 2000a; Joyner et al. 2003; Pruski and Fiala-Médioni 2003). Indeed, thiotaurine may be used effectively as a biomarker of thioautotrophic symbioses (Pruski et al. 2000b).

Other host organisms, including some bivalve mollusks, apparently avoid sulfide toxicity via mitochondrial oxidation of sulfide. Powell and Somero (1986) first demonstrated mitochondrial sulfide oxidation in the coastal protobranch *S. reidi*. The authors showed that mitochondria isolated from the gill and foot of *S. reidi* exhibit ADP-stimulated oxygen uptake and ATP synthesis following the addition of sulfide. On the basis of the effects of cytochrome and reduced nicotinamide adenine dinucleotide (NADH) oxidase inhibitors, electrons from sulfide oxidation appear to enter the respiratory chain at cytochrome *c* in *S. reidi* mitochondria (Powell and Somero 1986). Further characterization of this system using $^{35}S$ showed that sulfide is oxidized exclusively to thiosulfate (O'Brien and Vetter 1990), a nontoxic intermediate that can function as the energy source in symbiotic carbon fixation. Subsequently, researchers have

demonstrated mitochondrial sulfide oxidation across a wide range of organisms, including polychaete worms, clams, fishes, and chickens (Grieshaber and Volkel 1998; Yong and Searcy 2001). These data lend credence to the hypothesis that mitochondria evolved from sulfide-oxidizing endosymbiotic bacteria (Searcy 1992).

Readers should consult several additional reviews (e.g., Cavanaugh 1994; Fisher 1996; Polz et al. 2000) for a more extensive discussion of the remarkable adaptations used by chemoautotrophic symbioses to sequester both oxygen and reduced chemicals across oxic-anoxic zones.

## Carbon Uptake and Transport

In addition to oxygen and reduced sulfur compounds, thioautotrophic symbionts utilizing the Calvin cycle require $CO_2$ for autotrophic carbon fixation. Acquisition of $CO_2$ is not trivial given that relative concentrations of the three distinct chemical species ($CO_2$, $HCO_3^{\mu}$, and $CO_3^{\mu 2}$) in the dissolved inorganic carbon (DIC) pool can vary considerably depending on pH ($pK_a$ of 6.4 for $CO_2$:$HCO_3^{\mu}$ at 25 °C; see the section "Habitat Chemistry" in this chapter). In general, the majority of DIC in seawater (pH ~ 8.0) is $HCO_3^{\mu}$. But at vents the typically lower pH of the mixed vent fluid and ambient bottom water generates higher concentrations of $CO_2$, giving organisms that use the Calvin cycle a distinct advantage.

The tubeworm *Riftia pachyptila* provides an interesting model in which to study the uptake and transport of DIC. Goffredi et al. (1997b) demonstrated that for *R. pachyptila*, pH plays an important role in DIC uptake. The acidity of diffuse vent fluid (pH ca. 6) around tubeworms ensures that $CO_2$ ($pK_a$ of 6.1 at in situ temperature and pressure of ca. 10 °C and 101.3 kPa; Dickson and Millero 1987) is the dominant DIC form in the vent environment. This contrasts with the vascular fluid of the worm, which has an alkaline pH of 7.1–7.5, apparently because of the action of $H^+$-ATPases (Goffredi et al. 1999; Goffredi and Childress 2001; Girguis et al. 2002). The alkaline pH inside *Riftia* results in rapid conversion of $CO_2$ to $HCO_3^{\mu}$, which, because of its negative charge, cannot diffuse out of the worm; this in effect creates a bicarbonate "trap" (Childress et al. 1993). Thus, a gradient of higher external [$CO_2$] to lower internal [$CO_2$] develops across the tubeworm plume and drives diffusion of DIC into the blood (Childress et al. 1993; Goffredi et al. 1997b; Scott 2003). Following diffusion into the plume, DIC (as $CO_2$ and $HCO_3^{\mu}$) is transported by the vascular system to the symbiont-containing trophosome. Here, carbonic anhydrase, the enzyme that reversibly converts $CO_2$ into $HCO_3^{\mu}$ in both prokaryotes and eukaryotes, may play a role in converting $HCO_3^{\mu}$ into $CO_2$, the DIC species used by RubisCO (Kochevar and Childress 1996; De Cian et al. 2003a, b). As discussed above for *Riftia*, DIC incorporation into symbiont biomass occurs via $CO_2$ fixation by a form II RubisCO of the Calvin-Benson cycle. Rapid $CO_2$ fixation rates create steep internal [$CO_2$] gradients between symbiont and host cytoplasm that may, in combination with the relatively low discrimination of form II RubisCO against $^{13}C$, result in a $^{13}C$-enriched signature of symbiont and host biomass (Robinson et al. 2003; Scott 2003).

In chemosynthetic endosymbioses, the host benefits by obtaining part or all of its nutrition from the symbiont, via two potential transfer mechanisms: the host may assimilate autotrophically fixed carbon that has been released by the symbiont and translocated to host cells in the form of soluble organic molecules, or the host may directly digest bacterial cells. Radiotracer analysis and microscopy have proven particularly useful in studying host nutrition. For example, Fisher and Childress (1986) showed a rapid (within hours) appearance of radiolabeled carbon in the symbiont-free tissues of the host clam *Solemya reidi* following exposure to $^{14}C$-labeled bicarbonate, suggesting release of fixed carbon by the symbiont population. In contrast, a slow (1–5 days) transfer of labeled organic carbon from methanotroph-containing tissue to symbiont-free tissue of a seep mussel exposed to $^{14}C$-labeled methane was inferred to be due to initial $^{14}CH_4$ incorporation by the symbionts with host digestion of symbionts occurring later (Fisher and Childress 1992). Electron microscopy showing symbionts being degraded in the basal region of bacteriocytes in other methane-based and dual chemoautotroph-methanotroph mussel symbioses supports this interpretation (Cavanaugh et al. 1992; Barry et al. 2002), as does the detection of lysosomal enzymes in the gills of the vent bivalves *Calyptogena magnifica* and *Bathymodiolus thermophilus* (Fiala-Médioni et al. 1994; Boetius and Felbeck 1995) and the shallow water clam *Lucinoma aequizonata* (Boetius and Felbeck 1995).

In the *R. pachyptila* tubeworm symbiosis, the transfer of carbon from symbiont to host appears to occur via both translocation and digestion (Bright et al. 2000). Felbeck (1985) and Felbeck and Turner (1995) documented a rapid (within seconds) appearance of labeled succinate and malate in trophosome tissue and in vascular and coelomic blood following exposure of whole worms (in pressure vessels) and plumes to $^{14}C$-bicarbonate. Subsequently, Felbeck and Jarchow (1998) showed that succinate, malate, and several other organic acids and sugars were excreted by purified suspensions of *R. pachyptila* symbionts, suggesting that these simple organic compounds might be important intermediates in the transfer of fixed carbon from symbionts to host. Corroborating these data, Bright et al. (2000), using pulse labeling analysis, showed that the bulk of organic carbon assimilated into *R. pachyptila* tissue is first released by metabolically active bacteria at the center of a trophosome lobule. However, these authors also showed that a smaller fraction of host carbon is obtained by digestion of bacterial cells at the lobule periphery (Bright et al. 2000). This evidence for digestion is supported by prior studies showing degenerative stages of bacteria within the *R. pachyptila* trophosome (Bosch and Grassé 1984; Hand 1987). In addition, relatively high lysozyme activity in *Riftia* tissue further suggests that digestion of symbionts plays a role in tubeworm nutrition (Boetius and Felbeck 1995).

### Nitrogen

The partners in a symbiosis must also acquire all of the other macro- and micronutrients, particularly nitrogen and phosphorus, for use in the biosynthesis of organic compounds. Currently, very little is known about how various forms (inorganic and organic) of phosphorus are transferred to and among different pools within chemoautotrophic endosymbioses. Most studies have focused on nitrogen metabolism, using a combination of enzyme characterizations and physiological experiments to elucidate nitrogen assimilation pathways. Nitrate ($NO_3^{\mu 2}$), which is abundant at vents (in situ concentrations of ~40 μM; Johnson et al. 1988), appears to be the predominant nitrogen source for vent symbioses. For example, Lee et al. (1999) demonstrated the activity of nitrate reductase, a bacterial enzyme involved in converting nitrate to ammonia for either assimilatory or respiratory purposes, in the vent tubeworms *Riftia pachyptila* and *Tevnia jerichonana* and the mussel *Bathymodiolus thermophilus*. In addition, the ammonia assimilation enzymes glutamine synthetase (GS) and glutamate dehydrogenase (GDH) were detected in these symbioses, and almost all GS activity in symbiont-containing tissue was shown to be due to enzyme produced by the bacterial symbiont and not the host (Lee et al. 1999). Supporting these data, physiological experiments on *R. pachyptila* kept in pressurized chambers showed that the symbiont population reduces nitrate to ammonia not for respiratory purposes but for incorporation into both symbiont and host biomass (Girguis et al. 2000). However, in *R. pachyptila*, high GS activity also occurred in symbiont-free branchial plume tissue, suggesting that the host may also be involved in assimilation of ammonia from the vent environment (Minic et al. 2001). But further enzymatic characterization of *Riftia* tissues demonstrated that the tubeworm depends on its symbionts for the de novo synthesis of pyrimidine nucleotides (Minic et al. 2001) as well as for the biosynthesis of polyamines (Minic and Herve 2003), suggesting that the trophosome is a primary site for nitrogen assimilation and metabolism.

In contrast, in the thioautotrophic symbiosis involving the shallow water clam *Solemya reidi*, inorganic nitrogen is readily assimilated in the form of ammonia (Lee and Childress 1994), which is abundant in the shallow water, nutrient-rich habitats of the clam (e.g., sewage outfalls). Ammonia incorporation rates are highest in the symbiont-containing gill tissue, and the sulfur-containing amino acid taurine appears to be a major end product of ammonia assimilation (Lee et al. 1997). The mechanisms by which chemosynthetic symbionts, particularly those contained within the cells of invertebrate hosts (such as *Solemya* and *Riftia*), acquire all of the other macro- and micronutrients for biosynthesis have yet to be characterized.

## Ecology and Evolution

### History

Prior to the use of molecular techniques, researchers considered vent taxa to be relic species. These organisms, whose strange morphologies suggest a primitive state, purportedly survived past extinction events due to the relative isolation of vents from the photic zone (McArthur and Tunnicliffe 1998). This perception of vents as ancient ecosystems is supported by the fossil record, which shows that over 80 % of vent species are found only at vent sites (Tunnicliffe 1991, 1992; Little et al. 1997; Little and Vrijenhoek 2003) and that the oldest vent site dates to the Silurian (~430 Mya; Little et al. 2004). But the fossil record for vents is relatively poor. There are only 19 known fossilized vent sites on the planet, perhaps because calcium carbonate structures dissolve relatively quickly in vent fluids (Hunt 1992; Kennish and Lutz 1999). Also, studying vent fauna evolution based on the morphological characters of fossils is problematic if much of the specimen has degraded or if the preserved character is plastic or isomorphic. In particular, vestimentiferan tubeworms are known for the phenotypic plasticity of their tubes (Southward et al. 1995; Black et al. 1998).

In contrast, molecular evidence suggests that vent taxa evolved more recently (22–150 Mya; later-Mesozoic and Cenozoic) and suggests an alternative hypothesis to vent taxa as living relics: vents were recently populated from shallow seeps or whale falls (Van Dover et al. 2002; Hurtado 2002). Indeed, the communities most similar to those of vents occur at seeps. Compared to the spatially and temporally patchy distribution of vent fossils (with most being concentrated in the Silurian and Devonian rocks of the Ural Mountains), seep fossils are ubiquitous (Little and Vrijenhoek 2003). At least 50, and perhaps as many as 200, fossilized seep sites dating from the Devonian to the Pleistocene have been uncovered. These specimens are much better preserved than most vent fossils and include extant vent taxa not yet uncovered at fossil vent sites (e.g., vesicomyids, thyasirids, mytilids, and solemyids; Little and Vrijenhoek 2003). This greater diversity supports the seeps-to-vents hypothesis. However, opponents argue that the vent fossil record has been greatly affected by high calcium carbonate dissolution rates (Little and Vrijenhoek 2003).

While the discrepancy between the evolutionary histories suggested by the fossil and molecular evidence needs to be resolved, it must also be stressed that these data are not evidence for chemosynthetic symbioses. In a unique study, CoBabe (1991) was able to deduce a chemosynthetic symbiosis by analyzing the organic matrix from fossil lucinid shells using stable carbon isotopes. This result is encouraging and suggests that both the age of these organisms and their symbiosis can be addressed using current methods.

### Organism Interactions

In the relatively featureless and nutrient poor deep sea, vent and seep environments are ecological oases (Laubier 1989). Initially, free-living chemoautotrophic bacteria were hypothesized to provide the bulk of primary production in these communities (Lonsdale 1977). Indeed, at some vent sites, suspended bacteria or bacteria in surface-attached mats are a large food source for

higher trophic levels (Humes and Lutz 1994; Van Dover 2000). But the dominant strategy for the major vent and seep fauna is symbiosis with chemoautotrophic bacteria (Cavanaugh 1994), and these symbioses significantly influence the ecology of the nonsymbiotic community. Not only are chemosynthetic symbioses a major and stable source of organic carbon (Sarrazin and Juniper 1999), but as biogenic structures, they also provide living space for a diversity of species in an otherwise two-dimensional landscape of basalt or sediment (Bergquist et al. 2003). For example, the tubes of chemosynthetic vestimentiferans support mussels, sponges, and limpets, many of which host their own chemosynthetic symbionts (Yamamoto et al. 2002; Bergquist et al. 2003; Bates et al. 2004).

Vent symbioses may also significantly impact the free-living bacterial community by providing increased surface area for attachment. Free-living bacteria that cluster phylogenetically with known chemoautotrophic and heterotrophic groups have been isolated from tubeworm surfaces (Lopez-Garcia et al. 2002; Yamamoto et al. 2002). On the Mid-Atlantic Ridge, a single phylotype of shrimp episymbionts, which appear to be transmitted among hosts via the environment, represented over 60 % of the free-living bacteria (Polz and Cavanaugh 1995). This suggests that the host inoculates inanimate surfaces continuously, increasing the probability of symbiont attachment relative to the free-living community (Polz and Cavanaugh 1995). Such environmental inoculation may also occur in tubeworm and lucinid clam symbioses, in which the symbionts also appear to be transmitted environmentally (Durand and Gros 1996; Durand et al. 1996; Di Meo et al. 2000; Nelson and Fisher 2000; McMullin et al. 2003).

## Transmission Strategies and Effects on Symbiosis

The transmission strategy of a symbiosis reveals much about the evolutionary dynamics between host and symbiont. Symbiont transmission can occur environmentally (through a free-living population of symbiotic bacteria), horizontally (between contemporary organisms sharing the same habitat), or vertically (from parent to offspring). Vertically transmitted endosymbionts are effectively disconnected from their free-living counterparts. These symbionts experience elevated rates of mutation and fixation of slightly deleterious alleles because of genetic drift (Wernegreen 2002). For the most part, these evolutionary effects are due to a vastly different selective regime inside the host and a severely depreciated population size (Ohta 1973); endosymbionts undergo a population bottleneck upon host colonization and another upon transmission (Mira and Moran 2002). But the asexuality and lack of recombination in endosymbionts exacerbate these genetic problems through what is known as "Muller's rachet" (Muller 1964; Moran 1996). In Muller's ratchet, wild-type recombinants cannot be introduced into the endosymbiont population (Moran and Baumann 1994; Dale et al. 2003); genetic drift therefore occurs quickly, and the population cannot recover after fixation of deleterious alleles.

In contrast, symbiont populations that are environmentally transmitted are effectively larger and more genetically heterogeneous than populations transmitted vertically. Comparisons of 16S rRNA gene evolution between free-living bacteria, in which significant recombination occurs (Dykhuizen and Green 1991; Levin and Bergstrom 2000), and symbiotic chemosynthetic bacteria revealed unexpected differences in rates of evolution depending on mode of transmission (Peek et al. 1998). While chemoautotrophic, maternally transmitted endosymbionts did exhibit rapid evolutionary rates, consistent with their small population sizes, environmentally transmitted symbionts evolved more slowly than their free-living counterparts (Peek et al. 1998). The authors suggest that this slower rate of evolution could be caused by purifying selection in a large population. These results, however, were based on one gene across many lineages; a true genomic analysis of evolution in chemosynthetic endosymbionts is necessary to extend these findings.

Because chemosynthetic symbionts have yet to be cultured and their hosts are difficult to maintain in the laboratory, the transmission strategy of a symbiosis has been inferred by phylogenetic analysis or PCR-based detection of bacteria in host reproductive tissues or gametes. If the symbionts are maternally transmitted and the symbioses stable, congruence of host and symbiont phylogenies should occur (e.g., Chen et al. 1999; Thao et al. 2000; Degnan et al. 2004) and bacterial symbionts should be found in ovaries or oviducts of the host. Using these techniques, vertical transmission has been proposed for the solemyid protobranchs (Cary 1994; Krueger et al. 1996) and vesicomyid clams (Endow and Ohta 1990; Cary and Giovannoni 1993; Peek et al. 1998; Hurtado et al. 2003). Interestingly, although bacteria have been detected via PCR in the gonads of female hosts, this does not necessarily imply direct bacterial endocytic localization in host eggs. Indeed, in *Solemya reidi*, the internal contents of oocytes do not contain bacteria, and instead the transmission mechanism is thought to occur via ingestion; the larvae ingest the bacteria, which are then engulfed by hemocytes in the larval perivisceral cavity and transported to the developing gill (Gustafson and Reid 1988). The oligochaetes also exhibit an interesting mechanism of vertical transmission. During oviposition, the eggs appear to be infected with the symbiotic bacteria via the adult's genital pad (Giere and Langheld 1987). During the development of the larvae, many of the bacteria exist intracellularly, but as the animal matures, the symbionts take their primarily extracellular form.

However, of the putatively vertically transmitted symbioses, only associations involving vesicomyid clams show phylogenetic congruence between host and symbiont (Peek et al. 1998; Hurtado et al. 2003). Cospeciation does not appear to have occurred in the solemyid protobranchs (Durand et al. 1996; Krueger and Cavanaugh 1997) or in the mytilid mussels (McKiness 2004). When evaluating phylogenetic congruence, however, other factors that influence a phylogenetic reconstruction, such as geographic constraints, must be taken into account. Also, robust phylogenies with adequate taxa sampling for both host and symbiont are necessary; incomplete phylogenies may be hindering analyses of the solemyid and mytilid symbioses.

Lack of PCR-based evidence and phylogenetic incongruence has been used to infer an environmental mode of transmission for several of the chemosynthetic symbioses. For instance, the lucinid clams exhibit environmental transmission (Durand and Gros 1996; Gros et al. 1996, 1998, 2003a, b). Researchers have even been able to exchange symbionts between lucinid species without affecting the development of the juvenile animal (Gros et al. 2003a). In addition, vent tubeworms appear to acquire their symbionts from the environment (Distel and Cavanaugh 1994; Feldman et al. 1997; Laue and Nelson 1997; Di Meo et al. 2000; Nelson and Fisher 2000; McMullin et al. 2003), as evidenced in part by the presence of functional genes for sensing and responding to the environment as well as a flagellin gene in the *Riftia* symbiont (Hughes et al. 1997, 1998; Millikan et al. 1999). Indeed, the 16S rRNA phylotype has been detected in vent environments via both PCR and in situ hybridization, suggesting the vent tubeworm symbionts are environmentally transmitted (Harmer et al. 2004). While environmental transmission of tubeworm symbionts seems to be a potentially risky strategy, given the stochastic nature of environmental transmission and the complete dependence of the adult tubeworms on their symbionts for nutrition, detection of "wild" symbionts in conjunction with the phylogenetic evidence supports environmental transmission in this species.

The mechanism of transmission for the mytilid mussels remains largely unresolved; on the basis of varying evidence, researchers have suggested both vertical and environmental transmission. Vertical transmission in *Bathymodiolus thermophilus*, the thioautotroph-hosting mussel, was suggested in 1993, but evidence supporting this report is not yet published (Cary and Giovannoni 1993). In contrast, a recent study based on genetic and ultrastructural data of the chemoautotrophic symbionts of *B. azoricus*, a MAR mussel hosting both thioautotrophs and methanotrophs, indicated environmental acquisition of the chemoautotrophic symbionts (Won et al. 2003a; DeChaine et al. 2004). In addition, McKiness (2004) provided the first assessment of cospeciation between symbiont and host in *Bathymodiolus* mussels, analyzing molecular data for both methanotrophic and chemoautotrophic symbionts and testing phylogenetic congruence with the hosts. The results showed weak support for vertical transmission of the chemoautotrophic symbionts but provided no evidence for vertical transmission of the methanotrophs.

## Biogeography and Population Genetics

The view of hydrothermal vents as deep-sea islands frames questions of vent biogeography and population genetics. Compared to the relatively uniform and stable environment of the abyssal deep sea, hydrothermal vents are ephemeral, dynamic, and geographically fragmented. A chain of vents along a mid-ocean ridge resembles a chain of islands in an archipelago. However, genetic data for many vent species do not cleanly fit an "island" or "stepping-stone" model of biogeography (Vrijenhoek et al. 1998). Some host taxa do exhibit a decline in gene flow with increasing distance between sites (Black et al. 1994), as a stepping-stone model would predict (Kimura and Weiss 1964), while others show a more widespread gene flux (Karl et al. 1996) or appear to encounter barriers to dispersal other than distance (Black et al. 1998). These differences should be resolved with a greater understanding of the major variables affecting vent biogeography, including larval development and dispersal, symbiont distribution, oceanic flow, and past and current bathymetry. This section focuses predominantly on host biogeography because research on the population genetics and biogeography of bacterial symbionts is lacking. Understanding host population dynamics, however, does provide valuable insight into the distribution of the chemosynthetic symbionts to which most vent fauna are tightly linked.

Vent habitats are highly ephemeral and sensitive to variations in tectonic activity, hydrothermal inputs, and geologic events. Consequently, the persistence of vent organisms, which are predominantly sessile as adults, depends on successful larval dispersal to new sites. The dispersal strategy of larvae can significantly impact the biogeography of the adult organism (Lalou and Brichet 1982; Fustec et al. 1987). On the basis of laboratory studies and comparisons with shallow water species, researchers infer that some vent larvae are planktotrophic, while others are lecithotrophic (Lutz et al. 1980; Turner et al. 1985; Young et al. 1996; Marsh et al. 2001). Although both forms are pelagic, planktotrophic larvae are positively buoyant and feed in the water column, while lecithotrophic larvae are negatively buoyant and nonfeeding (Poulin et al. 2001). Larvae of the large vesicomyid clam *Calyptogena magnifica* typify a planktotrophic dispersal strategy successfully exploiting the vent plume to carry them many kilometers (Pradillon et al. 2001; Mullineaux et al. 2002). Although planktotrophic larvae risk being carried off the ridge axis by cross currents, *C. magnifica* apparently encounters no significant barriers to dispersal across the equator on the East Pacific Rise (EPR; Karl et al. 1996). Conversely, lecithotrophic larvae are less affected by crosscurrents but, because they are nonfeeding, have relatively short larval stages and therefore limited time for dispersal. For example, in laboratory studies, larval *Riftia pachyptila* exhibits a lecithotrophic strategy, surviving a maximum of 38 days (Marsh et al. 2001). Assuming flow rates characteristic of EPR currents, this interval suggests a maximum dispersal distance of 100 km (Marsh et al. 2001); however, the in situ dispersal distance is unknown given that *Riftia* larvae have not been detected in the wild.

The geology and tectonic activity associated with mid-ocean ridges also impact the biogeography of vent organisms. For instance, Iceland, an active site of crust formation, rises out of the ocean along the northern MAR, forming a barrier that prevents dispersal along the ridge axis (Tyler and Young 2003). Given that Iceland interrupted the MAR approximately 55 Mya, the ridge axis north of Iceland constitutes one of the most isolated vent systems on the planet, perhaps representing a new biogeographic province (Bilyard and Carey 1980; Dunton 1992; Svavarsson et al. 1993). Similar dispersal barriers are seen among vent fields abutting the Azores Rise in the Atlantic (Tyler and Young 2003) and also evident between the EPR and the

northeast Pacific vent fields (Tunnicliffe 1988; Tunnicliffe and Fowler 1996). These barriers are insurmountable and may provide the conditions for allopatric speciation of both host and symbiont.

Research on EPR bathymodiolid mussels and their symbionts provides a good example of how larval dispersal strategy, current regime, and bathymetry interact to structure biogeography (Lonsdale 1977; Corliss et al. 1979). Except at northern Pacific sites, which are separated from the EPR by the North America landmass, vent communities on the Pacific ridge axis appear relatively uniform. For example, the mussel *Bathymodiolus thermophilus*, which undergoes a planktotrophic larval stage, occurs over a distance of 4,900 km (from 13°N to 32°S) on the EPR. Mussel populations along 13°N and 11°S are genetically indistinguishable, indicating no population subdivision (Craddock et al. 1995; Won et al. 2003). Deep ocean currents that flow primarily NNW and SSE along the axis (Marsh et al. 2001) may facilitate dispersal of larval *B. thermophilus*, contributing to the homogeneity observed along the EPR. However, there is some genetic structure in the EPR mytilid populations; the westward currents across the ridge axis at 15°N and the Easter Microplate are obstacles for planktotrophic larvae. At 15°N, a westward current flows across the ridge axis, partially isolating the 17°S population from the other northern populations. Further south, at the Easter Microplate, mussel populations are severely divergent (Won et al. 2003). Although morphologically indistinguishable, mussels north and south of the Microplate are genetically distinct (Won et al. 2003). The Easter Microplate therefore appears to be a significant topographic obstacle for larval dispersal. Such a feature can produce cross-axis currents, like those at 15°N, that may sweep bathymodiolid larvae (which are positively buoyant) off the ridge axis (Fujio and Imasato 1991; Mullineaux et al. 1995). The degree to which such barriers also impact the genetic diversity and biogeography of chemosynthetic symbiont populations remains an open question.

## Summary

Scientific understanding of chemosynthetic symbioses continues to expand. The spectacular discovery of hydrothermal vents highlighted the importance of chemosynthetic bacteria both in food webs and in symbioses with eukaryotes and provided the impetus to examine less exotic environments for such associations. As other oxic-anoxic environments (e.g., freshwater) and the invertebrates and protists that inhabit them are explored, new symbioses will undoubtedly be discovered. Further, chemosynthetic bacteria that use other sources of energy (e.g., hydrogen) may be found in similar associations.

Current studies of these fascinating symbioses involve a range of experimental and diagnostic tools, including physiological assays in specialized growth chambers, enzyme characterizations, immunodetections, and stable isotope analyses. Increasingly, molecular techniques, such as PCR-based gene probing, FISH, and 16 rRNA phylogenetic analysis, are used to complement traditional methods. These studies provide valuable insight into the population dynamics, evolutionary history, and carbon and nutrient metabolism of symbionts. In addition, projects are currently underway to sequence the genomes of some of the chemosynthetic symbionts described in this review (e.g., symbionts of *Riftia pachyptila* and *Solemya velum*). Genomic analysis, in conjunction with new technologies to manipulate symbioses under in situ conditions (e.g., via vascular catheters; Felbeck et al. 2004) and to sample the physical environment (e.g., electrochemical sampling; Luther et al. 2001), will contribute significantly to our understanding of symbiont biology. Scientists are now poised to reveal how interactions with the host and the abiotic environment impact symbiotic chemosynthetic bacteria over both ecological and evolutionary timescales.

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## References

Alt JC (1995) Subseafloor processes in mid-ocean ridge hydrothermal systems. In: Humphris SE, Zierenberg RA, Mullineaux LS, Thomson RE (eds) Seafloor hydrothermal systems: physical, chemical, biological, and geological interactions. American Geophysical Union, Geophysical Monograph 91

Anderson A, Childress J, Favuzzi J (1987) Net uptake of $CO_2$ driven by sulfide and thiosulfite oxidation in the bacterial symbiont-containing clam *Solemya reidi*. J Exp Biol 133:1–131

Anthony C (1982) The biochemistry of methylotrophs. Academic, London

Arp AJ, Childress JJ, Fisher CRJ (1984) Metabolic and blood gas transport characteristics of the hydrothermal vent bivalve *Calyptogena magnifica*. Physiol Zool 57:648–662

Arp AJ, Childress JJ, Fisher CR (1985) Blood gas transport in *Riftia pachyptila*. Bull Biol Soc Wash 6:289–300

Arp AJ, Childress JJ, Vetter RD (1987) The sulphide-binding protein in the blood of the vestimentiferan tube-worm, *Riftia Pachyptila*, is the extracellular haemoglobin. J Exp Biol 128:139–158

Bailly X, Jollivet D, Vanin S, Deutsch J, Zal F, Lallier F, Toulmond A (2002) Evolution of the sulfide-binding function within the globin multigenic family of the deep-sea hydrothermal vent tubeworm *Riftia pachyptila*. Mol Biol Evol 19:1421–1433

Bailly X, Leroy R, Carney S, Collin O, Zal F, Toulmond A, Jollivet D (2003) The loss of the hemoglobin $H_2S$-binding function in annelids from sulfide-free

habitats reveals molecular adaptation driven by Darwinian positive selection. Proc Natl Acad Sci USA 100:5885–5890

Barry JP, Buck KR, Kochevar RK, Nelson DC, Fujiwara Y, Goffredi SK, Hashimoto J (2002) Methane-based symbiosis in a mussel, *Bathymodiolus platifrons*, from cold seeps in Sagami Bay, Japan Invertebr. Biol 121:47–54

Bauer-Nebelsick M, Bardele CF, Ott JA (1996) Electron microscopic studies on *Zoothamnium niveum* (Hemprich & Ehrenberg, 1831) Ehrenberg 1838 (Oligohymenophora, Peritrichida), a ciliate with ectosymbiotic, chemoautotrophic bacteria. Eur J Protistol 32:202–215

Bennett BA, Smith CR, Glaser B, Maybaum HL (1994) Faunal community structure of a chemoautotrophic assemblage on whale bones in the deep northeast Pacific Ocean. Mar Ecol Progr Ser 108:205–223

Bergquist DC, Ward T, Cordes EE, McNelis T, Howlett S, Kosoff R, Hourdez S, Carney R, Fisher CR (2003) Community structure of vestimentiferan-generated habitat islands from Gulf of Mexico cold seeps. J Exp Mar Biol Ecol 289:197–222

Bilyard GR, Carey AG (1980) Zoogeography of Western Beaufort Sea Polychaeta (Annelida). Sarsia 65:19–25

Black MB, Lutz RA, Vrifenhoek RC (1994) Gene flow among vestimentiferan tube worm (*Riftia pachyptila*) populations from hydrothermal vents of the Eastern Pacific. Mar Biol 120:33–39

Black MB, Trivedi A, Maas PAY, Lutz RA, Vrijenhoek RC (1998) Population genetics and biogeography of vestimentiferan tube worms. Deep Sea Res Pt II Top Stud Oceanogr 45:365–382

Boetius A, Felbeck H (1995) Digestive enzymes in marine-invertebrates from hydrothermal vents and other reducing environments. Mar Biol 122:105–113

Bosch C, Grassé PP (1984) Cycle partiel des bactéries chimioautotrophes symbiotiques et eurs rapports avec les bacteriocytes chez Riftia pachyptila Jones (Pogonophore Vestimentifére). II: L'evolution des bactéries symbotiques et des bacteriocytes. Comptes Rendus L'Acad. Sci Ser III: Sciences de la Vie—Life Sciences 299:413–319

Boss KJ, Turner RD (1980) The giant white clam from the Galapagos Rift, *Calyptogena magnifica* species novum. Malacologia 20:161–194

Bright M, Keckeis H, Fisher CR (2000) An autoradiographic examination of carbon fixation, transfer and utilization in the *Riftia pachyptila* symbiosis. Mar Biol 136:621–632

Brigmon RL, De Ridder C (1998) Symbiotic relationship of *Thiothrix* spp. with an echinoderm. Appl Environ Microbiol 64:3491–3495

Brooks JM, Kennicutt MC 2nd, Fisher CR, Macko SA, Cole K, Childress JJ, Bidigare RR, Vetter RD (1987) Deep-sea hydrocarbon seep communities: evidence for energy and nutritional carbon sources. Science 238:1138–1142

Buck KR, Barry JP, Simpson AGB (2000) Monterey Bay cold seep biota: Euglenozoa with chemoautotrophic bacterial epibionts. Eur J Protistol 36:117–126

Campbell BJ, Stein JL, Cary SC (2003) Evidence of chemolithoautotrophy in the bacterial community associated with *Alvinella pompejana*, a hydrothermal vent polychaete. Appl Environ Microbiol 69:5070–5078

Cary SC, Giovannoni SJ (1993) Transovarial inheritance of endosymbiotic bacteria in clams inhabiting deep-sea hydrothermal vents and cold seeps. Proc Natl Acad Sci USA 90:5695–5699

Cary SC, Warren W, Anderson E, Giovannoni SJ (1993) Identification and localization of bacterial endosymbionts in hydrothermal vent taxa with symbiont-specific polymerase chain reaction amplification and in situ hybridization techniques. Mol Mar Biol Biotechnol 2:51–62

Cary SC (1994) Vertical transmission of a chemoautotrophic aymbiont in the protobranch bivalve, *Solemya reidi*. Mol Mar Biol Biotechnol 3:121–130

Cary SC, Cottrell MT, Stein JL, Camacho F, Desbruyères D (1997) Molecular identification and localization of filamentous symbiotic bacteria associated with the hydrothermal vent annelid *Alvinella pompejana*. Appl Environ Microbiol 63:1124–1130

Cavanaugh CM, Gardiner SL, Jones ML, Jannasch HW, Waterbury JB (1981) Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila* Jones: possible chemoautotrophic symbionts. Science 213:340–342

Cavanaugh CM (1983) Symbiotic chemoautotrophic bacteria in marine invertebrates from sulfide-rich habitats. Nature 302:58–61

Cavanaugh CM (1985) Symbioses of chemoautotrophic bacteria and marine invertebrates from hydrothermal vents and reducing sediments. Biol Soc Wash Bull 6:373–388

Cavanaugh CM, Levering PR, Maki JS, Mitchell R, Lidstrom ME (1987) Symbiosis of methylotrophic bacteria and deep-sea mussels. Nature 325:346–348

Cavanaugh CM, Abbott MS, Veenhuis M (1988) Immunochemical localization of ribulose-1,5-bisphosphate carboxylase in the symbiont-containing gills of *Solemya velum* (Bivalvia:Mollusca). Proc Natl Acad Sci USA 85:7786–7789

Cavanaugh CM, Wirsen C, Jannasch HJ (1992) Evidence for methylotrophic symbionts in a hydrothermal vent mussel (Bivalvia:Mytilidae) from the Mid-Atlantic ridge. Appl Environ Microbiol 58:3799–3803

Cavanaugh CM (1993) Methanotroph-invertebrate symbioses in the marine environment: ultrastructural, biochemical, and molecular studies. In: Murrell JC, Kelly DP (eds) Microbial growth on C1 compounds intercept

Cavanaugh CM (1994) Microbial symbiosis: patterns of diversity in the marine environment. Am Zool 34:79–89

Cavanaugh CM, Robinson JJ (1996) $CO_2$ fixation in chemoautotroph-invertebrate symbioses: expression of form I and form II RuBisCO. In: Lidstrom ME, Tabita FR (eds) Microbial growth on C1 compounds. Kluwer, The Netherlands

Chen XA, Li S, Aksoy S (1999) Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus Glossina and its bacteriome-associated endosymbiont, Wigglesworthia glossinidia. J Mol Evol 48:49–58

Childress JJ, Fisher CR, Brooks JM, Kennicutt MC 2nd, Bidigare R, Anderson AE (1986) A methanotrophic marine molluscan (Bivalvia, Mytilidae) symbiosis: mussels fueled by gas. Science 233:1306–1308

Childress JJ, Fisher CR, Favuzzi JA, Kochevar RE, Sanders NK, Alayse AM (1991) Sulfide-driven autotrophic balance in the bacterial symbiont-containing hydrothermal vent tubeworm, *Riftia pachyptila* Jones. Biol Bull 180:135–153

Childress JJ, Fisher CR (1992) The biology of hydrothermal vent animals: physiology, biochemistry, and autotrophic symbioses. Oceanogr Mar Biol Annu Rev 30:337–441

Childress JJ, Lee RW, Sanders NK, Felbeck H, Oros DR, Toulmond A, Desbruyères D, Kennicutt MC, Brooks J (1993) Inorganic carbon uptake in hydrothermal vent tubeworms facilitated by high environmental $pCO_2$. Nature 362:147–169

CoBabe EA (1991) Lucinid Bivalve evolution and the detection of chemosymbiosis in the fossil record (Ph.D. thesis). Harvard University, Cambridge, MA

Colaco A, Dehairs F, Desbruyères D (2002) Nutritional relations of deep-sea hydrothermal fields at the Mid-Atlantic ridge: a stable isotope approach. Deep Sea Res Pt I Oceanogr Res Pap 49:395–412

Conway N, Capuzzo J, Fry B (1989) The role of endosymbiotic bacteria in the nutrition of *Solemya velum*: evidence from a stable isotope analysis of endosymbionts and hosts. Limnol Oceanogr 34:249–255

Conway N, Capuzzo JM (1991) Incorporation and utilization of bacterial lipids in the *Solemya velum* symbiosis. Mar Biol 108:277–292

Conway NM, McDowell Capuzzo JE (1992) High taurine levels in the *Solemya velum* symbiosis. Comp Biochem Physiol B 102:175–185

Conway N, Kennicutt MC, Van Dover CL (1992) Stable isotopes in the study of marine chemosynthesis-based ecosystems. In: Lajtha K, Michener R (eds) Stable isotopes in ecology. Blackwell, Oxford, UK

Corliss JB, Dymond J, Gordon LI, Edmond JM, Herzen RPV, Ballard RD, Green K, Williams D, Bainbridge A, Crane K, Van Andel TH (1979) Submarine thermal springs on the Galapagos Rift. Science 203:1073–1083

Craddock C, Hoeh WR, Lutz RA, Vrijenhoek RC (1995) Extensive gene flow among mytilid (*Bathymodiolis thermophilus*) populations from hydrothermal vents of the Eastern Pacific. Mar Biol 124:137–146

Dale C, Wang B, Moran N, Ochman H (2003) Loss of DNA recombinational repair enzymes in the initial stages of genome degeneration. Mol Biol Evol 20:1188–1194

Dando PR, Southward AJ (1986) Chemoautotrophy in the bivalve molluscs of the genus Thyasira. J Mar Biol Assoc UK 66:915–929

Dando PR, Spiro B (1993) Varying nutritional dependence of the thyasirid bivalves *Thyasira sarsi* and *T equalis* on chemoautotrophic symbiotic

bacteria, demonstrated by isotope ratios of tissue carbon and shell carbonate. Mar Ecol Progr Ser 92:151–158

De Bary A (1879) Die Erscheinung der Symbiose. Verlag von Karl J Trubner, Strasburg

de Burgh ME, Singla CL (1984) Bacterial colonization and endocytosis on the gill of a new limpet species from a hydrothermal vent. Mar Biol 84:1–6

de Burgh ME, Juniper SK, Singla CL (1989) Bacterial symbiosis in northeast Pacific vestimentifera—a TEM study. Mar Biol 101:97–105

De Cian MC, Andersen AC, Bailly X, Lallier FH (2003a) Expression and localization of carbonic anhydrase and ATPases in the symbiotic tubeworm *Riftia pachyptila*. J Exp Biol 206:399–409

De Cian MC, Bailly X, Morales J, Strub JM, Van Dorsselaer A, Lallier FH (2003b) Characterization of carbonic anhydrases from *Riftia pachyptila*, a symbiotic invertebrate from deep-sea hydrothermal vents. Prot Struct Funct Genet 51:327–339

Degnan PH, Lazarus AB, Brock CD, Wernegreen JJ (2004) Host-symbiont stability and fast evolutionary rates in an ant-bacterium association: cospeciation of Camponotus species and their endosymbionts, *Candidatus blochmannia*. System Biol 53:95–110

DeNiro MJ, Epstein S (1979) Relationship between the oxygen isotope ratios of terrestrial plant cellulose, carbon dioxide, and water. Science 204:51–53

Desbruyères D, Gaill F, Laubier L, Prieur D, Rau GH (1983) Unusual nutrition of the "Pompeii worm" *Alvinella pompejana* (polychaetous annelid) from a hydrothermal vent environment: SEM, TEM, $^{13}C$ and $^{15}N$ evidence. Mar Biol 75:201–205

Desbruyères D, Gaill F, Laubier L, Fouquet Y (1985) Polychaetous annelids from hydrothermal vent ecosystems: an ecological overview. Biol Soc Wash Bull 6:103–116

Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res Pt A Oceanogr Res Pap 34:1733–1743

Di Meo CA, Wilbur AE, Holben WE, Feldman RA, Vrijenhoek RC, Cary SC (2000) Genetic variation among endosymbionts of widely distributed vestimentiferan tubeworms. Appl Environ Microbiol 66:651–658

Distel DL, Cavanaugh CM (1994) Independent phylogenetic origins of methanotrophic and chemoautotrophic bacterial endosymbioses in marine bivalves. J Bacteriol 176:1932–1938

Distel DL, Lee HKW, Cavanaugh CM (1995) Intracellular coexistence of methano-and thioautotrophic bacteria in a hydrothermal vent mussel. Proc Natl Acad Sci USA 92:9598–9602

Distel D (1998) Evolution of chemoautotrophic endosymbioses in bivalves. Bioscience 48:277–286

Dubilier N, Giere O, Grieshaber MK (1995) Morphological and ecophysiological adaptations of the marine oligochaete *Tubificoides benedii* to sulfidic sediments. Am Zool 35:163–173

Dubilier N, Windoffer R, Giere O (1998) Ultrastructure and stable carbon isotope composition of the hydrothermal vent mussels Bathymodiolus brevior and B. sp. affinis brevior from the North Fiji Basin, western Pacific. Mar Ecol Progr Ser 165:187–193

Dubilier N, Amann R, Erseus C, Muyzer G, Park SY, Giere O, Cavanaugh CM (1999) Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochete Olavius loisae (Annelida). Mar Ecol Progr Ser 178:271–280

Dubilier N, Mulders C, Ferdelman T, de Beer D, Pernthaler A, Klein M, Wagner M, Erseus C, Thiermann F, Krieger J, Giere O, Amann R (2001) Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. Nature 411:298–302

Dufour SC, Felbeck H (2003) Sulphide mining by the superextensile foot of symbiotic thyasirid bivalves. Nature 426:65–67

Dunton K (1992) Arctic biogeography: the paradox of the marine benthic fauna and flora. Trends Ecol Evol 7:183–189

Duperron S, Nadalig T, Caprais J, Sibuet M, Fiala-Medioni A, Amann R, Dubilier N (2004) Dual symbiosis in a Bathymodiolus mussel from a methane seep on the Gabon continental margin (South East Atlantic): 16S rRNA phylogeny and distribution of the symbionts in the gills. Appl Environ Microbiol 71(4):1694–1700

Durand P, Gros O (1996) Bacterial host specificity of Lucinacea endosymbionts: interspecific variation in 16S rRNA sequences. FEMS Microbiol Lett 140:193–198

Durand P, Gros O, Frenkiel L, Prieur D (1996) Phylogenetic characterization of sulfur-oxidizing bacterial endosymbionts in three tropical Lucinidae by 16S rDNA sequence analysis. Mol Mar Biol Biotechnol 5:37–42

Dykhuizen DE, Green L (1991) Recombination in *Escherichia coli* and the definition of biological species. J Bacteriol 173:7257–7268

Elderfield H, Schultz A (1996) Mid-ocean ridge hydrothermal fluxes and the chemical composition of the ocean. Annu Rev Earth Planet Sci 24:191–224

Elsaied H, Kimura H, Naganuma T (2002) Molecular characterization and endosymbiotic localization of the gene encoding D-ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO) form II in the deep-sea vestimentiferan trophosome. Microbiology 148:1947–1957

Endow K, Ohta S (1989) The symbiotic relationship between bacteria and the mesogastropod snail, *Alviniconcha hessleri*, collected from hydrothermal vents of the Mariana back-arc basin. Bull Jpn Soc Microb Ecol 3:73–82

Endow K, Ohta S (1990) Occurrence of bacteria in the primary oocytes of vesicomyid clam *Calyptogena soyoae*. Mar Ecol Progr Ser 64:309–311

Felbeck H (1981) Chemoautotrophic potential of the hydrothermal vent tube worm, *Riftia pachyptila* Jones (Vestimentifera). Science 213:336–338

Felbeck H, Childress JJ, Somero GN (1981) Calvin-Benson cycle and sulfide oxidation enzymes in animals from sulfide-rich habitats. Nature 293:291–293

Felbeck H (1983) Sulfide oxidation and carbon fixation by the gutless clam *Solemya reidi*: an animal-bacteria symbiosis. J Comp Physiol 152:3–11

Felbeck H, Liebezeit G, Dawson R, Giere O (1983) $CO_2$ fixation in tissues of marine oligochaetes (*Phallodrilus leukodermatus* and *P. planus*) containing symbiotic, chemoautotrophic bacteria. Mar Biol 75:187–191

Felbeck H (1985) $CO_2$ fixation in the hydrothermal vent tube worm *Riftia pachyptila* (Jones). Physiol Zool 58:272–281

Felbeck H, Distel DL (1991) Prokaryotic symbionts of marine invertebrates. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer K-H (eds) The prokaryotes. Springer, New York

Felbeck H, Turner PJ (1995) $CO_2$ transport in catheterized hydrothermal vent tubeworms, *Riftia pachyptila* (Vestimentifera). J Exp Zool 272:95–102

Felbeck H, Jarchow J (1998) Carbon release from purified chemoautotrophic bacterial symbionts of the hydrothermal vent tubeworm *Riftia pachyptila*. Physiol Zool 71:294–302

Felbeck H, Arndt C, Hentschel U, Childress JJ (2004) Experimental application of vascular and coelomic catheterization to identify vascular transport mechanisms for inorganic carbon in the vent tubeworm, *Riftia pachyptila*. Deep Sea Res Pt I Oceanogr Res Pap 51:401–411

Feldman R, Black M, Cary C, Lutz R, Vrijenhoek R (1997) Molecular phylogenetics of bacterial endosymbionts and their vestimentiferan hosts. Mol Mar Biol Biotechnol 6:268–277

Fenchel T, Finlay BJ (1989) Kentrophoros—a mouthless ciliate with a symbiotic kitchen garden. Ophelia 30:75–93

Fiala-Médioni A (1984) Ultrastructural evidence of abundance of intracellular symbiotic bacteria in the gill of bivalve mollusks of deep hydrothermal vents. Comptes Rendus L'Acad Sci Ser III Sciences de la Vie—Life Sciences 298:487–492

Fiala-Médioni A, Boulègue J, Ohta S, Felbeck H, Mariotti A (1993) Source of energy sustaining the Calyptogena populations from deep trenches in subduction zones off Japan. Deep Sea Res 40:1241–1258

Fiala-Médioni A, Michalski J-C, Jollès J, Alonso C, Montreuil J (1994) Lysosomic and lysozyme activities in the gill of bivalves from deep hydrothermal vents. Comptes Rendus L'Acad Sci Ser III Sciences de la Vie—Life Sciences 317:239–244

Fiala-Médioni A, McKiness ZP, Dando P, Boulegue J, Mariotti A, Alayse-Danet AM, Robinson JJ, Cavanaugh CM (2002) Ultrastructural, biochemical, and immunological characterization of two populations of the mytilid mussel *Bathymodiolus azoricus* from the Mid-Atlantic Ridge: evidence for a dual symbiosis. Mar Biol 141:1035–1043

Fisher CR, Childress JJ (1986) Translocation of fixed carbon from symbiotic bacteria to host tissues in the gutless bivalve, *Solemya reidi*. Mar Biol 93:59–68

Fisher C, Childress J, Arp A, Brooks J, Distel D, Favuzzi J, Felbeck H, Hessler R, Johnson K, Kennicut M 2nd, Macko S, Newton A, Powell M, Somero G, Soto T (1988) Microhabitat variation in the hydrothermal vent mussel, *Bathymodiolus thermophilus*, at the Rose Garden vent on the Galapagos Rift. Deep Sea Res 35:1769–1791

Fisher CR (1990) Chemoautotrophic and methanotrophic symbioses in marine invertebrates. Rev Aquat Sci 2:399–436

Fisher CR, Childress JJ (1992) Organic carbon transfer from methanotrophic symbionts to the host hydrocarbon seep mussel. Symbiosis 12:221–235

Fisher CR (1993) Oxidation of methane by deep-sea mytilids in the Gulf of Mexico. In: Oremland RS (ed) Biogeochemistry of global change: radiatively active trace gases. Chapman and Hall, New York

Fisher CR, Brooks JM, Vodenichar JS, Zande JM, Childress JJ, Burke RA Jr (1993) The co-occurrence of methanotrophic and chemoautotrophic sulfur-oxidizing bacterial symbionts in a deep-sea mussel. Marine Ecology 14:277–289, Pubblicazioni Della Stazione Zoologica Di Napoli I

Fisher CR, Childress JJ, Macko SA, Brooks JM (1994) Nutritional interactions in Galapagos Rift hydrothermal vent communities: inferences from stable carbon and nitrogen isotope analyses. Mar Ecol Progr Ser 103:45–55

Fisher CR (1995) Toward an appreciation of hydrothermal vent animals: their environment, physiological ecology, and tissue stable isotope values. In: Seafloor hydrothermal systems: physical, chemical, biological, and geological interactions. American Geophysical Union

Fisher CR (1996) Ecophysiology of primary production at deep-sea vents and seeps. Biosystem Ecol Ser 11:313–336

Fox M, Juniper SK, Vali H (2002) Chemoautotrophy as a possible nutritional source in the hydrothermal vent limpet *Lepetodrilus fucensis*. Cahiers Biol Mar 43:371–376

Freytag JK, Girguis PR, Bergquist DC, Andras JP, Childress JJ, Fisher CR (2001) A paradox resolved: sulfide acquisition by roots of seep tubeworms sustains net chemoautotrophy. Proc Natl Acad Sci USA 98:13408–13413

Fry B, Gest H, Hayes JM (1983) Sulfur isotopic compositions of deep-sea hydrothermal vent animals. Nature 306:51–52

Fry B, Sherr EB (1984) $\delta^{13}C$ measurements as indicators of carbon flow in marine and freshwater ecosystems. Contrib Mar Sci 27:13–47

Fujio SZ, Imasato N (1991) Diagnostic calculation for circulation and water mass movement in the deep Pacific. J Geophys Res Oceans 96:759–774

Fujiwara Y, Kato C, Masui N, Fujikura K, Kojima S (2001) Dual symbiosis in the cold-seep thyasirid clam *Maorithyas hadalis* from the hadal zone in the Japan Trench, western Pacific. Mar Ecol Progr Ser 214:151–159

Fujiwara Y, Uematsu K (2002) Microdistribution of two endosymbionts in gill tissue from a hadal thyasirid clam *Maorithyas hadalis*. Cahiers Biol Mar 43:341–343

Fustec A, Desbruyères D, Juniper SK (1987) Deep-sea hydrothermal vent communities at 13°N on the East Pacific Rise: microdistribution and temporal variations. Biol Oceanogr 4:121–164

Gearing JN, Gearing PJ, Rudnick DT, Requejo AG, Hutchins MJ (1984) Isotopic variability of organic-carbon in a phytoplankton-based, temperate estuary. Geochim Cosmochim Acta 48:1089–1098

Giere O (1981) The gutless marine oligochaete *Phallodrilus leukodermatus*: structural studies on an aberrant tubificid associated with bacteria. Mar Ecol Progr Ser 5:353–357

Giere O (1985) The gutless marine tubificid *Phallodrilus planus*, a flattened oligochaete with symbiotic bacteria. Zool Scripta 14:279–286

Giere O, Langheld C (1987) Structural organization, transfer and biological fate of endosymbiotic bacteria in gutless oligochaetes. Mar Biol 93:641–650

Giere O, Krieger J (2001) A triple bacterial endosymbiosis in a gutless oligochaete (Annelida): ultrastructural and immunocytochemical evidence. Invertebr Biol 120:41–49

Girguis PR, Lee RW, Desaulniers N, Childress JJ, Pospesel M, Felbeck H, Zal F (2000) Fate of nitrate acquired by the tubeworm *Riftia pachyptila*. Appl Environ Microbiol 66:2783–2790

Girguis PR, Childress JJ, Freytag JK, Klose K, Stuber R (2002) Effects of metabolite uptake on proton-equivalent elimination by two species of deep-sea vestimentiferan tubeworm, *Riftia pachyptila* and Lamellibrachia cf luymesi: proton elimination is a necessary adaptation to sulfide-oxidizing chemoautotrophic symbionts. J Exp Biol 205:3055–3066

Glockner FO, Babenzien HD, Wulf J, Amann R (1999) Phylogeny and diversity of *Achromatium oxaliferum* system. Appl Microbiol 22:28–38

Goericke R, Montoya JP, Fry B (1994) Physiology of isotopic fractionation in algae and cyanobacteria. In: Lajtha K, Michener RH (eds) Stable isotopes in ecology and environmental science. Blackwell, Oxford, UK

Goffredi SK, Childress JJ, Desaulniers NT, Lallier FH (1997a) Sulfide acquisition by the hydrothermal vent tubeworm *Riftia pachyptila* appears to be via uptake of $HS^-$, rather than $H_2S$. J Exp Biol 200:2069–2616

Goffredi SK, Childress JJ, Desaulniers NT, Lee RW, Lallier FH, Hammond D (1997b) Inorganic carbon acquisition by the hydrothermal vent tubeworm *Riftia pachyptila* depends upon high external $pCO_2$ and upon proton-equivalent ion transport by the worm. J Exp Biol 200:883–896

Goffredi SK, Childress JJ, Lallier FH, Desaulniers NT (1999) The ionic composition of the hydrothermal vent tube worm *Riftia pachyptila*: evidence for the elimination of $SO_4^{2-}$ and $H^+$ and for a $Cl^-/HCO^{3-}$ shift. Physiol Biochem Zool 72:296–306

Goffredi SK, Childress JJ (2001) Activity and inhibitor sensitivity of ATPases in the hydrothermal vent tubeworm *Riftia pachyptila*: a comparative approach. Mar Biol 138:259–265

Grassle JF (1985) Hydrothermal vent animals: distribution and biology. Science 229:713–717

Gray ND, Howarth R, Rowan A, Pickup RW, Jones JG, Head IM (1999) Natural communities of *Achromatium oxaliferum* comprise genetically, morphologically, and ecologically distinct subpopulations. Appl Environ Microbiol 65:5089–5099

Grieshaber MK, Volkel S (1998) Animal adaptations for tolerance and exploitation of poisonous sulfide. Annu Rev Physiol 60:33–53

Gros O, Darrasse A, Durand P, Frenkiel L, Moueza M (1996) Environmental transmission of a sulfur-oxidizing bacterial gill endosymbiont in the tropical lucinid bivalve *Codakia orbicularis*. Appl Environ Microbiol 62:2324–2330

Gros O, De Wulf-Durand P, Frenkiel L, Moueza M (1998) Putative environmental transmission of sulfur-oxidizing bacterial symbionts in tropical lucinid bivalves inhabiting various environments. FEMS Microbiol Lett 160:257–262

Gros O, Liberge M, Felbeck H (2003a) Interspecific infection of aposymbiotic juveniles of *Codakia orbicularis* by various tropical lucinid gill endosymbionts. Mar Biol 142:57–66

Gros O, Liberge M, Heddi A, Khatchadourian C, Felbeck H (2003b) Detection of the free-living forms of sulfide-oxidizing gill endosymbionts in the lucinid habitat (Thalassia testudinum environment). Appl Environ Microbiol 69:6264–6267

Gustafson RG, Reid RGB (1988) Association of bacteria with larvae of the gutless protobranch bivalve *Solemya reidi* (Cryptodonta, Solemyidae). Mar Biol 97:389–401

Hand SC (1987) Trophosome ultrastructure and the characterization of isolated bacteriocytes from invertebrate-sulfur bacteria symbioses. Biol Bull 173:260–276

Head IM, Gray ND, Clarke KJ, Pickup RW, Jones JG (1996) The phylogenetic position and ultrastructure of the uncultured bacterium *Achromatium oxaliferum*. Microbiol SGM 142:2341–2354

Hentschel U, Felbeck H (1993) Nitrate respiration in the hydrothermal vent tubeworm *Riftia pachyptila*. Nature 366:338–340

Hentschel U, Cary SC, Felbeck H (1993) Nitrate respiration in chemoautotrophic symbionts of the bivalve *Lucinoma aequizonata*. Mar Ecol Progr Ser 94:35–41

Herry A, Le Pennec M (1987) Endosymbiotic bacteria in the gills of the littoral bivalve molluscs *Thyasira flexuosa* (Thyasiridae) and *Lucinella divaricata* (Lucinidae). Symbiosis 4:25–36

Hughes DS, Felbeck H, Stein JL (1997) A histidine protein kinase homolog from the endosymbiont of the hydrothermal vent tubeworm *Riftia pachyptila*. Appl Environ Microbiol 63:3494–3498

Hughes DS, Felbeck H, Stein JL (1998) Signal transduction and motility genes from the bacterial endosymbionts of *Riftia pachyptila*. Cahiers Biol Mar 39:305–308

Humes AG, Lutz RA (1994) Aphotopontius acanthinus, new species (Copepoda, Siphonostomatoida), from deep-sea hydrothermal vents on the East Pacific Rise. J Crustac Biol 14:337–345

Hunt S (1992) Structure and composition of the shell of the archaeogastropod limpet *Lepetodrilus elevatus-elevatus* (Mclean, 1988). Malacologia 34:129–141

Hurtado LA (2002) Evolution and biogeography of hydrothermal vent organisms in the Eastern Pacific Ocean, PhD thesis, Rutgers University, New Brunswick

Hurtado LA, Mateos M, Lutz RA, Vrijenhoek RC (2003) Coupling of bacterial endosymbiont and host mitochondrial genomes in the hydrothermal vent clam *Calyptogena magnifica*. Appl Environ Microbiol 69:2058–2064

Jacobs DK, Lindberg DR (1998) Oxygen and evolutionary patterns in the sea: onshore/offshore trends and recent recruitment of deep-sea faunas. Proc Natl Acad Sci USA 95:9396–9401

Johnson KS, Childress JJ, Hessler RR, Sakamoto-Arnold CM, Beehler CL (1988) Chemical and biological interactions in the Rose Garden hydrothermal vent field, Galapagos spreading center. Deep Sea Res 35:1723–1744

Johnson M, Diouris M, Le Pennec M (1994) Endosymbiotic bacterial contribution in the carbon nutrition of *Loripes lucinalis* (Mollusca: Bivalvia). Symbiosis 17:1–13

Jones ML (1981) *Riftia pachyptila* Jones: observations on the vestimentiferan worm from the Galapagos Rift. Science 213:333–336

Jones JL, Gardiner SL (1985) Light and scanning electron microscope studies of spermatogenesis in the vestimentiferan tube worm *Riftia pachyptila* (Pogonophora: Obturata). Trans Am Microscop Soc 104:1–18

Jørgensen BB, Postgate JR (1982) Ecology of the bacteria of the sulfur cycle with special reference to anoxic oxic interface environments. Philos Trans R Soc Lond Ser B Biol Sci 298:543–561

Joyner JL, Peyer SM, Lee RW (2003) Possible roles of sulfur-containing amino acids in a chemoautotrophic bacterium-mollusc symbiosis. Biol Bull 205:331–338

Julian D, Gaill F, Wood E, Arp AJ, Fisher CR (1999) Roots as a site of hydrogen sulfide uptake in the hydrocarbon seep vestimentiferan *Lamellibrachia* sp. J Exp Biol 202:2245–2257

Karl SA, Schutz S, Desbruyères D, Lutz R, Vrijenhoek RC (1996) Molecular analysis of gene flow in the hydrothermal vent clam (*Calyptogena magnifica*). Mol Mar Biol Biotechnol 5:193–202

Kennicutt MC, Burke RA, MacDonald IR, Brooks JM, Denoux GJ, Macko SA (1992) Stable isotope partitioning in seep and vent organisms: chemical and ecological significance. Chem Geol 101:293–310

Kennish MJ, Lutz RA (1999) Calcium carbonate dissolution rates in deep-sea bivalve shells on the East Pacific Rise at 21°N: results of an 8-year in situ experiment. Palaeogeogr Palaeoclimatol Palaeoecol 154:293–299

Kimura M, Weiss GH (1964) The stepping stone model of population structure and the decrease of genetic correlation with distance. Genetics 49:561–576

Kochevar RE, Childress JJ (1996) Carbonic anhydrase in deep-sea chemoautotrophic symbioses. Mar Biol 125:375–383

Krieger J, Giere O, Dubilier N (2000) Localization of RubisCO and sulfur in endosymbiotic bacteria of the gutless marine oligochaete *Inanidrilus leukodermatus* (Annelida). Mar Biol 137:239–244

Krueger DM (1996a) Ecology and evolution of *Solemya* spp., marine bivalves living in symbiosis with chemoautotrophic bacteria, PhD thesis, Harvard University Cambridge, MA

Krueger DM, Dubilier N, Cavanaugh CM (1996) Chemoautotrophic symbiosis in the tropical solemyid *Solemya occidentalis* (Bivalvia: Protobranchia): ultrastructural and phylogenetic analysis. Mar Biol 126:55–64

Krueger DM, Cavanaugh CM (1997) Phylogenetic diversity of bacterial symbionts of Solemya hosts based on comparative sequence analysis of 16S rRNA genes. Appl Environ Microbiol 63:91–98

Lalou C, Brichet E (1982) Ages and implications of East Pacific Rise sulfide deposits at 21°N. Nature 300:169–171

Laubier L (1989) Ecosystèmes benthiques profonds et chimiosynthèse bactérienne: Sources hydrothermales et suintement froids (Deep benthic ecosystems and bacterial chemosynthesys: hydrothermal wells and cold seepages). In: Denis M (ed) Oceanology: today and in the near future

Laue BE, Nelson DC (1994) Characterization of the gene encoding the autotrophic ATP sulfurylase from the bacterial endosymbiont of the hydrothermal vent tubeworm *Riftia pachyptila*. J Bacteriol 176:3723–3729

Laue BE, Nelson DC (1997) Sulfur-oxidizing symbionts have not co-evolved with their hydrothermal vent tubeworm hosts: an RFLP analysis. Mol Mar Biol Biotechnol 6:180–188

Lee R, Childress J (1994) Assimilation of inorganic nitrogen by marine invertebrates and their chemoautotrophic and methanotrophic symbionts. Appl Environ Microbiol 60:1852–1858

Lee RW, Childress JJ, Desaulniers NT (1997) The effects of exposure to ammonia on ammonia and taurine pools of the symbiotic clam *Solemya reidi*. J Exp Biol 200:2797–2805

Lee RW, Robinson JJ, Cavanaugh CM (1999) Pathways of inorganic nitrogen assimilation in chemoautotrophic bacteria-marine invertebrate symbioses: expression of host and symbiont glutamine synthetase. J Exp Biol 202:289–300

Le Pennec M, Hily A (1984) Anatomy, structure and ultrastructure of the gill of a deep-sea hydrothermal vent mytilid. Oceanol Acta 7:517–523

Levin BR, Bergstrom CT (2000) Bacteria are different: observations, interpretations, speculations, and opinions about the mechanisms of adaptive evolution in prokaryotes. Proc Natl Acad Sci USA 97:6981–6985

Levin LA, Michener RH (2002) Isotopic evidence for chemosynthesis-based nutrition of macrobenthos: the lightness of being at Pacific methane seeps. Limnol Oceanogr 47:1336–1345

Li L, Kato C, Horikoshi K (1999) Microbial diversity in sediments collected from the deepest cold-seep area, the Japan Trench. Mar Biotechnol 1:391–400

Lilley MD, Butterfield DA, Olson EJ, Lupton JE, Macko SA, McDuff RE (1993) Anomalous $CH_4$ and $NH^{4+}$ concentrations at an unsedimented mid-ocean-ridge hydrothermal system. Nature 364:45–47

Little CTS, Herrington RJ, Maslennikov VV, Morris NJ, Zaykov VV (1997) Silurian hydrothermal-vent community from the southern Urals Russia. Nature 385:146–148

Little CL (2002) The fossil record of hydrothermal vent communities. Cahiers Biol Mar 43:313–316

Little CTS, Thorseth IH (2002) Hydrothermal vent microbial communities: a fossil perspective. Cahiers Biol Mar 43:317–319

Little CTS, Vrijenhoek RC (2003) Are hydrothermal vent animals living fossils? Trends Ecol Evol 18:582–588

Little CTS, Danelian T, Herrington RJ, Haymon RM (2004) Early Jurassic hydrothermal vent community from the Franciscan Complex, California. J Paleontol 78:542–559

Lonsdale P (1977) Clustering of suspension-feeding macrobenthos near abyssal hydrothermal vents at oceanic spreading centers. Deep Sea Res 24:857

Lopez-Garcia P, Gaill F, Moreira D (2002) Wide bacterial diversity associated with tubes of the vent worm *Riftia pachyptila*. Environ Microbiol 4:204–215

Luther GWI, Rozan TF, Taillefert M, Nuzzio DB, Di Meo C, Shank TM, Lutz RA, Cary SC (2001) Chemical speciation drives hydrothermal vent ecology. Nature 410:813–816

Lutz RA, Jablonski D, Rhoads DC, Turner RD (1980) Larval dispersal of a deep-sea hydrothermal vent bivalve from the Galapagos Rift. Mar Biol 57:127–133

MacAvoy SE, Macko SA, Joye SB (2002) Fatty acid carbon isotope signatures in chemosynthetic mussels and tubeworms from Gulf of Mexico hydrocarbon seep communities. Chem Geol 185:1–8

Marsh AG, Mullineaux LS, Young CM, Manahan DT (2001) Larval dispersal potential of the tubeworm *Riftia pachyptila* at deep-sea hydrothermal vents. Nature 411:77–80

McArthur AG, Tunnicliffe V (1998) Relics and antiquity revisited in the modern vent fauna. In: Mills R, Harrison K (eds) Modern ocean floor processes and the geological record. Geological Society of London, London

McKiness ZP (2004) Evolution of endosymbioses in deep-sea bathymodioline mussels (Mollusca:Bivalvia) (Ph.D. thesis). Harvard University, Cambridge, MA

McMullin ER, Hourdez S, Schaeffer SW, Fisher CR (2003) Phylogeny and biogeography of deep-sea vestimentiferan tubeworms and their bacterial symbionts. Symbiosis 34:1–41

Metivier B, Voncosel R (1993) Acharax alinae n sp., a giant solemyid (Mollusca, Bivalvia) from the Lau Basin. Comptes Rendus L'Acad Sci Ser III Sciences de la Vie—Life Sciences 316:229–237

Michener RH, Schell DM (1994) Stable isotope ratios as tracers in marine aquatic food webs. In: Lajtha K, Michener RH (eds) Stable isotopes in ecology and environmental science. Blackwell, Oxford, UK

Millero FJ, Plese T, Fernandez M (1987) The dissociation of hydrogen-sulfide in seawater. Limnol Oceanogr 33:269–274

Millikan DS, Felbeck H, Stein JL (1999) Identification and characterization of a flagellin gene from the endosymbiont of the hydrothermal vent tubeworm *Riftia pachyptila*. Appl Environ Microbiol 65:3129–3133

Minagawa M, Wada E (1984) Stepwise enrichment of $^{15}$N along food chains—further evidence and the relationship between delta $^{15}$N and animal age. Geochim Cosmochim Acta 48:1135–1140

Minic Z, Simon V, Penverne B, Gaill F, Herve G (2001) Contribution of the bacterial endosymbiont to the biosynthesis of pyrimidine nucleotides in the deep-sea tubeworm *Riftia pachyptila*. J Biol Chem 276:23777–23784

Minic Z, Herve G (2003) Arginine metabolism in the deep-sea tube worm *Riftia pachyptila* and its bacterial endosymbiont. J Biol Chem 278:40527–40533

Mira A, Moran NA (2002) Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. Microb Ecol 44:137–143

Miura T, Nedachi M, Hashimoto A (2002) Sulphur sources for chemoautotrophic nutrition of shallow water vestimentiferan tubeworms in Kagoshima Bay. J Mar Biol Assoc UK 82:537–540

Moran N, Baumann P (1994) Phylogenetics of cytoplasmically inherited microorganisms of arthropods. Trends Ecol Evol 9:15–20

Moran NA (1996) Accelerated evolution and Muller's rachet in endosymbiotic bacteria. Proc Natl Acad Sci USA 93:2873–2878

Moran NA (2003) Tracing the evolution of gene loss in obligate bacterial symbionts. Curr Opin Microbiol 6:512–518

Muller HJ (1964) The relation of recombination to mutational advance. Mut Res 1:2–9

Mullineaux LS, Wiebe PH, Baker ET (1995) Larvae of benthic invertebrates in hydrothermal vent plumes over Juan de Fuca Ridge. Mar Biol 122:585–596

Mullineaux LS, Speer KG, Thurnherr AM, Maltrud ME, Vangriesheim A (2002) Implications of cross-axis flow for larval dispersal along mid-ocean ridges. Cahiers Biol Mar 43:281–284

Nelson D, Fisher C (1995) Chemoautotrophic and methanotrophic endosymbiotic bacteria at deep-sea vents and seeps. In: Karl D (ed) Microbiology of deep-sea hydrothermal vents. CRC Press, Boca Raton

Nelson DC, Hagen KD, Edwards DB (1995) The gill symbiont of the hydrothermal vent mussel *Bathymodiolus thermophilus* is a psychrophilic, chemoautotrophic, sulfur bacterium. Mar Biol 121:487–495

Nelson K, Fisher CR (2000) Absence of cospeciation in deep-sea vestimentiferan tubeworms and their bacterial endosymbionts. Symbiosis 28:1–15

Niemczuk K, Kondracki M (2004) Q fever as zoonosis. Med Weter 60:129–131

O'Brien J, Vetter RD (1990) Production of thiosulphate during sulphide oxidation by mitochondria of the symbiont-containing bivalve *Solemya reidi*. J Exp Biol 149:133–148

Ohta T (1973) Slightly deleterious mutant substitutions in evolution. Nature 246:96–98

Ott JA, Novak R, Schiemer F, Hentschel U, Nebelsick M, Polz M (1991) Tackling the sulfide gradient: a novel strategy involving marine nematodes and chemoautotrophic ectosymbionts. Mar Ecol 12:261–279, Pubblicazioni Della Stazione Zoologica Di Napoli I

Ott JA, Bright M, Schiemer F (1998) The ecology of a novel symbiosis between a marine peritrich ciliate and chemoautotrophic bacteria. Mar Ecol 19:229–243, Pubblicazioni Della Stazione Zoologica Di Napoli I

Peek AS, Vrijenhoek RC, Gaut BS (1998) Accelerated evolutionary rate in sulfur-oxidizing endosymbiotic bacteria associated with the mode of symbiont transmission. Mol Biol Evol 15:1514–1523

Pimenov NV, Kalyuzhnaya MG, Khmelenina VN, Mityushina LL, Trotsenko YA (2002) Utilization of methane and carbon dioxide by symbiotrophic bacteria in gills of mytilidae (Bathymodiolus) from the rainbow and Logachev hydrothermal fields on the Mid-Atlantic Ridge. Microbiology 71:587–594

Podar M, Mullineaux L, Huang HR, Perlman PS, Sogin ML (2002) Bacterial group II introns in a deep-sea hydrothermal vent environment. Appl Environ Microbiol 68:6392–6398

Polz MF, Felbeck H, Novak R, Nebelsick M, Ott JA (1992) Chemoautotrophic, sulfur-oxidizing symbiotic bacteria on marine nematodes: morphological and biochemical characterization. Microb Ecol 24:313–329

Polz MF, Distel DL, Zarda B, Amann R, Felbeck H, Ott JA, Cavanaugh CM (1994) Phylogenetic analysis of a highly specific association between ectosymbiotic, sulfur-oxidizing bacteria and a marine nematode. Appl Environ Microbiol 60:4461–4467

Polz MF, Cavanaugh CM (1995) Dominance of one bacterial phylotype at a Mid-Atlantic Ridge hydrothermal vent site. Proc Natl Acad Sci USA 92:7232–7236

Polz MF, Robinson JJ, Cavanaugh CM, Van Dover CL (1998) Trophic ecology of massive shrimp aggregations at a Mid-Atlantic Ridge hydrothermal vent site. Limnol Oceanogr 43:1631–1638

Polz MF, Harbison C, Cavanaugh CM (1999) Diversity and heterogeneity of epibiotic bacterial communities on the marine nematode Eubostrichus dianae. Appl Environ Microbiol 65:4271–4275

Polz MF, Ott JA, Bright M, Cavanaugh CM (2000) When bacteria hitch a ride. ASM News 66:531–539

Poulin E, von Boletzky S, Feral JP (2001) Combined ecological factors permit classification of developmental patterns in benthic marine invertebrates: a discussion note. J Exp Mar Biol Ecol 257:109–115

Powell MA, Somero GN (1986) Adaptations to sulfide by hydrothermal vent animals: sites and mechanisms of detoxification and metabolism. Biol Bull 171:274–290

Pradillon F, Shillito B, Young CM, Gaill F (2001) Deep-sea ecology: developmental arrest in vent worm embryos. Nature 413:698–699

Prior SD, Dalton H (1985) The effect of copper ions on membrane content and methane monooxygenase activity in methanol-grown cells of *Methylococcus capsulatus* (Bath). J Gen Microbiol 131:155–163

Pruski AM, Fiala-Médioni A, Fisher CR, Colomines JC (2000a) Composition of free amino acids and related compounds in invertebrates with symbiotic bacteria at hydrocarbon seeps in the Gulf of Mexico. Mar Biol 136:411–420

Pruski AM, Fiala-Médioni A, Prodon R, Colomines JC (2000b) Thiotaurine is a biomarker of sulfide-based symbiosis in deep-sea bivalves. Limnol Oceanogr 45:1860–1867

Pruski AM, Fiala-Médioni A (2003) Stimulatory effect of sulphide on thiotaurine synthesis in three hydrothermal vent species from the East Pacific Rise. J Exp Biol 206:2923–2930

Rau GA, Hedges JI (1979) Carbon-13 depletion in a hydrothermal vent mussel: suggestion of a chemosynthetic food source. Science 203:648–649

Rau GH (1981) Hydrothermal vent clam and tube worm $^{13}$C/$^{12}$C: further evidence of non photosynthetic food sources. Science 213:338–340

Robinson JJ, Cavanaugh CM (1995) Expressions of form I and form II RubisCO in chemoautotrophic symbioses: implications for the interpretation of stable carbon isotope values. Limnol Oceanogr 40:1496–1502

Robinson JJ, Polz MF, Fiala-Medioni A, Cavanaugh CM (1998) Physiological and immunological evidence for two distinct C-1-utilizing pathways in *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae), a dual endosymbiotic mussel from the Mid-Atlantic Ridge. Mar Biol 132:625–633

Robinson JJ, Scott KM, Swanson ST, O'Leary MH, Horken K, Tabita FR (2003) Kinetic isotope effect and characterization of form II RubisCO from the chemoautotrophic endosymbionts of the hydrothermal vent tubeworm *Riftia pachyptila*. Limnol Oceanogr 48:48–54

Rouxel O, Fouquet Y, Ludden JN (2004) Subsurface processes at the lucky strike hydrothermal field, Mid-Atlantic Ridge: evidence from sulfur, selenium, and iron isotopes. Geochim Cosmochim Acta 68:2295–2311

Sarrazin J, Juniper SK (1999) Biological characteristics of a hydrothermal edifice mosaic community. Mar Ecol Progr Ser 185:1–19

Schiemer F, Novak R, Ott J (1990) Metabolic studies on thiobiotic free-living nematodes and their symbiotic microorganisms. Mar Biol 106:129–137

Schmaljohann R, Flügel HJ (1987) Methane-oxidizing bacteria in pogonophora. Sarsia 72:91–98

Schweimanns M, Felbeck H (1985) Significance of the occurrence of chemoautotrophic bacterial endosymbionts in lucinid clams from Bermuda. Mar Ecol Progr Ser 24:113–120

Scott KM (2003) A $d^{13}C$-based carbon flux model for the hydrothermal vent chemoautotrophic symbiosis *Riftia pachyptila* predicts sizeable $CO_2$ gradients at the host-symbiont interface. Environ Microbiol 5:424–432

Scott KM, Schwedock J, Schrag DP, Cavanaugh CM (2004) Influence of form IA RubisCO and environmental dissolved inorganic carbon on the delta 13C of the clam-chemoautotroph symbiosis *Solemya velum*. Environ Microbiol 6(12):1210–1219

Searcy DG (1992) Origins of mitochondria and chloroplasts from sulfur-based symbioses. In: Hartman H, Matsuno K (eds) The origin and evolution of the cell world. Scientific Press, Singapore

Sibuet M, Olu K (1998) Biogeography, biodiversity and fluid dependence of deep-sea cold-seep communities at active and passive margins. Deep Sea Res II 45:517–567

Smith CR, Baco AR (2003) Ecology of whale falls at the deep-sea floor. Oceanogr Mar Biol 41:311–354

Southward AJ, Southward EC, Dando PR, Rau GH, Felbeck H, Flugel H (1981) Bacterial symbionts and low $^{13}C/^{12}C$ ratios in tissues of Pogonophora indicate unusual nutrition and metabolism. Nature 293:616–620

Southward EC (1982) Bacterial symbionts in Pogonophora. J Mar Biol Assoc UK 62:889–906

Southward AJ, Southward EC (1988) Pogonophora: tube worms dependent on endosymbiotic bacteria ISI Atlas of science. Animal & Plant Sciences 1:203–207

Southward EC, Tunnicliffe V, Black M (1995) Revision of the species of Ridgeia from North-East Pacific hydrothermal vents, with a redescription of *Ridgeia piscesae* Jones (Pogonophora: Obturata = Vestimentifera). Can J Zool 73:282–295

Spiro B, Greenwood PB, Southward AJ, Dando PR (1986) $^{13}C/^{12}C$ ratios in marine invertebrates from reducing sediments: confirmation of nutritional importance of chemoautotrophic endosymbiotic bacteria. Mar Ecol Progr Ser 28:233–240

Stanley SM (1970) Shell form and life habits of the Bivalvia. Geol Soc Am Mem 125:119–121

Stein J, Cary S, Hessler R, Ohta S, Vetter R, Childress J, Felbeck H (1988) Chemoautotrophic symbiosis in a hydrothermal vent gastropod. Biol Bull 174:373–378

Stumm W, Morgan JJ (1996) Aquatic chemistry, 3rd edn. Wiley-Interscience, New York

Svavarsson J, Stromberg JO, Brattegard T (1993) The deep-sea asellote (Isopoda, Crustacea) fauna of the Northern Seas: species composition, distributional patterns and origin. J Biogeogr 20:537–555

Temara A, de Ridder C, Kuenen JG, Robertson LA (1993) Sulfide-oxidizing bacteria in the burrowing echinoid, *Echinocardium cordatum* (Echinodermata). Mar Biol 115:179–185

Thao M, Moran N, Abbot P, Brennan E, Burckhardt D, Baumann P (2000) Cospeciation of psyllids and their primary prokaryotic endosymbionts. Appl Environ Microbiol 66:2898–2905

Trask JL, Van Dover CL (1999) Site-specific and ontogenetic variations in nutrition of mussels (*Bathymodiolus* sp.) from the Lucky Strike hydrothermal vent field, Mid-Atlantic Ridge. Limnol Oceanogr 44:334–343

Tunnicliffe V (1988) Biogeography and evolution of hydrothermal-vent fauna in the eastern Pacific Ocean. Proc R Soc Lond B 233:347–366

Tunnicliffe V (1991) The biology of hydrothermal vents: ecology and evolution. Oceanogr Mar Biol 29:319–407

Tunnicliffe V, Fowler C (1996) Influence of sea-floor spreading on the global hydrothermal vent fauna. Nature 379:531–533

Turner RD, Lutz RA, Jablonski D (1985) Modes of molluscan larval development at deep-sea hydrothermal vents. Biol Soc Wash Bull 6:167–184

Tyler PA, Young CM (2003) Dispersal at hydrothermal vents: a summary of recent progress. Hydrobiologia 503:9–19

Vacelet J, Boury-Esnault N, Fiala-Medioni A, Fisher CR (1995) A methanotrophic carnivorous sponge. Nature 377:296

Vacelet J, Fiala-Médioni A, Fisher CR, Boury-Esnault N (1996) Symbiosis between methane-oxidizing bacteria and a deep-sea carnivorous cladorhizid sponge. Mar Ecol Progr Ser 145:77–85

Van Dover CL, Fry B, Grassle JF, Humphris S, Rona PA (1988) Feeding biology of the shrimp *Rimicaris exoculata* at hydrothermal vents on the Mid-Atlantic Ridge. Mar Biol 98:209–216

Van Dover CL, Fry B (1994) Microorganisms as food resources at deep-sea hydrothermal vents. Limnol Oceanogr 39:51–57

Van Dover C (2000) The ecology of deep-sea hydrothermal vents. Princeton University Press, Princeton

Van Dover CL, Humphris SE, Fornari D, Cavanaugh CM, Collier R, Goffredi SK, Hashimoto J, Lilley M, Reysenbach AL, Shank TM, Von Damm KL, Banta A, Gallant RM, Götz D, Green D, Hall J, Harmer TL, Hurtado LA, Johnson P, McKiness ZP, Meredith C, Olson E, Pan IL, Turnipseed M, Won Y, Young CR 3rd, Vrijenhoek RC (2001) Biogeography and ecological setting of Indian Ocean hydrothermal vents. Science 294:818–822

Van Dover C (2002) Trophic relationships among invertebrates at the Kairei hydrothermal vent field (Central Indian Ridge). Mar Biol 141:761–772

Van Dover CL, German CR, Speer KG, Parson LM, Vrijenhoek RC (2002) Marine biology: evolution and biogeography of deep-sea vent and seep invertebrates. Science 295:1253–1257

Vereshchaka AL, Vinogradov GM, Lein AY, Dalton S, Dehairs F (2000) Carbon and nitrogen isotopic composition of the fauna from the Broken Spur hydrothermal vent field. Mar Biol 136:11–17

Vrijenhoek R, Shank T, Lutz R (1998) Gene flow and dispersal in deep-sea hydrothermal vent animals. Cahiers Biol Mar 39:363–366

Waser NAD, Harrison PJ, Nielsen B, Calvert SE, Turpin DH (1998) Nitrogen isotope fractionation during the uptake and assimilation of nitrate, nitrite, ammonium, and urea by a marine diatom. Limnol Oceanogr 43:215–224

Watanabe A (2004) Various clinical types of Q-fever disease. Int Med 43:12

Weber RE, Vinogradov SN (2001) Nonvertebrate hemoglobins: functions and molecular adaptations. Physiol Rev 81:569–628

Weiser W (1959) Eine ungewöhnliche Assoziation zwischen Blaualagen und freilebenden marinen. Nematoden Österr Bot Zeitschr 106:81–87

Wernegreen JJ (2002) Genome evolution in bacterial endosymbionts of insects. Nat Rev Genet 3:850–861

Windoffer R, Giere O (1997) Symbiosis of the hydrothermal vent gastropod *Ifremeria nautilei* (Provannidae) with endobacteria: structural analyses and ecological considerations. Biol Bull 193:381–392

Wirsen CO, Sievert SM, Cavanaugh CM, Molyneaux SJ, Ahmad A, Taylor LT, DeLong EF, Taylor CD (2002) Characterization of an autotrophic sulfide-oxidizing marine *Arcobacter* sp. that produces filamentous sulfur. Appl Environ Microbiol 68:316–325

Woldehiwet Z (2004) Q fever (coxiellosis): epidemiology and pathogenesis. Res Vet Sci 77:93–100

Won YJ, Hallam SJ, O'Mullan GD, Pan IL, Buck KR, Vrijenhoek RC (2003a) Environmental acquisition of thiotrophic endosymbionts by deep-sea mussels of the genus Bathymodiolus. Appl Environ Microbiol 69:6785–6792

Won Y, Young CR, Lutz RA, Vrijenhoek RC (2003b) Dispersal barriers and isolation among deep-sea mussel populations (Mytilidae:Bathymodiolus) from eastern Pacific hydrothermal vents. Mol Ecol 12:169–184

Yamamoto H, Fujikura K, Hiraishi A, Kato K, Maki Y (2002) Phylogenetic characterization and biomass estimation of bacterial endosymbionts associated with invertebrates dwelling in chemosynthetic communities of hydrothermal vent and cold seep fields. Mar Ecol Progr Ser 245:61–67

Yamanaka T, Mizota C, Satake H, Kouzuma F, Gamo T, Tsunogai U, Miwa T, Fujioka K (2003) Stable isotope evidence for a putative endosymbiont-based lithotrophic *Bathymodiolus* sp. mussel community atop a serpentine seamount. Geomicrobiol J 20:185–197

Yong R, Searcy DG (2001) Sulfide oxidation coupled to ATP synthesis in chicken liver mitochondria. Comp Biochem Physiol Pt B 129:129–137

Young CM, Vazquez E, Metaxas A, Tyler PA (1996) Embryology of vestimentiferan tube worms from deep-sea methane/sulphide seeps. Nature 381:514–516

Zal F, Lallier FH, Wall JS, Vinogradov SN, Toulmond A (1996) The multi-hemoglobin system of the hydrothermal vent tube worm *Riftia pachyptila*.

1: reexamination of the number and masses of its constituents. J Biol Chem 271:8869–8874

Zal F, Green BN, Lallier FH, Toulmond A (1997) Investigation by electrospray ionization mass spectrometry of the extracellular hemoglobin from the polychaete annelid *Alvinella pompejana*: an unusual hexagonal bilayer hemoglobin. Biochemistry 36:11777–11786

Zal F, Leize E, Lallier FH, Toulmond A, Van Dorsselaer A, Childress JJ (1998) S-sulfohemoglobin and disulfide exchange: the mechanisms of sulfide binding by *Riftia pachyptila* hemoglobins. Proc Natl Acad Sci USA 95:8997–9002

Zal F, Leize E, Oros DR, Hourdez S, Van Dorsselaer A, Childress JJ (2000) Haemoglobin structure and biochemical characteristics of the sulphide-binding component from the deep-sea clam *Calyptogena magnifica*. Cahiers Biol Mar 41:413–423

Zhang JZ, Millero FJ (1993) The products from the oxidation of $H_2S$ in seawater. Geochim Cosmochim Acta 57:1705–1718

# Erratum: *Vibrio fischeri*: A Bioluminescent Light-Organ Symbiont of the Bobtail Squid *Euprymna scolopes*

Unfortunately the title of chapter 20 is incorrect. The correct title is:
*Vibrio fischeri*: A Bioluminescent Light-Organ Symbiont of the Bobtail Squid *Euprymna scolopes*

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